Intramural Activities Volume I
October 1, 1990- September 31, 1991

91 annual report

Division Of

Cancer Etiology

NATIONAL CANCER INSTITUTE

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health

Intramural Activities Volume I
October 1, 1990- September 31, 1991

91 annual report

Division Of

Cancer Etiology

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health

NATIONAL CANCER INSTITUTE

ANNUAL REPORT

DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

TABLE OF CONTENTS

ANNUAL REPORT
OF THE
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

Richard H. Adamson, Ph.D., Director

October 1, 1990 through September 30, 1991

OVERVIEW

The Division of Cancer Etiology (DCE) 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 six 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, the Laboratory of Viral Carcinogenesis, and the Laboratory of Tumor Cell Biology). The Chemical and Physical Carcinogenesis Program consists of two extramural components (the Chemical and Physical Carcinogenesis Branch and the 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 Division has been structured in such a way as to maximize 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. Four years ago an Associate Director for Biological Carcinogenesis joined the DCE staff. He is currently also serving as Acting Chief, Laboratory of Molecular Virology.

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 stabilization in overall contract support after several years of reductions. This was realized by reducing activities which provide materials and services, and by continuing various cost-recovery mechanisms. For example, in the Biological Carcinogenesis Branch all resource contracts are functioning in the cost-recovery, or "payback" mode. These

include 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 Radiolabeled Carcinogen Reference Standard 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 decreased, support for investigator-initiated research grants has continued to increase, and the Cooperative Agreement is now being utilized as an additional instrument of support. 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 and Development Center (FCRDC)

   Research conducted by the contractor began in the early 1970s as a series of unrelated tasks or projects. Today, the carcinogenesis program at the FCRDC has gradually evolved into a coordinated effort on the mechanisms of action of viral and chemical carcinogens. FCRDC is the focus of NCI research on acquired immunodeficiency syndrome (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 FCRDC: the Laboratory of Comparative Carcinogenesis, the Laboratory of Molecular Oncology, the Laboratory of Viral Carcinogenesis and a section of the Laboratory of Tumor Cell Biology.

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 and branches. 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 third cycle of site visits to the Division's intramural operation began in October 1987 and was completed with the review on May 30-31, 1991, of the Laboratory of Tumor Cell Biology. The fourth cycle will begin with site visits to the Laboratory of Tumor Virus Biology, Laboratory of Viral Carcinogenesis, and the Biostatistics Branch, all of which are scheduled for the fall of 1991.

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. One workshop which was Institute-sponsored but organized by the Biological Carcinogenesis Branch, was entitled "Cancer Vaccines." This workshop, held partly in response to congressional interest and partly because of scientific advances, concluded that a sufficient base of knowledge and technical skill exists to mandate a vigorous and broadly-based cancer vaccine program at the NCI. Another workshop was also held partly in response to congressional interest and scientific advances. It was entitled "Animal Models of Retrovirus-Associated Malignancies" and was focused on the neoplastic potential of various retroviruses and the different molecular mechanisms by which viruses might be involved in the etiology of both lymphoid malignancies and sarcomas. In collaboration with the Organ Systems Program of the Division of Cancer Biology, Diagnosis and Centers, DCE participated in sponsoring an international workshop on the epidemiology and biology of multiple myeloma. As a result of such workshops, several requests for applications (RFAs) have been approved by the Board of Scientific Counselors and/or issued during the past year, including "Vaccines for Human Cancers of Viral Etiology," "Domestic Animal Models for Retrovirus-Associated Human Cancers," and "Epidemiology of Cancer in U.S. Minority Populations." As a result of previous workshops held in 1989, four RFAs approved by the Board of Scientific Counselors during FY 1990 were funded this past year. The RFAs are: "Viral Oncogenic and Pathogenesis of Hepatocellular Carcinoma"; "New Approaches to Understanding Transformation by SV40 Virus, Polyomavirus, and Adenoviruses"; "Human T-cell Lymphotropic Viruses in Human Neoplasia"; and "Mechanisms of Viral-Induced AIDS-Associated Neoplasias."

The Small Grants Program for Cancer Epidemiology was reannounced in August 1988 following intensive evaluation and approval by the DCE Board of Scientific Counselors. The program is serving a useful purpose especially for young investigators, recruiting doctoral students, fellows, and junior faculty into cancer epidemiology. Several recent changes increase the flexibility of the grants: allowable direct costs increased to $50,000 and the maximum project period increased to three years. Competitive renewal is permitted. The amended purposes of the program, all relevant to cancer epidemiology, include: planning a complex study; developing or validating a laboratory or statistical procedure; obtaining rapid funding; analyzing previously collected data, including meta-analysis; resolving problems of methodology; and

providing essential resources for an investigator to mount a study of an emerging issue. The Small Grants Program is an important innovation and the quality of applications continues to improve.

The objectives of the extramural research programs are accomplished through a variety of extramural support mechanisms including traditional individual research project grants (R01), program project grants (P01), First Independent Research Support and Transition (FIRST) awards (R29), conference grants (R13), Cooperative Agreements (U01), contracts (N01), small business innovative research (SBIR) contracts (N43/44) and grants (R43/44), academic research enhancement awards (R15), outstanding investigator awards (R35) and the new Method to Extend Research in Time (MERIT) award (R37).

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, 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

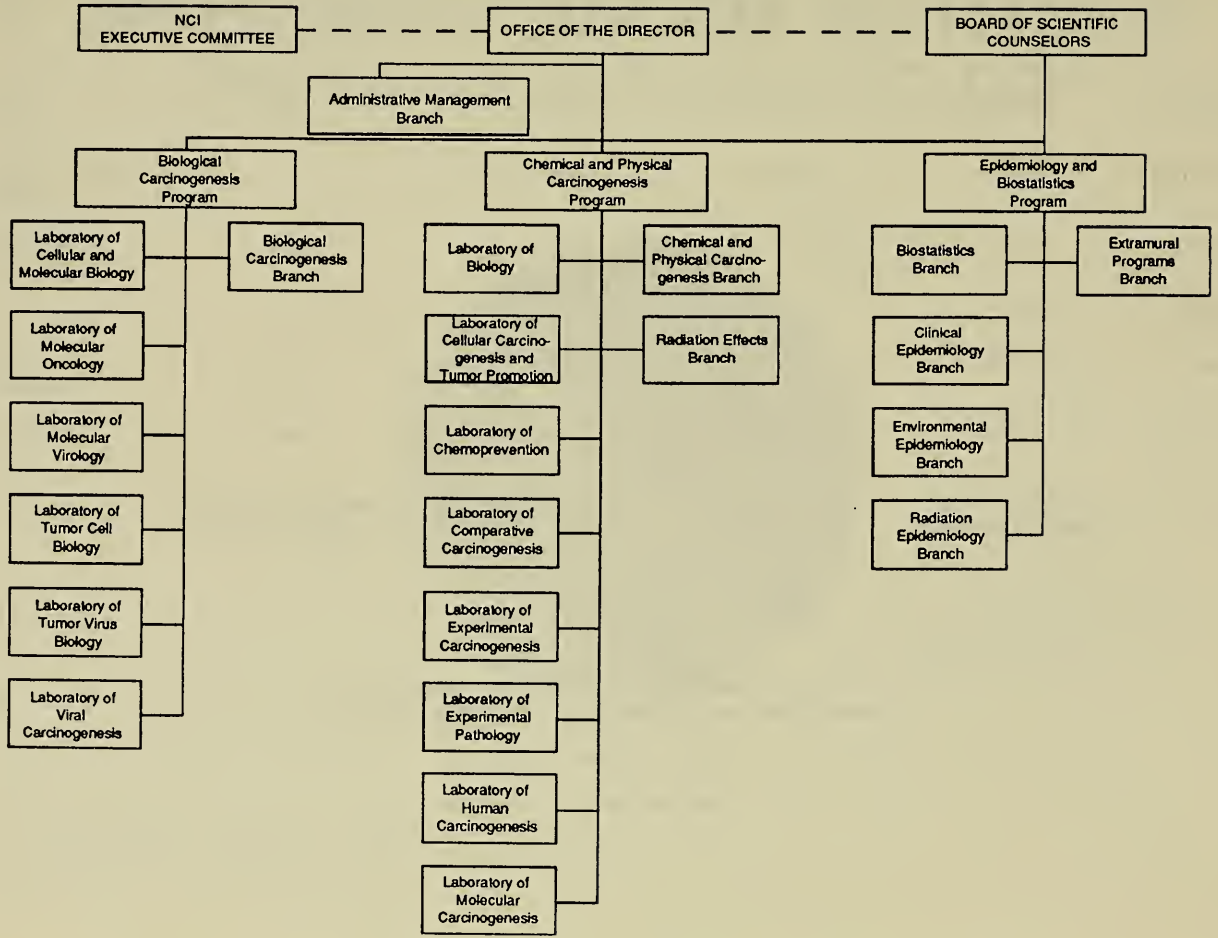


TABLE I

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER ETIOLOGY

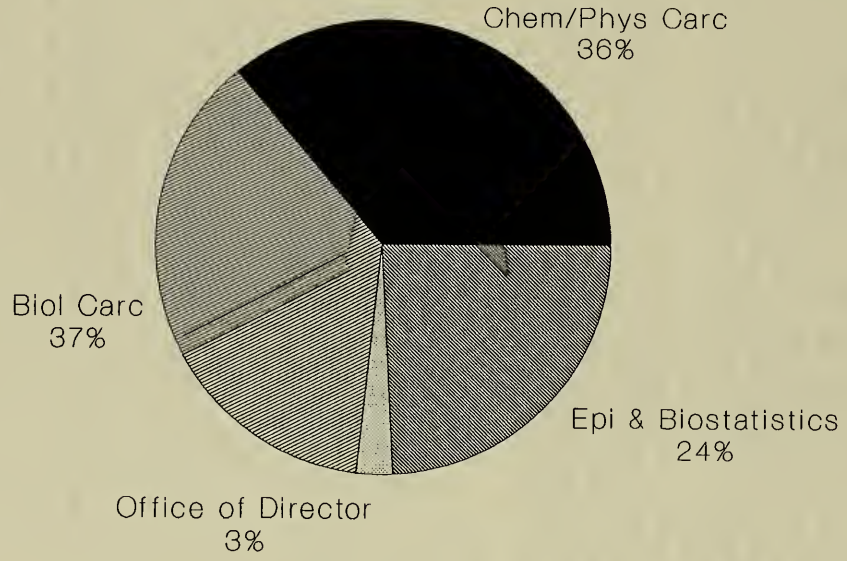
Tables of Mechanisms by Organizational Unit Based On
Estimated Current Level of Expenditures
(Dollars in Thousands)

FY 1991 Estimate

	Office of the Division Director	Chemical and Physical Carcinogenesis	Biological Carcinogenesis	Epidemiology and Biostatistics	Total
In House	4,166	19,602	26,013	15,297	65,078
Research & Development	4,203	4,188	3,700	23,552	35,643
RFA	0	1,709	6,846	1,075	9,630
Cooperative Agreements	502	1,690	0	231	2,423
Research Project Grants	0	94,411	88,141	41,300	223,852
TOTAL	8,871	121,600	124,700	81,455	336,626

NOTE: Obligations for NCI FCRDC are included in the program intramural and OD budgets. SBIR Contracts are included in OD.

NATIONAL CANCER INSTITUTE
DIVISION OF CANCER ETIOLOGY



Distribution of Funds, FY 1991 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. It is also responsible for directing and coordinating AIDS vaccine research and development efforts for NCI. 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, molecular genetics, biochemistry, mathematics, biometry, and biostatistics.

Fundamental information on biological carcinogenesis has been acquired by studying human and 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 viruses may themselves be directly responsible for some malignant transformations in man. Investigations 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 (promotors or long terminal repeats) on cellular oncogenes; or through the interaction of viral protein products with endogenous growth regulatory substances or with a variety of environmental factors, such as hormones, chemicals and radiation.

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 or inactivation of tumor suppressor genes 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 lifestyle in the causation or prevention of cancer, in particular the role of macro- and micronutrients in the diet, dietary mutagens/carcinogens, alcohol consumption and tobacco use, special emphasis has been given to projects that have 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 other studies, cancer risks associated with fluoridation of drinking water and with living in proximity to nuclear power generating plants have been evaluated. Especial emphasis is being placed on gaining a better understanding of the differences between whites and various minority groups with regard to cancer incidence and survival. Efforts to develop safe and effective cancer vaccines were also intensified during the past year. In addition, major studies on the viral etiology of cancer, cancer incidence in the workplace, effects of low-level radiation including radon, and environmental pollutants in air, water, and soil are under investigation. Studies on the pathogenesis, natural history, transmission, and cofactors for human immunodeficiency virus (HIV) infection have received major emphasis during the past year, as has HIV-associated malignancies.

BIOLOGICAL CARCINOGENESIS PROGRAM

During the past fiscal year the Biological Carcinogenesis Program continued to make research advances in the study of DNA viruses associated with the development of cancers, and in the study of growth factors and oncogenes. Laboratory studies of viruses that have been shown to cause certain cancers have focused particularly on the molecular biology of these viruses, including their interactions with oncogenes.

Highlights of the year include the development of a new, highly efficient cDNA cloning system. Research on HIV has elucidated the ways in which this virus may cause Kaposi's sarcoma to develop and the mechanisms by which it stimulates its growth. Studies have continued to elucidate the regions of HIV-1 that could be used as the basis for a vaccine, and dogs and macaques have been immunized. In the field of papillomavirus research, the interactions of the viral E7 and E6 proteins with the cellular RB and p53 proteins, respectively, are providing important clues to the carcinogenic roles of these viruses.

New Technology: A New cDNA Cloning System

A high efficiency cDNA cloning system has been developed that can direct the orientation of inserts in γ-plasmid composite vectors with large cloning capacities. Cleavage of the vector DNA by *SfiI* creates two different nonsymmetrical 3′ extensions at the ends vector arms. Using a linker-primer and an adaptor, cDNA is prepared so it has two different sticky ends which can be ligated to those of the vector arms. When the cDNA and the vector arms are mixed, both the molecules can assemble without self-circularization due to base-pairing specificity. Ligation of the cDNA-vector mixture produces the concatemers from which phage clones carrying a single cDNA insert in the

desired orientation can be formed very efficiently by *in vitro* packaging. This system provides: (1) high cloning efficiency [10^7 -10^8 clones/μg poly(A)$^+$RNA], (2) low background (more than 90% of the clones contain inserts) (3) directional insertion of cDNA fragments into the vectors, (4) presence of a single insert in each clone, (5) accommodation of long inserts (up to 10 kb) (6) a mechanism for rescue of the plasmid part for the λ genome, and (7) a straightforward protocol for library preparations. Screenings of cDNA libraries constructed by this method demonstrated that cDNAs of up to 6.4 kb, containing complete coding sequences, could be isolated at high efficiency. Thus, this cloning system should be useful for the isolation of cDNAs of relatively long transcripts, present even at low abundance in cells.

HIV and AIDS-KS

HIV-1 tat is capable of providing a growth stimulus to AIDS-KS cells in a complex dose-dependent manner. The HIV-1 tat molecule contains multiple independent segments that are capable of adhering to AIDS-KS cells, as well as other cell types, and have been used to identify the existence of a specific high-affinity receptor molecule on AIDS-KS cells. The identification of this and other possible receptor molecules is in progress, as is the possible contribution of AIDS-KS cell adhesion molecules to pathogenesis. Several drugs and compounds have been evaluated for their effect on KS cell induced biological functions. The effect of SP-PG (a peptidoglycan natural product) on AIDS-KS cell growth and induction of vascular permeability or angiogenesis was promising. It was target specific with an effect on AIDS-KS and endothelial cells but not in fibroblasts.

Specific, high-affinity receptors have been demonstrated for a peptide from the HIV-1 transactivator tat protein. Those receptors could be saturated using an excess of unlabelled tat peptide. The tat peptide induced proliferation of the cultured AIDS-KS cells. These receptors are likely to mediate the proliferation of the AIDS-KS cells induced by the tat protein.

A 30 kd growth factor was isolated and purified; it supports the long-term growth of AIDS-KS cells from human activated CD4+ T cells (mitogen-stimulated normal peripheral blood mononuclear leukocytes as well as retrovirus-infected/transformed cells).

Previous studies on AIDS-KS have elucidated some of the mechanisms in the formation of the KS lesion, and demonstrated that the HIV-1 gene product, Tat, may play a role in KS pathogenesis. However, the reasons for the very high risk of KS development in HIV-1 infected homosexual or bisexual men are unclear. In the early stages of HIV-1 infection, AIDS-KS patients often show signs of immunoactivation and are only marginally immunosuppressed, suggesting that immune stimulation may play a pivotal role ln the development of AIDS-KS. The HTLV cell line which is used as the source of conditioned media (CM) for the long-term culture of the AIDS-KS cells expressed several cytokines normally produced during T cell activation, suggesting that T cell activation products may play a role *in vivo* in the pathogenesis of KS. To clarify the biological bases of these clinical-epidemiological and *in vitro* observations, and to evaluate the role of immunoactivation in AIDS-KS, CM from mitogen-activated primary immune cells were investigated to see if they could induce proliferation of cells derived from KS lesions of AIDS patients and of other cells of mesenchymal origin. The CM reproducibly stimulated AIDS-KS and adult

aortic smooth muscle cell growth. Protein and mRNA analyses indicated that several cytokines were expressed by two types of activated T cells (as evidenced by HTLV-II CM and phytohemagglutinin (PHA)-T CM). IL-1α and -β, TNF-α and -β, PDGF and, to a lesser extent, IL-6 and granulocyte-macrophage colony stimulating factor promoted the growth of AIDS-KS cells at concentrations shown to be biologically active in other systems. A synthetic CM (made by combining the cytokines at the same concentration present in PHA-CM and HTLV-II CM, in vitro CM) stimulated a growth response very similar or identical to that obtained with CM from activated primary T cells. These results demonstrated that cytokines released from activated primary lymphocytes can induce growth of AIDS-KS cells and of normal mesenchymal cells present in the KS lesion, and that the combination of individually submitogenic levels of several cytokines had additive or synergistic growth effects on mesenchymal cells.

HIV-1; Vaccine-Related Studies

The principal neutralizing determinant of HIV, the V3 loop, elicits highly type-specific neutralizing antibodies, complicating its use in vaccine preparations. To study the influence of viral heterogeneity on neutralization, neutralizing serotypes among Zairian isolates have been studied. Immunologic analysis of V3 loop sequences has been carried out using synthetic peptides and hyperimmune goat sera to the V3 loops in homologous and heterologous competition ELISA assays. As expected, highly related immunologic reactivity was associated with conservation of loop amino acid sequence. Nevertheless, in cross-neutralization analyses of Zairian isolates with matched sera, neutralizing serotypes did not correlate with homology in the V3 loop, suggesting the participation of alternate epitopes in broad neutralization. The role of envelope conformation in neutralization was also emphasized by studies on a chimeric virus composed of the V3 loop of the MN isolate substituted into the envelope of HXB2D. A panel of Zairian sera neutralized this chimeric virus with titers 20-fold higher compared to those against the MN isolate itself. Competition studies with V3 peptides showed this increase in titer could be attributed to better presentation and recognition of either the V3 loop itself or an alternate epitope. Thus, the context in which the V3 loop is presented is crucial for neutralization. The chimeric virus approach is being pursued both for enhancing immunogenicity and for further analysis of neutralizing epitopes. Additional studies aimed at elucidating the principal neutralizing determinant of HIV-2 have implicated a role for the homologous V3 region in this related virus.

A successful vaccine for AIDS may induce a cell mediated immune response in addition to neutralizing antibody. Initial studies of rhesus macaques infected with HIV-2 and HIV-2 accessory gene mutants have shown that while the animals are not immune compromised, they have a poor proliferative response to HIV-2 antigens, and lack circulating cytotoxic T-lymphocytes (CTLs) specific for HIV-2. Thus, the low viral load in these animals cannot be attributed to a strong immune response. These animals also lack neutralizing antibodies. However, HIV-2-specific CTL clones were obtained from several of the animals. These will be useful for mapping HIV-2 CTL epitopes for use in subsequent subunit vaccines.

Dogs have been vaccinated with adenovirus constructs carrying the HIV-1 *env* gene. A strong neutralizing antibody response was seen in these animals

following inoculation with two successive adenovirus constructs of different serotype. Four SIV peptides representing highly conserved and seroprevalent regions of HIV-1 gp120 and gp41 were mixed and used as immunogens in three macaques. The two animals with the highest neutralizing titers resisted a challenge of 100 infectious particles of SIV. Macaques have also been immunized with two doses of a vaccinia virus expressing the envelope proteins of the pathogenic molecular clone of SIV/Mne.

Papillomavirus

The "high risk" human papillomaviruses (HPV) include HPV-16 and HPV-18. These viruses can be differentiated from "low risk" papillomavirus such as HPV-6 and HPV-11 by their ability to efficiently immortalize primary human keratinocytes. Efficient immortalization of these cells requires the combination of both the E6 and E7 gene products which are now considered oncoproteins. The E7 oncoprotein shares properties with the E1A transforming protein of adenovirus E1A and the large T antigen encoded by the polyomaviruses in that it can complex with a group of cellular proteins which includes the product of the retinoblastoma tumor suppressor gene (RB). The biochemical and biological properties of the high risk and low risk HPVs were studied and compared. Chimeric proteins were constructed between these high risk and low risk E7 proteins and their biochemical and biological properties were studied. Biological properties important to cellular transformation were mapped to the amino terminus of the HPV-16 E7 protein. This amino terminus is required for efficient immortalization, high affinity binding to the retinoblastoma gene product, and the abrogation of the TGF-beta mediated transcriptional repression of c-*myc* expression. In contrast, both the HPV-6 and HPV-16 E7 proteins can efficiently transactivte the adenovirus E2 promoter. Additional experiments have been carried out to characterize the cellular proteins associated with HPV-16 E7. In addition to pRB binding HPV-16 E7, another protein with the same electrophoretic ability as the adenovirus E1A associated protein, p107, was noted. A series of additional cellular proteins has been observed and their identities are under investigation.

The ability of the HPV-16 E7 protein to abrogate the TGF-beta-induced transcriptional repression of c-*myc* expression is dependent on the intact pRB binding site on E7. This has implicated pRB, or another cellular protein capable of binding E7 through this same domain, as an essential component in the pathway of transcriptional modulation of the c-*myc* gene by TGF-beta. Transient expression of pRB also leads to the repression of c-*myc* expression.

The human papillomavirus E6 protein, which binds p53, stimulates the degradation of p53 *in vitro*. The E6-promoted degradation of p53 is ATP-dependent and involves the ubiquitin-dependent protease system. Selective degradation of cellular proteins with negative regulatory function (such as p53) provides a novel mechanism of action for dominant acting oncoproteins.

Approximately 85% of human cervical carcinomas can be demonstrated to contain HPV DNA sequences. Five HPV-positive cell lines were studied, and it was found that the Rb and p53 genes are wild type in these cell lines. This result is consistent with the hypothesis that the normal functions of the tumor suppressor gene products, pRB and p53, are abrogated as a consequence of the complex formation with the HPV E6 and E7 oncoproteins. In contrast,

mutations were identified in the p53 and Rb genes expressed in the two HPV-negative cervical carcinoma cell lines examined. In Rb, the mutations mapped to the domain involved in complex formation with the oncoproteins of the DNA tumor virus. Mutations in the Rb genes that affect this portion of the protein have been noted in a variety of different human cancers. Mutations in the p53 gene were likewise in regions commonly mutated in human cancers. These results support the hypothesis that the inactivation of the normal function of the tumor suppressors, pRB and p53, are important steps in human cervical carcinogenesis, either by mutation or as a consequence of complex formation with the virally encoded oncoproteins.

CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM

Chemical carcinogenesis is a multistage process that begins with exposure, usually to complex mixtures of chemicals found in the human environment. Once inside the body, carcinogens are frequently subject to competing metabolic pathways of activation and inactivation, although some reactive environmental chemicals can act directly. Differences amongst individuals in metabolism together with differences in DNA repair capacity and response to tumor promoters govern the relative risk of an individual. The initial genetic change(s) that occurs as the result of the chemical-DNA interaction is termed tumor initiation. Initiated cells are irreversibly altered in such a way as to be at a greater risk of malignant conversion than normal cells. The effects of tumor promoters facilitate the expansion of one initiated cell into many. This selective growth advantage (known as "clonal expansion") results in formation of a focus of preneoplastic cells. These cells are more vulnerable to progression towards tumorigenesis because they present a larger, more rapidly proliferating, target population for the further action of chemical carcinogens, oncogenic viruses or other cofactors. Additional genetic changes occur and consequently the accumulation of mutations, which may activate oncogenes and inactivate tumor suppressor genes, leads to malignant conversion, tumor progression and metastasis. The underlying genetic mechanisms which regulate chemical carcinogenesis are becoming increasingly well understood, and the insights generated have assisted in the development of methodologies designed to assess human cancer risk and individual susceptibility factors. The results of these latter studies are further intended to mold strategies for cancer prevention.

Cytochromes P450

The cytochromes P450 are a family of isozymes capable of oxidizing a wide variety of both endogenous and exogenous compounds. Two characteristics of these enzymes make it possible for a limited number of isozymes to metabolize a vast and varied array of chemical compounds. The first is the generally broad substrate and regio-specificity presumably due to relatively nonspecific substrate binding characteristics and mutiple binding orientations. The second is a versatile active oxygenating species that is capable of oxidizing a variety of functional groups. The goal of this research is to explore the mechanisms of oxygen activation, substrate oxidation and the topology of the P450 active sites. Methods used in the project include recombinant DNA techniques, determination of enzyme and isotope effect kinetics, and kinetic analysis of both wild type and mutant enzymes. Four general areas are being investigated: 1) regulation of cytochrome P450s and their genes by inducing

agents and during development, 2) the structure-function relationships of P450s to determine those amino acid residues in the P450 that are required for substrate binding and catalytic activities, 3) catalytic activities of human P450s, and 4) the mechanisms and diagnosis of human P450 polymorphisms.

Studies on Food-Derived Mutagens/Carcinogens and Cytochrome P450

Heterocyclic arylamines (HAAs) are formed in meats such as beef, chicken and fish upon cooking at normal, household temperature. These compounds are potent promutagens in the Ames *Salmonella* mutagenicity assay and a number of these compounds have been shown to be carcinogenic in rodent bioassays. 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) has been recently shown to be carcinogenic to cynomolgus monkeys. It has also been shown, using recombinant cytochromes P450-expressed in human cells, that cytochrome P450IA2 is the predominant cytochrome P450 responsible for the activation of these compounds via N-oxidation. Further studies have shown that N-hydroxy-IQ covalently binds to DNA directly and following further activation by O-acetyl- and sulfotransferases. Adducts of IQ have been examined in rodents and monkeys and found to be identical to those formed *in vitro* upon reaction of N-hydroxy-IQ with DNA, and those found in *Salmonella*. In addition to *in vitro* studies of HAA metabolism, studies are underway on the *in vivo* metabolism and distribution of HAAs in monkeys. Studies concerning repair in specific genes using the UvrABC excinuclease and alkaline hydrolysis are also underway. Studies are being carried out in isolated genomic DNA, plasmids and cultured cells. With this methodology, investigators are seeing heterogeneity in initial adduct formation in different gene regions and differences in adduct levels with N-hydroxy-IQ and N-hydroxy-MeIQx.

Studies of Tumor Promotion

Protein kinase C is the receptor for the phorbol esters, the best studied class of tumor promoters in the mouse skin model. The mechanisms of protein kinase C activators which fail to induce phorbol ester-like responses are being characterized at the cellular and whole animal levels. The patterns of response are being correlated with the selectivity of the agents for cloned protein kinase C isozymes. One class of compounds which is receiving particular attention is that of the short chain monoesters of 12-deoxyphorbol, exemplified by prostratin. Although prostratin is a weak protein kinase C activator, chronic treatment of mouse skin with prostratin blocks the inflammatory and hyperplastic response of skin to subsequent treatment with the strong tumor promoter phorbol 12-myristate 13-acetate. These properties predict that prostratin will function as an anti-promoter. Ingenol is being examined because it possesses all elements of the phorbol ester pharmacophore except for the presence of a hydrophobic domain. It was found that ingenol still retains weak activity as a protein kinase C agonist. Although all tumor promoting phorbol esters are inflammatory, the converse is not true. Studies have revealed that resiniferatoxin, an ultra-inflammatory phorbol ester, acts through a distinct mechanism, stimulating unique receptors on sensory neurons involved in pain and neurogenic inflammation. Studies are continuing to characterize these receptors, developing strategies to clone them and screening for the existence of endogenous analogs which may act as the normal physiological mediators of this pathway.

Regulation of Epidermal Growth and Relationship to Early Events in Carcinogenesis

Elevation of the medium [Ca^{2+}] of cultured keratinocytes induces terminal differentiation. The expression of differentiation-specific proteins is preceded by increases in intracellular free Ca^{2+}, inositol phosphate turnover, as well as increases in levels of phospholipase C and diacylglycerol. Protein kinase C is necessary for the induction and maintenance of Ca^{2+}-induced terminal differentiation. However, protein kinase C isozyme mRNA levels were not affected by an increase in the concentration of extracellular Ca^{2+}. Neoplastic keratinocytes are defective in their terminal differentiation response to Ca^{2+} and to phorbol ester tumor promoters. The kinase inhibitor staurosporine induces terminal differentiation in neoplastic, as well as normal, keratinocytes. In grafts of papilloma cell lines to the backs of nude mice, treatment with staurosporine inhibits papilloma development. A cell culture model of initiated epidermis in which the growth of initiated keratinocytes is inhibited by coculture with normal keratinocytes was used to assay promoters and antipromters. Several promoters for which protein kinase C is the receptor, as well as several non-phorbol ester-type promoters, allow growth of initiated foci in cocultures. Papillomas induced by introduction of an activated ras^{Ha} oncogene into dermal hair follicle cells could not be distinguished from papillomas induced by ras^{Ha} in cells prepared from epidermis. A procedure was developed for preparing hair follicle buds, which requires a contribution from the dermis for hair development. The activated oncogenes ras^{Ha} and *fos* can cooperate to produce carcinomas in grafted keratinocytes. In the *ras/fos* carcinomas, levels of transcripts for the proteases transin and urokinase were increased compared to those seen in ras^{Ha}-induced papillomas. Inbred SENCAR mice, compared to outbred SENCARs, are more sensitive to promotion by 12-O-tetradecanoylphorbol-13-acetate, and to initiation by 7,12-dimethylbenz[a-]anthracene and urethane, but not by N-methyl-N′-nitro-N-nitrosoguanidine.

Oncogenes and Tumor Suppressor Genes

Five families of activated proto-oncogenes, *ras*, *raf*, *jun*, *erb*-2 (*neu*) and *myc* have so far been associated with human bronchogenic carcinoma. Human bronchial epithelial cells *in vitro* are being used to investigate the functional role of these specific oncogenes and growth regulatory genes in carcinogenesis and tumor progression. Overexpression of *erb*B-2 leads to neoplastic transformation of human bronchial epithelial cells. The molecular mechanisms of *ras* and *raf/myc* induction of neoplastic transformation is the major focus of current and future studies. The role of *ras* and *raf* in the transduction of signals from ligand activated cellular membrane receptors to the nucleus and subsequent altered expression of growth and terminal differentiation genes is being investigated. It is hypothesized that receptor-mediated phosphorylation of the *raf* kinase causes both an increase in its activity and a translocation to the nucleus. Possible substrates of the activated *raf* kinase include PI kinase and nuclear regulatory proteins, *myc* and p53. The effects of mutant p53 gene on cell growth and terminal squamous differentiation is being assessed. The cooperativity of mutant p53 and *ras* genes in the "immortalization" and neoplastic transformation of normal human bronchial epithelial cells will also be determined. Since the Ha-*ras* or *myc/raf* transformed bronchial epithelial cells are highly invasive and metastatic in athymic nude mice, we plan to investigate the regulation and

expression of genes considered to be involved in tumor metastsis including Nm23, collagenase IV and TIMP-2.

The role of *ras* (especially in K-*ras*) and of *neu* in chemically induced tumors in rodents continue to be investigated in efforts to distinguish between activating mutations that are the direct result of chemical reactions of carcinogenic agents with DNA encoding these specific genes, and stochastic events that occur independently of the inducing carcinogen during tumor progression. Studies on rat renal mesenchymal tumors have been especially informative in this regard. Another theme has been extrapolation of activating mutational events in experimental tumors to human cancers that involve activation of the corresponding human oncogene; human pediatric renal tumors, as well as mucinous carcinoma of the ovary, endometrial carcinoma of the uterus, the "intestinal" variant of human gastric carcinomas and prostatic carcinoma are currently under investigation.

Mutations in the evolutionarily conserved codons of the p53 tumor suppressor gene are common in diverse types of human cancer. The p53 mutational spectrum differs among cancers of the colon, lung, esophagus, breast, liver, brain, lymphomas, and leukemias. Transitions predominate in cancers of the colon, brain and lymphoid malignancies, whereas G:C to T:A transversions are the most frequent substitutions observed in cancers of the lung and liver. Mutations at A:T base pairs are seen more frequently in esophageal carcinomas than in other solid tumors. The distributions of mutations in the p53 sequence are also distinct. Most transitions in colorectal carcinomas, brain tumors, leukemias and lymphomas are at CpG dinucleotide mutational hotspots (codons 175, 248, 273, and 282). G to T transversions in lung, breast and esophageal carcinomas are dispersed among numerous codons, whereas in liver tumors from geographic areas in which both aflatoxin B_1 and hepatitis B virus are cancer risk factors, most mutations are at one nucleotide pair of codon 249. These differences may reflect the etiological contributions of both exogenous and endogenous factors in human carcinogenesis.

Studies on Rat Liver Epithelial Cells

Rat liver epithelial (RLE) cells share many cellular markers with the "oval" cell population found in adult rat liver after a certain type of hepatic injury. It has been hypothesized that these cell types are derived from a common stem cell compartment present in the adult liver. Investigation of patterns of cytokeratin expressions in adult and fetal livers and during early stages of "oval" cell proliferation revealed that cytokeratin 14 was transiently expressed in early fetal hepatocytes and oval cells. The expression of cytokeratin 14 in adult rat liver was found in small epithelial cells in the periductal space. These results indicate that cytokeratin 14 may be a marker for early progeny derived from the hepatic stem cell compartment. Evidence was recently obtained that epithelial cell lines obtained from adult pancreas have, similar to RLE cells, an extended capacity for proliferation *in vitro*, and share close lineage relationship to the RLE cells. These data indicate a common cell of origin for primitive cells isolated from rat liver and pancreas. Genetic analysis of aflatoxin B_1-induced RLE transformants revealed a consistent poin mutation consisting of a transition from G to A within codon 173 (CGC to GAG) in the tumor suppressor p53 gene. This mutation resulted in a change of the encoded amino acid from histidine to arginine.

Studies on Silica

Neoplastic transformation was obtained in the BALB/3T3/A31-1-1 mouse embryo cell line by exposure of the cells to three different samples of crystalline silica (quartz) with different toxicity and surface reactive properties. The incidence of induced neoplastic transformed foci differed for the three samples, but for all three it was dose-dependent at lower tested dust concentrations and near plateau level at higher concerntrations. The induced transformed foci were subcultured and cryopreserved for molecular analysis. They were all rapidly tumorigenic in nude mice. The silica-transformed cells and the resulting tumors are being analyzed for karyotypic alterations and for the identification of activated oncogenes.

The mechanisms of silica toxicity were studied in series of biological assay systems, using cultured cells, DNA *in vitro* and a plasmid shuttle vector. A major toxic mechanism was found to be dependent on the production of reactive oxygen radicals catalyzed by divalent and trivalent iron cations. These mechanisms were studied using inhibitors of reactive oxygen species, iron chelators and specific iron cations. The underlying hypothesis is that the highly toxic hydroxyl radical is produced by a cascade of reactions triggered by silica surfaces, probably through their iron impurities.

Nucleophile/NO Complexes

Nucleophile/NO complexes have been found to have a number of interesting biological activities. Members of this compound class are potent vasorelaxants both *in vivo* and *in vitro*. One of them is a good inhibitor of platelet aggregation. Several have the ability to damage DNA in a suitably oxidizing environment, with the corollary property of being mutagenic in *Salmonella typhimurium*. The biological activity is correlated to a greater or lesser extent with the rate and extent of spontaneous nitric oxide release these complexes engender. The nucleophile/NO complexes should be useful both as research tools and for possible drug design strategies.

Studies on Nickel Genotoxicity

Molecular mechanisms of nickel (Ni) genotoxicity were studied. The working hypothesis tested assumes that Ni derivatives initiate tumors through active oxygen species. Increased contents of a DNA oxidation product, 8-hydroxy-2′-deoxyguanosine (8-OHdG) were found in DNA isolated from NRK-52 and NIH 3T3 cells and from kidneys of mice exposed to Ni(II). Moreover, the magnitude of 8-OH-dG increase by Ni was greatest in kidneys of BALB/c mice, i.e., mice which were also more susceptible to renal lipid peroxidation (LPO) by Ni than C3H, B6C3F1, and C57BL mice. No such concurrence was observed in NRK-52 and NIH 3T3 cells, indicating that LPO and nucleobase oxidation might constitute two independent phenomena. This hypothesis was also tested on pure 2′-deoxyguanosine (dG), DNA, and nuclear chromatin isolated from the human-derived K562/S cell line. Three major discoveries were made in these systems: 1) activation of oxygen peroxide by Ni is facilitated by L-histidine (His), a principal *in vivo* Ni carrier, and by tetraglycine (TG), a model Ni-binding peptide; 2) Ni + H_2O_2 modifies all four DNA bases; besides 8-OH-dG, 10 more potentially mutagenic products were identified; 3) nuclear proteins enhance Ni-catalyzed attack of H_2O_2 or O_2 on DNA. The relatively high sensitivity of guanine to attack by Ni-mediated oxygen radicals is consistent with

correspondingly strong complex formation between Ni and dG. It is also consistent with growing evidence that G:C base pairs are the major site of point mutation in the K-*ras* oncogene isolated from Ni-induced renal tumors in rats. Further, both TG and His enhanced DNA-protein cross-linking in kidneys of rats injected with Ni, a phenomenon characteristic for oxygen radical attack on cell nuclei. Studies on the mechanisms of yet another genotoxic effect by Ni subsulfide (Ni_3S_2), deamination of 5-methyl-2'deoxycytidine (5MedC), disclosed that the deamination is caused by O_2 activated through auto-oxidation of the sulfur moiety of Ni3S2. Hence, sulfur is capable of increasing Ni genotoxicity. This may account for the uniquely high carcinogenicity of Ni_3S_2 compared with other Ni derivatives.

Transforming Growth Factor-Beta (TGF-Beta)

Mammalian cells express three distinct TGF-beta isoforms, called TGF-beta's 1, 2, and 3. There is already substantial evidence of differential control of expression of these three TGF-beta isoforms both *in vitro* and *in vivo*. Insight into the molecular mechanisms of transcriptional control of TGF-beta expression in mammalian cells was obtained by comparative analyses of the promoter regions of the genes for TGF-beta's 1, 2, 3. The 5' flanking regions of the three genes are distinctly different. The promoters for the TGF-beta 2 and 3 genes contain TATAA boxes just upstream of the start sites, whereas initiation of transcription of the TGF-betal gene is thought to result from a cluster of SPI binding sites. Selective expression may result from the use of AP-1 sites in the TGF-betal promoter, whereas both the TGF-beta2 and 3 promoters contain CRE and AP-2 sites which suggest responsiveness to cyclic AMP. Correlating with the observed high levels of expression of TGF-beta3 in muscle, it has been found that the TGF-beta3 promoter is selectively regulated during the differentiation of myoblasts into myotubes through novel sites. Recent studies demonstrate that while several oncogenes such as *jun*, *fos src*, *abl*, and *ras*, selectively activate TGF-betal expression through its AP-1 sites, the product of the tumor suppressor gene, Rbl, activates expression of all three TGF-beta promoters through retinoblastoma response elements (RCE) sites. This finding suggests that a general mechanism of growth regulation by RB, a nuclear protein, might involve control of the expression of a secreted peptide which acts to control growth via its interaction with cell-membrane receptors.

Normal genital epithelial cells possess an intracellular control mechanism directed against HPV gene transcription; however, cellular functions down-regulating HPV expression are absent in genital carcinoma cells, suggesting that this loss represents an important step in the development of cancer. Thus, a major goal has been to identity host factors that modulate HPV gene expression and to define the underlying molecular mechanisms. The beta transforming growth factors (TGFβs) are members of a family of polypeptides that modulate cell proliferation and gene expression in diverse cells. Thus, the effect of TGFβs on cell growth and HPV gene expression was determined in a series of immortal genital epithelial cell lines at different stages of malignant progression. TGFβ1 completely inhibited clonal growth in secondary cultures of normal genital epithelial cells derived from either foreskin or cervix. Different cell lines derived by transfection and immortalization of normal cells with HPV-16 DNA varied significantly in their response to TGFβ1, with some being more resistant than normal cells. Cell lines of late passage often exhibited a further increase in resistance. Two immortalized cell lines

that were malignantly transformed after transfection with the v-Ha-*ras* oncogene or the HSV-2 DNA formed colonies in TGFβ1 containing medium with a frequency greater than the parental lines. Three cervical carcinoma-derived tumor lines grew clonally in medium containing TGFβ1. Thus, loss of responsiveness to TGFβ1 often precedes or accompanies malignant development in cultured genital epithelial cells.

The effect of TGFβ1 on the expression of the HPV-16 early genes E6 and E7 was examined in an HPV-16-immortalized cervical cell line that was partially resistant to TGFβ1 in clonal growth assays. TGFβ1 treatment for 24 hours markedly decreased levels of E6 and E7 protein expression. When cultures were maintained in the presence of TGFβI, E7 expression remained low to undetectable for 48 hours, but expression was partially restored when cultures were switched to fresh medium. In contrast, E6 expression remained undetectable even after TGFβ1 was removed. TGFβ1 did not alter expression of involucrin, a marker of squamous differentiation in cervical epithelium. TGFβ1 regulates expression of a wide variety of cellular genes, and regulation may occur at multiple levels. These results are the first report that TGFβ1 inhibits transcription of viral genes.

A major new project that has been started during the past year is an attempt to establish an intensive effort directed at the chemoprevention of prostate cancer. Prostate cancer is the second cause of cancer death in the male population. The lack of progress in treatment and management of this disease is hampered by inadequate experimental animal models. The purpose of this new project is to develop and establish an efficient and reproducible animal model system for inducing prostate carcinogenesis; analyze the molecular and cellular events following the development of prostate cancer and to identify agents that prevent and/or suppress prostate cancer incidence. Induction of prostate carcinogenesis was accomplished by initiation with N-methyl-nitrosourea (NMU) and promotion with testosterone propionate (TP) in Lobund/Wistar rats of varying age ranging from 2-4 months old. Partial success was obtained in growing primary cultures of normal rat prostate epithelium for 3 weeks in chemically defined media supplemented with exogenous nutrients and growth promoting substances. Using 3 month old rats, increased levels of TGF-betal and beta3 message and protein in the prostate gland at 3-6 days following castration was demonstrated. This effect was reversed by treatment with TP. In collaboration with Dr. Morris Pollard, Lobund Laboratory, a decreased incidence of prostate cancer in MNU/TP-treated animals fed with N-(4-Hydroxyphenyl) retinamide was demonstrated for the first time. Thus, an experimental basis for further studies of prevention of prostate cancer was established. In the coming year, such studies will be pursued intensively, both at the molecular level, as well as in whole animals, with the practical goal of developing a clinically acceptable agent for chemoprevention of cancer in men at high risk.

EPIDEMIOLOGY AND BIOSTATISICS PROGRAM

The Epidemiology and Biostatistics Program is the focus within the NCI for epidemiologic and biostatistical research in cancer etiology. The Program is responsible for intramural, collaborative, and grant-supported investigations into the distribution, causes, and natural history of cancer, as well as the means for preventing cancer. Comprehensive approaches are employed, covering

the gamut of environmental and host determinants of cancer. The Program also conducts and supports the development of new and innovative methodologic approaches in epidemiology and biostatistics, multidisciplinary investigations, and biostatistical and mathematical research on carcinogenic mechanisms and risk assessment.

Descriptive studies continued to examine geographic variation and clustering of cancer, and to relate these cancer patterns to demographic and exposure characteristics of the affected populations. Published this year was an updated atlas of maps illustrating cancer mortality trends from 1950-1980 among nonwhite populations. The atlas serves as a companion volume to a similar publication of mortality trends among whites. As noted for whites, the mortality patterns for most cancer sites among nonwhites showed increasing geographic uniformity over time. Noteworthy exceptions to this pattern include the emergence in the 1970's of high rates for prostate cancer among black men in the South Atlantic states, rising rates of stomach cancer among Native Americans in the Southwest, and limited declines in cervical cancer among black women in the Southeast.

The possible carcinogenicity of fluoride compounds added to drinking water supplies was suggested by a recent animal experiment. This concern prompted an evaluation of 36 years of U.S. cancer mortality and 15 years of cancer incidence data in relation to the fluoridation status of drinking water supplies on a county-by-county basis. Based on results of the animal study, osteosarcomas of the bone were singled out for detailed analysis. No consistent evidence was found for a relationship between any malignancy and the pattern of fluoridation, and no trends in bone cancer or osteosarcoma incidence could be correlated with the intake of fluoridated drinking water.

Diet, Nutrition, and Cancer

International correlations, studies of migrant populations, a variety of cohort and case-control studies, and experimental evidence indicate that dietary and nutritional factors play an important role in cancer etiology. These factors continued to be investigated, with an emphasis on biochemical measurements to clarify the influence of micronutrients and other dietary constituents.

A large case-control study of breast cancer among women of Asian ancestry in California and Hawaii found that the place of birth of the subject and her grandparents had a major impact on risk, as did the rural or urban nature of the place where the subject lived prior to migration. The age at migration did not affect risk unless migration occurred prior to age 15, suggesting that the childhood-adolescent years are crucial in determining risk or that relevant lifestyles (e.g., diet/nutrition) are not altered unless migration occurs early in life.

A case-control study of endometrial cancer in five U.S. areas found that obesity is a major predictor of risk. The timing and distribution of weight gain and nutritional intake data will be examined in relation to risk. Information on fat storage, using blood samples and fat biopsies, will be used to supplement interview data, as will results of micronutrient and hormonal assays.

A case-control study of invasive and *in situ* cervical cancer in five U.S. communities found no association with the intake of carotenoids, vitamin A, or vitamin C. In a study of invasive cervical cancer in Latin America, however, higher risk was associated with lower dietary intake of vitamin C and β-carotene. An effect for low β-carotene is supported by serologic analyses among the early-stage cases. Neither study found an effect of low dietary or serum folate levels, as reported by some investigators.

Because of the current interest in β-carotene as a chemopreventive agent, investigators at the University of Hawaii examined data from a population-based case-control study among the multiethnic populations of Hawaii for the food sources of this carotenoid. Sources of β-carotene were mainly carrots, papaya, pumpkin, sweet potatoes, and mangoes. With the exception of papaya, which was positively associated with prostate cancer risk among men aged 70 years and older, consumption of yellow-orange fruits and vegetables, tomatoes, dark green vegetables, and cruciferous vegetables was not associated with prostate cancer risk. The association with papaya was consistent across various ethnic groups, and persisted even after adjustment for confounders.

In a case-control study of stomach cancer in Italy, conducted in collaboration with the Center for the Study and Prevention of Cancer in Florence and other institutions, increased risks for both intestinal and diffuse type stomach cancer were associated with certain traditional soups and meats, while decreased risks were linked to the intake of fresh fruits and vegetables (notably garlic) and vitamins C and E.

Infectious Agents

Epidemiologic data suggest that cervical neoplasia is related to a sexually-transmitted infectious agent. Recent laboratory and epidemiologic studies have identified human papillomaviruses (HPV) as the candidate agents that are most likely involved in this disease. A prevalent case-control study of women with cervical intraepithelial neoplasia (CIN) revealed that over 80 percent of the cases had detectable HPV, compared with 18 percent of controls. Although the number of genital HPV types exceed 20, only a few (notably types 16, 18, 31, 33, and 51) were found in the great majority of women with high-grade CIN, confirming earlier reports. In a correlational study of normal subjects, HPV infection was associated with other cervical neoplasia risk factors, including lifetime number of sexual partners, oral contraceptive use, and black race (independent of the measured behavioral factors). Another survey found that HPV prevalence was inversely correlated with socioeconomic status.

The first prospective data, though still preliminary, suggest that HPV infection precedes and predicts the appearance of cervical neoplasia. Eleven (52%) of the first 21 cases of incident dysplasia in a large cohort study had detectable HPV in cervical smears taken a year before diagnosis, when they were judged to be cytologically normal, compared to 63 control women (16%). The presence of HPV thus appears to indicate either abnormal cytology missed by screening or an increased risk of developing new disease.

Based on descriptive epidemiologic data, adult T-cell leukemia (ATL) has been etiologically linked to human T-cell lymphotropic virus type 1 (HTLV-I) exposure in early life. Recently, the infective dermatitis syndrome of childhood was linked to HTLV-I infection in Jamaicans, suggesting a precursor

syndrome related to the immunodeficiency induced by the virus. In collaboration with laboratory investigators, molecular analysis of ATL cases demonstrated an overexpression of the immune-modulating transforming growth factor beta (TGF-β), supporting the concept that viral-related immunosuppression plays an etiologic role in ATL. The finding of elevated spontaneous lymphocyte proliferation in seropositive subjects may represent an intermediate pathway by which HTLV-I amplifies its target cells.

Infection by HTLV-II is emerging as a major problem among drug abusers, including addicts from the early 1970's. Spontaneous lymphocyte proliferation occurs among HTLV-II infected subjects, though at a lower level than among persons infected with HTLV-I. Reservoirs of HTLV-II have been identified among American Indian populations in the southeastern and western United States, Brazil, and Panama. In one hospital survey of intravenous drug abusers, HTLV-II infection was not associated with any specific disease.

Efforts continued to estimate the magnitude of the human immunodeficiency virus (HIV) epidemic and project its impact on cancer in the United States. Using backcalculation modeling, some 700,000 persons were infected with HIV by 1985, and perhaps one million by 1987. In homosexual and hemophilia cohorts, an increased risk of developing non-Hodgkin's lymphoma (NHL) was observed, which markedly accelerated eight years after seroconversion. It is now projected that over 4,000 excess cases of NHL (i.e., in excess of expected numbers from the pre-AIDS era) will occur in 1994, constituting approximately 10 percent of all NHL. Except for Kaposi's sarcoma, no clear excesses of other cancers related to HIV infection have been uncovered.

Investigators at the University of Southern California examined biopsy material of lymph nodes from HIV-infected patients with persistent generalized lymphadenopathy (PGL) and controls for the presence of Epstein-Barr virus (EBV) to determine the association between EBV-related lymphoproliferation and the risk of NHL. EBV DNA was not found in any of the benign lymph node biopsies from control subjects, but was detected from about a third of the PGL biopsies. EBV positivity was significantly associated with the concurrent presence of EBV-positive NHL at another site, or subsequent development of EBV-positive lymphoma. The presence of EBV DNA in benign PGL biopsies was thus found to be a significant risk factor for the development of EBV-positive HIV-associated NHL.

Tobacco and Alcohol

Research continued to define the roles of tobacco and alcohol on the risk of various cancers. In a 20-year follow-up of almost 18,000 men from the upper midwest and northeast regions of the United States, dose-response relationships for cigarette smoking were identified for prostate cancer and lymphatic and unspecified leukemias. In a large-scale case-control study conducted in Los Angeles, Iowa, and New Jersey, cigarette smoking was found to account for almost three out of four cancers of the renal pelvis and ureter.

Investigators at Loma Linda University followed a cohort of Seventh-Day Adventists (SDAs) for six years, during which time incident cancers were detected through annual mailings and record linkage with the two population-based tumor registries in California. In comparison with the non-smokers, SDAs who were ex-smokers experienced relative risks of 2.0 for leukemia, 2.2

for myeloid leukemia, and 3.0 for myeloma. Risks increased with number of cigarettes smoked daily and with duration of smoking. These data suggest that cigarette smoking may induce malignant transformation in the bone marrow.

A large-scale case-control study revealed a 35-fold excess risk of oral cancer following heavy use of tobacco and alcoholic beverages. In addition, an increased risk of oral cancer was associated with the regular use of mouthwash with high (>25%) alcohol content, with the risk increasing in proportion to the duration and frequency of mouthwash use. This finding suggests that alcohol may produce a carcinogenic effect through topical mechanisms.

Occupational/Environmental Carcinogens

Exposures in the workplace are often heavier and more prolonged than those typically encountered by the general public. Epidemiologic studies of workers thus offer unique opportunities to identify environmental causes of cancer. A number of recent investigations have focused on cancer risks associated with agricultural pesticide exposure. In a population-based case-control study in Nebraska, farmers using the phenoxyacetic acid herbicide, 2,4-D, experienced an elevated risk of NHL, consistent with findings from an earlier study in Kansas. In addition, farmers who used organophosphate insecticides had a two-fold risk of NHL, which was independent of the risk from exposure to 2,4-D.

A case-control study of leukemia in Iowa and Minnesota revealed elevated risks among farmers, particularly if they used insecticides on animals. Risks of over twofold occurred from the use of various of insecticides, including carbaryl, coumaphos, dichlorvos, famphur, methoxychlor, nicotine, pyrethrins, and toxaphene. The risk of leukemia from the use of insecticides on crops was generally lower, possibly because of limited exposures.

Excesses of NHL and multiple myeloma were observed in a cohort employed at an aircraft maintenance facility, where a variety of organic solvents are used. These excesses will be evaluated in an extended follow-up of the cohort. Lung cancer was found to be elevated in silicotic workers employed in dusty trades in North Carolina. In a large proportionate mortality study of embalmers and funeral directors, a significant excess of lymphatic and hematopoietic cancers was observed among blacks and whites. An excess of nasopharyngeal cancer was also noted for the first time, consistent with the excess risk seen among industrial workers exposed to formaldehyde. An investigation, which includes a case-control interview plus environmental and biologic monitoring, is underway to further evaluate cancer risks among embalmers.

Non-melanoma skin cancer is the most common neoplasm in the U.S. Caucasian population. Among a cohort of 73,366 nurses, investigators at Harvard University observed a steep increase of basal cell carcinoma (BCC) in the northeast and north central states, as well as the western and southern states. The incidence rate of BCC among cohort members 60 to 64 years of age in Florida rose more than one percent per year. The age-adjusted relative risk for BCC was about 50 percent higher in California and twice as high in Florida than in the northeast states.

Genetic Susceptibility

Families at high risk of developing cancer and heritable disorders predisposing to neoplasia are studied in an interdisciplinary approach to identify genetic mechanisms of cancer susceptibility. After a two-decade search, a collaborative study with the Massachusetts General Hospital Cancer Center identified the p53 tumor suppressor gene as the site of the inherited defect in the Li-Fraumeni syndrome (LFS). This disorder features an autosomal dominant pattern of diverse cancers, particularly sarcomas of bone and soft tissue, breast cancer, acute leukemia, brain tumor, and adrenocortical carcinoma. In the five LFS families that were examined, all showed germ-line point mutations in the p53 gene. In one family, the mutation co-segregated with cancer in four relatives. In tumor specimens of family members, the wild-type p53 allele was deleted in the cancer cells, as expected in recessive tumor suppressor genes. These findings should lead to new means for prevention, early detection and perhaps treatment of LFS, as well as a broader understanding of carcinogenesis.

At the University of California at Berkeley, investigators performed genetic linkage analysis of 23 families with a high risk for early onset of breast cancer. A tight linkage to a locus on the long arm of chromosome 17q21 was found among women less than 46 years of age at disease onset, but which became weaker and ultimately disappeared among women with progressively older age at onset. This finding supports the hypothesis that familial breast cancer is a genetically heterogeneous disease, and that the early-onset pattern represents a major subgroup defined by a susceptibility gene on chromosome 17. Future research will concentrate on identifying the putative breast cancer gene.

Familial melanoma and the dysplastic nevus syndrome (DNS) continued to receive attention. An updated analysis revealed relative risks of 500 for subsequent melanoma in those family members with a prior melanoma. Melanoma risks for those members with DNS, but not melanoma, was 100-fold higher than the general population, and the actuarial estimate of cumulative lifetime risk of melanoma in DNS patients approached 100 percent. A case-control study of dysplastic nevi found that most affected patients also had first degree relatives with dysplastic nevi.

Follow-up studies have continued of a family with 10 cases of renal cancer and a translocation between chromosomes 3 and 8. Studies have shown chromosome 3p rearrangements or deletions in nearly all renal cancer tissues of non-familial cases, suggesting its importance to the development of this neoplasm. In the past year, all five surviving cases of renal carcinoma in the t(3;8) family developed recurrent disease. Chromosome and molecular genetics analyses of fresh tumor tissue unexpectedly showed that the derivative 8 chromosome, with the rearranged distal 3p, had been deleted. Work is in progress to identify the critical gene(s) involved in the origins of hereditary renal cancer.

Radiation

Populations exposed to ionizing radiation are studied to clarify cancer risks associated with low-dose exposures. Diagnostic x-rays were not causally related to adult leukemia or lymphoma in a study of members of prepaid health plans. However, there was some increase in risk for multiple myeloma with increasing number of x-rays. In a Swedish study, prenatal x-rays were

associated with an increased risk of childhood cancer among twins. However, cohort studies of leukemia risk in twins born in Connecticut and Sweden did not reveal an excess, despite the greater exposure to prenatal x-rays of twins than single-born children.

High doses of iodine-131 to treat hyperthyroidism or thyroid cancer were not convincingly linked to increased risks of leukemia, indicating that the carcinogenic potential of this exposure is less than x-rays or gamma rays. Stomach cancer was the only malignancy for which there was a suggestion of dose-related risk.

New risk models, taking into account population mobility and occupancy, suggest that indoor radon may be less of a public health hazard than previously estimated. No association with levels of indoor radon was found for lung cancer in a case-control study of women in China, and a modest association seen among Swedish women disappeared when adjusted for occupancy. These results suggest that models for extrapolating risks from high exposures, based on miner data, to low-level exposures should be viewed cautiously. An ongoing study in Missouri, which includes innovative approaches to dosimetry, should provide new insights on levels of risk from residential radon.

In a study of populations living near nuclear facilities in the United States, excesses were not found for childhood leukemia or other cancers, in contrast to findings from the United Kingdom. Risks were slightly higher before the facilities began operations than afterward, providing little evidence that environmental releases of radiation influenced mortality rates in these areas.

Medications

The Program continued to evaluate the carcinogenic effects of hormones, cytotoxic drugs, and other medicinal compounds. Analyses of cancer incidence in a large cohort of Swedish women treated with non-contraceptive estrogens revealed a significantly increased risk of endometrial cancer, a slightly decreased risk of cervical cancer, and no increase in risk of cancers of the ovary, pancreas, large bowel, or kidney. The risk of liver and biliary tract cancers was significantly lower than expected, while the risk of breast cancer was slightly elevated. Breast cancer risk increased with duration of use, reaching 1.7 after nine years. Unlike the effect on endometrial cancer risk, the excess risk of breast cancer was not decreased by the addition of progestin to the regimen.

In a prospective study of a cohort of nurses, investigators at Harvard University found that past users of replacement estrogen were not at increased risk (RR=0.98) of breast cancer, including those with more than 10 years of use (RR=0.70). However, the risk of breast cancer was significantly elevated among current users (RR=1.36). A stronger relationship was observed with increasing age but not with increasing duration of use. Among women who did not consume alcohol, breast cancer risk was not increased by current use of postmenopausal hormones. Among alcohol consumers, however, current hormone users were at increased risk of breast cancer (RR=1.56).

Investigations continued of the health risks related to various methods of contraception. Users of barrier methods were found to be at a reduced risk of invasive cervical cancer, presumably because of concomitant use of spermicides

with anti-viral activity, in a U.S. case-control study. In a study in Latin America, the use of injectable contraceptives was associated with an increased risk of cervical cancer. Because of reports linking the extended use of oral contraceptives to increased risk of breast cancer, a multicenter case-control study, focusing on premenopausal women, is underway in three U.S. regions.

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, on the molecular biology, natural history, and transmission of HIV, and on the development of a vaccine for AIDS. 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 have been carried out under interagency agreements with the U.S. Environmental Protection Agency (EPA) and National Institute for Occupational Safety and Health (NIOSH). 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 17 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.

Staff in the Office of the Director 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 interest 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 Registry of Experimental Cancers is directed and maintained by staff of the Office of the Director. 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 13,383 (725 since last year) single or group accessions from investigators outside the NCI, and approximately 76,668 records have been coded. Forty-eight investigators have come to the Registry for study and consultation on single or multiple visits. The Director General of the World Health Organization (WHO) designated the Registry of Experimental Cancers as the WHO Collaborating Center for Reference on Tumors of Laboratory

Animals on October 26, 1976, and the Pan American Health Organization (PAHO) renewed this collaboration on July 19, 1983. This is the only such repository in the world to be so designated by the WHO. The Registry facilitates communication between U.S. scientists and those of other countries, now numbering 153, which are members of WHO.

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. The NCI is the most frequent source of nominations to NTP's bioassay program. Another information dissemination activity involves preparation, under contract, of the "Survey of Compounds Which Have Been Tested for Carcinogenic Activity." This document has been published, under contract, by NCI since 1951. During this reporting period, the sixteenth volume, covering the years 1989-1990, was completed. After clearance and printing by the Government Printing Office, it will be distributed to over 600 users worldwide. In order to compile this latest volume, which includes information on 676 chemicals and 686 citations, an average of 625 journals as well as all pertinent computerized data bases, both national and international, were screened. This project was recompeted and an award was made for an additional four years, which will continue the "Survey of Compounds Which Have Been Tested for Carcinogenic Activity" for the years 1991-1994.

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 Carcinogenic Risks 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 50 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 lifestyle 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. 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 14 surveys have been published and the fifteenth 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, DC. 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 (CDC): Studies on the Human Health Consequences of Polybrominated Biphenyl (PBB) Contamination of Farms in Michigan

The cohort organized to monitor the effects of the 1973 animal feed error remains relatively stable at 4,000 individuals. Enrollment of newborn and other individuals continues to balance the <1% annual loss due to deaths and "dropouts." A major activity that began this year was the development of a 14-page questionnaire that will permit the health recharacterization of the cohort. The original enrollment questionnaire of 1976-1977 has been updated to permit the collection of a 15-year experience as to lifestyle, health history and life experiences. At the request of CDC, eight questions were added to the questionnaire concerning potential exposure to additives in interior paints. Each questionnaire will be filled out in a personal interview. At the same time, blood samples will be drawn from any willing donors. These samples will be archived until funds are available for an analytical work-up and their PBB, PCB and pesticide levels can then be compared to previous samples. To date, some 32 scientific manuscripts have been attributed directly or indirectly to the cohort study and reflects the degree of collaborative investigations.

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

No studies were funded during this reporting period under this interagency agreement. However, dialog between NCI and EPA staff continued to identify projects of interest to both agencies and suitable for support under this mechanism.

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

The first year of a new four-year contract began on September 30, 1990. The incumbent was awarded both assay tasks through an intensive competitive process. As described above, the contract supports the NCI chemical nomination process to the National Toxicology Program, investigates compounds of interest to this Division and sister Agencies and supplies data to the Chemical Carcinogenesis Research Information System (CCRIS) data base of the National Library of Medicine's TOXNET. It is anticipated that in the next fiscal year, several antineoplastic and anti-AIDS compounds will be tested for another NCI Division. A manuscript on five acrylates has been accepted for publication and three other manuscripts are in various degrees of completeness.

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

Efforts continued this year on six projects. In February, the Division's Board of Scientific Counselors approved four "feasibility" projects. One of these, "The Effects of Diesel Fumes on Underground Miners," goes well beyond the rather simple approach of the usual feasibility study (it includes industrial hygiene sampling) and requires matching funds of $85,000 from NIOSH. This "feasibility" project has been so successful that it will undergo extensive evaluation to permit the development of the epidemiological leads it has uncovered to date. The "Industry and Occupational Coding of Death Certificates" project, to which 40% of the interagency agreement funding has been allocated, is under review as to structure, scope and alternate funding mechanisms. The Acrylonitrile study appears not to need additional funding in FY 92 at this point and the "Biological Markers of Occupational Bladder Cancers" project is progressing at an accelerated pace. The joint staff meeting, held in late spring of each year, continues to fulfill its objectives of assessing progress on each ongoing project and providing a consensus for future directions.

Technical Resources, Inc.: A 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 to the International Agency for Research on Cancer (IARC)

During this reporting period, contractor staff prepared for and attended three Chemical Selection Working Group (CSWG) meetings and six Chemical Selection Planning Group (CSPG) meetings. Summary sheets on 18 candidate chemicals for nomination for carcinogenicity testing by the National Toxicology Program were prepared for consideration by the CSWG members. In addition, information sheets on 20 compounds were prepared for presentation to the CSPG members for evaluation as potential candidates for summary sheet development and further evaluation by the CSWG. The National Cancer Institute continues to be a primary source for nomination of candidate chemicals to the National Toxicology Program. The contractor completed class studies on adhesives and cyanoacrylates. Information was gathered on dental materials, household pesticides, flavorings and spices, arts and crafts materials, and fluorinated aromatics, for evaluation as possible candidates for future class studies. Contractor staff also provided support for two working group meetings of the

International Agency for Research on Cancer (IARC) by providing information for Sections 1 and 2 (Chemical and Physical Data and Production, Use and Occurrence) for a total of 24 monographs. A contractor representative attended the IARC meeting on the evaluation of Occupational Exposures in Insecticide Application and Some Pesticides and chaired the Chemistry Subgroup of that meeting. The Chemical Carcinogenesis Research Information System (CCRIS) data base, which is part of the National Library of Medicine's TOXNET System, was maintained and updated. During this reporting period, 50 new chemicals were added to the carcinogenicity file, which now contains entries on over 850 unique chemicals; 200 were added to the mutagenicity file for a total of more than 2000 chemicals; the tumor promotor file, with the addition of 20 new entries, now contains 120 chemicals and the tumor inhibitor file, with the addition of 30 chemicals, now has a total of 70 chemicals. The NCI/NTP Bioassay Report Summary Handbook was updated by the addition of 20 new summaries. This contract was recompeted and renewed for an additional five years.

Chemical Carcinogenesis in Nonhuman Primates

Staff of the Office of the Director direct a large project on chemical carcinogenesis in nonhuman primates. During the 30 years of this ongoing project, the carcinogenic potential of 44 compounds has been tested. These include the antineoplastic and immunosuppressive agents; food additives, food components and environmental contaminants; classical rodent carcinogens; nitroso compounds; and viruses. In 1985 a heterocyclic amine found in cooked meat, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was introduced in the monkey colony. In a group of 20 animals receiving 10 mg/kg of IQ, three are dead with hepatocellular carcinoma and two are alive with tumor. In another group of 20 animals receiving 20 mg/kg of IQ, 13 are dead with hepatocellular carcinoma and two are alive with tumor. Dosing of two other heterocyclic amines, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was started in 1987 and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in 1990. So far, there is no evidence of tumor development in either group. DNA adducts have been measured for both IQ and PhIP in peripheral blood cells and various organs, particularly in the liver and heart. Ongoing studies are focusing on metabolic activation of IQ and MeIQx in monkeys and the high cardiac adduct levels of IQ and PhIP. The possibility exists that they may cause myocardial damage unrelated to neoplasia. Several collaborative studies have been initiated this year which utilize monkey tissues from different treatment groups. These include: 1) ultrastructural and histochemical studies of myocardium from monkeys dosed with IQ, 2) histopathological study of adriamycin-induced myocardial degeneration, 3) evaluation of p53 suppressor gene mutations in PCR amplified DNA from aflatoxin-induced hepatocellular carcinomas, and 4) histopathological and histochemical studies of DENA induced liver tumors and precursor lesions.

OFFICE OF THE DIRECTOR

CONTRACTS ACTIVE DURING FY 91

Institution/Principal Investigator/ Contract Number	Title
Centers for Disease Control Rebecca Schilling Y02-CP-00550Biphenyls (PBBs)	Studies on the Human Health Consequences of Polybrominated Contamination of Farms in Michigan
Environmental Protection Agency (EPA) W. Farland and F. Ulvedal Y01-CP-80205	Performance of Collaborative Studies in the Area of Environmental Cancer
Microbiological Associates Inc. John Harbell/Anita Bigger Richard San N01-CP-05620	In Vitro Evaluation of Chemical Candidates for In Vivo Testing - Mouse Lymphoma Assay and Salmonella Typhimurium Assay
National Institute for Occupational Safety and Health (NIOSH) Roy Fleming Y01-CP-60505	Conduct of Research on Occupational Carcinogenesis
Technical Resources, Inc. Beverly Campbell N01-CP-05648	Survey of Compounds Which Have Been Tested for Carcinogenic Activity
Technical Resources, Inc. Andy Chen N01-CP-05619	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 International Agency for Research on Cancer
Smithsonian Institution John Harshbarger N01-CP-51031	Operation of a Registry of Tumors in Lower Animals
Hazelton Laboratories America, Inc. Dan W. Dalgard N01-CP-51013	Induction, Biological Markers and Therapy of Tumors in Lower Animals

GRANTS ACTIVE DURING FY 91

International Agency for Research on Cancer Harri Vainio 5-U01-33193-06	IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP03509-28 OD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	R. H. Adamson	Director	OD, DCE	NCI
	R. H. Corbitt	Biological Lab. Tech.	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Division of Cancer Etiology

SECTION

Office of the Director

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	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.)*

During the thirty years of the ongoing monkey carcinogenesis project, the carcinogenic potential of forty-four compounds has been tested. These include antineoplastic and immunosuppressive agents; food additives, food components and environmental contaminants; classical rodent carcinogens; nitroso-compounds; and viruses. In 1985 a heterocyclic amine found in cooked meat, 2-amino-3-methyl-3H-imidazo-[4,5-f]quinoline (IQ) was introduced in the monkey colony. In a group of twenty animals receiving 10 mg/kg of IQ, three are dead with hepatocellular carcinoma and two are alive with tumor. In another group of twenty animals receiving 20 mg/kg of IQ, thirteen are dead with hepatocellular carcinoma and two are alive with tumor. Dosing of two other heterocyclic amines, 2-amino-3,8-dimethyl[4,5-f]-quinoxaline (MeIQx) was started in 1987 and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in 1990. So far, there is no evidence of tumor development in either group. DNA adducts have been measured for both IQ and PhIP in peripheral blood cells and various organs, particularly in liver and heart. Ongoing studies are focusing on metabolic activation of IQ and MeIQx in monkeys and the high cardiac adduct levels of IQ and PhIP. The possibility exists that they may cause myocardial damage unrelated to neoplasia. Several collaborative studies have been initiated this year which utilize monkey tissues from different treatment groups. These include: 1) ultrastructural and histochemical studies of myocardium from monkeys dosed with IQ; 2) histopathological study of adriamycin induced myocardial degeneration; 3) evaluation of p53 suppressor gene mutations in PCR amplified DNA from aflatoxin-induced hepatocellular carcinomas; and 4) histopathological and histochemical studies of DENA induced liver tumors and precursor lesions.

Project Description

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

U. P. Thorgeirsson	Expert	OD, DCE	NCI
R. H. Adamson	Director	OD, DCE	NCI
R. H. Corbitt	Biological Lab. Tech.	OD, DCE	NCI

Objectives:

At present the major objectives of the project are as follows:

1. To evaluate in nonhuman primates the carcinogenic potential of heterocyclic amines found in cooked meat. The three heterocyclic amines (IQ, MeIQx, and PhIP) that are under study in the monkey colony have been shown to be highly mutagenic in the Ames test and carcinogenic in rodents. Due to the biological similarities to humans, the nonhuman primate represents an important model to assess the carcinogenic risk of these widely consumed cooked food components. The measurements of high cardiac DNA adduct levels in the IQ and PhIP treated monkeys led to a hypothesis that these compounds may lead to myocardial damage unrelated to neoplasia.

2. To utilize the histological material that has been collected since the beginning of this 30 year old chemical carcinogenesis project. The following collaborative projects are ongoing: 1) ultrastructural and histochemical studies of myocardium from autopsied IQ treated monkeys with hepatocellular carcinomas; 2) histopathological study on adriamycin-induced myocardial degeneration; 3) diethylnitrosamine-induced liver tumors and precursor lesions; 4) evaluation of p53 suppressor gene mutations in PCR amplified DNA extracted from histological sections of aflatoxin B_1-induced liver tumors; and 5) incidence and histopathological study of hyalinized islets of Langerhans in the monkey colony. So far, 31 cases have been found in the test animals. Incidence is not related to a specific compound, age, sex, or species.

Methods Employed:

The present colony, which consists of 446 animals (May 1, 1991) is comprised of four species: Macaca mulatta (rhesus), Macaca fascicularis cynomolgus), Cercopithecus aethiops (African green) and Galago crassicaudatus (bushbabies). Thirty-nine of the monkeys are adult breeders which supply infants (17 per year), who are reared by their mothers and weaned at approximately 4 months of age. The majority of the monkeys are housed in an isolated facility which contains only animals committed to this study.

The administration of test compounds is continued until a tumor is diagnosed, or until a predetermined exposure period is completed. The compounds are administered subcutaneously, intravenously, intraperitoneally, or orally. For oral administration to newborn monkeys, the compound is added to the Similac formula at the time of feeding. When the monkeys are 6 months old, compounds given orally are incorporated into a vitamin mixture which is given on a slice of bread. An alternate way of giving compounds orally is to incorporate them into baited foods or administer by intubation. The antineoplastic and immunosuppressive agents are administered at doses likely to be given in a clinical situation. Environmental contaminants are given at levels 10- to 40-fold higher than the estimated human exposure level. The remainder of the test chemicals are administered at maximally tolerated doses, judged by clinical observations, weight, blood chemistry, and hematology.

To detect early evidence of tumorigenesis, serum samples are obtained every three months from the monkeys receiving heterocyclic amines for measurements of alpha-fetoprotein.

The substances that are currently, or have been, under investigation include antineoplastic and immunosuppressive agents (procarbazine, adriamycin, 1-methylnitrosourea (MNU), melphalan, azathioprine and cyclophosphamide), food additives (cyclamate, saccharin, butter yellow); food components (aflatoxin B_1, cycad, sterigmatocystin, IQ, MeIQx, PhIP); environmental contaminants (DDT, arsenic, cigarette tobacco smoke condensate); "classical" rodent carcinogens (urethane, 3-methylcholanthrene, 2-acetylaminofluorene (2-AAF), copper chelate of N-OH-AAF (dibenzpyrene and dibenzanthracene); nitroso-compounds (dimelthylnitrosamine, diethylnitrosamine, dipropylnitrosamine, 1-nitrosopiperidine, N-methyl-N'-nitro-N-nitrosoguanidine).

To monitor hematological and clinical parameters, blood samples are collected and physical examination done on all of the monkeys in the colony at least every six months.

Annual survey of 10% of the monkey colony is made for changes in antibody levels to various viral agents, such as herpes B, SIV, and measles. Tuberculin testing is done quarterly on the entire colony.

Surgical procedures are performed as required. They include caesarean section, laparoscopy, endoscopy and biopsy.

Necropsies are performed on all the dead animals. Fresh tissues are collected from tumors and different organs, and the remainder of the organs are fixed in formalin for histopathological studies.

Major Findings:

1. After 5-6 years of dosing with IQ, 50% of the monkeys have developed hepatocellular carcinoma. In a group of 20 animals receiving 10 mg/kg, 3 are dead and two alive with tumor. In another group of 20 animals receiving 20 mg/kg, 13 are dead and two are alive with tumor.

2. Preliminary data from ultrastructural studies on myocardium from the autopsied IQ monkeys showed some evidence of focal myocardial degeneration. Ongoing collaborative studies with cardiac pathologists at the Armed Forces Institute of Pathology are focused on looking further into the possibility of IQ-induced myocardial damage.

3. Retrospective histopathological study of pancreatic lesions found in test animals of the monkey colony has revealed thirty-one cases of completely or partially hyalinized islets of Langerhans. These lesions are not related to specific compounds, age, or species.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP04548-19 OD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Registry of Experimental Cancers/WHO Collab. Ctr. for Tumors of Lab. Animals

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

PI:	Harold L. Stewart	Scientist Emeritus	OD	DCE	NCI
Others:	Umberto Saffiotti	Acting Head	OD	DCE	NCI
	Annabel G. Liebelt	Expert	OD	DCE	NCI
	Gwendolyn Green	Program Assistant	OD	DCE	NCI

COOPERATING UNITS *(if any)*
Data Management Branch, DCRT, NIH- Ms. Aileen Kelly
Department of Laboratory Services, Baltimore V.A. Medical Center-Drs. J.J. Berman and G.W. Moore

LAB/BRANCH
Office of the Director

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.1	2.1	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 objectives of the Registry of Experimental Cancers are the storage and retrieval of pathological 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's designation as The World Health Organization Collaborating Centre for Reference on Tumours of Laboratory Animals was renewed for four years. During the current year the Registry has acquired 725 single or group accessions reaching a total of 13,383 (an accession usually corresponds to an animal autopsy). The number of diagnostic records added this year was 2606, bringing the total of records coded since the establishment of the Registry to 76,668 (a record usually corresponds to a diagnosis). During this year 48 investigators have come to the Registry for study and consultation on a single or multiple visits. A total of 191 study sets were loaned to investigators throughout the world. An extensive review on mouse pituitary tumors was completed by Dr. A.G. Liebelt, with inclusion of new histological materials and new documentation (photographs, tables). This review was used to update the relevant chapter in "Pathology of Tumours in Laboratory Animals, Vol. II-Tumours in the Mouse," to be published by the International Agency for Research on Cancer. A new activity was undertaken to provide search and retrieval capabilities to the Registry's computer database. In collaboration with the Division of Computer Research and Technology, NIH, and with the Department of Laboratory Services, Baltimore Veterans Administration Hospital, a pilot computer program was developed using a sample of 1000 entries from the Registry's database. Rapid and effective retrieval and indexing capabilities were obtained. The program is currently being further refined and extended.

PROJECT DESCRIPTION

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

Harold L. Stewart	Scientist Emeritus	OD	DCE	NCI
Umberto Saffiotti	Acting Head	OD	DCE	NCI
Annabel G. Liebelt	Expert	OD	DCE	NCI
Gwen Green	Program Assistant	OD	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 photo-micrographs 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. Data on experimental animals (species, strain, sex, age and treatment), tissues and histopathological diagnoses, and relevant comments are entered into a computerized database, on the WYLBUR system, maintained at the Division of Computer Research and Technology, NIH. The Registry accesses material from investigators at NCI, other institutes of NIH, other Governmental agencies, industrial laboratories, and universities in the U.S. and abroad.

The Registry prepares study sets consisting of histologic slides (up to 200 per set) with accompanying syllabus materials. The study sets are developed on cancers of particular organ/systems or particular rodents. The study sets available are: 1) Comparative Pathology of Hematopoietic and Lymphoreticular neoplasms; 2) Induced and Spontaneous Tumors of Small Intestine and Peritoneum in Mice; 3) Neoplastic and Nonneoplastic Lesions of the Lymphoreticular Tissue in Mice; 4) Induced Tumors of the Liver in Rats; 5) Tumors and Nonneoplastic Proliferative Lesions of the Lungs of Mice; 6) Mammary Tumors in Mice; 7) Pulmonary Metastases in Mice; 8) Neoplasms and Other Lesions of Praomys (Mastomys) Natalensis; 9) Malignant Schwannomas of Rats; 10) Tumors of the Harderian Gland of Mice; 11) Induced Tumors of Kidney of Rats; 12) Spontaneous Gastric Adenomatosis, Polyps and Diverticula; Duodenal Plaques of Mice; 13) Tumors of Adrenal Gland of the Rat; 14) Malignant Schwannomas of Mice; 15) Comparative Pathology of Malignant Tumors of the Soft Tissues and a Few Miscellaneous Tumors of Several Animal Species; and 16) Liver Tumors in Mice.

Major Activities:

Since 1976, the Registry of Experimental Cancers has been designated by The Director General of the World Health Organization (WHO) as the WHO Collaborating Centre for Reference on Tumours of Laboratory Animals. This is the only such

registry in the world to be so designated by the WHO. This designation was renewed in December 1990 for a further period of four years, with the concurrence of the Assistant Secretary for Health, DHHS. The Registry will expand communications between U. S. scientists and those of other countries, now numbering 153, which are members of the WHO.

A total of 725 new single or group accessions have been processed during the current year bringing the total number of accessions to 13,383. The number of diagnostic entry records added this year was 2606, bringing the total number of records in the Registry to 76,668. Of these 725 new accessions, that is autopsy cases, Dr. W. Banfield selected 232 cases (approximately 696 entry records) from 5100 slides and Dr. J.A. Lumadue selected 98 cases (approximately 490 entry records).

Between April 6, 1990 and May 7, 1991, 191 study sets were sent to scientists in the United States and abroad. In addition study sets Nos. 1-16 of histologic slides with accompanying syllabi were sent to be part of the auto-tutorial program at the 41st Annual Meeting of the American Association for Laboratory Animal Science in Milwaukee, WI from October 14-18, 1990.

This year there have been single or multiple visits by 48 investigators who came to the Registry for study and consultation. There have been numerous other consultations through correspondence and telephone calls.

During this year the pathology of mouse pituitary tumors was extensively reviewed by Dr. Annabel G. Liebelt, who prepared the revision of the chapter "Pituitary Tumours in the Mouse" for the second edition of the IARC Scientific Publication "Pathology of Tumours in Laboratory Animals, Volume II - Tumours in the Mouse." Many of the histological diagnoses and new photographs were obtained from materials either already in the Registry collection or from materials entered by Dr. Liebelt into the Registry this year. The classification and characterization of spontaneous and induced adenomas and carcinomas of the pituitary gland in mice as well as other laboratory animals has been reviewed. Likewise comprehensive tables and references have been developed, including recent data on mouse pituitary from studies using immunological and ultrastructural techniques.

A new activity was undertaken to add versatile and effective retrieval and search capabilities to the computer program that stores the Registry database. With the collaboration of Ms. Aileen Kelly, Data Management Branch, DCRT, NIH, and of Drs. J.J. Berman and G.W. Moore, Department of Laboratory Services, Baltimore Veteran's Administration (V.A.) Medical Center, a pilot program was developed using a sample of 1000 entries from the database, which were copied at DCRT on a diskette for use on personal computers. The HYPAD program, used for pathology files at the V.A., was adapted for data search and retrieval in Registry files. Coded entries were used to replace lengthy fields with a few bytes in the computer memory. Rapid retrieval and indexing was obtained by all search parameters (e.g., animal species, strain, tissue, diagnosis, autopsy number). This program is being further refined for application to the entire database. It is expected to provide easy access to the Registry files through personal computers. It will greatly accelerate retrieval of pathology information and will also provide quality control on the accuracy of the entries in the database.

Publications:

Liebelt AG. Tumours of the pituitary gland. In: Turusov VS, ed. Pathology of tumours in laboratory animals. vol. II. tumours of the mouse. 2nd ed. Lyon, International Agency for Research on Cancer (In Press)

Sass B, Banfield WG, Saffiotti U. Detection and cellular localization of lead by electron probe analysis in the diagnosis of suspected lead poisoning in Rhesus monkeys. Toxicol Pathol 1990;19:30-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05576-04 OD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression of ras and Collagenase in Primary Tumors vs. Metastases

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

PI:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	A. R. Mackay	Visiting Associate	OD, DCE	NCI
	J. L. Hartzler	Biologist	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	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.)*

This project has been completed. It originated from a previous observation of a simultaneous induction of the metastatic phenotype and increased type IV collagenolytic activity in ras transfected NIH/3T3 cells. Here a N-nitrosomethylurea induced rat mammary carcinoma model was used to study the relationship between ras levels, metalloproteinase expression and metastatic behavior. When comparing normal and malignant rat breast tissues, there was no direct relationship between ras DNA levels and neoplastic development, or between metastatic and nonmetastatic rat mammary carcinomas. Two major gelatinolytic metalloproteinases (gelatinases) of 65 and 92kDa were present in the carcinomas, but only the 65kDa gelatinase was detected in normal breast tissues and a rat fibroma. Type IV collagenolytic activity in tissue lysates from the carcinomas was two to three times higher than that of normal breasts, but primary tumors did not differ from their corresponding metastases.

In this carcinogenically induced metastatic rat mammary carcinoma model, we have demonstrated that ras amplification is not necessary for the development of the malignant or metastatic phenotype. Furthermore, we have discovered through a glucocorticoid promoter mediated induction of the p21 ras protein that increased ras expression does not lead to an increase in metastatic capacity.

Project Description

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

U. P. Thorgeirsson	Expert	OD, DCE	NCI
A. R. Mackay	Visiting Associate	OD, DCE	NCI
J. R. Hartzler	Biologist	OD, DCE	NCI

Objectives:

1. To study the relationship between ras expression and type IV collagenolytic activity in vivo in an autochthonous metastatic tumor model.

2. To see if ras levels correspond with the degree of malignancy in a rat mammary carcinoma model.

3. To quantitate type IV collagenolytic activity in normal and malignant breast tissue lysates and correlate with the expression of two gelatinases which have been proposed to be type IV collagenolytic.

4. To study what effect stimulation of ras expression has on metastatic propensity.

Methods Employed:

1. The mammary tumors (usually several per animal) were induced through a single injection of NMU (30 μg/g body weight) into Sprague Dawley or Buffalo rats at 50 days. Tumors were observed in about 90% of the rats within 3-6 months. In the majority of the rats the mammary tumors did not produce metastases during the observation period of 9-12 months. Several metastatic NMU-induced tumors were obtained. Three of these tumors persistently produced the same patterns of metastases when transplanted into syngeneic rats. Comparison of individual metastases with a parent primary mammary tumor was accomplished through resection of ten separate lung metastases and a primary tumor from a rat treated with NMU 9 months earlier. The tumors were then transplanted subcutaneously into nude mice for expansion and nucleic acid isolation.

2. DNA was extracted from the NMU-induced tumors and normal rat mammary glands. For detection of H-ras-specific sequences, DNA was digested with Bam-H1 or Hind III, electrophoresed through 0.8% agarose gels and transferred to nitrocellulose filters by the Southern blotting technique. The filters were hybridized with 10^6 cpm/ml of nick-translated ^{32}P-labelled c-H-ras specific probe. The blot hybridization mixture contained 3 x SSC, 0.05 M Tris (pH 7.5), 5 x Denhardt's solution, and 1 mM EDTA and 50% formamide. The blots hybridized with the ras probe were washed three times in 2 x SSC, 0.1% SDS at room temperature and three times in 0.1 x SSC, 0.1% SDS at 60°C. The blots were then dried and exposed to Kodak XR-5 film at -70°C for 1-2 days.

Slot blot analysis was performed on 10 μg, 1 μg or 0.1 μg of denatured DNA from the NMU-induced tumors which was passed through nitrocellulose filters and hybridized with a ^{32}P-labeled c-H-ras probe as described for Southern blot.

3. Assay for type IV collagenolytic activity in culture supernatants and tissue lysates. The 433 cells were washed three times with PBS, and incubated in serum-free medium which was collected after 48 hours and concentrated through ammonium sulfate (0-60%) precipitation, then dissolved and dialyzed against a collagenase buffer (50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 5 mM $CaCl_2$) overnight at 4°C. Tissue lysates were prepared by pulverizing the frozen tissues in a mortar and homogenizing in a lysate buffer composed of 0.05 M Tris-HCl (pH 7.4), 0.1 M $CaCl_2$ and 0.25% Triton X-100. The homogenates were centrifuged at 10,000 rpm for 10 minutes, and the supernatants dialyzed against the collagenase buffer. The tissue lysates were stored at -70°C until assayed.

   Biosynthetically [^{14}C] proline-labeled EHS type IV collagen was used as a substrate in the assay measuring collagen IV degrading activity. The samples were first incubated with trypsin (10 μg/ml) for 10 minutes at 37°C; then soybean trypsin inhibitor (50 μg/ml), N-ethylmaleimide (3.8 mM) and aprotinin (1000 kallikrein inhibiting units/ml) were added and the mixture incubated for 16 hours at 37°C. Parallel samples were assayed in the presence of 10 mM EDTA to ensure that only the metalloproteinase activity was tested. The reaction was terminated by adding trichloroacetic acid (0.6%) - tannic acid (0.03%) and placing the samples on ice for 30 minutes. The undigested substrate was removed by centrifugation and the radioactivity of the supernatants was measured in a β-scintillation counter. The enzyme activity was expressed either as cpm/10^7 cells or cpm/mg protein.

4. Gelatin zymograms. Metalloproteinase activity was assessed using SDS gels that contained gelatin substrate copolymerized with acrylamide. Preparation of the gel and the running buffer was the same as for regular SDS gels except for the addition of 0.1% gelatin to the 7.4% acrylamide separating gel. The electrophoresis was performed under nonreducing conditions at a constant current of 9 mA at 4°C. Samples of serum-free culture supernatants, containing equal amounts of protein (5 μg), were analyzed. Following electrophoresis the gels were washed three times in 50 mM tris-HCl (pH 7.4) containing 2% Triton X-100 for 30 min, and three times in 50 mM tris-HCl (pH 7.4) for 5 min. After rinsing, the gels were incubated in a buffer containing 50 mM tris-HCl (pH 7.4), 0.2 M NaCl, 5 mM $CaCl_2$, 0.01% Triton X-100, 0.02% NaN_3 at 37°C overnight, depending on the intensity of the gelatinolytic activity. The gelatin gels were stained and fixed for 1 hr shaking at room temperature using 0.1% amido black in a mixture of acetic acid:methanol:water (1:3:6) and then destained in the same mixture without amido black.

5. Attachment assay. The effect of dexamethasone treatment on attachment of the 433 cells to different substrates was studied in vitro. Tissue culture wells were coated with type I collagen, type IV collagen, laminin or fibronectin, each substrate 10 μg/well. The 433 cells with and without six days of treatment with dexamethasone (2×10^{-6}M) were labeled with Tran ^{35}S amino acid mixture (10 μCi/ml) for 40 hr, washed and 10^6 cells added to each well. At 15 min, 30 min, 1 hr, 2 hr, 3 hr, and 6 hr the medium was removed and the wells washed gently with PBS. The attached cells were lysed with 0.2 N NaCl, 10 mM tris-HCl (pH 8.0), 0.1 M EDTA (pH 7.8) and 1% SDS and the radioactivity measured in a scintillation counter.

Major Findings:

1. There was no correlation between ras DNA levels and the malignant or metastatic capacity of NMU-induced rat mammary carcinomas.

2. Dexamethasone induced ras expression did not result in increased metastatic behavior of 433 ras transfectant carrying a glucocorticoid promoter.

3. Tissue lysates of the malignant breast tumors possessed up to three times higher type IV collagenolytic activity than the normal breasts.

Publication:

Ballin M, Mackay AR, Hartzler JL, Nason A, Pelina P, Thorgeirsson UP. Ras levels and metalloproteinase activity in normal versus neoplastic rat mammary tissues. Clin Exp Metastasis 1991;9:179-89

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05608-03 OD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Purification and Further Characteristics of Tumor Cell Gelatinases

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

PI:	A. R. Mackay	Visiting Associate	OD, DCE	NCI
Others:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
	J. L. Hartzler	Biologist	OD, DCE	NCI
	M. D. Pelina	Pre-IRTA Fellow	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	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.)*

Type IV collagenolytic activity has been closely linked to the malignant phenotype. In this study two gelatinolytic metalloproteinases (gelatinases) of 65kDa and 92kDa which have been proposed to degrade native full-length type IV collagen were purified from a tumor cell line. Analysis of their substrate specificities was performed. Both enzymes degraded pepsinized type IV collagen and type V collagen under native conditions. However, native full-length type IV collagen was a poor substrate for both enzymes. This evidence, in combination with zymogram analysis, demonstrated that they had a similar substrate specificity for precleaved forms of type IV collagen, type V collagen, and denatured collagens. These data suggest that the 65 and 92kDa tumor cell gelatinases are not true type IV collagenases. cDNA clones for both enzymes have been isolated and characterized and are currently used to assess the distribution of gelatinases in tumors. In addition to the 65 and 92kDa gelatinases, cDNA clones have been isolated and characterized for two other metalloproteinase family members, i.e., type I collagenase and stromelysin. cDNA clones for two inhibitors of metalloproteinases, TIMP-1 and TIMP-2, have also been isolated. In situ hybridization studies of a variety of human tumors will be performed to evaluate the role of specific metalloproteinases in tumor invasion.

The effect of matrix components laminin, fibronectin, and type IV collagen on tumor cell gelatinase expression is currently under study. Laminin, laminin fragment E-8 and laminin synthetic peptides containing either the sequence I-K-V-A-V or Y-I-G-S-R did not influence 65kDa or 92kDa gelatinase activity. Fibronectin and EHS type IV collagen also did not influence tumor cell gelatinases. However, in the presence of pepsinized human placental type IV collagen 185kDa, gelatinolytic activity appeared in culture supernatants from tumor cells. This activity is presently being characterized.

Project Description

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

A. R. Mackay	Visiting Fellow	OD, DCE	NCI
U. P. Thorgeirsson	Expert	OD, DCE	NCI
J. L. Hartzler	Biological Lab. Worker	OD, DCE	NCI
M. D. Pelina	Pre-IRTA Fellow	OD, DCE	NCI

Objectives:

1. To further characterize the substrate specificities of two tumor cell gelatinases of 65kDa and 92kDa.

2. To obtain cDNA clones for four members of the metalloproteinase family (type I collagenase, 65kDa and 92kDa gelatinases, stromelysin) and their inhibitors (TIMP-1 and TIMP-2).

3. To assess the distribution of mRNA for these genes during tumor progression in vivo.

4. To assess the effects of matrix components on tumor cell gelatinase expression.

Methods Employed:

1. Cell cultures. Cell lines were grown in their respective media using antibiotics. Culture supernatants were collected following 48 hour incubation in serum-free conditions.

2. Gelatinase purification. Concentrated HL-60 supernatant was applied to gelatin-Sepharose affinity column equilibrated with 50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35 (pH 7.6). Gelatinases were eluted in the same buffer containing 5% dimethyl sulphoxide and 10% glycerol. The eluate was concentrated by ultrafiltration and dialyzed against the above buffer at 4°C overnight.

3. Degradation studies. Soluble type IV collagen or type V collagen substrates were incubated with purified enzymes in solution at temperatures ranging from 25 to 37°C for 16 h. Degradation products were analyzed by SDS-PAGE followed by silver staining.

4. Substrate gel electrophoresis. 7.5% SDS PAGE gels were prepared containing either gelatin (0.1%) or type IV collagen (0.04%) substrates. Electrophoresis was performed under non-reducing conditions. Enzyme activity was evaluated following incubation in collagenase buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, pH 7.6) and staining with Coomasie blue.

5. cDNA clones. Clones were obtained by either colony screening procedures or by polymerase chain reaction technology (PCR). The clones were obtained by using specific oligonucleotides corresponding to the published cDNA sequences for the two gelatinases, type I collagenase, stromelysin, TIMP-1 and TIMP-2. The clones were subcloned into the vector PGEM 3z.

6. Matrix studies. The matrix components laminin, type IV collagen, fibronectin, as well as a laminin fragment (E-8) and laminin synthetic peptides containing either the sequence I-K-V-A-V or Y-I-G-S-R were incubated in solution at a variety of concentrations with tumor cells in vitro. Supernatants were collected and tested for proteolytic activity in substrate zymograms.

Major Findings:

1. Matrix components laminin, laminin fragments, and fibronectin did not modulate tumor cell gelatinase expression when incubated with tumor cells in solution.

2. A 185kDa gelatinolytic band was detected in supernatants of tumor cell lines in the presence of pepsinized human placental and EHS type IV collagen, but was not seen in the presence of full-length EHS type IV collagen.

Publication:

Mackay AR, Hartzler JL, Pelina MD, Thorgeirsson UP. Studies on the ability of 65kDa and 92kDa tumor cell gelatinases to degrade type IV collagen. J Biol Chem 1990;265:21929-34.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05609-03 OD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gelatinase/Type IV Collagenase Response in Normal and Neoplastic Cells to TPA

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

PI:	A. R. Mackay	Visiting Associate	OD, DCE	NCI
Others:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
	M. D. Pelina	Pre-IRTA Fellow	OD, DCE	NCI
	J. L. Hartzler	Biologist	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.3	1.2

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 effect of the tumor promotor, 12-O-tetradeconoyl-phorbol-13 acetate (TPA) on the expression of 65kDa and 92kDa gelatinolytic metalloproteinases (gelatinases) was studied in a variety of normal and malignant cell lines. Enzyme activity was assessed by gelatin zymography. At mRNA levels the 65kDa gelatinase was expressed equally by normal fibroblasts and tumor cells and was minimally affected by TPA treatment. In contrast, the 92kDa gelatinase which was constitutively expressed only by tumor cell lines was stimulated to a varying degree by TPA in all tumor cell lines. TPA-mediated stimulation of the 92kDa gelatinase was abolished by inhibitors of protein kinase C (PKC), as well as by inhibitors of protein synthesis and RNA transcription. Analysis of transcription binding proteins containing c-fos capable of binding to type I collagenase and stromelysin AP-1 consensus sequences in lysates from TPA treated cells was undertaken. No relationship was observed between AP-1 levels and 65kDa gelatinase expression. However, AP-1 binding activity did follow a similar pattern to the expression of 92kDa gelatinase. Interstitial collagenase mRNA expression, known to contain an AP-1 site in a positive regulatory element, appeared to follow the same pattern of expression as 92kDa gelatinase following TPA treatment. Two physiologically relevant cytokines, thought to act via the PKC pathway, also induced cell line specific selective stimulation of 92kDa gelatinase activity. These effects were blocked by inhibitors of protein synthesis, RNA transcription and PKC. The project has been extended to evaluate the effects of TPA on the RNA expression of two inhibitors of metalloproteinases, TIMP-1 and TIMP-2.

Project Description

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

A. R. Mackay	Visiting Fellow	OD, DCE	NCI
U. P. Thorgeirsson	Expert	OD, DCE	NCI
M. D. Pelina	Pre-IRTA Fellow	OD, DCE	NCI
J. L. Hartzler	Biological Lab. Worker	OD, DCE	NCI

Objectives:

1. To investigate whether the two major gelatinases of 65 and 92kDa, expressed by tumor cells, are induced by the tumor promoter TPA.

2. To determine if the TPA-mediated gelatinase effect differs between normal and neoplastic cells.

3. To assess the levels of AP-1 binding jun/fos complexes in normal and tumor cells following TPA treatment and to relate this to the expression of the two gelatinases.

4. To examine the effect of two cytokines, IL-1 and TNFα, that act through the PKC pathway, on the 65 and 92kDa gelatinolytic activity.

5. To compare gelatinase and TIMP mRNA expression with gelatinolytic activity in different normal and neoplastic cell lines. If an alternate regulatory mechanism is suspected, it will be pursued further.

Methods Employed:

1. Cell cultures. Cell lines were grown in their respective recommended media containing antibiotics. Routinely, cells were grown on 150 mm petri dishes. Upon reaching subconfluency, cells were washed three times in PBS then incubated in the absence of serum with 1 x 10^{-8} M TPA (16 plates) or with medium alone (16 plates). Culture supernatants and cells were harvested separately at 1, 6, 12, 24 and 48 hours following TPA addition. Lysates were prepared from the cell pellets for a) enzyme activity, and b) jun/fos complex assay. Cell supernatants were used for enzyme activity.

2. Cell lysates. Cell pellets were rinsed in PBS three times. Pellets were frozen and thawed three times at -70°C. Cells were then resuspended in an equal volume of 50 mM Tris HCL 1% Triton X-100 (pH 7.5). Cells were sonicated for 5 seconds at level 4. Cell debris was removed and the lysates stored at -80°C.

3. Substrate gel electrophoresis. Preparation of gel and running buffer was the same as for regular SDS PAGE gels except for the addition of 0.1% gelatin or 0.4% type IV collagen to 7.5% acrylamide separating gels. Electrophoresis was carried out under nonreducing conditions.

Gels were washed in 2% Triton X-100 to displace SDS, rinsed with 50 mM Tris (pH 7.5) and incubated at 37°C for 16 hours in 0.05 M Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 1% TX100 (pH 7.4). Enzyme activity was visualized by negative staining after incubation of the gels with 1% amido black in acetic acid:methanol:water (1:3:6) and destained in the same mixture without amido black.

4. Gel retardation assays were performed using type I collagenase and stromelysin AP-1 binding site oligonucleotides for the determination of active jun/fos complexes. 4 μg of cell lysates were mixed with 0.4 ng of ^{32}P end-labelled oligonucleotide plus 3 μg of poly DIC to remove nonspecific protein binding. Samples were incubated for 20 minutes and run on 4% DNA acrylamide gels. DNA shifts were assessed following autoradiography.

Major Findings:

1. Adult and embryonic lung fibroblasts, endothelial cells, and tumor cells expressed 65kDa gelatinase activity to similar levels. This activity was unresponsive to TPA or cytokines IL-1 and TNFα.

2. 92kDa gelatinase activity was enhanced by TPA and cytokine treatment in tumor cell lines.

3. TPA mediated stimulatory effect on 92kDa gelatinase was also observed at the mRNA level.

4. TPA and cytokine mediated effects required RNA transcription, protein synthesis, and activation of PKC as determined by inhibitor studies.

5. Type 1 collagenase and TIMP-1 mRNA expression were also stimulated by TPA.

6. TIMP-2 mRNA expression was not affected by TPA.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05641-02 OD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Use of Nonhuman Primates to Study Progression of Hepatocellular Carcinoma

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

PI:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	S. S. Thorgeirsson	Chief	LEC	NCI
	R. H. Adamson	Director	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	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.)*

Diethylnitrosamine (DENA) has proven to be the most potent and persistent hepatocarcinogen in the monkey carcinogenesis project with a latency period of 1-2 years when administered intraperitoneally at a dose of 40 mg/kg. Here DENA is used as a model compound to induce hepatocellular carcinoma and follow progression of the carcinogenic process. Of seven monkeys that were placed on DENA in January 1990, two died within a few months as a result of massive liver necrosis due to DENA toxicity. The five remaining animals tolerated the DENA well. Laparoscopies performed at 1-2 month intervals revealed considerable variability in the hepatic response; some were affected by DENA earlier and more severely than others. Generally, the liver became pale and tannish in color within two months of dosing. The frequent needle biopsies induced scarring of the liver capsule and shrinking of the lobes involved in both test animals and the two controls that received only the vehicle, PBS. Hyperplastic nodules were observed in some of the animals within 6-8 months. After one year the livers of two of the monkeys appeared cirrhotic and entirely replaced with nodules of varying size. One of the two animals developed intestinal hemorrhage and severe lethargy and was sacrificed after 16 months of dosing with DENA. The entire liver of this animal was riddled with nodules with intervening fibrous bands. The nature of the liver nodules is being evaluated. The two control animals that are receiving only the vehicle for DENA, i.e. PBS, have not revealed any abnormalities except for fibrous reaction and shrinking of the liver lobes due to the frequent needle biopsies.

Project Description

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

U. P. Thorgeirsson	Expert	OD,	DCE	NCI
S. S. Thorgeirsson	Chief		LEC	NCI
R. H. Adamson	Director	OD,	DCE	NCI

Objectives:

The main objective is to use nonhuman primates to study molecular and cellular events during the carcinogenic and metastatic process. The relevance for using the nonhuman primate model is obvious, considering proximity to humans, as well as the possibility of following the malignant progression of the tumors in the same animal over an extended time period. It has been well established in the NCI nonhuman primate carcinogenesis program that DENA is a highly potent inducer of hepatocellular carcinoma. This study is mainly focused on the cellular target and the genetic changes induced by DENA. In the rodent model of chemical carcinogenesis it was recently revealed that a stem cell compartment which exists in the liver may play a significant role in the development of liver tumors. The DENA-induced liver tumors in the monkey represent an important model to assess the role of the stem cell compartment in nonhuman primates, which may more closely reflect human carcinogenesis than any other models available. With respect to DENA, the optimum carcinogenic dose and route for inducing metastatic hepatocellular carcinomas has been defined in this monkey colony. Considering that DENA is a highly predictable carcinogen (almost 100% tumor incidence) with a 1-2 year latency period in the monkeys, this model offers a unique opportunity to study various genetic and biochemical markers associated with malignancy and tumor progression. Due to the relatively large size of the monkeys, it is feasible to obtain sequential liver biopsies and blood samples which will allow close monitoring of the various parameters studied.

Methods Employed:

1. DENA is administered at 40 mg/kg to four cynomolgus monkeys, 3-4 years old, through intraperitoneal injections every 14 days. Two monkeys of the same age serve as controls receiving only PBS which is the vehicle for DENA. Dosing with DENA will be continued until the demise of the animal.

2. Laparoscopies and examination of the liver surface are performed every other month using a videocamera. Once hyperplastic nodules and tumors develop, their location is recorded by the videocamera and followed by subsequent laparoscopies. Hyperplastic nodules will be sampled and biopsies will be obtained from the same tumor nodule repeatedly. Liver needle biopsies obtained at laparoscopy are used for the following:

light and electron-microscopy; use of immunohistochemistry and in situ hybridization techniques on frozen tissue sections to locate and quantitate the expression of various tumor markers; isolation of DNA to search for gene amplification and oncogene activation, using the PCR technique.

3. Blood is collected monthly for the following: studying DNA adducts in white blood cells, using ^{32}P post-labeling technique; serum for measurements of AFP levels; serum for protein analysis by two dimensional gel electrophoresis; semiquantitative assessment of proteolytic activity (especially metalloproteinase and plasminogen activator) in serum, using substrate zymography.

4. Upon sacrificing and autopsying the monkeys, the primary tumors and metastatic nodules are dissected individually and studied for various parameters, especially for gene amplification and cytogenetic abnormalities.

Major Findings:

1. Two of seven monkeys have developed macronodular cirrhosis. The histology of the nodules is under evaluation in one of the monkeys that was sacrificed due to intestinal hemorrhage, but there was not gross evidence of invasive or metastatic liver malignancy.

2. Hyperplastic nodules were observed at laparoscopy after 6-8 months of dosing with DENA.

3. Two monkeys died within a few months from massive liver necrosis due to DENA-induced hepatotoxicity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05698-01 OD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Identification and Characterization of New Endothelial Proteinase Inhibitors

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

PI:	N. R. Chilukuri	Sr. Staff Fellow	OD, DCE	NCI
Others:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
	D. E. Gomez	Visiting Fellow	OD, DCE	NCI
	J. L. Hartzler	Biologist	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	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.)*

Proteinase inhibitors are of potential therapeutic importance as regulators of tumor cell mediated enzymatic degradation of the extracellular matrix. The classes of proteinases most commonly associated with tumor invasion are: metalloproteinases, plasminogen activators and cathepsins. Tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitors (PAI) have been shown to suppress tumor cell invasion in vitro and metastatic propensity in vivo. Recently, a trypsin-like enzyme has been purified from ovarian cancer and shown to activate other proteinases associated with malignancy, such as collagenases and plasminogen activators.

Endothelial cells are known to express high constitutive levels of TIMP and PAI in vitro. The present study is mainly focused on a search for new endothelial proteinase inhibitors and their possible role in blocking tumor dissemination. Conditioned medium from human umbilical vein endothelium was screened for trypsin inhibitors using reverse zymography technique. There is preliminary evidence for the expression of trypsin inhibitors by endothelial cells. Four trypsin resistant proteins of 15, 21, 31, and 40kDa were isolated from endothelial cell conditioned medium by trypsin affinity chromatography. Considering the possible role of a tumor derived trypsin-like enzyme(s) as activator(s) of the major matrix degrading proteinases, trypsin inhibitors expressed by endothelial cells may block their action and thus interfere with the proteolytic cascade involved in tumor cell invasion.

Project Description

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

N. R. Chilukuri	Sr. Staff Fellow	OD, DCE	NCI
U. P. Thorgeirsson	Expert	OD, DCE	NCI
D. E. Gomez	Visiting Fellow	OD, DCE	NCI
J. R. Hartzler	Biologist	OD, DCE	NCI

Objectives:

1. The primary objective of this project is to identify and characterize new proteinase inhibitors expressed by endothelial cells.

2. We have considered the hypothesis that reported TIMP-mediated antimetastatic effect may be due to a growth inhibitory effect of the inhibitor. The objective here is to find out if TIMP-1 and TIMP-2, as well as other, yet unidentified proteinase inhibitors, can act as growth inhibitors.

3. The final objective is to assess endothelial proteinase inhibitor expression in response to normal vs. malignant cells and low metastatic vs. high metastatic tumor cells.

Methods Employed:

1. Cell cultures. Human umbilical vein endothelial cells were used for the production of conditioned medium for identification of new proteinase inhibitors. Cells were grown to subconfluency in Ham's F12K medium containing 30% fetal calf serum, heparin and endothelial growth supplement. Prior to the collection of conditioned medium the endothelial cell monolayers were washed three times with PBS, incubated in Ham's F12K alone for 2 h to get rid of remaining traces of fetal calf serum, and finally in Ham's F12K alone which was collected as conditioned medium after 24 h.

2. Reverse zymography. Preparation of the gels and running buffer was the same as for regular SDS-PAGE, except for incorporation into the gel of one of the following substrates: 0.1% gelatin; 0.4% collagens, type I, III, or IV; 0.1% elastin, or 0.1% casein. Electrophoresis was carried out under nonreducing conditions. The gels were rinsed in 2% Triton X-100, then incubated with a substrate digesting enzyme solution in PBS at room temperature. Inhibitor activity was identified by positive staining of undigested bands following incubation with 1% amido black in acetic acid:methanol:water (1:3:6) and destaining in the same mixture without amido black.

3. Trypsin affinity chromatography. Endothelial cell secreted proteins were isolated by ammonium sulphate extraction procedure and subjected to trypsin affinity chromatography. The bound proteins to trypsin affinity matrix were eluted with 0.1 M glycine HCl (pH 2.5) buffer and

immediately adjusted to pH 7.4 with 1 M tris HCl, pH 8.8. The proteins were concentrated using YM10 centricon "microconcentrator" and analyzed for trypsin inhibitory activity by reverse zymography.

Major Findings:

Four proteins with trypsin inhibitory activity were isolated from endothelial conditioned medium, using trypsin affinity chromatography. They were identified by reverse gelatin zymography, which revealed trypsin resistant protein bands at molecular weight of 15, 21, 31, and 40kDa.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05699-01 OD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Plasmin System Basement Membrane Degradation and Tumor Invasion

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

PI:	A. R. Mackay	Visiting Associate	OD, DCE	NCI
Others:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
	R. H. Corbitt	Biological Lab. Technician	OD, DCE	NCI
	J. L. Hartzler	Biologist	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.3	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.)*

Current hypotheses suggest that both the plasmin system and metalloproteinases are involved in tumor invasion of basement membranes. Plasmin has been reported to degrade BM glycoproteins and activate collagenolytic metalloproteinases. Alternatively, it has been suggested that it is not necessary to postulate involvement of specific type IV collagenases in tumor cell-mediated degradation of type IV collagen. In this study, we demonstrated that plasmin can directly degrade native and denatured type IV collagen in solution and in tissue sections. Tumor cell lines secreted plasminogen activators into culture supernatants that activated exogenous plasminogen to degrade type IV collagen in zymograms and to remove type IV collagen immunoreactivity from tissue sections. Inhibition of metalloproteinase activity in culture supernatants by the addition of EDTA did not interfere with plasminogen mediated type IV collagen degradation. Thus, we propose that a metalloproteinase independent route for type IV collagen degradation exists, dependent on plasminogen conversion to plasmin.

We are currently evaluating whether this metalloproteinase independent collagenolytic activity facilitates tumor invasion of extracellular matrices in vitro.

Project Description

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

A. R. Mackay	Visiting Associate	OD, DCE	NCI
U. P. Thorgeirsson	Expert	OD, DCE	NCI
R. H. Corbitt	Biological Lab. Technician	OD, DCE	NCI
J. L. Hartzler	Biologist	OD, DCE	NCI

Objectives:

1. To investigate the role of the plasmin system in degradation of basement membrane type IV collagen.

2. To determine whether a metalloproteinase independent route for type IV collagen degradation exists.

3. To determine whether tumor cells utilize a metalloproteinase independent pathway for *in vitro* invasion of basement membranes.

Methods Employed:

1. Cell cultures. Cell lines were grown in their respective recommended media containing antibiotics. Serum free culture supernatants were harvested following 48 hour incubation of serum-free media with subconfluent cells.

2. Substrate gel electrophoresis. 7.5% SDS PAGE gels were prepared containing gelatin (0.1%), type IV collagen (0.04%), or plasminogen (15 μg/ml). Electrophoresis was performed under non-reducing conditions. Enzyme activity was observed following gel incubation for 16 hours at 37°C by staining with Coomassie blue.

3. Degradation of basement membrane type IV collagen *in situ*. 10 μm frozen sections of mouse hind leg musculature were placed on glass slides and incubated with enzyme preparations for varying times and conditions. Following incubation basement membrane type IV collagen in the tissue sections was localized by immunofluorescence using a monospecific polyclonal antibody to mouse type IV collagen.

4. Type IV collagen degradation in solution. Soluble type IV collagen was incubated with enzymes in solution for different times and under native or denaturing conditions. Following incubation samples were analyzed by SDS PAGE and degradation products analyzed by silver staining of the gels.

Major Findings:

1. Plasmin degraded native type IV collagen in solution to large molecular weight fragments.

2. Urokinase plasminogen activator plus plasminogen degraded type IV collagen in tissue sections under metalloproteinase inhibitory conditions.

3. Tumor cells elaborated plasminogen activators into culture supernatants, resulting in type IV collagen degradation in the presence of exogenous plasminogen in solution and in tissue sections. This degradation occurred under metalloproteinase inhibitory conditions.

Publication:

Mackay AR, Corbitt RH, Hartzler JL, Thorgeirsson UP. Basement membrane type IV collagen degradation: evidence for the involvement of a proteolytic cascade independent of metalloproteinases. Cancer Res 1990;50:5997-01.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05700-01 OD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Tumor-Endothelial Cell Interactions; In Vitro and In Vivo Models

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

PI:	U. P. Thorgeirsson	Expert	OD,	DCE	NCI
Others:	D. E. Gomez	Visiting Fellow	OD,	DCE	NCI
	H. C. Bisgaard	Visiting Fellow	LEC,	DCE	NCI
	A. R. Mackay	Visiting Associate	OD,	DCE	NCI
	J. L. Hartzler	Biologist	OD,	DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	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.)*

Cancer cells must interact with endothelial cells both when they enter and exit the vascular wall. The interaction with endothelial cells may determine whether or not the cancer cells can complete the metastatic process and seed in a distant organ. In this study we used a syngeneic rat model to study in vivo the initial stages of metastatic development which involves tumor cell arrest in the microvasculature and interaction with vascular endothelium. Rat liver epithelial (RLE) cells transformed with raf or raf/myc oncogenes produced spontaneous metastases in syngeneic animals. After introduction of the E. coli lacZ gene, the oncogene transformed rat cells could be visualized as single cells in different organs through histochemical staining for beta galactosidase. Time course experiments following intravenous injections of the raf/myc transfectant demonstrated that a large number of cells arrested in the lungs within minutes after the injection, but relatively few cells developed into microscopic and grossly visible metastases. Spontaneous micro metastases consisting of few cells could be visualized in the lungs six days following subcutaneous injection.

For in vitro studies of tumor-endothelial cell interactions, we have established a new approach to isolated pure primary cultures of rat endothelial cells. This involves a magnetic immunobead isolation technique using dynal beads coated with endothelial specific antibodies. So far, pure cultures of sinusoidal liver endothelial cells were isolated using OX-43 monoclonal endothelial antibody coated beads. Studies on cocultures of the rat tumor cells and endothelial cells have revealed modulation of metalloproteinase activity of tumor cells by endothelial cells and vice versa.

Project Description

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

U. P. Thorgeirsson	Expert	OD,	DCE	NCI
D. E. Gomez	Visiting Fellow	OD,	DCE	NCI
H. C. Bisgaard	Visiting Fellow	LEC,	DCE	NCI
A. R. Mackay	Visiting Associate	OD,	DCE	NCI
J. L. Hartzler	Biologist	OD,	DCE	NCI

Objectives:

Relatively little is known about the role endothelial cells play in either facilitating or preventing spread of cancer. Our first objective was to establish an animal model which would allow us to study in vivo the initial stages of the metastatic process. The second objective was to establish a new technique for isolation of pure primary cultures of endothelial cells from the same animal model for in vitro studies. Thirdly, since proteinases may play a critical role in facilitating vascular invasion, it was important to study how proteolytic activity is modulated during the interaction of cancer cells and endothelial cells. Specifically, 1) do endothelial cells inhibit tumor cell metalloproteinase activity and thus prevent them from degrading basement membrane? and 2) does cancer cell attachment to endothelium mediate activation of endothelial cells and thus assist the tumor cells in their effort to degrade the underlying basement membrane?

The two long-term objectives of this project are: 1) to identify markers on tumor endothelial cells that differ from normal endothelium and 2) to understand what kind of tumor-endothelial interactions are essential for completion of the metastatic cascade.

Methods Employed:

1. Perfusion of rat liver: The hepatic portal vein in an anaesthetized rat was located and perfused with 500 ml of Hanks balanced salt solution without Ca++ or Mg++ followed by 300 ml of Williams E, Hepes buffered medium containing 200 mg of collagenase. The liver was removed and placed on a petri dish and in a small amount of the plating medium. The liver was gently shaken to release the cells. The crude material was centrifuged at 800 rpm for 5 minutes to pellet the bulk of the hepatocytes. The supernatant was used in the selection of endothelial cells.

2. Preparation of OX-43 coated beads: Rat specific endothelial OX-43 antibody was dissolved in 0.5 M borate solution (pH 9.5) at a concentration of 150 μg protein/ml. Equal volumes of beads were added to the antibody solution and both were incubated for 24 hr at 22°C by slow end-over-end rotation. The beads were collected with a magnet and washed three times with 0.01 M PBS containing 0.1% BSA.

3. Attachment of beads to rat liver endothelium: The supernatant from the perfused liver was incubated with the OX-43 coated magnetic beads at a ratio of 1:40 for one hour at 4°C. After the incubation the cells attached to the beads were magnetically separated from the unattached cells and washed three times. The beads were separated from the sorted cells using a 14 N 2-mercaptoethanol solution.

4. Detection of micrometastasis using tumor cells possessing a reporter gene: RLE cells transformed with raf/myc (J2 cells) or raf (T2 cells) oncogenes were further transduced with the E. Coli lacZ gene as a reporter gene, the expression of which can be visualized by histochemical staining for ß-D-galactosidase (ß-gal). After intravenous injection of the transfectants into syngeneic rats, they were sacrificed at different time points. Lungs and liver were fixed immediately in glutaraldehyde and stained for ß-gal.

5. Tumor-endothelial cell interactions: Serum-free conditioned media were collected from J2 and T2 rat cells cocultured with human umbilical vein endothelial cells. Media was also collected from RLE or tumor cells cultured on monolayers of microwave fixed endothelial cells. Endothelial plasma membranes, as well as luminal plasmalemma-derived vesicles, were obtained using formaldehyde and dithiothreitol. Membranes and vesicles were allowed to interact with the tumor cells for 24 hours before collection of the conditioned media. Enzymatic activity was assessed using 7.4% SDS-PAGE gels copolymerized with either 0.1% gelatin or 0.04% human placental type IV collagen.

Major Findings:

1. Cocultures of endothelial cells and J2 or T2 tumor cells revealed enhanced expression of a 92kDa gelatinase and concomitant reduction of a 65kDa gelatinase when compared to the gelatinolytic activity of endothelial cells alone.

2. Minimal gelatinolytic activity was detected in conditioned medium of J2 tumor cells. Following incubation with endothelial cell membranes gelatinolytic activity was detected at the following m.w.: 116kDa, 97kDa, 92kDa, and 65-63kDa. Endothelial cell membranes incubated with conditioned media alone from RLE, J-2 or T-2 cell lines had no effect on the gelatinolytic activity.

3. RLE parent cell line expressed gelatinolytic activity at 65kDa, 90-92kDa and approximately 200kDa. Following incubation with endothelial cell membranes a marked increase in the 65kDa gelatinase activity as well as the appearance of 116, 105 and 95kDa gelatinolytic bands. These bands were not detected in lysates of endothelial cell membrane preparation alone. Gelatinolytic activity of T2 tumor cell line was not modified by the presence of endothelial cell membranes; neither was the activity of RLE, J-2 and T-2 cells in the presence of endothelial cell membrane vesicles or microwave fixed endothelial cells.

4. The 116kDa gelatinase induced by the endothelial cell membranes was found to be a metalloproteinase, inhibited by Ethylenediamine-tetraacetic acid and 1-10 phenanthroline, but not by N-ethylmaleimide, E64, soybean trypsin inhibitor or Diisopropylfluorophosphate. Partial inhibition was noted with pepstatin and Phenylmethylsulfonylfluoride. Human placental type IV collagen gels showed that the 116kDa band also possessed type IV collagenolytic activity.

5. Experimental and spontaneous metastases were observed following inoculation of the J2 (raf/myc transfectant) and T2 (raf transfectant) in both newborn syngeneic rats and nude mice. Following intravenous injections individual tumor cells were detected in the lungs through histochemical staining for ß-gal. After five days microscopic pulmonary metastases were detected. Following subcutaneous injections individual tumor cells and micrometastases were detected in the lungs after six days and grossly visible metastases were seen after 30 days.

6. Isolation of pure cultures of endothelial cells was accomplished through immunomagnetic bead separation technique. Beads coated with Ulex Europaeus Lectin I antibody sorted out human umbilical vein endothelial cells from a mixed culture of endothelial cells and tumor cells. Similarly, beads coated with a rat specific endothelial antibody, OX-43, separated pure population of endothelial cells from perfused liver homogenates. The rat liver endothelial cells revealed a typical cobblestone growth pattern in culture. Since they do not express factor VIII their positive identification was made through demonstrating incorporation of fluorescein labeled low density lipoprotein and expression of angiotensin converting enzyme. The rat liver endothelial cells were propagated in culture for several weeks without loss of their cobblestone growth pattern.

ANNUAL REPORT OF
THE BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

The Biological Carcinogenesis Program provides most of the National Cancer Institute's research program on the viral etiology of cancer as well as much of the oncogene research. The Program provides intramural laboratory research and grant-supported extramural research on the roles of viruses and oncogenes in the etiology of cancer and the molecular mechanisms by which they cause cancer.

During the first half of this century, the only proven cancer viruses were animal viruses. Between 1960-1990, several human viruses have been shown to cause or contribute to the causation of cancer in humans, including Epstein-Barr virus, hepatitis B virus, papillomavirus, human T-cell lymphotropic virus, and human immunodeficiency virus. In the Biological Carcinogenesis Program, each of these viruses has continued to be a focus of intense research into the causes of human cancer.

Dr. Edward Tabor continued to direct the Program as the Associate Director for Biological Carcinogenesis. The seven components of the Program are: Laboratory of Molecular Oncology (Chief, Dr. Takis Papas), Laboratory of Viral Carcinogenesis (Chief, Dr. Stephen O'Brien), Laboratory of Cellular and Molecular Biology (Chief, Dr. Stuart Aaronson), Laboratory of Tumor Cell Biology (Chief, Dr. Robert Gallo), Laboratory of Molecular Virology (Acting Chief, Dr. Edward Tabor), Laboratory of Tumor Virus Biology (Chief, Dr. Peter Howley), and the Biological Carcinogenesis Branch (Chief, Dr. Jack Gruber).

The only major organizational change during this year was the transfer of the Hormone Action and Oncogenesis Section from the Laboratory of Experimental Carcinogenesis (LEC), Chemical and Physical Carcinogenesis Program, to the Laboratory of Molecular Virology in the Biological Carcinogenesis Program, effective May 1, 1991. The move was made on the recommendation of the LEC site visit and was endorsed by the full Board of Scientific Counselors of the Division of Cancer Etiology. The move included eight doctoral level scientists, two technicians, and one student.

A two-day site visit of the Laboratory of Tumor Cell Biology was held May 30 and 31, 1991.

INTRAMURAL RESEARCH

New Technology

A high efficiency cDNA cloning system has been developed that can direct the orientation of inserts in λ-plasmid composite vectors with large cloning capacities. Cleavage of the vector DNA by *SfiI* creates two different

nonsymmetrical 3′ extensions at the ends of the vector arms. Using a linker-primer and an adaptor, cDNA is prepared so it has two different sticky ends which can be ligated to those of the vector arms. When the cDNA and the vector arms are mixed, both the molecules can assemble without self-circularization due to base-pairing specificity. Ligation of the cDNA-vector mixture produces the concatemers from which phage clones carrying a single cDNA insert in the desired orientation can be formed very efficiently by in vitro packaging. This system provides: (1) high cloning efficiency [10^7-10^8 clones/μg poly(A)$^+$RNA], (2) low background (more than 90% of the clones contain inserts), (3) directional insertion of cDNA fragments into the vectors, (4) presence of a single insert in each clone, (5) accommodation of long inserts (up to 10 kb), (6) a mechanism for rescue of the plasmid part for the λ genome, and (7) a straightforward protocol for library preparations. Screenings of cDNA libraries constructed by this method demonstrated that cDNAs of up to 6.4 kb, containing complete coding sequences, could be isolated at high efficiency. Thus, this cloning system should be useful for the isolation of cDNAs of relatively long transcripts, present even at low abundance in cells.

An efficient unidirectional phagemid cDNA expression cloning system for mammalian transforming genes was developed. By means of this system, a cDNA library was constructed from an NIH/3T3 transformant induced by mouse hepatocellular carcinoma DNA. Transfection of NIH/3T3 cells by the DNA library led to the detection of several transformed foci, from which identical plasmids with transforming ability could be rescued. Structure and sequence analysis of the cDNA clones revealed that the oncogene was created by recombinational events involving an unknown gene and the mouse homologue of the B-raf proto-oncogene. This rearrangement led to amino terminal truncation of the B-raf gene product. This genetic rearrangement was also detected in two primary transformants independently induced by the original tumor DNA, implying that the recombination event generating the oncogene occurred in vivo within the tumor rather than in vitro during DNA transfection or cDNA library construction. This phenotypic expression cloning strategy should have wide applicability to the isolation of cDNAs of biologic interest.

HIV and AIDS-KS

HIV-1 tat is capable of providing a growth stimulus to AIDS-KS cells in a complex dose-dependent manner. The HIV-1 tat molecule contains multiple independent segments that are capable of adhering to AIDS-KS cells, as well as other cell types, and have been used to identify the existence of a specific high-affinity receptor molecule on AIDS-KS cells. The identification of this and other possible receptor molecules is in progress, as is the possible contribution of AIDS-KS cell adhesion molecules to pathogenesis. Several drugs and compounds have been evaluated for their effect on KS cell induced biological functions. The effect of SP-PG (a peptidoglycan natural product) on AIDS-KS cell growth and induction of vascular permeability or angiogenesis was promising. It was target specific with an effect on AIDS-KS and endothelial cells but not in fibroblasts.

Specific, high-affinity receptors have been demonstrated for a peptide from the HIV-1 transactivator tat protein. Those receptors could be saturated using an excess of unlabelled tat peptide. The tat peptide induced

proliferation of the cultured AIDS-KS cells. These receptors are likely to mediate the proliferation of the AIDS-KS cells induced by the tat protein.

A 30 kd growth factor was isolated and purified; it supports the long-term growth of AIDS-KS cells from human activated CD4+ T cells (mitogen-stimulated normal peripheral blood mononuclear leukocytes as well as retrovirus-infected/transformed cells).

Previous studies on AIDS-KS have elucidated some of the mechanisms in the formation of the KS lesion, and demonstrated that the HIV-1 gene product, Tat, may play a role in KS pathogenesis. However, the reasons for the very high risk of KS development in HIV-1 infected homosexual or bisexual men are unclear. In the early stages of HIV-1 infection, AIDS-KS patients often show signs of immunoactivation and are only marginally immunosuppressed, suggesting that immune stimulation may play a pivotal role in the development of AIDS-KS. The HTLV cell line which is used as the source of conditioned media (CM) for the long-term culture of the AIDS-KS cells expressed several cytokines normally produced during T cell activation, suggesting that T cell activation products may play a role in vivo in the pathogenesis of KS. To clarify the biological bases of these clinical-epidemiological and in vitro observations, and to evaluate the role of immunoactivation in AIDS-KS, CM from mitogen-activated primary immune cells were investigated to see if they could induce proliferation of cells derived from KS lesions of AIDS patients and of other cells of mesenchymal origin. The CM reproducibly stimulated AIDS-KS and adult aortic smooth muscle cell growth. Protein and mRNA analyses indicated that several cytokines were expressed by two types of activated T cells (as evidenced by HTLV-II CM and phytohemagglutinin (PHA)-T CM). IL-1α and -β, TNF-α and -β, PDGF and, to a lesser extent, IL-6 and granulocyte-macrophage colony stimulating factor promoted the growth of AIDS-KS cells at concentrations shown to be biologically active in other systems. A synthetic CM (made by combining the cytokines at the same concentration present in PHA-CM and HTLV-II CM, in vitro CM) stimulated a growth response very similar or identical to that obtained with CM from activated primary T cells. These results demonstrated that cytokines released from activated primary lymphocytes can induce growth of AIDS-KS cells and of normal mesenchymal cells present in the KS lesion, and that the combination of individually submitogenic levels of several cytokines had additive or synergistic growth effects on mesenchymal cells.

Cultured AIDS-KS cells reacted with a vascular smooth muscle actin (SMC-actin) specific antibody. These cells also expressed SMC-actin messenger ribonucleic acid (mRNA). Furthermore, these AIDS-KS cells appeared, by transmission electron microscopy, morphologically similar to a fetal type of vascular smooth muscle cell. In addition, spindle cells derived directly from KS lesions were also positive for SMC-actin. Thus, the AIDS-KS spindle-like cells have properties of primitive vascular cells, probably more closely related to smooth muscle cells.

Cultured or primary fresh AIDS-KS cells transplanted subcutaneously into the backs of athymic nude mice induced angiogenesis and caused the development of KS-like lesions on day 5 post-transplantation. This model has been duplicated in another animal; when AIDS-KS cells were transplanted into hairless guinea pigs, they also induced a similar highly vascular lesion.

The in vitro growth of AIDS-KS cells was significantly enhanced by several glucocorticoids, viz., hydrocortisone and dexamethasone, an effect that was inhibited by the addition of a steroid antagonist, U486. By comparison, the mineralocorticoid aldosterone had little or no effect. In contrast to their effect on AIDS-KS cells, these glucocorticoids did not augment the growth of normal vascular endothelial cells, fibroblasts, or aortic smooth muscle cells either alone or when used as a supplement to appropriate growth factors. The results suggest the existence of a specific interaction between certain corticosteroids and factors involved in the regulation of AIDS-KS cell growth and they provide a basis for more detailed studies of the mechanisms involved in corticosteroid-associated induction or promotion of KS lesion development in man. They also emphasize the need for judicious use of corticosteroids in patients at risk for developing KS.

Among other clinical symptoms, edema, pleural effusions, and continuous diarrhea are frequently observed in AIDS-associated KS patients. The mechanism(s) responsible for these changes is not clear, although it has been suggested that these are due to a simple mechanical obstruction of the lymphatic system capillaries. However, long-term cultured AIDS-KS cells can induce a biphasic vascular hyperpermeability response in athymic nude mice. In vivo studies to evaluate the factor(s) produced by AIDS-KS cells which induces a vascular permeability response were performed in nude mice and hairless guinea pigs. To assay vascular permeability, dye was injected intravenously (IV) at various intervals after an initial subcutaneous or intradermal injection of AIDS-KS cells or serum-free AIDS-KS CM. An AIDS-KS cell-related late phase response was observed 12 hours later. The activity in the late phase was nondializable and stable at least for 3 weeks at 4°C. A similar vascular permeability response was also induced by the inoculation of freshly isolated primary AIDS-KS cells cultured for a short time without any additional growth factors. These findings indicate that AIDS-KS cells have been activated in the lesion and that such a phenotype has been maintained during growth in culture. This soluble mediator(s) is, therefore, likely to be involved in the regulation of the edematous response in AIDS-KS lesion development. Preliminary molecular evidence suggests that vascular permeability factor is expressed by cultured AIDS-KS cells, and that this factor may be the prime candidate for the induction of increased vascular permeability by AIDS-KS cells.

HIV-1 Gene Expression, Antiviral Strategies, Vaccines

Defective proviruses are likely to be common in infected individuals. Genetic recombination among these proviruses and/or phenotypic mixing of the viruses may result in viruses with altered phenotype. This can be demonstrated in vitro by co-infection with more than one defective virus or co-transfection with defective proviral DNAs. Analysis of molecular clones of two defective viruses showed that one of these viruses (LW12.3) was defective in vpr and vif genes and showed preference for infecting macrophages. The other (MN-ST) had defect in vpu gene and in gag p6 and infected both T-lymphocytes and macrophages. These partially defective viruses were able to trans complement each other resulting in viruses with widened host range and increased cytopathic effects. Similarly, envelope defective HIV-1 proviruses could be complemented by providing competent envelope in trans.

Replication-incompetent defective proviruses may also revert to replication-competent proviruses. For example, when T-lymphocytes harboring Tat defective HIV-1 provirus were subjected to ultraviolet irradiation, replication-competent virus was rescued. The progeny infectious virus was composed of a mixture containing the original mutant, new mutants, and true revertants with the wild-type genotype. These results emphasize that the environmental factors can contribute to HIV-1 pathogenesis in more than one way.

The genetic information carried by the mature retrovirus particles is the RNA genome. Convincing evidence has now been obtained that virus particles of HIV-1 contain viral DNA in addition to the viral RNA. This viral DNA, an intermediate product of reverse transcription of viral RNA, is tightly associated with the reverse transcriptase (RT). Indeed, this particular intermediate DNA form co-purifies with RT through rather stringent purification procedures. This observation encourages certain interesting speculations. Is it possible that this viral DNA enclosed in the virion is the agent of latent infection, particularly of quiescent and nondividing cells (e.g., macrophages)? Productive infection with retroviruses requires integration of viral genome into the cellular chromosomes during cell division. This pathway may not be available in non- or minimally-dividing cells. Could it be that viral DNA:RT brought in by the infecting HIV-1 virions set up extrachromosomal replication of viral DNA in such cells? This could explain the unusually large quantities of unintegrated viral DNA observed in macrophages of infected brains. Thus, HIV-1 could adopt different strategies of replication, depending on the metabolic state of the cell or its state of differentiation.

The retroviral life cycle provides several targets for intervention. The transactivation (tat) function of HIV-1 is mediated by interaction of tat with the tar element. An expression vector directing the synthesis of transcripts with multiple tar elements inhibited HIV-1 long terminal repeat directed gene expression in mammalian cells. To further evaluate the validity and usefulness of this approach, permanent lymphocytic cell lines stably expressing multiple tar elements have been created. These cell lines can now be challenged with HIV-1 to determine if this approach would successfully prevent HIV-1 infection. To further expand the usefulness, multiple tar elements have been inserted into a retroviral vector as a gene therapy delivery vehicle and lymphocytic cell lines infected with and expressing integrated multi-tar elements are being obtained for a challenge test with HIV-1.

The fission yeast Schizosaccharomyces pombe has been utilized as a model system to study transcription factors of HIV-1. Yeast replicating vectors containing HIV-1 LTR CAT and deletions of the HIV-LTR promoters were transfected in S. pombe cells. In a separate experiment, an SV40 promoter transcribing the tat gene was used in a cotransfection assay. The results show that the HIV-LTR promoter is functional in yeast and that RNA synthesis initiates from two sites. Deletion analysis of the promoter suggests NF-kB binding sequences are required for promoter activity.

The antisense approach was used to target HIV-1 rev function. Rev is a second transactivator of viral expression ensuring balanced production of viral transcripts and its action is mediated by the rev response element (RRE). Phosphorothioate derivatized antisense oligonucleotides corresponding to the

first 90 nucleotides of RRE strongly inhibited HIV-1 replication and syncytia induction in human lymphocytic cells without accompanying cellular toxicity.

The in vitro inhibition of HIV-1 by deferoxamine (DFX) was demonstrated. Some of the hepatocellular carcinoma lines in which the effect of DFX had been studied contained integrated hepatitis B virus, which has an unusual reverse transcriptional step in its replication; this suggested testing DFX for antiretroviral activity. Duplicate cultures of H9 cells infected with HIV-1 were studied. At day 7, coded samples of supernatants were tested. In this blinded study, DFX inhibited the expression of p24 antigen and substantially reduced detectable levels of gag and env genes in H9 cell cultures after 7 days. The inhibition was dose-dependent; 30 μM DFX had the same effect on p24 expression as 187 μM AZT (azidothymidine; zidovudine) (50 μg/ml).

An important aspect of the HIV-1 variability is its effect on immune recognition with implications for vaccine design. A neutralizing escape mutant provirus was previously described which contained a point mutation in gp41 away from the major neutralizing epitope (V3 loop). Analysis of this mutant showed that resistance was unlikely due to the direct effect on the antibody binding site but probably resulted from a global change in the tertiary or quaternary structure of the envelope. A new escape mutant has been obtained which also contains a point mutation upstream of the V3 loop in gp120.

To assess the detailed role of the V3 loop in immune recognition and response, chimeric proviruses containing the exchanges of V3 loops, and parts thereof, of HIV-1(IIIB) and HIV-1(MN) have been constructed. One such chimera which contains the V3 loop of HIV-1(MN) substituting the V3 loop of HIV-1(IIIB) is neutralized well by sera which fail to neutralize either parental virus, indicating that not only the loop content but its context is also important for neutralization. Analysis of these chimera should identify motifs within the V3 loop whose recognition results in neutralization.

Recent analysis of the DNA sequence of the envelope region of several HIV-1 isolates from a cohort of individuals from Zaire showed that the V3 loop of these isolates are much more closely related than expected--a divergence of 2-6 amino acid residues among the 32 residues of the loop compared with 10-20 residue differences for other African isolates. These results suggest that the V3 loop is not always as variable as the regions adjacent to it, and that varied changes within the V3 loop may be under functional constraints. This raises the hope that a broadly protective immune response may be attainable by eliciting reactivity toward a finite number of loop genotypes.

The principal neutralizing determinant of HIV, the V3 loop, elicits highly type-specific neutralizing antibodies, complicating its use in vaccine preparations. To study the influence of viral heterogeneity on neutralization, neutralizing serotypes among Zairian isolates have been studied. Immunologic analysis of V3 loop sequences has been carried out using synthetic peptides and hyperimmune goat sera to the V3 loops in homologous and heterologous competition ELISA assays. As expected, highly related immunologic reactivity was associated with conservation of loop amino acid sequence. Nevertheless, in cross-neutralization analyses of Zairian isolates with matched sera, neutralizing serotypes did not correlate with homology in the V3 loop, suggesting the participation of alternate epitopes in broad

neutralization. The role of envelope conformation in neutralization was also emphasized by studies on a chimeric virus composed of the V3 loop of the MN isolate substituted into the envelope of HXB2D. A panel of Zairian sera neutralized this chimeric virus with titers 20-fold higher compared to those against the MN isolate itself. Competition studies with V3 peptides showed this increase in titer could be attributed to better presentation and recognition of either the V3 loop itself or an alternate epitope. Thus, the context in which the V3 loop is presented is crucial for neutralization. The chimeric virus approach is being pursued both for enhancing immunogenicity and for further analysis of neutralizing epitopes. Additional studies aimed at elucidating the principal neutralizing determinant of HIV-2 have implicated a role for the homologous V3 region in this related virus.

A successful vaccine for AIDS may induce a cell mediated immune response in addition to neutralizing antibody. Initial studies of rhesus macaques infected with HIV-2 and HIV-2 accessory gene mutants have shown that while the animals are not immune compromised, they have a poor proliferative response to HIV-2 antigens, and lack circulating cytotoxic T-lymphocytes (CTLs) specific for HIV-2. Thus, the low viral load in these animals cannot be attributed to a strong immune response. These animals also lack neutralizing antibodies. However, HIV-2-specific CTL clones were obtained from several of the animals. These will be useful for mapping HIV-2 CTL epitopes for use in subsequent subunit vaccines.

Dogs have been vaccinated with adenovirus constructs carrying the HIV-1 env gene. A strong neutralizing antibody response was seen in these animals following inoculation with two successive adenovirus constructs of different serotype. The antibody response declined over 12 weeks, but was readily boosted with purified recombinant envelope protein. These encouraging results form the basis for a vaccine trial in chimpanzees. If sufficiently high neutralizing antibody titers are achieved, the chimpanzees will be subjected to a live virus challenge. The cell mediated immune response in these chimpanzees will also be assessed. In additional studies the immune responses obtained following immunization of macaques with additional vectors carrying HIV or simian immunodeficiency virus (SIV) genes are being assessed.

Three separate recombinant or peptide vaccines have been shown to protect macaques from an intravenous challenge with simian immunodeficiency virus (SIV) or type-D retrovirus (SRV-2). Four SIV peptides representing highly conserved and seroprevalent regions of HIV-1 gp120 and gp41 were mixed and used as immunogens in three macaques. The two animals with the highest neutralizing titers resisted a challenge of 100 infectious particles of SIV. Macaques have also been immunized with two doses of a vaccinia virus expressing the envelope proteins of the pathogenic molecular clone of SIV/Mne. Macaques immunized with a vaccinia virus expressing the envelope (env) proteins of the clone SRV-2/WASHINGTON were protected against a homologous challenge with 10^6 infectious particles of SRV-2.

HTLV-I

Extensive studies were conducted to continue exploring the role of extracellular tax1 protein, and to clarify its role in the HTLV-I associated diseases, adult T-cell leukemia (ATL) and tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM). Lymphocytes from both ATL and TSP/HAM

patients were shown to display abnormal proliferation properties in culture. Purified, soluble taxl protein can be taken up by, and stimulate proliferation of, uninfected phytohemagglutinin-treated human peripheral blood lymphocytes (PBLs). Taxl was 40-70% as active as IL-2 in stimulating proliferation of PBLs. Furthermore, purified HTLV-I taxl protein can be taken up by 70Z/3 lymphoid cells and localized in both the nuclear and cytoplasmic compartments. Introduction of the taxl protein into the growth medium of 70Z/3 cells resulted in the rapid and transient induction of NF-kB binding activity in the nuclear fraction. Western blot and competitive radioimmunoassays demonstrated that taxl protein was present in the tissue culture media of HTLV-I-transformed cell lines.

Papillomavirus

The "high risk" human papillomaviruses (HPV) include HPV-16 and HPV-18. These viruses can be differentiated from "low risk" papillomavirus such as HPV-6 and HPV-11 by their ability to efficiently immortalize primary human keratinocytes. Efficient immortalization of these cells requires the combination of both the E6 and E7 gene products which are now considered oncoproteins. The E7 oncoprotein shares properties with the E1A transforming protein of adenovirus E1A and the large T antigen encoded by the polyomaviruses in that it can complex with a group of cellular proteins which includes the product of the retinoblastoma tumor suppressor gene (RB). The biochemical and biological properties of the high risk and low risk HPVs were studied and compared. Chimeric proteins were constructed between these high risk and low risk E7 proteins and their biochemical and biological properties were studied. Biological properties important to cellular transformation were mapped to the amino terminus of the HPV-16 E7 protein. This amino terminus is required for efficient immortalization, high affinity binding to the retinoblastoma gene product (RB), and the abrogation of the TGF-beta mediated transcriptional repression of c-myc expression. In contrast, both the HPV-6 and HPV-16 E7 proteins can efficiently transactivate the adenovirus E2 promoter. We have carried out additional experiments characterizing the cellular proteins associated with HPV-16 E7. In addition to pRB binding HPV-16 E7, another protein with the same electrophoretic ability as the adenovirus E1A associated protein, p107, was noted. A series of additional cellular proteins have been observed and their identities are under investigation.

The ability of HPV-16 E7 protein to abrogate the TGF-beta-induced transcriptional repression of c-myc expression is dependent on the intact pRB binding site on E7. This has implicated pRB, or another cellular protein capable of binding E7 through this same domain, as an essential component in the pathway of transcriptional modulation of the c-myc gene by TGF-beta. Transient expression of pRB also leads to the repression of c-myc expression.

The human papillomavirus E6 protein, which binds p53, stimulates the degradation of p53 in vitro. The E6-promoted degradation of p53 is ATP-dependent and involves the ubiquitin-dependent protease system. Selective degradation of cellular proteins with negative regulatory function (such as p53) provides a novel mechanism of action for dominant acting oncoproteins.

Approximately 85% of human cervical carcinomas can be demonstrated to contain HPV DNA sequences. Five HPV-positive cell lines were studied, and it was found that the Rb and p53 genes are wild type in these cell lines. This

result is consistent with the hypothesis that the normal functions of the tumor suppressor gene products, pRB and p53, are abrogated as a consequence of the complex formation with the HPV E6 and E7 oncoproteins. In contrast, mutations were identified in the p53 and Rb genes expressed in the two HPV-negative cervical carcinoma cell lines examined. In Rb, the mutations mapped to the domain involved in complex formation with the oncoproteins of the DNA tumor virus. Mutations in the Rb genes that affect this portion of the protein have been noted in a variety of different human cancers. Mutations in the p53 gene were likewise in regions commonly mutated in human cancers. These results support the hypothesis that the inactivation of the normal function of the tumor suppressors, pRB and p53, are important steps in human cervical carcinogenesis, either by mutation or as a consequence of complex formation with the virally encoded oncoproteins.

Oncogenes

Work continued on studies to explain the mechanisms by which oncogenes (and closely related growth factors) regulate cell function and growth.

TGF-α

The expression of transforming growth factor-α (TGF-α) and its relation to the hepatitis B virus (HBV) antigens were evaluated in human hepatocellular carcinoma (HCC) and adjacent non-neoplastic liver from 33 patients from the United States and China. TGF-α was detected by immunoperoxidase staining in HCC from 27 of 33 (82%) patients; the frequencies were similar in patients from the US and from China. TGF-α was detected in HCC more frequently in patients whose adjacent non-neoplastic liver had detectable hepatitis B surface antigen (HBsAg) and/or hepatitis B core antigen (HBcAg), than in those whose adjacent liver lacked HBsAg and HBcAg. Detection of TGF-α was not affected by tumor size, histologic type, or grade. TGF-α was detected in adjacent non-neoplastic liver from 31/33 patients (94%). Co-expression of TGF-α and HBsAg in the same hepatocytes could be demonstrated by specific staining of consecutively-cut sections in 17 of 32 patients (53%). HBV is etiologically associated with many cases of HCC; its localization within the same cells as TGF-α, a growth factor associated with hepatocarcinogenesis in animals, suggests a possible interaction between them during hepatocarcinogenesis in humans.

PDGF

Human platelet-derived growth factor (PDGF) is an important connective tissue cell mitogen comprised of two related chains encoded by distinct genes. The B chain is the homologue of the v-sis oncogene product. Properties that distinguish these ligands include greater transforming potency of the B chain and more efficient secretion of the A chain. By a strategy involving the generation of PDGF A and B chimeras, these properties were mapped to distinct domains of the respective molecules. It was also demonstrated that increased transforming efficiency segregated with the ability to activate both α and β PDGF receptors. These findings genetically map PDGF amino acid residues 105 to 144 as responsible for subtle conformational alterations critical to B PDGF receptor interaction and provide a mechanistic basis for the greater transforming potency of the PDGF B chain.

Binding of PDGF to cell surface receptors stimulates a variety of biochemical and biological responses. Two receptor gene products (designated α and β) have been identified, and the different binding affinities of various PDGF isoforms for these receptors are prime determinants of the spectrum of responses observed. The β receptor binds PDGF-BB, but not PDGF-AA, while the α receptor binds PDGF-AA and PDGF-BB. These different ligand binding specificities were used to investigate the PDGF-AA binding site in the human αPDGF receptor by constructing chimeric molecules between the human αPDGF and βPDGF receptors. The results demonstrate that amino acids 1-340 of the αPDGF receptor comprise the region that confers PDGF-AA binding specificity. This region corresponds to immunoglobulin-like subdomains 1, 2, and 3 of its external domain. The tyrosine kinase domains of PDGF and colony-stimulating factor-1 (CSF-1)/c-fms receptors are interrupted by kinase inserts (ki) which vary in length and amino acid sequence. Taken together, data derived in the studies of ki domains indicate that they have evolved to play very similar roles in the known signalling functions of PDGF and CSF-1 receptors.

PDGF promotes the growth of oligodendrocyte type-2 astrocyte (O-2A) glial progenitor cells and allows their timely differentiation into oligodendrocytes, the CNS myelin-forming cells. We demonstrate that basic FGF is a potent mitogen for brain O-2A progenitor cells, but blocks their differentiation into oligodendrocytes. Treatment with basic FGF also influences the level of expression of PDGF receptors on O-2A progenitor cells. These cells express only the α chain PDGF receptor, and the levels of αPDGF receptors decrease as the cells differentiate. In contrast, basic FGF maintains a high level of functionally responsive αPDGF receptors in O-2A progenitors. Thus, basic FGF activates a signalling pathway that can positively regulate PDGF receptors in O-2A progenitor cells. In this way basic FGF or an FGF-like factor may modulate the production of myelin-forming cells in the CNS.

ETS1

The mouse ets-1 gene is similar in organization to the human ETS1 gene. The positions of the introns (points of disruption in the predicted open reading frame) are identical. Sequence analysis of the promoter region of the mouse ets-1 gene and alignment with that of the human ETS1 gene reveal several highly-conserved domains, suggesting functional significance. At least two of these conserved sequences can be shown to interact with nuclear proteins by mobility gel shift analyses. These interactions possibly define a new class of regulatory proteins, as analyses of the DNA sequence do not reveal any consensus sites for protein binding.

The c-ets-1 protooncogene and the c-ets-2 gene encode related nuclear chromatin-associated proteins that bind DNA in vitro. To investigate the possibility that ets1 and ets2 proteins are transcriptional activators, the ability of these proteins to transactivate promoter/enhancer sequences was studied in transient co-transfection experiments. The HTLV-I LTR was found to be transactivated by both ets1 and ets2 proteins. An ets-responsive sequence between positions -117 and -160 of the LTR was identified by analyses of a series of 5′ deletion mutants of the HTLV-I LTR and of dimerized versions of specific motifs of the LTR enhancer region. Ets1 protein was found to bind specifically to the -117 to -160 regulatory sequence. These results show that ets1 and ets2 are sequence-specific transcriptional activators. In view of

the high level expression of ets1 in lymphoid cells, ets1 protein could be part of the transcription complex that mediates the response to tax1 and the control of HTLV-I replication.

raf

The c-raf-1 gene is selectively and consistently mutated in a mouse model for lung cancer and is also frequently mutated and overexpressed in human lung carcinoma. Overexpression can contribute to malignant transformation; wild-type forms of raf and ras were shown to synergize in transformation. Raf-1 protein kinase was shown to be an essential component of growth signaling pathways used by many growth factors and from this position controls the mitogenic and oncogenic signal flow of most oncogenes.

Rb

An expression plasmid of the human Rb gene, a tumor suppressor, under control of the human β-actin promoter, was transfected into the bladder carcinoma line, HTB9, which lacks Rb gene expression. Marker-selected transfectants that expressed Rb protein were identified by western blotting and immunohistochemical staining using an anti-Rb monoclonal antibody. In selected clones, stable Rb expression persisted over several months under standard culture conditions. The tumorigenicity of cells expressing Rb was markedly decreased. Tumors that formed in nude mice were much smaller and had a longer latency period but were indistinguishable microscopically from those produced by parental cells. Slower growing tumors were shown to produce Rb protein. These findings support the concept that the Rb gene acts as both a growth and tumor suppressor in bladder cancer cells.

EXTRAMURAL RESEARCH

The extramural activities of the Biological Carcinogenesis Program have continued in the support and management of research on the roles of viruses and oncogenes in the etiology of cancer. Two successful workshops were held: "Cancer Vaccines" and "Animal Models of Retrovirus-Associated Malignancies."

Two new RFAs are expected to be issued by the end of this fiscal year: 1) "Cancer Vaccines" and 2) "Animal Models of Retrovirus-Associated Malignancies."

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05646-02 ADBC

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Oncogene Expression in Cell Lines Derived from Human Hepatocellular Carcinoma

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

PI:	Edward Tabor	Associate Director	BCP, NCI
Others:	Mahmood Farshid	Biotechnology Fellow	OAD, BCP, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Office of the Associate Director, Biological Carcinogenesis Program

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	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 possible role of oncogenes in the development of human hepatocellular carcinoma (HCC) has not been well defined. We have examined several cell lines from human HCC, as well as a control (non-HCC) cell line (WI-38) for levels of expression of oncogenes.

The expression of thirteen oncogenes (c-myc, H-ras, K-ras, N-ras, raf, myb, abl, N-myc, erb-A, erb-B, fos, src, and mos) and two tumor suppressor genes (Rb and p53) were studied by northern blot hybridization in three human HCC/HB cell lines (PLC/PRF/5, Hep3B, and Hep G2) and in a human embryonic lung fibroblast cell line (WI-38) to look for differences that might be associated with the presence (PLC/PRF/5 and Hep3B) or absence (Hep G2) of integrated hepatitis B virus (HBV) DNA. Most of the oncogenes were expressed at a higher level (based on band intensity) in HCC/HB cell lines than in WI-38 cell lines (exceptions: K-ras, fos, and Rb were expressed at the same level in all four cell lines; p53 was not detectable in Hep3B). Certain oncogenes (c-myc, H-ras, raf, src) were expressed at different levels among the three HCC/HB cell lines, unrelated to the presence or absence of integrated HBV-DNA.

Additional studies were conducted using additional human HCC cell lines: 2.2.15 (which is derived from Hep G2 transfected with HBV) and the three Japanese lines: HLE, HLF, and HuH7. The data for these lines are still being analyzed.

PROJECT DESCRIPTION

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

Edward Tabor	Associate Director	BCP, NCI
Mahmood Farshid	Biotechnology Fellow	OAD, BCP, NCI

Objectives:

To determine the level of expression of oncogenes in human hepatocellular carcinoma (HCC) cell lines, and to determine whether these differ in those cell lines with and without hepatitis B virus (HBV) DNA.

Methods Employed:

Cell culture, RNA extraction and purification, northern blot hybridization, and dot blot hybridization.

Major Findings:

Most of the oncogenes were expressed at a higher level in HCC/HB cell lines than in WI-38 cell lines. The level of expression of the oncogenes varied in the different HCC/HB cell lines and was not associated with the presence or absence of HBV DNA.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05647-02 ADBC

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Inhibition of Hepatocellular Carcinoma by Desferoxamine

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

PI:	Edward Tabor	Associate Director	BCP, NCI
Others:	C.M. Kim	Biologist	OAD, BCP, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Office of the Associate Director, Biological Carcinogenesis Program

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.2	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.)*

Inhibition of several human hepatocellular carcinoma (HCC) cell lines by deferoxamine mesylate (desferrioxamine) (DFX) was evaluated and compared with growth of the cells in the absence of DFX and with the growth of human lung fibroblast (WI-38) cells. PLC/PRF/5 or HepG2 maintained for 7 days in 30 μM or 60 μM DFX did not increase in number and by day 7 produced little or no alpha-fetoprotein (AFP). Cell growth without DFX reached confluence at day 7 and by day 7 had AFP = 30-60 ng/ml (PLC/PRF/5) and >1,000 ng/ml (HepG2) in the supernate. (Cell growth and AFP production in 3 μM DFX were mildly reduced.) Titers of hepatitis B surface antigen (HBsAg) produced by the PLC/PRF/5 cells were reduced 1-2 logs in 30 μM and 60 μM DFX compared to cells grown without DFX. WI-38 cells grown with 30 μM or 60 μM DFX grew at 50% of the rate of WI-38 cells without DFX. DFX inhibits the growth of PLC/PRF/5 and HepG2 cells in an apparently dose-related manner (30 μM or 60 μM DFX arresting cell growth and AFP production) compared to reducing the growth rate of WI-38 cells. The inhibition of PLC/PRF/5 and HepG2 cells may be due, in part, to mechanisms other than iron chelation.

Many additional studies have been conducted to further evaluate these observations. These have included attempts to overcome or prevent inhibition with different iron salts, and evaluating whether inhibition occurs in the same way in six other human HCC cell lines. The inhibition of HCC cell lines by deferoxamine occurred similarly in all human HCC cell lines tested, regardless of the country of origin (South Africa, United States, Japan) or the presence or absence of HBV DNA in the cell line. In no case could equimolar concentrations of iron (ferric or ferrous salts) prevent or reverse the inhibitory effect of deferoxamine.

PROJECT DESCRIPTION

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

Edward Tabor	Associate Director	BCP, NCI
C. M. Kim	Biologist	OAD, BCP, NCI

Objectives:

To evaluate whether deferoxamine mesylate (DFX) can inhibit the growth of human hepatocellular carcinoma (HCC) cell lines, as suggested by preliminary reports in the literature, and to characterize that inhibition and its mechanisms.

Methods Employed:

Cell culture and enzyme immunoassays.

Major Findings:

1. DFX inhibits the growth of hepatocellular carcinoma and hepatoblastoma cell lines in an apparently dose-related manner.

2. The inhibition does not depend on the presence or absence of integrated hepatitis B virus DNA.

3. The inhibition may be due, in part, to mechanisms other than iron chelation.

Publication:

Tabor, E, Kim, CM. Inhibition of human hepatocellular carcinoma and hepatoblastoma cell lines by deferoxamine. J Med Virol 34:45-50 (1991).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05693-01 ADBC

PERIOD COVERED

October 1, 1990 through September 30, 1991

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

Development of Systems to Detect PCR Contamination

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

PI:	Edward Tabor	Associate Director	BCP, NCI
Others:	Yoshihiro Okada	Visiting Associate	OAD, BCP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Office of the Associate Director, Biological Carcinogenesis Program

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	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 polymerase chain reaction (PCR) can be used to detect small quantities of hepatitis B virus (HBV) DNA, generally by testing for one or two gene sequences, in liver and serum from patients without detectable markers of HBV. It is sometimes necessary to distinguish the detection of HBV DNA from false-positive results due to contamination by carry-over of previously amplified PCR products. Occasional use of "multi-target" PCR using primer sets for portions of the three HBV genes, C, S, and X, simultaneously can verify the lack of contamination by carry-over and enhance the accuracy of PCR.

PROJECT DESCRIPTION

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

Edward Tabor	Associate Director	BCP, NCI
Yoshihiro Okada	Visiting Associate	OAD, BCP, NCI

Objectives:

To develop a method to distinguish positive reaction by polymerase chain reaction (PCR) assay from contamination by amplified sequence.

Methods Employed:

Polymerase chain reaction (PCR), Southern blot hybridization. Special variations on PCR such as double amplification with internal and external primers and multi-target PCR.

Major Findings:

Using multi-target PCR, a new method to amplify simultaneously three different gene sequences, contamination of the PCR process can be distinguished from true positive reactions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05694-01 ADBC

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunohistochemical Studies of Human Hepatocellular Carcinoma

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

PI:	Edward Tabor	Associate Director	BCP, NCI
Others:	C. C. Hsia	Visiting Scientist	OAD, BCP, NCI
	A. Di Bisceglie	Visiting Scientist	DD, NIDDKD
	C. Axiotis	Expert	CC, NIH

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Office of the Associate Director, Biological Carcinogenesis Program

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	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 expression of transforming growth factor-α (TGF-α) and its relation to the hepatitis B virus (HBV) antigens were evaluated in human hepatocellular carcinoma (HCC) and adjacent non-neoplastic liver from 33 patients from the United States and China. TGF-α was detected by immunoperoxidase staining in HCC from 27 of 33 (82%) patients; the frequencies were similar in patients from the US and from China. TGF-α was detected in HCC more frequently in patients whose adjacent non-neoplastic liver had detectable hepatitis B surface antigen (HBsAg) and/or hepatitis B core antigen (HBcAg), than in those whose adjacent liver lacked HBsAg and HBcAg. Detection of TGF-α was not affected by tumor size, histologic type, or grade.

TGF-α was detected in adjacent non-neoplastic liver from 31/33 patients (94%). Co-expression of TGF-α and HBsAg in the same hepatocytes could be demonstrated by specific staining of consecutively-cut sections in 17 of 32 patients (53%). HBV is etiologically associated with many cases of HCC; its localization within the same cells as TGF-α, a growth factor associated with hepatocarcinogenesis in animals, suggests a possible interaction between them during hepatocarcinogenesis in humans.

PROJECT DESCRIPTION

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

Edward Tabor	Associate Director	BCP, NCI
C. C. Hsia	Visiting Scientist	OAD, BCP, NCI
A. Di Bisceglie	Visiting Scientist	DD, NIDDKD
C. Axiotis	Expert	CC, NIH

Objectives:

To study the role of oncogenes in human hepatocellular carcinoma (HCC) tissue and to determine the relationship to hepatitis viral antigens.

Methods Employed:

Immunohistochemical staining using immunoperoxidase and the avidin-biotin system; other tissue staining.

Major Findings:

1. TGF-α was detected in adjacent non-neoplastic liver from 31/33 patients (94%). Co-expression of TGF-α and HBsAg in the same hepatocytes could be demonstrated by specific staining of consecutively-cut sections in 17 of 32 patients (53%).

2. Detection of TGF-α was not affected by tumor size, histologic type, or grade.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05695-01 ADBC

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
PCR for Detection of Hepatitis B Virus DNA in Sera from Patients with HCC

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

PI:	Edward Tabor	Associate Director	BCP, NCI
Others:	Yoshihiro Okada	Visiting Associate	OAD, BCP, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Office of the Associate Director, Biological Carcinogenesis Program

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)
□ (a) Human subjects ☒ (b) Human tissues □ (c) Neither
□ (a1) Minors Serum Only
□ (a2) Interviews

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

The presence of hepatitis B virus (HBV) DNA was investigated in the sera from 25 Japanese patients with hepatocellular carcinoma (HCC) using the polymerase chain reaction (PCR) method. Two stages of PCR amplifications (30 cycles each) for three different gene segments of HBV DNA, the PreC/C, S and X genes, were performed using the outer and inner primer pairs. The three target sequences were amplified separately (standard PCR) or simultaneously ("multi-target" PCR).

Fifteen of the 16 hepatitis B surface antigen (HBsAg)-negative sera were negative for all three gene segments; the remaining one serum was positive only for the PreC/C gene segment. By contrast, 8 of 9 HBsAg-positive sera were positive for all three gene segments; the remaining one serum could not be determined to be positive or negative.

Additional serologic studies (e.g., HBeAg, anti-HBc) were also conducted.

PROJECT DESCRIPTION

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

Edward Tabor	Associate Director	BCP, NCI
Yoshihiro Okada	Visiting Associate	OAD, BCP, NCI

Objectives:

To determine whether the hepatitis B virus (HBV) plays a role in the etiology of hepatocellular carcinoma in patients with no serologic evidence of HBV infection, using polymerase chain reaction to detect HBV DNA.

Methods Employed:

Polymerase chain reaction (PCR), Southern blot hybridization. Special variations on PCR such as double amplification with internal and external primers and multi-target PCR.

Major Findings:

HBV cannot be shown to play a role in most HCC patients in Japan who lack serologic evidence of HBV, using PCR for HBV DNA in serum.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05696-01 ADBC

PERIOD COVERED

October 1, 1990 through September 30, 1991

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

Role of Pre-S2 Promoter of HBV Studied in Hepatocellular Carcinoma Cell Line

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

PI:	Edward Tabor	Associate Director	BCP, NCI
Others:	Young-Sok Lee	Visiting Associate	OAD, BCP, NCI
	Seong-Jin Kim	Visiting Associate	LC, NCI

COOPERATING UNITS *(if any)*

Genetic Engineering Research Institute, KIST, Seoul, KOREA (Dr. Young-Ik Lee)

LAB/BRANCH

Office of the Associate Director, Biological Carcinogenesis Program

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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 S open reading frame of the hepatitis B virus (HBV) comprises preS1 region, preS2 region, and S structural gene region, which code the large, middle and major surface antigen polypeptides, respectively, in accordance with translation from the three in-frame initiation codons. In order to investigate the most important sequence within the preS2 promoter region, plasmids were created with serial deletion mutants directing the expression of the chloramphenicol acetyl transferase gene and the transcriptional activities were analyzed by transfection assays in four human hepatocellular carcinoma or hepatoblastoma cell lines (HepG2, PLC/PRF/5, Hep3B, and 2.2.15). Deletion mutant analysis revealed that the promoter from sequence 2861 to 2887 had highest activity. Two binding sites for nuclear proteins were observed by DNase I footprinting assay but multiple shifted DNA bands were identified by gel retardation assay suggesting that there are multiple binding sites for nuclear protein in the promoter sequence between nucleotides 2920 and 2951.

PROJECT DESCRIPTION

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

Edward Tabor	Associate Director	BCP, NCI
Young-Sok Lee	Visiting Associate	OAD, BCP, NCI
Seong-Jin Kim	Visiting Associate	LC, NCI

Young-Ik Lee, Genetic Engineering Research Institute, Seoul, KOREA

Objectives:

To determine which portions of the Pre-S2 promoter are most important for HBV gene expression, to evaluate the interactions with nuclear protein(s), and to determine the effects of various cytokines on its functions.

Methods Employed:

Cell cultures, PCR, DNA splicing, transfection, CAT-assays, DNA footprinting, gel retardation assay, northern blot hybridization.

Major Findings:

Deletion mutant analysis revealed that the promoter from sequence 2861 to 2887 had highest activity. Two binding sites for nuclear proteins were observed by DNase I footprinting assay but multiple shifted DNA bands were identified by gel retardation assay suggesting that there are multiple binding sites for nuclear protein in the promoter sequence between nucleotides 2920 and 2951.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05697-01 ADBC

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Inhibition by Desferrioxamine of In Vitro Replication of HIV-1

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: Edward Tabor Associate Director BCP, NCI

COOPERATING UNITS *(if any)*
Center for Biologics Evaluation and Research, Food and Drug Administration
(Jay S. Epstein and Indira K. Hewlett)

LAB/BRANCH
Office of the Associate Director, Biological Carcinogenesis Program

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.2	.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.)*

Some of the hepatocellular carcinoma lines in which we had studied the effect of deferoxamine (desferrioxamine; DFX) contained integrated hepatitis B virus, which has an unusual reverse transcription step in its replication; this prompted us to test DFX for antiretroviral activity.

Duplicate cultures of H9 cells infected with HIV-1 were studied. At day 7, coded samples of supernatants were tested.

In this blinded study, DFX inhibited the expression of p24 antigen and substantially reduced detectable levels of gag and env genes in H9 cell cultures after 7 days. The inhibition was dose-dependent; 30 μm DFX had the same effect on p24 expression as 187 μM AZT (azidothyimidine [AZT]; zidovudine) (50 μg/ml). Cultures grown in medium lacking DFX and AZT produced substantial concentrations of p24, and the signals for *gag* and *env* sequences were strongly positive. Viability of the H9 cells was above 70% at day 7 in all cultures. Three independent experiments were done, with similar results for p24 expression. Evaluation of *gag* and *env* were available only in one experiment.

DFX could have inhibited HIV-1 by interfering with the RNA-dependent DNA synthesis that occurs early in each infectious cycle. The observation of in vitro inhibition of HIV-1 by DFX suggests a new mechanism of viral inhibition.

PROJECT DESCRIPTION

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

Edward Tabor Associate Director BCP, NCI

Objectives:

Some of the hepatocellular carcinoma lines in which we had studied the effect of DFX contained integrated hepatitis B virus, which has an unusual reverse transcription step in its replication; this prompted us to test DFX for antiretroviral activity.

Methods Employed:

Cell culture, western blot hybridization, PCR.

Major Findings:

In repeatable, blinded studies, DFX inhibited HIV-1 to essentially the same extent as did AZT.

Publication:

Tabor E, Epstein JS, Hewlett IK, Lee SF. Inhibition by desferrioxamine of in vitro replication of HIV-1. Lancet 1991; 337:795.

ANNUAL REPORT OF

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

October 1, 1990 to September 30, 1991

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 this knowledge to the prevention of cancer in man.

Many ongoing investigations within the laboratory derive from our research on retroviruses. 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 cancer of many species. Certain retroviruses, the so-called "replication-defective" transforming viruses, have arisen by a recombination with cellular genes or proto-oncogenes. Investigations within the laboratory have provided strong evidence that proto-oncogenes are frequent targets of genetic alterations that convert them to oncogenes and lead 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. Our findings that proto-oncogenes can encode growth factors as well as growth factor receptors have strongly implicated subversion of normal growth factor-activated pathways of cell proliferation in the neoplastic process. Thus, today, a considerable portion of our research effort concerns these important genes and the cascade of biochemical events involved in mitogenic signalling. The aim of these studies is the identification of new oncogenes, as well as strategies that may eventually be useful in blocking neoplastic progression.

The lentiviruses, a subfamily of the retroviruses, have been implicated as the causative agents of nonneoplastic diseases of certain animal species and of acquired immunodeficiency syndrome (AIDS) in man. Investigations in our laboratory utilize animal lentiviruses as models for treatment and prevention of AIDS.

1. Growth Factors

Platelet-derived growth factor/*sis*. Human platelet-derived growth factor (PDGF) is an important connective tissue cell mitogen comprised of two related chains encoded by distinct genes. The B chain is the homologue of the v-*sis* oncogene product. Properties that distinguish these ligands include greater transforming potency of the B chain and more efficient secretion of the A chain. By a strategy involving the generation of PDGF A and B chimeras, we mapped these properties to distinct domains of the respective molecules. We further demonstrated that increased transforming efficiency segregated with the ability to activate both α and β PDGF receptors. These findings genetically map PDGF amino acid residues 105 to 144 as responsible for subtle conformational alterations critical to B PDGF receptor interaction and provide a mechanistic basis for the greater transforming potency of the PDGF B chain.

Deletion scanning mutagenesis within the transforming region of the v-*sis* oncogene was used to dissect structure-function relationships. Mutations affecting codons within a domain encoding amino acids 136 through 148 had no effect upon homodimer formation or recognition by antisera which detect determinants dependent upon native

intrachain disulfide linkages, yet the same mutations completely abolished transforming activity. A PDGF B monoclonal antibody that prevents its interaction with PDGF receptors recognized v-*sis*, A142 (deletion of codon A142) and A148 but not A136, A137, or A139 mutants. These findings mapped the epitope recognized by this monoclonal antibody to include amino acid residues 136 to 139. Furthermore, mutations in the codon 136 to 148 domain caused markedly impaired ability to induce PDGF receptor tyrosine phosphorylation. Thus, subtle conformational alterations in this small domain were shown to critically affect PDGF receptor recognition and/or functional activation.

Genetic alterations that constitutively activate crictical genes in mitogenic signalling pathways have been causally implicated in the neoplastic process. Intervention with the pathologic expression of this important subset of genes might be most readily approached in the case of growth factors, if functional activation of their receptors were confined to a cell surface location. Recent studies with the v-*sis* oncogene, whose human homologue encodes the PDGF B chain, have demonstratated that autocrine activation of PDGFRs occurs internally. However, activated receptors must achieve a cell surface location in order to functionally couple with intracellular mitogenic signalling pathways. Thus, agents such as neutralizing PDGF antibody or suramin, which specifically interfere with ligand-receptor interactions at the cell surface, are capable of partially or completely reverting the v-*sis*/PDGF B transformed phenotype in cell culture. We sought to demonstrate a functional autocrine loop involving PDGF and its receptors in human malignancies. In tumor cells expressing PDGF ligand(s) and receptor(s), immunoblot analysis established tyrosine phosphorylation of PDGF receptors (PDGFRs) in the absence of any exogenous ligand, implying chronic receptor activation. Exposure to suramin resulted in diminished receptor autophosphorylation and/or upregulation of receptor protein. In a subset of such tumor lines, there was marked reduction in DNA synthesis in response to suramin or PDGF neutralizing antiserum. These findings demonstrate that autocrine PDGF stimulation contributes significantly to proliferation of some human tumors and that agents which interfere with ligand-receptor interactions at the cell surface can significantly intervene in this process.

Keratinocyte growth factor (KGF). Human KGF is an epithelial cell-specific mitogen which is secreted by normal stromal fibroblasts. We demonstrated that KGF is as potent as EGF in stimulating proliferation of primary or secondary human keratinocytes in tissue culture. Exposure of KGF- or EGF-stimulated keratinocytes to 1.0 mM calcium, an inducer of differentiation, led to cessation of cell growth. However, immunologic analysis of early and late markers of terminal differentiation, K1 and filaggrin, respectively, revealed striking differences in keratinocytes propagated in the presence of these growth factors. With KGF, the differentiation response was associated with expression of both markers, whereas their appearance was retarded or blocked by EGF. TGFα, which also interacts with the EGF receptor, gave a similar response to that observed with EGF. These findings functionally distinguish KGF from the EGF family and support the role of KGF in the normal proliferation and differentiation of human epithelial cells.

EGF/transforming growth factor α (TGFα). Alterations in the EGF/TGFα-responsive mitogenic pathway are frequently detected in malignancies. In particular, the EGF-receptor (EGFR) molecule has been found overexpressed in a number of human tumors, and TGFα is produced by a large array of tumor cells. Gene transfer experiments have previously demonstrated that expression of either TGFα or EGFR alone is not sufficient to induce the transformed phenotype in NIH/3T3 cells.

Alterations affecting the EGF/TGFα-responsive mitogenic pathway are frequently detected in malignancies. In particular, the epidermal growth factor receptor has been found overexpressed in a number of human tumors. Production and secretion of

transforming growth factor type α has also been shown in several tumor cells but not in their normal counterparts. We described the establishment of *in vitro* model systems to study the transforming potential of these molecules and summarized our current understanding of the mechanisms involved in transformation by genes encoding a growth factor and a growth factor receptor.

EGF and TGFα bind to a common cell surface receptor that mediates their diverse biological activities. NIH/3T3 fibroblasts, transfected with either full-length EGF precursor (preproEGF) or proTGFα cDNA, displayed distinct patterns of growth in culture. PreproEGF induced focal transformation, and transfectants grew in a chemically defined medium (CDM) at low cell density in the absence of added EGF. In contrast, TGFα failed to cause focal transformation, and transfectants grew in CDM in the absence of added growth factors only when seeded at high cell density. The 53-amino acid EGF portion of the preproEGF translation product was essential for its effects. These results indicate that constitutive expression of preproEGF is sufficient to establish autocrine growth of NIH/3T3 expressing low levels of EGF receptors. At high cell density, where paracrine as well as autocrine effects of these growth factors would be evident, TGFα transfectants displayed at least as high or higher levels of EGF receptor (EGFR) tyrosine phosphorylation than preproEGF transfectants. Since quantitative levels of ligand expression did not account for differences in their transforming properties, preproEGF must be more efficient than proTGFα in binding and/or activating EGF receptors in an autocrine manner.

The precursor for TGFα, pro-TGFα, is a cell surface glycoprotein that can establish contact with EGF receptors on adjacent cells. To examine whether the pro-TGFα/EGF receptor pair can simultaneously mediate cell adhesion and promote cell proliferation, we have expressed pro-TGFα in a bone marrow stromal cell line. Expression of pro-TGFα allows these cells to support long term attachment of an EGF/interleukin-3-dependent hematopoietic progenitor cell line that expresses EGF receptors but is unable to adhere to normal stroma. This interaction is inhibited by soluble EGF receptor ligands. Further, the hematopoietic progenitor cells replicate their DNA while they are attached to the stromal cell layer and become foci of sustained cell proliferation. Thus, pro-TGFα and the EGF receptor can function as mediators of intercellular adhesion and this interaction may promote a mitogenic response. We propose the term "juxtacrine" to designate this form of stimulation between adjacent cells.

Hepatocyte growth factor (HGF). A heparin-binding mitogen was isolated from conditioned medium of human embryonic lung fibroblasts. It exhibited broad target-cell specificity whose pattern was distinct from that of any known growth factor. It rapidly stimulated tyrosine phosphorylation of a 145-kDa protein in responsive cells, suggesting that its signalling pathways involved activation of a tyrosine kinase. Purification identified a major polypeptide with an apparent molecular mass of 87 kDa under reducing conditions. Partial amino acid sequence analysis and cDNA cloning revealed that it was a variant of HGF, a mitogen thought to be specific for hepatic cells and structurally related to plasminogen. Recombinant expression of the cDNA in COS-1 cells established that it encoded the purified growth factor. Its site of synthesis and spectrum of targets imply that this growth factor may play an important role as a paracrine mediator of the proliferation of melanocytes and endothelial cells, as well as cells of epithelial origin.

II. Growth Factor Receptors

PDGF. Binding of platelet-derived growth factor (PDGF) to its cell surface receptors stimulates a variety of biochemical and biological responses. Two receptor gene products (designated α and β) have been identified, and the different binding affinities of various PDGF isoforms for these receptors are prime determinants of the spectrum of responses observed. The β receptor binds PDGF-BB,

but not PDGF-AA, while the α receptor binds PDGF-AA and PDGF-BB. We utilized these different ligand binding specificities to investigate the PDGF-AA binding site in the human αPDGF receptor by constructing chimeric molecules between the human αPDGF and βPDGF receptors. Our results demonstrate that amino acids 1-340 of the αPDGF receptor comprise the region that confers PDGF-AA binding specificity. This region corresponds to immunoglobulin-like subdomains 1, 2, and 3 of its external domain.

The tyrosine kinase domains of PDGF and colony-stimulating factor-1 (CSF-1)/c-*fms* receptors are interrupted by kinase inserts (ki) which vary in length and amino acid sequence. To define the role of the ki in the human αPDGF receptor (αPDGFR), we generated deletion mutants, designated αRAki-1 and αRAki-2, which lacked 80 (710 to 789) and 95 (695 to 789) amino acids of the 104-amino-acid ki region, respectively. Their functional characteristics were compared with those of the wild-type αPDGFR following introduction into a naive hematopoietic cell line, 32D. Biochemical responses, including PDGF-stimulated PDGFR tyrosine phosphorylation, phosphatidylinositol (PI) turnover, and receptor-associated PI-3 kinase activity, were differentially impaired by the deletions. Despite a lack of any detectable receptor-associated PI-3 kinase activity, 32D cells expressing αRAki-1 showed only partially impaired chemotactic and mitogenic responses and were capable of sustained proliferation in vitro and in vivo under conditions of autocrine stimulation by the c-*sis* product. 32D transfectants expressing the larger ki deletion (αRAki-2) showed markedly decreased or abolished biochemical and biological responses. However, insertion of the highly unrelated smaller c-*fms* (685 to 750) ki domain into αRAki-2 restored each of these activities to wild-type αPDGFR levels. Since the CSF-1R does not normally induce PI turnover, the ability of the c-*fms* ki domain to reconstitute PI turnover in the αRAki-2 transfectant provides evidence that the ki domain of the αPDGFR does not directly couple with this pathway. Taken together, all of these findings imply that their ki domains have evolved to play very similar roles in the known signalling functions of PDGF and CSF-1 receptors.

erbB-2. The *erbB-2* gene product, gp185^{erbB-2}, displays a potent transforming effect when overexpressed in NIH/3T3 cells. In addition, it possesses constitutively high levels of tyrosine kinase activity in the absence of exogenously added ligand. We demonstrated that its carboxy-terminal domain exerts an enhancing effect on *erbB-2* kinase and transforming activities. A premature termination mutant of the *erbB-2* protein lacking the entire carboxy-terminal domain (*erbB-2*$^{\Delta 1050}$) showed a 40-fold reduction in transforming ability and a lowered in vivo kinase activity for intracellular substrates. When the carboxy-terminal domain of *erbB-2* was substituted for its analogous region in the EGFR (EGFR/*erbB-2*^{COOH} chimera), it conferred *erbB-2*-like properties of the EGFR, including transforming ability in the absence of EGF, elevated constitutive autokinase activity in vivo and in vitro, and constitutive ability to phosphorylate PLC-γ. Conversely, a chimeric *erbB-2* molecule bearing an EGFR carboxy-terminal domain (*erbB-2*/EGFR^{COOH} chimera) showed reduced transforming and kinase activity with respect to the wild type *erbB-2* and was only slightly more efficient than the *erbB-2*$^{\Delta 1050}$ mutant. Thus, we concluded that the carboxy-terminal domains of *erbB-2* and EGFR exert different regulatory effects on receptor kinase function and biological activity. The upregulation of gp 185^{erbB-2} enzymatic activity exerted by its carboxy-terminal domain can explain, at least in part, its constitutive level of kinase activity.

The EGFR and gp185^{erbB-2} are closely related tyrosine kinases. Despite extensive sequence and structural homology, these two receptors display quantitative and qualitative differences in their ability to couple with mitogenic signalling pathways. By employing chimeric molecules between EGFR and *erbB-2*, we found that determinants responsible for the specificity of mitogenic signal transduction are located in the amino terminal half of the tyrosine kinase domain of either receptor. In the EGFR, mutational analysis within this subdomain revealed that deletion of residues 660-667 impaired the receptor mitogenic activity without affecting its

tyrosine kinase properties. Therefore this sequence is likely to contribute to the specificity of substrate recognition by the EGFR kinase.

While the normal human *erb*B-2 gene is potently transforming when overexpressed in NIH/3T3 cells, its rat homolog, the *neu* gene, seems to acquire transforming properties only upon alteration of its coding sequence. We compared the effects of different levels of expression of normal *erb*B-2 and *neu* in NIH/3T3 cells. Results revealed that the normal rat *neu* gene acts as a potent oncogene when sufficiently overexpressed in NIH/3T3 cells.

Overexpression of the *erb*B-2/*neu* gene is frequently detected in human cancers. When overexpressed in NIH/3T3 cells, the normal *erb*B-2 product, gp185^{erbB-2}, displays potent transforming ability as well as constitutively elevated levels of tyrosine kinase activity in the absence of exogenously added ligand. To investigate the basis for its chronic activation, we sought evidence of a ligand for gp185^{erbB-2} either in serum or produced by NIH/3T3 cells in an autocrine manner. We demonstrated that a putative ligand for gp185^{erbB-2} is not contained in serum. Chimeric molecules composed of the extracellular domain of gp185^{erbB-2} and the intracellular portion of the epidermal growth factor receptor (EGFR) did not show any transforming ability or constitutive autophosphorylation when they were expressed in NIH/3T3 cells. However, they were able to transduce a mitogenic signal when triggered by a monoclonal antibody directed against the extracellular domain of *erb*B-2. These results provide evidence against the idea that an *erb*B-2 ligand is produced by NIH/3T3 cells. Furthermore, we obtained direct evidence of the constitutive enzymatic activity of gp185^{erbB-2} by demonstrating that the *erb*B-2 kinase remained active in a chimeric configuration with the extracellular domain of the EGFR, in the absence of any detectable ligand for the EGFR. Thus, under conditions of overexpression, the normal gp185^{erbB-2} is a constitutively active kinase able to transform NIH/3T3 cells in the absence of ligand.

In another study, we demonstrated that the gp185^{erbB-2} carboxy-terminal domain exerts an enhancing effect on *erb*B-2 kinase and transforming activities. A premature termination mutant of the *erb*B-2 protein, lacking the entire carboxy-terminal domain (erbB-2^{A1050}), showed a 40-fold reduction in transforming ability and a lowered in vivo kinase activity for intracellular substrates. When the carboxy-terminal domain of *erb*B-2 was substituted for its analogous region in the EGFR (EGFR/*erb*B-2^{COOH} chimera), it conferred *erb*B-2-like properties to the EGFR, including transforming ability in the absence of EGF, elevated constitutive autokinase activity in vivo and in vitro, and constitutive ability to phosphorylate phospholipase C-γ. Conversely, a chimeric *erb*B-2 molecule bearing an EGFR carboxyterminal domain (*erb*B-2/EGFR^{COOH} chimera) showed reduced transforming and kinase activity with respect to the wild-type *erb*B-2 and was only slightly more efficient than the *erb*B-2^{A1050} mutant. Thus, we conclude that the carboxy-terminal domains of *erb*B-2 and EGFR exert different regulatory effects on receptor kinase function and biological activity. The upregulation of gp185^{erbB-2} enzymatic activity exerted by its carboxy-terminal domain can explain, at least in part, its constitutive level of kinase activity.

KGF (FGF) receptors. We reported saturable specific binding of ^{125}I-KGF to surface receptors on intact Balb/MK mouse epidermal keratinocytes. ^{125}I-KGF binding was competed efficiently by acidic FGF (aFGF) but with 20-fold lower efficiency by basic FGF (bFGF). The pattern of ^{125}I-acidic FGF binding and competition on Balb/MK keratinocytes and NIH/3T3 fibroblasts suggests that these cell types possess related but distinct FGF receptors. Scatchard analysis of ^{125}I-KGF binding suggested major and minor high affinity receptor components (K_D=400 and 25 pM, respectively) as well as a third high capacity/low affinity heparin-like component. Covalent affinity cross-linking of ^{125}I-KGF to its receptor on Balb/MK cells revealed two species of

115 and 140 kDa. KGF also stimulated the rapid tyrosine phosphorylation of a 90-kDa protein in Balb/MK cells but not in NIH/3T3 fibroblasts. Together these results indicate that BALB/MK keratinocytes possess high affinity KGF receptors to which the FGFs may also bind. However, these receptors are distinct from the receptor(s) for aFGF and bFGF on NIH/3T3 fibroblasts, which fail to interact with KGF.

An expression cloning strategy was devised to isolate the KGF receptor complementary DNA. NIH/3T3 fibroblasts, which secrete this epithelial cell-specific mitogen, were transfected with a keratinocyte expression complementary DNA library. Among several transformed foci identified, one demonstrated the acquisition of specific high-affinity KGF binding sites. The pattern of binding competition by related FGFs indicated that this receptor had high affinity for acidic FGF as well as KGF. The rescued 4.2-kilobase complementary DNA was shown to encode a predicted membrane-spanning tyrosine kinase related to but distinct from the basic FGF receptor. This expression cloning approach may be generally applicable to the isolation of genes that constitute limiting steps in mitogenic signalling pathways.

We isolated five different variants of the FGF receptor from human fibroblasts and showed that all were derived from a single genetic locus. Four of these variants encode transmembrane receptors and can be divided into two subgroups that differed from one another with respect to the number (two or three) of immunoglobulin (Ig)-like domains. Within each subgroup, one receptor differed from the other by the presence of a two-codon insertion. Thus, all of the variations among the four isoforms are localized to their ligand-binding domains. The fifth isoform encodes a molecule truncated just 3' to the first Ig-like domain and thus could be secreted from the cell. The transcripts encoding the long and short isoforms were found to be expressed in many cell types, but their relative levels of expression varied greatly depending on the cell type. These findings indicated that alternative splicing generates diverse FGF receptor isoforms in human cells.

HGF receptor. HGF is an 87-kDa protein resembling plasminogen that has been purified from human and rabbit plasma and rat platelets principally on the basis of its high affinity for heparin. Its mitogenic activity was first characterized on hepatocytes, where it was thought to participate in liver regeneration. The same factor was independently purified from human fibroblast culture medium and shown to act on melanocytes and a variety of epithelial and endothelial cell types. Preliminary investigation of the HGF mitogenic signalling mechanism revealed the tyrosine phosphorylation of a 145-kDa protein (p145) in intact target cells. Electrophoretic mobility under reduced and nonreduced conditions and immunoblot analysis suggested that p145 could be the beta subunit of c-*met* protein kinase. Covalent affinity cross-linking using [^{125}I]-labeled ligand and immunoprecipitation of the crosslinked species with anti-c-*met* protein antisera led us to conclude that the HGF receptor is the c-*met* proto-oncogene product.

III. Growth Factor Signalling Pathways

PDGF promotes the growth of oligodendrocyte type-2 astrocyte (O-2A) glial progenitor cells and allows their timely differentiation into oligodendrocytes, the CNS myelin-forming cells. We demonstrate that basic FGF is a potent mitogen for brain O-2A progenitor cells, but blocks their differentiation into oligodendrocytes. Treatment with basic FGF also influences the level of expression of PDGF receptors on O-2A progenitor cells. These cells express only the α chain PDGF receptor, and the levels of PDGF α receptors decrease as the cells differentiate. In contrast, basic FGF maintains a high level of functionally responsive PDGF α receptors in O-2A progenitors. Thus, basic FGF activates a signalling pathway that can positively regulate PDGF receptors in O-2A progenitor cells. In this way basic FGF or an FGF-like factor may modulate the production of myelin-forming cells in the CNS.

The c-*fms* proto-oncogene encodes the receptor for macrophage-colony-stimulating factor (CSF-1). Expression vectors containing either normal or oncogenic point-mutated human c-*fms* genes were transfected into interleukin 3 (IL-3)-dependent 32D cells in order to determine the effects of CSF-1 signalling in this murine clonal myeloid progenitor cell line. CSF-1 was shown to trigger proliferation in association with monocytic differentiation of the 32D-c-*fms* cells. Monocytic differentiation was reversible upon removal of CSF-1, implying that CSF-1 was required for maintenance of the monocyte phenotype but was not sufficient to induce an irrevocable commitment to differentiation. Human CSF-1 was also shown to be a potent chemoattractant for 32D-c-*fms* cells, suggesting that CSF-1 may serve to recruit monocytes from the circulation to tissue sites of inflammation or injury. Although c-*fms* did not release 32D cells from factor dependence, point-mutated c-*fms*[S301,F969] (Leu-301 → Ser, Tyr-969 → Phe) was able to abrogate their IL-3 requirement and induce tumorigenicity. Il-3-independent 32D-c-fms[301,F969] cells also displayed a mature monocyte phenotype, implying that differentiation did not interfere with progression of these cells to the malignant state. All of these findings demonstrated that a single growth factor receptor can specifically couple with multiple intracellular signalling pathways and play a critical role in modulating cell proliferation, differentiation, and migration.

IV. Inhibition of Growth Factor Signalling Pathways

Retinoblastoma (RB) gene. The product of the human RB gene is a nuclear phosphoprotein which is thought to function as a tumor suppressor. Mutations of the RB gene frequently occur in human bladder carcinoma. To investigate the significance of the functional loss of this gene in bladder cancer, an RB expression plasmid (pBARB), under control of the human β-actin promoter, was transfected into the bladder carcinoma line, HTB9, which lacks RB gene expression. Marker-selected transfectants that expressed RB protein were identified by western blotting and immunohistochemical staining using an anti-RB monoclonal antibody. In selected clones, stable RB expression persisted over several months under standard culture conditions with 10% serum. However, RB expression caused major alterations of HTB9 growth properties both in vitro and in vivo. RB+ transfectants lacked the ability to form colonies in semisolid medium and their growth rate was significantly decreased in 3% serum-containing medium. In addition, their tumorigenicity was markedly decreased. Tumors that formed in nude mice were much smaller and had a longer latency period but were indistinguishable microscopically from those produced by parental cells. Slower growing tumors were shown to be RB+, as measured by their nuclear RB protein staining and by a normal RB pattern on western blots. These findings support the concept that the RB gene acts as both a growth and tumor suppressor in bladder cancer cells.

MyoD. *Myo*D is a gene involved in the control of muscle diferentiation. We show that *Myo*D causes growth arrest when expressed in cell lines drived from tumors or transformed by different oncogenes. MyoD-induced growth inhibition was demonstrated by reduction in the efficiency of colony formation and at the single-cell level. We further showed that MyoD growth inhibition can occur in cells that are not induced to activate muscle differentiation markers. The inhibitory activity of MyoD was mapped to the 68-amino acid segment necessary and sufficient for induction of muscle differentiation, the basic helix-loop-helix motif. Mutants with alterations in the basic region of MyoD that fail to bind or do not activate a muscle-specific enhancer inhibited growth; mutants with deletions in the helix-loop-helix region failed to inhibit growth. Thus, inhibition of cell growth by MyoD seems to occur by means of a parallel pathway to the one that leads to myogenesis. We conclude that MyoD is a prototypic gene capable of functionally activating intracellular growth inhibitory pathways.

V. Oncogenes

arg. We have previously described partial genomic sequences of *arg*, a human gene related to c-*abl*, and have shown that it is expressed as a 12-kilobase transcript and is located at chromosome position 1q24-25. In this study we elucidated the complete coding sequence of *arg* by characterization of cDNA clones. Analysis of the predicted amino acid sequence of *arg* revealed that it is indeed closely related to that of c-*abl*. The two proteins are strikingly similar with regard to overall structural architecture as well as the amino acid sequences of their tyrosine kinase and *src* homologous 2 and 3 domains. In addition, *arg*, like c-*abl*, is expressed as two transcripts that result from a process of alternative splicing and encode alternative protein forms that differ only in their amino termini. The two genes define the Abelson subfamily of cytoplasmic tyrosine kinases and share a common homolog in *Drosophila*.

dbl. Proto-dbl is a human proto-oncogene whose oncogenic activation was initially detected by DNA transfection. We found a significant sequence similarity between the predicted proto-dbl product and the products of *CDC24*, a *Saccharomyces cerevisiae*, a cell division cycle gene required for correct budding and establishment of cell polarity, and *bcr*, a gene implicated in the pathogenesis of chronic myelogenous leukemia (CML). Of 925 residues of the predicted proto-*dbl* protein, a stretch of 238 residues showed 29% and 22% identity over a region of similar length of the *CDC24* and *bcr* proteins, respectively. When evolutionarily conservative substitutions were taken into account, the similarities were 68.8% and 71.6% for proto-*dbl*/*CDC24* and proto-*dbl*/*bcr* gene products, respectively. Moreover, all three sequences were predicted to be markedly hydrophilic over this region. Very small deletions within the conserved region completely abolished transforming activity of *dbl*, while extensive deletion outside of this region had no effect. Even substitutions over a small stretch of close similarity with the other proteins substantially impaired transforming activity. Cells transformed by the *dbl* oncogene, like *cdc24* mutants arrested at the nonpermissive temperature, form multinucleate cells. Our findings indicate that the conserved region is an essential domain that may reflect important functional similarities among these otherwise highly divergent molecules.

VI. Metastatic Phenotype

The role of oncogenes in the acquisition of invasive and metastatic capabilitites is controversial. Interactions with basement membranes are critical in the process of tumor invasion and metastasis. We compared the ability of 3T3 cells transformed by oncogenes involved in various stages of signal transduction to invade a reconstituted basement membrane *in vitro* and to grow in a three dimensional basement membrane gel (matrigel). Cell lines transformed by various oncogenes and oncoviruses (v-*sis* [a growth factor], v-*erb*-B [a truncated EGF receptor], Moloney sarcoma virus [v-*mos*, a protein kinase homologue], mutated c-*ras* oncogenes [G protein homologues], FBJ virus [v-*fos*, a nuclear protein]) were investigated. All transformed cell lines were able to invade in the chemoinvasion assay, where a layer of matrigel is coated onto chemotaxis filters. FBJ/3T3 were the least invasive and SSV/3T3 the most invasive. Control 3T3 cells could not cross the matrigel barrier. All transformed cells grew on matrigel forming invasive, branching colonies, whereas control 3T3 were unable to grow in matrigel. Cells transfected with the v-*erb*-B gene grew as multilayers inside matrigel. Invasiveness and growth on matrigel were accompanied by a high chemotactic response to laminin (LN) in all transformed lines. These results suggest that invasion and growth on matrigel, together with migration to LN, are induced by a large spectrum of oncogenes. When 3T3 cells were transfected with v-*sis* oncogene under the transcriptional control of the metallothionein (MMT) promoter and exposed to Zn++, their *in vitro* invasiveness was

specifically increased by around threefold. These findings provide further evidence supporting a direct role of the v-*sis* oncogene in the invasive phenotype.

VII. New Technology

We have developed a high efficiency cDNA cloning system which can direct the orientation of inserts in λ-plasmid composite vectors with large cloning capacities. Cleavage of the vector DNA by *Sfi*I creates two different nonsymmetrical 3' extensions at the ends of the vector arms. Using a linker-primer and an adaptor, cDNA is prepared so it has two different sticky ends which can be ligated to those of the vector arms. When the cDNA and the vector arms are mixed, both the molecules can assemble without self-circularization due to base-pairing specificity. Ligation of the cDNA-vector mixture produces the concatemers from which phage clones carrying a single cDNA insert in the desired orientation can be formed very efficiently by in vitro packaging. This system provides: (1) high cloning efficiency [10^7-10^8 clones/μg poly(A)$^+$RNA], (2) low background (more than 90% of the clones contain inserts), (3) directional insertion of cDNA fragments into the vectors, (4) presence of a single insert in each clone, (5) accommodation of long inserts (up to 10 kb), (6) a mechanism for rescue of the plasmid part from the λ genome, and (7) a straightforward protocol for library preparations. Screenings of cDNA libraries constructed by this method demonstrated that cDNAs of up to 6.4 kb, containing complete coding sequences, could be isolated at high efficiency. Thus, this cloning system should be useful for the isolation of cDNAs of relatively long transcripts, present even at low abundance in cells.

An efficient unidirectional phagemid cDNA expression cloning system for mammalian transforming genes was developed. By means of this system, a cDNA library was constructed from an NIH/3T3 transformant induced by mouse hepatocellular carcinoma DNA. Transfection of NIH/3T3 cells by the DNA library led to the detection of several transformed foci, from which identical plasmids with transforming ability could be rescued. Structure and sequence analysis of the cDNA clones revealed that the oncogene was created by recombinational events involving an unknown gene and the mouse homologue of the B-*raf* proto-oncogene. This rearrangement led to amino terminal truncation of the B-*raf* gene product. This genetic rearrangement was also detected in two primary transformants independently induced by the original tumor DNA, implying that the recombination event generating the oncogene occurred *in vivo* within the tumor rather than *in vitro* during DNA transfection or cDNA library construction. This phenotypic expression cloning strategy should have wide applicability to the isolation of cDNAs of biologic interest.

Procedure for carrier detection in ataxia-telangiectasia (A-T). An assay for A-T heterozygotes, i.e., healthy carriers of the A-T gene(s) requiring only a small sample (3.5 ml) of peripheral blood, was developed. Frequencies of chromatid aberrations in phytohemagglutinin-stimulated blood lymphocytes collected by demecolcine from 0.5 hour to 1.5 hours after X-irradiation with 58 roentgens were twofold to threefold higher in A-T heterozygotes than in clinically normal controls and twofold to threefold higher in A-T patients (homozygotes) than in A-T gene carriers. The persistence of chromatid breaks and gaps in lymphocytes following radiation-induced DNA damage during G_2 suggests a deficiency or deficiencies in DNA repair that may be the defect at the molecular level that results in the enhanced radiosensitivity and cancer proneness characterizing A-T gene carriers and patients. The assay should facilitate chromosome analysis and prove useful for detecting unaffected, cancer-prone A-T carriers in known A-T families. Furthermore, this G_2 assay may be applicable to screening of the general population for individuals genetically predisposed to cancer and for detecting other healthy heterozygous carriers of cancer-prone genes.

Chromatid radiosensitivity in relation to DNA repair and cancer susceptibility. Skin fibroblasts or peripheral blood lymphocytes from individuals with genetic disorders predisposing to cancer or with familial cancer show a higher than normal incidence of chromatid breaks and gaps when irradiated during the G_2 phase of the cell cycle. The incidence is also higher in human tumor cells and cells transformed in culture than in normal controls. This enhanced G_2 chromatid radiosensitivity is thus associated with both genetic susceptibility to cancer and neoplastic transformation. It is observed only in cells harvested at least 1.5 hours after irradiation and appears to result from a deficiency(ies) in DNA repair during the G_2 phase. Results show that this assay can identify individuals with genes for cancer proneness in families with any one of three cancer-prone genetic diseases (A-T, dysplastic nevus syndrome (DNS) and xeroderma pigmentosum) or hereditary/familial cancers.

Abnormal responses to X-irradiation during the G_2 phase have also been observed in skin fibroblasts and/or stimulated peripheral blood lymphocytes from individuals with cancer-prone diseases (Bloom's syndrome, Fanconi's anemia, familial polyposis, Gardner's syndrome) and hereditary/familial cancers (retinoblastoma, Wilms' tumor, nevoid basal cell carcinoma, breast cancer, acute myelogenous leukemia, osteosarcoma, liposarcoma, medullosarcoma).

VIII. Virus Studies

Epstein-Barr Virus. The association of EBV with nasopharyngeal carcinoma (NPC) has been known for some time but the precise role of EBV in this cancer is poorly understood, due partly to the lack of an in vitro system for studying NPC cells and the effect of EBV on epithelial cells. Biopsies of NPC tumors have revealed expression of the EBV latent membrane protein in 65% of cases, suggesting that in at least some NPC tumors LMP may contribute to cell transformation. Transfection of an immortalized, nontumorigenic keratinocyte cell line (RHEK-1) with the latent membrane protein gene causes a striking morphological transformation. The originally flat, polygonal colonies change to bundles of spindle-shaped cells that form multilayer foci and cytokeratin expression is down-regulated. Results suggest that latent membrane protein expression may be an important causal factor in the development of NPC.

CR2, a membrane glycoprotein, is one of a number of cell surface proteins which bind activation and processing fragments of the complement system. CR2, which is found on normal B lymphocytes, follicular dendritic cells in lymphoid organs, and epithelial cells, interacts preferentially with C4dg, the terminal activation/processing fragment of the third complement component. Attachment of C3dg to CR2 brings complement activators bearing covalently bound C3dg into direct membrane contact with CR2-bearing cells. Epstein-Barr virus, a human herpesvirus, also binds to CR2 on B lymphocytes. Attachment of EBV is followed by infection. CR2 was purified and the binding properties of its ligands analyzed. Monoclonal antibodies were developed and used to probe the structural correlates of CR2 functions. CR2 has been molecularly cloned and its primary amino acid sequence deduced. Our data indicate that it shares characteristic structural features with a number of other complement and noncomplement cell membrane and plasma proteins. Several of the complement-associated proteins in this family possess regulatory functions; they are encoded by linked genes which have been localized to band q32 on chromosome 1. CR2 was expressed in primate and rodent cells by transfection of cDNA in antigenically and functionally intact form. It has also been expressed in soluble form and its structure, electron microscopic appearance and binding characteristics analyzed in detail.

Lentiviruses.

Equine infectious anemia virus (EIAV). A full-length molecular clone of EIAV was isolated from a persistently infected canine fetal thymus cell line, Cf2Th. The clone, designated CL22, upon transfection of equine dermis cells, yielded infectious EIAV particles (CL22-V) that replicated in vitro in both Cf2Th cells and an equine dermis cell strain. Horses infected with CL22-V developed an antibody response to viral proteins and possessed viral DNA in peripheral blood mononuclear cells, as determined by polymerase chain reaction assays. In addition, horses infected with CL22-V became persistently infected and were capable of transmitting the infection by transfer of whole blood to uninfected horses. However, CL22-V, like the parental canine cell-adapted virus, did not cause clinical signs in infected horses. Reverse transcriptase assays of CL22-V and virulent EIAV-infected equine mononuclear cell cultures indicated that the lack of virulence of CL22-V was not due to an inability to infect and replicate in equine mononuclear cells in vitro.

The pattern of expression of the EIAV genome in a persistently infected canine line was determined. Five EIAV-specific transcripts (8.2, 5.0, 4.0, 2, and 1.8 kb) were detected by using subgenomic restriction enzyme fragments of EIAV DNA and EIAV-specific oligonucleotides as probes. The 8.2-kb mRNA could be shown to represent viral genomic RNA, whereas the smaller transcripts were generated by splicing events. Evidence was obtained that indicated that each subgenomic RNA species shared a common 5'-splice donor. The 5.0-kb mRNA was found to be expressed at relatively low levels, was difficult to detect consistently, and appeared to be generated by a single splicing event which linked the 5' exon to the 3' region of *pol*. The 4.0-kb transcript was concluded to be *env* mRNA on the basis of its hybridization pattern with the various probes and its abundance. The 2-kb species was found to be multiply spliced and was encoded by sequences derived from *orf2* but was not detected by probes representing 3'-*env*/3'-*orf* sequences. The 1.8-kb species was shown to consist of sequences representing *orf1*, part of *orf2*, and the 3'-*orf*/*env* and may represent the message for the EIAV *trans*-activator gene.

The EIAV *trans*-activator gene, *tat*, stimulates gene expression directed by the viral long terminal repeat (LTR). This function has been previously shown by us and others to be encoded by sequences within the middle region of the EIAV genome in which two short open reading frames, S1 and S2, reside. By using in vitro mutagenesis, we showed that disruption of S1, but not S2, completely abolished *trans*-activation. Addition of oligonucleotides complementary to S1 to cells transfected with a *tat* expression vector resulted in inhibition of *trans*-activation. EIAV cDNAs were isolated from a library of EIAV-infected cells constructed by using a eukaryotic expression vector. One cDNA clone which contained S1 sequences was able to *trans*-activate the EIAV LTR. Sequence analysis of this cDNA clone revealed that, in addition to S1, two other open reading frames were present. The cDNA still retained its activity when the latter two sequences were deleted.

The EIAV *tat* gene product, Tat, was shown to be encoded by at least three species of mRNA which differed in their ability to *trans*-activate the EIAV LTR upon expression in canine cells. The most active cDNA was monocistronic, consisting of three exons. The most abundant cDNA in the library contained four exons and was identical to a polycistronic transcript which contains open frames for Tat, putative Rev and truncated transmembrane proteins. Products consistent in size to those predicted for these latter two proteins could be detected in in vitro translation experiments. The third Tat message, another four-exon form, also potentially encodes an amino terminally truncated transmembrane protein open reading frame. In vitro mutagenesis experiments and analysis of subgenomic and partial cDNA clones confirmed and extended previous findings that S1 sequences are essential for transformation and that Tat translation initiates at a non-AUG codon in either the full length Tat message or in the genomic S1 open reading frame. The tat protein (8 kDa) was

detected in cells transfected with a Tat cDNA construct and in canine cells persistently infected with EIAV. The Tat activity of polycistronic mRNAs was lower than that of the monocistronic form, suggesting that the expression of the EIAV *trans*-activator may be subject to several levels of posttranscriptional control.

Caprine arthritis-encephalitis virus (CAEV). The pattern of expression of the CAEV genome in acutely infected tahr lung cells was found to be complex and temporally regulated. Employing northern analysis, five CAEV-specific transcripts, 9.0-, 6.5-, 5.0-, 2.5- and 1.4-kb, were detected. Nucleotide sequence analysis established the genetic structure of two species of cDNA, isolated from a library of CAEV-infected tahr cells, and suggested that they represent *rev*-like transcripts. One of these cDNA species was composed of three exons, the leader, an exon derived from the 5' region of *env*, and an exon which spanned the 3' *orf*. The second cDNA species consisted of four exons, three of which were identical to those of the former species. The additional exon (the third) was located at the 3' end of *pol*. These transcripts could potentially encode three proteins, a Rev-like protein, which is a fusion of 38 amino acids derived from the N-terminus of *env* and 91 residues from the 3' *orf*; a truncated form of the *env* transmembrane protein, and a novel protein, designated X, composed of 73 amino acids. Thus, CAEV, like other lentiviruses, displays a complex pattern of gene expression, characterized by alternative splicing and the production of potentially polycistronic transcripts.

IX. Cell Immortalization and Transformation

Immortalization of Epithelial Cell Line by Adenovirus 12 (ad 12)-SV40 Infection. An immortalized cell line was created from a primary culture of bronchial epithelia isolated from a patient with cystic fibrosis (CF). The culture was transformed with a hybrid virus, ad 12-SV40, that has been used successfully on a number of different epithelial tissues. The transformed bronchial epithelial cells have the following characteristics: (1) cAMP is stimulated by beta adrenergic agonists, (2) outwardly rectifying Cl^- channels are present on the apical cell membrane. These channels can be activated by depolarizing voltages but not by protein kinase A or C, (3) keratin is present by immunofluorescence, and this is consistent with the epithelial origin of the cells, (4) the SV40 large T antigen is present as demonstrated by immunofluorescence, (5) multiple karyotype analyses show modal chromosome number to be 80-90. There are an average of four chromosomes #7 per cell, and (6) the Phe508 deletion in the gene coding for CFTR is present on at least one chromosome. The cells can be grown in multiple passages, contain the abnormal regulation of the secretory Cl^- channel, and should be an appropriate substrate for studies of the mutant CF transmembrane regulatory (CFTR) protein and its interaction with the Cl^- channel.

Multistep Nature of In Vitro Epithelial Cell Carcinogenesis. In keeping with the multistep development of human cancer in vivo, a stepwise approach to neoplastic transformation in vitro presents a reasonable strategy. We have recently developed an in vitro multistep model suitable for the study of human epithelial cell carcinogenesis. Upon infection with ad 12-SV40 hybrid virus, primary human epidermal keratinocytes acquired an indefinite life span in culture but did not undergo malignant conversion. Subsequent addition of Kirsten murine sarcoma virus and human *ras* oncogene or chemical carcinogens (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or 4-nitroquinoline 1-oxide) to these cells induced morphological alterations and the acquisition of neoplastic properties. Subsequently, it was found that this line could be transformed neoplastically by a variety of retrovirus-containing H-*ras*, *bas*, *fes*, *fms*, *erb*B, and *src* oncogenes. In addition, we found that the immortalized human epidermal keratinocyte (RHEK-1) line can be transformed neoplastically by exposure to ionizing radiation. Thus, this in vitro system may be useful in studying the interaction of a variety of carcinogenic agents and human epithelial cells. These findings demonstrate the malignant transformation of human primary

epithelial cells in culture by the combined action of viruses, oncogenes, chemical carcinogens or x-ray irradiation and support a multistep process for neoplastic conversion.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04930-20 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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. H. Pierce	Research Microbiologist	LCMB	NCI
	J. S. Rhim	Research Microbiologist	LCMB	NCI
	T. Fleming	Senior Staff Fellow	LCMB	NCI
	A. Eva	Visiting Scientist	LCMB	NCI
	M. H. Kraus	Visiting Scientist	LCMB	NCI

COOPERATING UNITS *(if any)*

State of California Department of Health Services, Berkeley, CA (J. Riggs, R. Emmons and K. Walen)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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 purpose of this project is to demonstrate the effects of selected oncogenes in appropriate rodent hosts. In addition, the mechanisms of malignant transformation are analyzed by in vitro and in vivo correlations. Using Type C viral vectors, the oncogenes erbB-2, erbB-3, HGF and IL-4 are on test in nude athymic and euthymic mouse cohorts. In transplantation experiments (allografts and xenografts), comparisons in tumorigenicity between erbB-2 transfectants and cancers naturally expressing the oncogene have been done; similarities and differences have been determined. Other oncogenes tested in transplant experiments were HGF, erbB-3, INT4, v-abl, arg, PDGF, ect2 and Epstein-Barr virus (EBV) cDNA. Three new spontaneous human cancers (kidney carcinoma, lung carcinoma and ocular melanoma) have been transplanted and are being analyzed.

PROJECT DESCRIPTION

Names, 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. H. Pierce	Research Microbiologist	LCMB	NCI
J. S. Rhim	Research Microbiologist	LCMB	NCI
T. Fleming	Senior Staff Fellow	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
M. H. Kraus	Visiting Scientist	LCMB	NCI
G. Kruh	Senior Staff Fellow	LCMB	NCI

Objectives:

1. Explore in vivo effects of oncogenes, growth factors, receptors, viruses and other agents currently being investigated at LCMB as potential tumor inducers or promoters.

2. Study mechanisms of neoplastic transformation by correlating intrinsic changes in selected cells and cultures altered by above agents with their ability to produce malignant xenografts.

3. Participate in the acquisition, propagation, transplantation and search for oncogenes among human cancers from Washington, DC area and California hospitals.

Methods Employed:

Specified oncogenes, selected in consultation with other LCMB investigators and incorporated into viral vectors, are tested for pathogenicity (particularly carcinogenicity) by inoculation into normal (euthymic) and immunodeficient (athymic nude) siblings by several routes (intraperitoneal, subcutaneous, intramuscular, intracerebral), usually at birth. A recently initiated schedule involves mating athymic nude mice chronically infected with specified oncogenic viral constructs and subsequently sampling and monitoring their offspring for tumors and other effects of early embryonic infection.

In parallel with the viral experiments above, potential oncogenes are tested by transplantation of cultures or tissues in which the cDNAs are integrated into the cellular genomes. Host recipients are mice of three levels of immunodeficiency: (a) nu/nu, T-lymphocyte deficient; (b) NII nu/nu, T- and B-lymphocyte deficient; (c) NIII nu/nu, T-, B- and LAK-lymphocyte deficient.

Special studies on oncogenic transformation of primate cells and neoplastic behavior of spontaneously expressed oncogenes in human malignancies (in collaboration with Drs. Aaronson, Pierce, Rhim, Kraus, Fleming and Kruh) utilize similar techniques.

Major Findings:

Experiments with viral recombinants containing specified oncogenes included the following:

1. A continuation of monitoring long-range neoplasia in *erb*B-2 recipients and their offspring.

2. Neoplasia in sucklings newly inoculated with *erb*B-3 and HGF.

3. Immune response following inoculation of newborn mice with IL-4.

Except for the previously reported induction of vascular and cardiac neoplasms by *erb*B-2, these oncogenes have not exhibited highly prevalent in vivo effects.

Additional tests on normal breast epithelium (B5) transfected with the oncogene *erb*B-2 confirmed the phenomenon of simultaneous increase in tumorigenicity and oncogene expression following a single in vivo graft passage. Similar experiments were conducted with four spontaneous human breast cancer tissue cultures with naturally elevated levels of *erb*B-2 expression. These cultures, similar to the previously reported transfectants, readily transplanted to suckling, but not to weanling, nu/nu mice. In contrast to the transfectants, however, the spontaneous cancer-derived cultures did not become more tumorigenic following in vivo graft passage and did not exhibit additional augmentation in *erb*B-2 expression.

Two other oncogenes, HGF and *erb*B-3 were tested in cultures of the B5 epithelium. Transplants produced high levels of appropriate proteins but did not prove to be turmorigenic for nude athymic mice. A third oncogene, INT-4, is under current investigation using the B5 system.

The effect of selected oncogenes on the nontumorigenic hematopoietic line 32D was the subject of additional tests, which confirmed the neoplastic transformation induced by v-*abl* DNA, but showed that transfection with a closely related gene, *arg*, did not induce neoplastic transformantion.

As previously reported, the introduction of the B chain of the oncogene PDGF into fibroblast cultures results in much more malignant transformants than if the A chain is used. We have subsequently tested the theory that the difference may be due to lack of sufficient receptors for the A chain. It was shown that transfection with the A chain cDNA with the appropriate receptor α increases the tumorigenicity significantly over the A chain growth factor alone, but not to the level of the highly neoplastic B chain transfectants.

A recently isolated oncogene, *ect2*, was shown to be effective in transforming normal fibroblasts into sarcomagenic tumor lines.

Tests on immortalized nonhuman keratinocytes transfected with Epstein-Barr virus (EBV) DNA have confirmed that such transfections result in malignant, carcinoma graft-inducing tissue cultures.

Three newly established human cancer lines with unusual properties are under analysis:

1. HA2419, a kidney carcinoma, grows slowly, exhibits contact inhibition, and senesces early in vitro. In contrast, it is highly tumorigenic in vivo by graft for nu/nu mice and can be readily passaged serially in these animals.

2. BATN 73, a lung carcinoma, does not survive in vitro in any combination of media tested, but is transplantable in nu/nu mice where it has replicated serially for 10 graft passages spanning 24 months.

3. OM431, an ocular melanoma, was progressively neoplastic. This cell culture is unusually susceptible to cDNA transfection and therefore promising in the search for tumor suppressor genes.

Publications:

Pierce JH, Arnstein P, Di Marco E, Artrip J, Kraus MH, Lonardo F, Di Fiore PP, Aaronson SA. Oncogenic potential of *erb*B-2 in human mammary epithelial cells. Oncogene (In Press)

Rhim JS, Arnstein P, Fahraeus R, Rymo L, Klein G, Gradoville L, Miller G, Wang F, Kieff E. Interaction of EBV genes with human epithelial cells. In: Ablashi DV, Hwang A, Pagano J, Pearson G, Yang C, eds. Epstein-Barr virus and human disease. Clifton NJ: Humana Press (In Press)

Rubin AL, Arnstein P, Rubin H. Physiological induction and reversal of focus formation and tumorigenicity in NIH/3T3 cells. Proc Natl Acad Sci USA (In Press)

Zeithin PL, Lu L, Rhim JS, Cutting G, Stetten G, Kieffer KA, Craig R, Guggino WB. A cystic fibrosis bronchial epithelial cell line: immortalization by AD12-SV_{40} infection. Am J Resp Cell Mol Biol 1991;4:313-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04940-24 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

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:	J. S. Rubin	Senior Staff Fellow	LCMB	NCI
	T. Miki	Visiting Scientist	LCMB	NCI
	W. J. La Rochelle	Staff Fellow	LCMB	NCI
	M. A. Heidaran	Staff Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

Duke U. Med. Ctr. (D. Iglehart); CA Biotechnology (J. Abraham, J. Fiddes); U. Ma. (J. Greenberger); Baylor College Med. (W. Benedict); U. NC (B. Weissman); Hospital Gen. Gregorio Maranon, Madrid, Spain (J. Moscat)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	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.)*

Some of the highlights of the past year include: (1) Subtle conformational changes in a small v-sis/platelet-derived growth factor (PDGF)-protein domain abrogate PDGF receptor interaction and transforming activity; (2) autocrine PDGF stimulation contributes significantly to proliferation of some human tumors and agents which interfere with ligand-receptor interactions at the cell surface can significantly intervene in this process; (3) expression cDNA cloning of the keratinocyte growth factor (KGF) receptor by creation of a transforming autocrine loop; (4) broad-spectrum, heparin-binding human lung fibroblast heparin-derived mitogen is a variation of hepatocyte growth factor (HGF); (5) the HGF receptor is the c-met proto-oncogene product; (6) chimeric α and β PDGF receptors define three immunoglobulin-like domains of the α PDGF receptor that determine PDGF-AA binding specificity; (7) although the tyrosine kinase domains of PDGF and colony-stimulating factor-1 (CSF-1) are very similar, their kinase insert domains are highly unrelated but play very similar roles in the known signalling functions of PDGF and CSF-1 receptors; (8) the carboxy terminal domains of erbB-2 and epidermal growth factor (EGF) receptor exert different regulatory effects on intrinsic tyrosine kinase function and transforming activity; (9) CSF-1 induces proliferation, chemotaxis, and reversible monocytic differentiation in myeloid progenitor cells transfected with the human c-fms/CSF-1 receptor cDNA; (10) MyoD, a gene involved in the control of muscle differentiation, induces growth arrest independent of differentiation in normal and transformed cells; (11) development of a highly efficient cloning system for mammalian transforming genes; and (12) the retinoblastoma (RB) gene functions as a growth and tumor suppressor in human bladder carcinoma cells.

PROJECT DESCRIPTION

Names, 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
J. H. Pierce	Research Microbiologist	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
J. S. Rubin	Senior Staff Fellow	LCMB	NCI
M. H. Kraus	Visiting Scientist	LCMB	NCI
P. Arnstein	Veterinary Director	LCMB	NCI
P. P. Di Fiore	Visiting Scientist	LCMB	NCI
T. Miki	Visiting Scientist	LCMB	NCI
G. D. Kruh	Senior Staff Fellow	LCMB	NCI
T. P. Fleming	Senior Staff Fellow	LCMB	NCI
M. A. Heidaran	Staff Fellow	LCMB	NCI
R. Jensen	Biotechnology Fellow	LCMB	NCI
W. J. LaRochelle	Staff Fellow	LCMB	NCI
J.-C. Yu	IRTA Fellow	LCMB	NCI
M. Chedid	Visiting Fellow	LCMB	NCI
E. Gak	Visiting Fellow	LCMB	NCI

Objectives:

1. To identify oncogenes of human tumors and investigate their mechanisms of action.

2. To identify novel growth factors and define growth factor signalling pathways in normal and malignant cells.

Methods Employed:

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

Major Findings:

1. Growth Factors

Platelet-derived growth factor/*sis*. Human platelet-derived growth factor (PDGF) is an important connective tissue cell mitogen comprised of two related chains encoded by distinct genes. The B chain is the homologue of the v-*sis* oncogene product. Properties that distinguish these ligands include greater transforming potency of the B chain and more efficient secretion of the A chain. By a strategy involving the generation of PDGF A and B chimeras, we mapped these properties to distinct domains of the respective molecules. We further demonstrated that increased transforming efficiency segregated with the ability to activate both α and β PDGF receptors. These findings genetically map PDGF amino acid residues 105 to 144 as responsible for subtle conformational alterations critical to B PDGF receptor interaction and provide a mechanistic basis for the greater transforming potency of the PDGF B chain.

Deletion scanning mutagenesis within the transforming region of the v-*sis* oncogene was used to dissect structure-function relationships. Mutations affecting codons within a domain encoding amino acids 136 through 148 had no effect upon homodimer

formation or recognition by antisera which detect determinants dependent upon native intrachain disulfide linkages, yet the same mutations completely abolished transforming activity. A PDGF B monoclonal antibody that prevents its interaction with PDGF receptors recognized v-*sis*, A142 (deletion of codon A142) and A148 but not A136, A137, or A139 mutants. These findings mapped the epitope recognized by this monoclonal antibody to include amino acid residues 136 to 139. Furthermore, mutations in the codon 136 to 148 domain caused markedly impaired ability to induce PDGF receptor tyrosine phosphorylation. Thus, subtle conformational alterations in this small domain were shown to critically affect PDGF receptor recognition and/or functional activation.

Genetic alterations that constitutively activate crictical genes in mitogenic signalling pathways have been causally implicated in the neoplastic process. Intervention with the pathologic expression of this important subset of genes might be most readily approached in the case of growth factors, if functional activation of their receptors were confined to a cell surface location. Recent studies with the v-*sis* oncogene, whose human homologue encodes the PDGF B chain, have demonstratated that autocrine activation of PDGFRs occurs internally. However, activated receptors must achieve a cell surface location in order to functionally couple with intracellular mitogenic signalling pathways. Thus, agents such as neutralizing PDGF antibody or suramin, which specifically interfere with ligand-receptor interactions at the cell surface, are capable of partially or completely reverting the v-*sis*/PDGF B transformed phenotype in cell culture. We sought to demonstrate a functional autocrine loop involving PDGF and its receptors in human malignancies. In tumor cells expressing PDGF ligand(s) and receptor(s), immunoblot analysis established tyrosine phosphorylation of PDGF receptors (PDGFRs) in the absence of any exogenous ligand, implying chronic receptor activation. Exposure to suramin resulted in diminished receptor autophosphorylation and/or upregulation of receptor protein. In a subset of such tumor lines, there was marked reduction in DNA synthesis in response to suramin or PDGF neutralizing antiserum. These findings demonstrate that autocrine PDGF stimulation contributes significantly to proliferation of some human tumors and that agents which interfere with ligand-receptor interactions at the cell surface can significantly intervene in this process.

Keratinocyte growth factor (KGF). Human KGF is secreted by normal stromal fibroblasts. We demonstrated that KGF is as potent as EGF in stimulating proliferation of primary or secondary human keratinocytes in tissue culture. Exposure of KGF- or EGF-stimulated keratinocytes to 1.0 mM calcium, an inducer of differentiation, led to cessation of cell growth. However, immunologic analysis of early and late markers of terminal differentiation, K1 and filaggrin, respectively, revealed striking differences in keratinocytes propagated in the presence of these growth factors. With KGF, the differentiation response was associated with expression of both markers, whereas their appearance was retarded or blocked by EGF. TGFα, which also interacts with the EGF receptor, gave a similar response to that observed with EGF. These findings functionally distinguish KGF from the EGF family and support the role of KGF in the normal proliferation and differentiation of human epithelial cells.

EGF/transforming growth factor α (TGFα). Alterations in the EGF/TGFα-responsive mitogenic pathway are frequently detected in malignancies. In particular, the EGF-receptor (EGFR) molecule has been found overexpressed in a number of human tumors, and TGFα is produced by a large array of tumor cells. Gene transfer experiments have previously demonstrated that expression of either TGFα or EGFR alone is not sufficient to induce the transformed phenotype in NIH/3T3 cells.

Alterations affecting the EGF/TGFα-responsive mitogenic pathway are frequently detected in malignancies. In particular, the epidermal growth factor receptor has been found overexpressed in a number of human tumors. Production and secretion of transforming growth factor type α has also been shown in several tumor cells but not in their normal counterparts. We described the establishment of *in vitro* model systems to study the transforming potential of these molecules and summarized our current understanding of the mechanisms involved in transformation by genes encoding a growth factor and a growth factor receptor.

EGF and TGFα bind to a common cell surface receptor that mediates their diverse biological activities. NIH/3T3 fibroblasts, transfected with either full-length EGF precursor (preproEGF) or proTGFα cDNA, displayed distinct patterns of growth in culture. PreproEGF induced focal transformation, and transfectants grew in a chemically defined medium (CDM) at low cell density in the absence of added EGF. In contrast, TGFα failed to cause focal transformation, and transfectants grew in CDM in the absence of added growth factors only when seeded at high cell density. The 53-amino acid EGF portion of the preproEGF translation product was essential for its effects. These results indicate that constitutive expression of preproEGF is sufficient to establish autocrine growth of NIH/3T3 expressing low levels of EGF receptors. At high cell density, where paracrine as well as autocrine effects of these growth factors would be evident, TGFα transfectants displayed at least as high or higher levels of EGF receptor (EGFR) tyrosine phosphorylation than preproEGF transfectants. Since quantitative levels of ligand expression did not account for differences in their transforming properties, preproEGF must be more efficient than proTGFα in binding and/or activating EGF receptors in an autocrine manner.

Hepatocyte growth factor (HGF). A heparin-binding mitogen was isolated from conditioned medium of human embryonic lung fibroblasts. It exhibited broad target-cell specificity whose pattern was distinct from that of any known growth factor. It rapidly stimulated tyrosine phosphorylation of a 145-kDa protein in responsive cells, suggesting that its signalling pathways involved activation of a tyrosine kinase. Purification identified a major polypeptide with an apparent molecular mass of 87 kDa under reducing conditions. Partial amino acid sequence analysis and cDNA cloning revealed that it was a variant of HGF, a mitogen thought to be specific for hepatic cells and structurally related to plasminogen. Recombinant expression of the cDNA in COS-1 cells established that it encoded the purified growth factor. Its site of synthesis and spectrum of targets imply that this growth factor may play an important role as a paracrine mediator of the proliferation of melanocytes and endothelial cells, as well as cells of epithelial origin.

II. Growth Factor Receptors

PDGF. Binding of platelet-derived growth factor (PDGF) to its cell surface receptors stimulates a variety of biochemical and biological responses. Two receptor gene products (designated α and β) have been identified, and the different binding affinities of various PDGF isoforms for these receptors are prime determinants of the spectrum of responses observed. The β receptor binds PDGF-BB, but not PDGF-AA, while the α receptor binds PDGF-AA and PDGF-BB. We utilized these different ligand binding specificities to investigate the PDGF-AA binding site in the human αPDGF receptor by constructing chimeric molecules between the human αPDGF and βPDGF receptors. Our results demonstrate that amino acids 1-340 of the αPDGF receptor comprise the region that confers PDGF-AA binding specificity. This region corresponds to immunoglobulin-like subdomains 1, 2, and 3 of its external domain.

The tyrosine kinase domains of PDGF and colony-stimulating factor-1 (CSF-1)/c-*fms* receptors are interrupted by kinase inserts (ki) which vary in length and amino acid sequence. To define the role of the ki in the human αPDGF receptor (αPDGFR), we generated deletion mutants, designated αRAki-1 and αRAki-2, which lacked 80 (710 to 789) and 95 (695 to 789) amino acids of the 104-amino-acid ki region, respectively. Their functional characteristics were compared with those of the wild-type αPDGFR following introduction into a naive hematopoietic cell line, 32D. Biochemical responses, including PDGF-stimulated PDGFR tyrosine phosphorylation, phosphatidylinositol (PI) turnover, and receptor-associated PI-3 kinase activity, were differentially impaired by the deletions. Despite a lack of any detectable receptor-associated PI-3 kinase activity, 32D cells expressing αRAki-1 showed only partially impaired chemotactic and mitogenic responses and were capable of sustained proliferation in vitro and in vivo under conditions of autocrine stimulation by the c-*sis* product. 32D transfectants expressing the larger ki deletion (αRAki-2) showed markedly decreased or abolished biochemical and biological responses. However, insertion of the highly unrelated smaller c-*fms* (685 to 750) ki domain into αRAki-2 restored each of these activities to wild-type αPDGFR levels. Since the CSF-1R does not normally induce PI turnover, the ability of the c-*fms* ki domain to reconstitute PI turnover in the αRAki-2 transfectant provides evidence that the ki domain of the αPDGFR does not directly couple with this pathway. Taken together, all of these findings imply that their ki domains have evolved to play very similar roles in the known signalling functions of PDGF and CSF-1 receptors.

erbB-2. The *erb*B-2 gene product, gp185^{erbB-2}, displays a potent transforming effect when overexpressed in NIH/3T3 cells. In addition, it possesses constitutively high levels of tyrosine kinase activity in the absence of exogenously added ligand. We demonstrated that its carboxy-terminal domain exerts an enhancing effect on *erb*B-2 kinase and transforming activities. A premature termination mutant of the *erb*B-2 protein lacking the entire carboxy-terminal domain (*erb*B-2$^{\Delta 1050}$) showed a 40-fold reduction in transforming ability and a lowered in vivo kinase activity for intracellular substrates. When the carboxy-terminal domain of *erb*B-2 was substituted for its analogous region in the EGFR (EGFR/*erb*B-2^{COOH} chimera), it conferred *erb*B-2-like properties of the EGFR, including transforming ability in the absence of EGF, elevated constitutive autokinase activity in vivo and in vitro, and constitutive ability to phosphorylate PLC-γ. Conversely, a chimeric *erb*B-2 molecule bearing an EGFR carboxy-terminal domain (*erb*B-2/EGFR^{COOH} chimera) showed reduced transforming and kinase activity with respect to the wild type *erb*B-2 and was only slightly more efficient than the *erb*B-2$^{\Delta 1050}$ mutant. Thus, we concluded that the carboxy-terminal domains of *erb*B-2 and EGFR exert different regulatory effects on receptor kinase function and biological activity. The upregulation of gp 185^{erbB-2} enzymatic activity exerted by its carboxy-terminal domain can explain, at least in part, its constitutive level of kinase activity.

Overexpression of the *erb*B-2/*neu* gene is frequently detected in human cancers. When overexpressed in NIH/3T3 cells, the normal *erb*B-2 product, gp185^{erbB-2}, displays potent transforming ability as well as constitutively elevated levels of tyrosine kinase activity in the absence of exogenously added ligand. To investigate the basis for its chronic activation, we sought evidence of a ligand for gp185^{erbB-2} either in serum or produced by NIH/3T3 cells in an autocrine manner. We demonstrated that a putative ligand for gp185^{erbB-2} is not contained in serum. Chimeric molecules composed of the extracellular domain of gp185^{erbB-2} and the intracellular portion of the epidermal growth factor receptor (EGFR) did not show any transforming ability or constitutive autophosphorylation when they were expressed in NIH/3T3 cells. However, they were able to transduce a mitogenic signal when triggered by a monoclonal antibody directed against the extracellular domain of *erb*B-2. These results provide evidence against the idea that an *erb*B-2 ligand is

produced by NIH/3T3 cells. Furthermore, we obtained direct evidence of the constitutive enzymative activity of gp185^{erbB-2} by demonstrating that the *erb*B-2 kinase remained active in a chimeric configuration with the extracellular domain of the EGFR, in the absence of any detectable ligand for the EGFR. Thus, under conditions of overexpression, the normal gp185^{erbB-2} is a constitutively active kinase able to transform NIH/3T3 cells in the absence of ligand.

KGF receptor. We reported saturable specific binding of ^{125}I-KGF to surface receptors on intact BALB/MK mouse epidermal keratinocytes. ^{125}I-KGF binding was competed efficiently by acidic FGF (aFGF) but with 20-fold lower efficiency by basic FGF (bFGF). The pattern of ^{125}I-acidic FGF binding and competition on Balb/MK keratinocytes and NIH/3T3 fibroblasts suggests that these cell types possess related but distinct FGF receptors. Scatchard analysis of ^{125}I-KGF binding suggested major and minor high affinity receptor components (K_D=400 and 25 pM, respectively) as well as a third high capacity/low affinity heparin-like component. Covalent affinity cross-linking of ^{125}I-KGF to its receptor on Balb/MK cells revealed two species of 115 and 140 kDa. KGF also stimulated the rapid tyrosine phosphorylation of a 90-kDa protein in Balb/MK cells but not in NIH/3T3 fibroblasts. Together these results indicate that BALB/MK keratinocytes possess high affinity KGF receptors to which the FGFs may also bind. However, these receptors are distinct from the receptor(s) for aFGF and bFGF on NIH/3T3 fibroblasts, which fail to interact with KGF.

An expression cloning strategy was devised to isolate the KGF receptor complementary DNA. NIH/3T3 fibroblasts, which secrete this epithelial cell-specific mitogen, were transfected with a keratinocyte expression complementary DNA library. Among several transformed foci identified, one demonstrated the acquisition of specific high-affinity KGF binding sites. The pattern of binding competition by related FGFs indicated that this receptor had high affinity for acidic FGF as well as KGF. The rescued 4.2-kilobase complementary DNA was shown to encode a predicted membrane-spanning tyrosine kinase related to but distinct from the basic FGF receptor. This expression cloning approach may be generally applicable to the isolation of genes that constitute limiting steps in mitogenic signaling pathways.

HGF receptor. HGF is an 87-kDa protein resembling plasminogen that has been purified from human and rabbit plasma and rat platelets principally on the basis of its high affinity for heparin. Its mitogenic activity was first characterized on hepatocytes, where it was thought to participate in liver regeneration. The same factor was independently purified from human fibroblast culture medium and shown to act on melanocytes and a variety of epithelial and endothelial cell types. Preliminary investigation of the HGF mitogenic signalling mechanism revealed the tyrosine phosphorylation of a 145-kDa protein (p145) in intact target cells. Electrophoretic mobility under reduced and nonreduced conditions and immunoblot analysis suggested that p145 could be the beta subunit of c-*met* protein kinase. Covalent affinity cross-linking using [^{125}I]-labeled ligand and immunoprecipitation of the cross-linked species with anti-c-*met* protein antisera led us to conclude that the HGF receptor is the c-*met* proto-oncogene product.

III. Growth Factor Signalling Pathways

PDGF promotes the growth of oligodendrocyte type-2 astrocyte (O-2A) glial progenitor cells and allows their timely differentiation into oligodendrocytes, the CNS myelin-forming cells. We demonstrated that basic FGF is a potent mitogen for brain O-2A progenitor cells, but blocks their differentiation into oligodendrocytes. Treatment with basic FGF also influences the level of expression of PDGF receptors on O-2A progenitor cells. These cells express only the α chain PDGF receptor, and the levels of PDGF α receptors decrease as the cells differentiate. In contrast, basic

FGF maintains a high level of functionally responsive PDGF α receptors in O-2A progenitors. Thus, basic FGF activates a signalling pathway that can positively regulate PDGF receptors in O-2A progenitor cells. In this way basic FGF or an FGF-like factor may modulate the production of myelin-forming cells in the CNS.

The c-*fms* proto-oncogene encodes the receptor for macrophage-colony-stimulating factor (CSF-1). Expression vectors containing either normal or oncogenic point-mutated human c-*fms* genes were transfected into interleukin 3 (IL-3)-dependent 32D cells in order to determine the effects of CSF-1 signalling in this murine clonal myeloid progenitor cell line. CSF-1 was shown to trigger proliferation in association with monocytic differentiation of the 32D-c-*fms* cells. Monocytic differentiation was reversible upon removal of CSF-1, implying that CSF-1 was required for maintenance of the monocyte phenotype but was not sufficient to induce an irrevocable commitment to differentiation. Human CSF-1 was also shown to be a potent chemoattractant for 32D-c-*fms* cells, suggesting that CSF-1 may serve to recruit monocytes from the circulation to tissue sites of inflammation or injury. Although c-*fms* did not release 32D cells from factor dependence, point-mutated c-*fms*[S301,F969] (Leu-301 → Ser, Tyr-969 → Phe) was able to abrogate their IL-3 requirement and induce tumorigenicity. Il-3-independent 32D-c-fms[301,F969] cells also displayed a mature monocyte phenotype, implying that differentiation did not interfere with progression of these cells to the malignant state. All of these findings demonstrated that a single growth factor receptor can specifically couple with multiple intracellular signalling pathways and play a critical role in modulating cell proliferation, differentiation, and migration.

IV. Inhibition of Growth Factor Signalling Pathways

Retinoblastoma (RB) gene. The product of the human RB gene is a nuclear phosphoprotein which is thought to function as a tumor suppressor. Mutations of the RB gene frequently occur in human bladder carcinoma. To investigate the significance of the functional loss of this gene in bladder cancer, an RB expression plasmid (pBARB), under control of the human β-actin promoter, was transfected into the bladder carcinoma line, HTB9, which lacks RB gene expression. Marker-selected transfectants that expressed RB protein were identified by western blotting and immunohistochemical staining using an anti-RB monoclonal antibody. In selected clones, stable RB expression persisted over several months under standard culture conditions with 10% serum. However, RB expression caused major alterations of HTB9 growth properties both in vitro and in vivo. RB+ transfectants lacked the ability to form colonies in semisolid medium and their growth rate was significantly decreased in 3% serum-containing medium. In addition, their tumorigenicity was markedly decreased. Tumors that formed in nude mice were much smaller and had a longer latency period but were indistinguishable microscopically from those produced by parental cells. Slower growing tumors were shown to be RB+, as measured by their nuclear RB protein staining and by a normal RB pattern on western blots. These findings support the concept that the RB gene acts as both a growth and tumor suppressor in bladder cancer cells.

MyoD. *MyoD* is a gene involved in the control of muscle differentiation. We show that *MyoD* causes growth arrest when expressed in cell lines drived from tumors or transformed by different oncogenes. MyoD-induced growth inhibition was demonstrated by reduction in the efficiency of colony formation and at the single-cell level. We further showed that MyoD growth inhibition can occur in cells that are not induced to activate muscle differentiation markers. The inhibitory activity of MyoD was mapped to the 68-amino acid segment necessary and sufficient for induction of muscle differentiation, the basic helix-loop-helix motif. Mutants with alterations in the basic region of MyoD that fail to bind or do not activate a muscle-specific enhancer

inhibited growth; mutants with deletions in the helix-loop-helix region failed to inhibit growth. Thus, inhibition of cell growth by MyoD seems to occur by means of a parallel pathway to the one that leads to myogenesis. We conclude that MyoD is a prototypic gene capable of functionally activating intracellular growth inhibitory pathways.

V. *arg* Oncogene

arg. We have previously described partial genomic sequences of *arg*, a human gene related to c-*abl*, and have shown that it is expressed as a 12-kilobase transcript and is located at chromosome position 1q24-25. In this study we elucidate the complete coding sequence of *arg* by characterization of cDNA clones. Analysis of the predicted amino acid sequence of *arg* revealed that it is indeed closely related to that of c-*abl*. The two proteins are strikingly similar with regard to overall structural architecture as well as the amino acid sequences of their tyrosine kinase and *src* homologous 2 and 3 domains. In addition, *arg*, like c-*abl*, is expressed as two transcripts that result from a process of alternative splicing and encode alternative protein forms that differ only in their amino termini. The two genes define the Abelson subfamily of cytoplasmic tyrosine kinases and share a common homolog in *Drosophila*.

VI. Metastatic Phenotype

The role of oncogenes in the acquisition of invasive and metastatic capabilitites is controversial. Interactions with basement membranes are critical in the process of tumor invasion and metastasis. We compared the ability of 3T3 cells transformed by oncogenes involved in various stages of signal transduction to invade a reconstituted basement membrane *in vitro* and to grow in a three dimensional basement membrane gel (matrigel). Cell lines transformed by various oncogenes and oncoviruses (v-*sis* [a growth factor], v-*erb*-B [a truncated EGF receptor], Moloney sarcoma virus [v-*mos*, a protein kinase homologue], mutated c-*ras* oncogenes [G protein homologues], FBJ virus [v-*fos*, a nuclear protein]) were investigated. All transformed cell lines were able to invade in the chemoinvasion assay, where a layer of matrigel is coated onto chemotaxis filters. FBJ/3T3 were the least invasive and SSV/3T3 the most invasive. Control 3T3 cells could not cross the matrigel barrier. All transformed cells grew on matrigel forming invasive, branching colonies, whereas control 3T3 were unable to grow in matrigel. Cells transfected with the v-*erb*-B gene grew as multilayers inside matrigel. Invasiveness and growth on matrigel were accompanied by a high chemotactic response to laminin (LN) in all transformed lines. These results suggest that invasion and growth on matrigel, together with migration to LN, are induced by a large spectrum of oncogenes. When 3T3 cells were transfected with v-*sis* oncogene under the transcriptional control of the metallothionein (MMT) promoter and exposed to Zn++, their *in vitro* invasiveness was specifically increased by around threefold. These findings provide further evidence supporting a direct role of the v-*sis* oncogene in the invasive phenotype.

VII. Methodology

We have developed a high efficiency cDNA cloning system which can direct the orientation of inserts in λ-plasmid composite vectors with large cloning capacities. Cleavage of the vector DNA by *Sfi*I creates two different nonsymmetrical 3' extensions at the ends of the vector arms. Using a linker-primer and an adaptor, cDNA is prepared so it has two different sticky ends which can be ligated to those of the vector arms. When the cDNA and the vector arms are mixed, both the molecules can assemble without self-circularization due to base-pairing specificity. Ligation

of the cDNA-vector mixture produces the concatemers from which phage clones carrying a single cDNA insert in the desired orientation can be formed very efficiently by in vitro packaging. This system provides: (1) high cloning efficiency [10^7-10^8 clones/μg poly(A)$^+$RNA], (2) low background (more than 90% of the clones contain inserts), (3) directional insertion of cDNA fragments into the vectors, (4) presence of a single insert in each clone, (5) accommodation of long inserts (up to 10 kb), (6) a mechanism for rescue of the plasmid part from the λ genome, and (7) a straightforward protocol for library preparations. Screenings of cDNA libraries constructed by this method demonstrated that cDNAs of up to 6.4 kb, containing complete coding sequences, could be isolated at high efficiency. Thus, this cloning system should be useful for the isolation of cDNAs of relatively long transcripts, present even at low abundance in cells.

An efficient unidirectional phagemid cDNA expression cloning system for mammalian transforming genes was developed. By means of this system, a cDNA library was constructed from an NIH/3T3 transformant induced by mouse hepatocellular carcinoma DNA. Transfection of NIH/3T3 cells by the DNA library led to the detection of several transformed foci, from which identical plasmids with transforming ability could be rescued. Structure and sequence analysis of the cDNA clones revealed that the oncogene was created by recombinational events involving an unknown gene and the mouse homologue of the B-*raf* proto-oncogene. This rearrangement led to amino terminal truncation of the B-*raf* gene product. This genetic rearrangement was also detected in two primary transformants independently induced by the original tumor DNA, implying that the recombination event generating the oncogene occurred *in vivo* within the tumor rather than *in vitro* during DNA transfection or cDNA library construction. This phenotypic expression cloning strategy should have wide applicability to the isolation of cDNAs of biologic interest.

Publications:

Aaronson SA, Pierce JH. Molecular mimicry of growth factors by products of tumor viruses. Cancer Cells 1990;2:212-4.

Aaronson SA, Rubin JS, Finch PW, Wong J, Marchese C, Falco J, Taylor WG, Kraus MH. Growth factor regulated pathways in epithelial cell proliferation. Am Rev Respir Dis 1990;142:S7-10.

Aaronson SA, Tronick SR. Constitutive activation of growth factor signalling pathways in cancer cells. In: Broder S, ed. Molecular foundations of oncology. Baltimore: Williams & Wilkins (In Press)

Aaronson SA, Tronick SR. Growth factors. In: Holland JF, Frei III E, Bast Jr RC, Kufe DW, Morton DL, Weichselbaum RR. Cancer medicine. 3rd ed. Philadelphia: Lea & Febiger (In Press)

Bottaro DP, Rubin JS, Faletto DL, Chan A, Kmiecik TE, Vande Woude GF, Aaronson SA. Identification of hepatocyte growth factor receptor as the c-*met* protooncogene product. Science 1991;251:802-4.

Bottaro DP, Rubin JS, Ron D, Finch PW, Florio C, Aaronson SA. Characterization of the receptor for keratinocyte growth factor: evidence for multiple fibroblast gorwth factor receptors. J Biol Chem 1990;265:12767-70.

Crescenzi M, Fleming TP, Lassar AB, Weintraub H, Aaronson SA. MyoD induces growth arrest independent of differentiation in normal and transformed cells. Proc Natl Acad Sci USA 1990;87:8442-6.

Di Fiore PP, Segatto O, Aaronson SA. Cloning, expression and biological effects of the *erb*B2/*neu* gene in mammalian cells. In: Barnes D, Mather JP, Sato GH, eds. Peptide growth factors. Methods in enzymology: peptide growth factors, Part C. New York: Academic Press (In Press)

Di Fiore PP, Segatto O, Lonardo F, Fazioli F, Pierce J, Aaronson SA. The carboxy-terminal domains of *erb*B-2 and epidermal growth factor receptor exert different regulatory effects on intrinsic receptor tyrosine kinase function and transforming activity. Mol Cell Biol 1990;10:2749-56.

Di Marco E, Pierce JH, Aaronson SA, Di Fiore PP. Mechanisms by which EGF receptor and TGFα contribute to malignant transformation. Nat Immun Cell Growth Regul 1990;9:209-21.

Fleming TP, Matsui T, Molloy CJ, Artrip J, Aaronson SA. Demonstration of an activated PDGF autocrine pathway and its role in human tumor cell proliferation in vitro. Mol Cell Biol (In Press)

Fleming TP, Pech MW, Di Marco E, Di Fiore PP, Falco JP, Aaronson SA. Gene encoding growth factors as oncogenes. In: Michell RH, Putney JW Jr, eds. Current communications in molecular biology. New York, Cold Spring Harbor Press (In Press)

Giese N, LaRochelle WJ, May-Siroff M, Robbins KC, Aaronson SA. A small v-*sis*/platelet-derived growth factor (PDGF) B-protein domain in which subtle conformational changes abrogate PDGF receptor interaction and transforming activity. Mol Cell Biol 1990;10:5496-501.

Heidaran MA, Fleming TP, Bottaro DP, Bell GI, Di Fiore PP, Aaronson SA. Transformation of NIH/3T3 fibroblasts by an expression vector for the human epidermal growth factor precursor. Oncogene 1990;5:1265-70.

Heidaran MA, Pierce JH, Jensen RA, Matsui T, Aaronson SA. Chimeric α- and β-platelet-derived growth factor (PDGF) receptors define three immunoglobulin-like domains of the α-PDGF receptor that determine PDGF-AA binding specificity. J Biol Chem 1990;265:18741-4.

Heidaran MA, Pierce JH, Lombardi D, Ruggiero M, Gutkind JS, Matsui T, Aaronson SA. Deletion or substitution within the α platelet-derived growth factor receptor kinase insert domain: effects on functional coupling with intracellular signaling pathways. Mol Cell Biol 1991;11:134-42.

Kraus MH, Aaronson SA. Detection and isolation of novel protein tyrosine kinase genes employing reduced stringency hybridization. In: Hunter T, Sefton BM, eds. Methods in enzymology: tyrosine kinases. New York, Cold Spring Harbor Press (In Press)

Kruh GD, Perego R, Miki T, Aaronson SA. The complete coding sequence of *arg* defines the Abelson subfamily of cytoplasmic tyrosine kinases. Proc Natl Acad Sci USA 1990;87:5802-6.

LaRochelle WJ, Giese N, May-Siroff M, Robbins KC, Aaronson SA. Chimeric molecules map and dissociate the potent transforming and secretory properties of PDGF A and PDGF B. J Cell Sci 1990;13:31-42.

LaRochelle WJ, Giese N, May-Siroff M, Robbins KC, Aaronson SA. Molecular localization of the transforming and secretory properties of PDGF A and PDGF B. Science 1990;248:1541-4.

La Rochelle WJ, Giese N, Robbins KC, Aaronson SA. Variant PDGF ligands and receptors - structure/function relationship. News Physiol Sci (In Press)

Lonardo F, Di Marco E, King CR, Pierce JH, Segatto O, Aaronson SA, Di Fiore PP. The normal *erb*B-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. New Biol 1990;2:992-1003.

Marchese C, Rubin J, Ron D, Faggioni A, Torrisi MR, Messina A, Frati L, Aaronson SA. Human keratinocyte growth factor activity on proliferation and differentiation of human keratinocytes: differentiation response distinguishes KGF from EGF family. J Cell Physiol 1990;144:326-32.

McKinnon RD, Matsui T, Dubois-Dalcq M, Aaronson SA. bFGF modulates the PDGF-driven pathway of oligodendrocyte development. Neuron 1990;5:603-14.

Melchiori A, Carlone S, Allavena G, Aresu O, Parodi S, Aaronson SA, Albini A. Invasiveness and chemotactic activity of oncogene-transformed NIH/3T3 cell. Anticancer Res 1990;10:37-44.

Miki T, Fleming TP, Bottaro DP, Rubin JS, Ron D, Aaronson SA. Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. Science 1991;251:72-5.

Miki T, Fleming TP, Crescenzi M, Molloy CJ, Blam SB, Reynolds SH, Aaronson SA. Isolation of a mouse hepatoma oncogene cDNA using a novel phenotypic expression cloning system. Proc Natl Acad Sci USA (In Press)

Miki T, Matsui T, Heidaran MA, Aaronson SA. Automatic directional cloning: an efficient method to construct eukaryotic expression cDNA libraries. In: Alitalo KK, Huhtala M-L, Knowles J, Vaheri A, eds. Proceedings of the international symposium on recombinant systems in protein expression. Amsterdam: Elsevier Science Publishers BV, 1990;125-36.

Molloy CJ, Fleming TP, Bottaro DP, Aaronson SA. PDGF signaling pathways in malignancy. In: Papas T. eds. Advances in applied biotechnology series, volume 6: Oncogenesis in signal transduction and cell proliferation. Houston: Gulf Publishing, 1990;13-23.

Molloy CJ, Fleming TP, Bottaro DP, Cuadrado A, Pangelinan MJ, Aaronson SA. Oncogenes and signal transduction in malignancy. In: Brinkley W, Lechner J, Harris C, eds. Current communications in molecular biology. New York: Cold Spring Harbor Press, 1991:67-81.

Pierce JH, Arnstein P, Di Marco E, Artrip J, Kraus MH, Lonardo F, Di Fiore PP, Aaronson SA. Oncogenic potential of *erb*B-2 in human mammary epithelial cells. Oncogene (In Press)

Pierce JH, Di Marco E, Cox GW, Lombardi D, Ruggiero M, Varesio L, Wang L-M, Choudhury GG, Sakaguchi AY, Di Fiore PP, Aaronson SA. Macrophage-colony-stimulating factor (CSF-1) induces proliferation, chemotaxis and reversible monocytic differentiation in myeloid progenitor cells transfected with the human c-*fms*/CSF-1 receptor cDNA. Proc Natl Acad Sci USA 1990;87:5613-7.

Ron D, Zannini M, Lewis M, Wickner RB, Hunt LT, Graziani G, Tronick SR, Aaronson SA, Eva A. A region of proto-*dbl* essential for the transforming activity shows sequence similarity to a yeast cell cycle gene *CDC25* and the human breakpoint cluster gene, *bcr*. New Biol (In Press)

Rubin JS, Chan A M-L, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirschfield DW, Wong J, Miki T, Finch PW, Aaronson SA. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc Natl Acad Sci USA 1991;88:415-9.

Takahashi R, Hashimoto T, Xu H-J, Hu S-X, Matsui T, Bigo-Marshall H, Aaronson SA, Benedict WF. The retinoblastoma gene functions as a growth and tumor suppressor in human bladder carcinoma cells. Proc Natl Acad Sci USA (In Press)

Tronick SR, Aaronson SA. Oncogenes. In: Cossman J, ed. Molecular genetics and the diagnosis of cancer. Amsterdam: Elsevier Science Publishers BV (In Press)

Yu J-C, Heidaran MA, Pierce JH, Gutkind JS, Lombardi D, Ruggiero M, Aaronson SA. Tyrosine mutations within the αPDGFR kinase insert domain abrogate receptor-associated PI-3 kinase activity without affecting mitogenic or chemotactic signal transduction. Mol Cell Biol (In Press)

Patents:

Bottaro DP, Rubin JS, Chan AM-L, Aaronson SA. US Patent Application Serial No. 7,642,971. Identification of the HGF Receptor as the c-*met* Proto-oncogene Product.

Bottaro DP, Rubin JS, Faletto D, Chan A, Vande Woude GF, Aaronson SA. US Patent CIP (Pending): Hepatic Growth Factor Receptor is the *met* Proto-Oncogene.

Chan A, Rubin JS, Bottaro DP, Aaronson SA. US Patent CIP (Pending): A Non-Mitogenic Competitive HGF Antagonist.

King CR, Kraus MH, Aaronson SA. US Patent Application Serial No. 7,110,791 (Pending): A human Gene Related to but Distinct from EGF Receptor Gene.

Kraus MH, Aaronson SA. US Patent Application Serial No. 7,444,406 (Pending): DNA Segment Encoding a Gene for a Receptor Related to the Epidermal Growth Factor Receptor.

Kruh G, Perego R, Miki T, Aaronson SA. US Patent Application Serial No. 7,135,280 (Pending): The Complete Coding Sequences of *arg* Defines the Abelson Subfamily of Cytoplasmic Tyrosine Kinases.

Lacal JC, Aaronson SA. US Patent No. 890,510: Deletion Mutants and Monoclonal Antibodies Against *ras* Proteins.

La Rochelle WJ, Robbins KC, Aaronson SA. US Patent Application Serial No. 7,365,715 (Pending): Neutralizing Monoclonal Antibody to Human Platelet-derived Growth Factor Hetero- and Homodimers.

Matsui T, Heidaran MA, Miki T, Popescu N, La Rochelle WJ, Kraus MH, Pierce JH, Aaronson SA. US Patent Application Serial No. 7,308,282 (Pending): Type A Platelet-derived Growth Factor Receptor Gene.

Miki T, Aaronson SA, Fleming TP. US Patent Application Serial No. 7,386,053 (Pending): Efficient Directional Genetic Cloning System to Construct cDNA Libraries Containing Full-length Inserts at a High Frequency.

Rubin JS, Chan A M-L, Bottaro DP, Aaronson SA. US Patent Application Serial No. 7,582,063 (Pending): A Novel Broad Spectrum Human Lung Fibroblast-derived Mitogen.

Rubin JS, Finch PW, Aaronson SA. US Patent Application Serial No. 7,304,281 (Pending) and Foreign Patent Application No. PCT/US90/00418 (Pending): DNA Encoding a Growth Factor Specific for Epithelial Cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04941-19 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Biochemical Characterization of Retroviruses

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
	T. Miki	Visiting Scientist	LCMB	NCI

COOPERATING UNITS (if any)

Sackler School of Medicine, Tel Aviv, Israel (A. Yaniv, A. Gazit); Pan Data Inc., Rockville, Md. (J. Dahlberg); North Carolina State University, Raleigh, NC (L. Coggins, F. Fuller)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Gene Structure Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
4.0	1.0	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.)

Lentivirus infections are generally characterized by their chronic and degenerative clinical course in which the virus is able to persist despite a significant host immune response. Two animal models, equine infectious anemia virus (EIAV) and caprine arthritis-encephalitis virus (CAEV) are being studied in order to determine the molecular basis of viral pathogenesis. Analysis of cDNA libraries of cells chronically infected with EIAV revealed an array of transcripts that could encode viral structural or regulatory proteins. Studies focusing on cDNAs capable of trans-activating the EIAV LTR demonstrated that EIAV expresses at least three distinct tat mRNAs with complex structures. Sequence analysis and in vitro mutagenesis studies confirmed and extended earlier findings that the EIAV tat gene initiates translation at a non-Aug codon. The tat mRNAs were predicted to be polycistronic, containing open reading frames (orfs) downstream of tat that could encode a putative rev protein and/or two different versions of amino-terminally truncated forms of the transmembrane protein. cDNA constructs or subgenomic clones containing only the tat orf were reproducibly more active than polycistronic forms in trans-activation assays. The EIAV tat protein was detected for the first time as an 8 Kd species in cells transfected with tat cDNAs or in chronically infected cells. An infectious molecular EIAV clone was isolated but virus derived from it could not induce clinical symptoms, although it could persistently infect horses. Studies are in progress to isolate portions of the genome of the pathogenic EIAV "field strain" directly from blood cells of diseased horses by PCR techniques. Chimeras between these segments and the infectious clone will be generated and virus derived from them will be tested for pathogenicity. The pattern of expression of CAEV in acutely infected cells was found to be complex and temporally regulated. Nucleotide sequence analysis suggested that they represent rev-like transcripts which could potentially encode as well a truncated form of the env transmembrane protein and a novel protein, designated X, composed of 73 amino acids.

PROJECT DESCRIPTION

Names, 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
T. Miki	Visiting Scientist	LCMB NCI

Objectives:

The purposes of this project are to biochemically characterize animal lentiviruses in order to understand the mechanisms by which these viruses cause disease and to develop strategies to prevent and treat lentivirus infection.

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 polymerase chain reaction (PCR); immunological techniques (RIA, western and ELISA).

Major Findings:

Nucleotide sequence analysis of a cDNA library of equine infectious anemia virus (EIAV)-infected canine cells established a complex pattern of gene expression, characterized by alternatively spliced polycistronic transcripts. The EIAV *tat* gene product was shown to be encoded by at least three species of mRNA which differed in their ability to *trans*-activate the EIAV LTR upon expression in canine cells. The most active cDNA was monocistronic, consisting of three exons. The most abundant cDNA in the library contained four exons and contains open frames for *tat*, putative *rev* and truncated transmembrane proteins. Products consistent in size to those predicted for these latter two proteins could be detected in in vitro translation experiments. The third *tat* message, another four exon form, also potentially encodes an amino terminally truncated transmembrane protein open reading frame. In vitro mutagenesis experiments and analysis of subgenomic and partial cDNA clones confirmed and extended previous findings that S1 sequences are essential for transformation and that *tat* translation initiates at a non-AUG codon in either the full length *Tat* message or in the genomic S1 open reading frame. These findings led to the conclusion that the *tat* protein could be synthesized directly from the genomic RNA and could thus be the first viral protein expressed *de novo* upon infection. For the first time, the *tat* protein (8 kDa) was detected in cells transfected with a *tat* cDNA construct and in canine cells persistently infected with EIAV. The *tat* activity of polycistronic mRNAs was lower than that of the monocistronic form suggesting that the expression of the EIAV *trans*-activator may be subject to several levels of post-transcriptional control. We previously reported that virus derived from an infectious EIAV molecular clone could persistently infect horses and elicit an immune response but could not induce clinical symptoms of equine infectious anemia. In an attempt to generate a pathogenic EIAV molecular clone, segments of the viral genome have been isolated directly from blood cells of diseased animals by the PCR technique. These PCR clones, which, to date, are not full length, have been substituted for the homologous sequences in the infectious clone. These chimeras are being tested for infectivity in vitro and in vivo.

Analysis of the PCR clones have revealed a number of differences between them and the prototype EIAV clone derived from the tissue culture adapted strain.

The pattern of expression of the caprine arthritis-encephalitis virus genome (CAEV) in acutely infected tahr lung cells was found to be complex and temporally regulated. Employing northern analysis, five CAEV-specific transcripts, 9.0 6.5, 5.0, 2.5 and 1.4 kb, were detected. Nucleotide sequence analysis established the genetic structure of two species of cDNA, isolated from a library of CAEV-infected tahr cells, and suggested that they represent *rev*-like transcripts. One of these cDNA species was composed of three exons--the leader, an exon derived from the 5' region of *env*, and an exon which spanned the 3' *orf*. The second cDNA species consisted of four exons, three of which were identical to the those of the former species. The additional exon (the third) was located at the 3' end of *pol*. These transcripts could potentially encode three proteins, a *rev*-like protein, which is a fusion of 38 amino acids derived from the N-terminus of *env* and 91 residues from the 3' *orf*, a truncated form of the *env* transmembrane protein, and a novel protein, designated X composed of 73 amino acids. Thus, CAEV, like other lentiviruses, displays a complex pattern of gene expression, characterized by alternative splicing and the production of potentially polycistronic transcripts.

Publications:

Aaronson SA, Tronick SR. Constitutive activation of growth factor signalling pathways in cancer cells. In: Broder S, ed. Molecular foundations of oncology. Baltimore: Williams & Wilkins (In Press)

Aaronson SA, Tronick SR. Growth factors. In: Holland JF, Frei III E, Bast Jr RC, Kufe DW, Morton DL, Weichselbaum RR, eds. Cancer medicine. 3rd ed. Philadelphia: Lea & Febiger (In Press)

Eisemann A, Ahn JA, Graziani G, Tronick SR, Ron D. Alternative splicing generates at least five different isoforms of the human basic FGF receptor. Oncogene (In Press)

Gak E, Yaniv A, Ianconesca M, Tronick SR, Gazit A. An in vivo infectivity assay for cloned retroviruses lacking a susceptible cell culture. J Virol Methods (In Press)

Gak E, Yaniv A, Sherman L, Ianconesca M, Tronick SR, Gazit A. The lymphoproliferative disease virus (LPDV) of turkeys - nucleotide sequence analysis and transcriptional activity of the long terminal repeat. Virology (In Press)

Kalinski H, Yaniv A, Mashiah P, Miki T, Tronick SR, Gazit A. *Rev*-like transcripts of caprine arthritis-encephalitis virus. Virology (In Press)

Noiman S, Gazit A, Tori O, Sherman L, Miki T, Tronick SR, Yaniv A. Identification of sequences encoding the equine infectious anemia virus *tat* gene. Virology 1990;176:280-8.

Noiman S, Yaniv A, Sherman L, Tronick SR, Gazit A. Pattern of transcription of the genome of equine infectious anemia virus. J Virol 1990;64:1839-43.

Noiman S, Yaniv A, Tsach T, Miki T, Tronick SR, Gazit A. The *tat* protein of equine infectious anemia virus is encoded by at least three types of transcripts. Virology (In Press)

Ron D, Zannini M, Lewis M, Wickner RB, Hunt LT, Graziani G, Tronick SR, Aaronson SA, Eva A. A region of proto-*dbl* essential for the transforming activity shows sequence similarity to a yeast cell cycle gene *CDC24* and the human breakpoint cluster gene, *bcr*. New Biol (In Press)

Tronick SR, Aaronson SA. Oncogenes. In: Cossman J, ed. Molecular genetics and the diagnosis of cancer. Amsterdam: Elsevier Science Publishers BV (In Press)

Whetter L, Archambault D, Perry S, Gazit A, Coggins L, Yaniv A, Clabough D, Dahlberg J, Fuller F, Tronick SR. Equine infectious anemia virus derived from a molecular clone persistently infects horses. J Virol 1990;64:5750-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04976-14 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(60 characters or less. Title must fit on one line between the borders.)*

Mechanisms of Neoplastic Transformation in Cultured Human Cells

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

PI:	K. K. Sanford	Chief, In Vitro Carcin Sect	LCMB	NCI
Others:	K. H. Kraemer	Research Scientist	LMC	NCI
	R. E. Tarone	Mathematical Statistician	EPB	NCI
	R. Gantt	Chemist	REB/CPCP	NCI
	T. A. Waldmann	Chief	MET	NCI

COOPERATING UNITS *(if any)*

Howard U. College Med. (R. Parshad); Walter Reed Dept. Med. (R. Knight); U. CA, Irvine (E. Stanbridge); Lawrence Berkeley Laboratory (M. Stampfer); Hosp. U. PA (D. Guerry); NIEHS (C. Barrett); U. NC (B. Weissman); NIA (S. I. Rapoport)

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	2.0	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.)*

Accomplishments of the past year include the following findings:

(1) Enhanced chromatid damage in blood lymphocytes after G2 phase x-irradiation can provide a marker for the ataxia telangiectasia (A-T) gene useful for identifying unaffected carriers in A-T families. The persistence of chromatid breaks and gaps, representing unrepaired DNA strand breaks, following radiation-induced DNA damage during G2 phase of the cell cycle suggests deficient DNA repair. This may be the defect at the molecular level that results in the radiosensitivity and cancer-proneness of A-T gene carriers and patients. (2) Lymphoblastoid cell lines can be used in a similar cytogenetic assay to identify in a family with Gardner syndrome (GS) members with the GS gene. (3) Chromosome 11 (ch 11) complements the DNA repair deficiency in six human tumor cell lines. In one line, addition of the long arm of ch 11 was sufficient to restore repair efficiency. These results suggest that ch 11 carries a DNA repair gene. (4) Deficient DNA repair was acquired spontaneously or induced by ras oncogene in diverse lines of human epithelial cells in culture prior to or in association with their neoplastic transformation by virus or chemical carcinogen. (5) 13-cis-retinoic acid, shown previously to reduce the frequency of skin cancers in xeroderma pigmentosum patients, was found to protect against x-ray-induced chromatid damage in cultured blood lymphocytes. These observations suggest that 13-cis-retinoic acid directly or indirectly acts as a scavenger of the peroxide or (.OH) radicals generated during ionizing radiation, thereby providing protection against x-ray-induced damage.

PROJECT DESCRIPTION

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

K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
J. H. Robbins	Research Scientist	D	NCI
K. H. Kraemer	Research Scientist	LMC	NCI
R. E. Tarone	Mathematical Statistician	BB/EBP	NCI
R. Gantt	Chemist	REB/CPCP	NCI
T. A. Waldmann	Chief	MET	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 deficient DNA repair and genetic predisposition to cancer. Efforts are also directed toward following in human epithelial cells in culture the progression of changes leading to neoplastic transformation with particular emphasis on the acquisition of DNA repair deficiencies and the role of the *ras* oncogene. Specific goals are: (1) to develop a more rapid assay for genetic predisposition to cancer using skin fibroblasts, lymphoblastoid cell lines or peripheral blood lymphocytes. This assay will be useful for identifying clinically normal carriers of genes for cancer proneness; (2) to determine, at the molecular and cytogenetic levels, consequences of radiation-induced DNA damage and repair; (3) to determine the relationship between DNA repair deficiency, genetic instability and neoplastic transformation in vitro and in vivo; and (4) to identify genes for DNA repair in mouse and human cells.

Methods Employed:

Chromatid breaks and gaps persisting after G_2-phase x-irradiation, exposure to fluorescent light or other DNA-damaging agents are quantified in skin fibroblasts, stimulated peripheral blood lymphocytes or lymphoblastoid cell lines. 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. A complementary measurement of DNA repair capacity in human cells is being developed using flow cytometry.

Major Findings:

1. Enhanced chromatid damage in blood lymphocytes after G_2 phase x-irradiation can provide a marker for the ataxia telangiectasia (A-T) gene useful for identifying unaffected carriers in A-T families. The persistence of chromatid breaks and gaps, representing unrepaired DNA strand breaks, following radiation-induced DNA damage during the G_2 phase of the cell cycle suggests deficient DNA repair. This may be the defect at the molecular level that results in the radiosensitivity and cancer-proneness of A-T gene carriers and patients.

2. Lymphoblastoid cell lines, in addition to skin fibroblasts and peripheral blood, can be used in a similar cytogenetic assay to identify in a family with Gardner syndrome (GS) members with the GS gene.

3. Chromosome 11 (ch 11) complements the DNA repair deficiency in six human tumor cell lines. In one cell line, addition of the long arm of ch 11 was sufficient to restore repair efficiency. These results suggest that ch 11 carries a DNA repair gene.

4. Deficient DNA repair, manifest as persistent chromatid breaks and gaps after G_2 phase x-irradiation, was acquired spontaneously or induced by *ras* oncogene in diverse lines of human epithelial cells in culture prior to or in association with their neoplastic transformation by virus or chemical carcinogen.

5. 13-*cis*-retinoic acid, shown previously to reduce the frequency of skin cancers in xeroderma pigmentosum patients, was found to protect against x-ray-induced chromatid damage in cultured blood lymphocytes. These observations suggest that 13-*cis*-retinoic acid directly or indirectly acts as a scavenger of the peroxide or (.OH) radicals generated during ionizing radiation, thereby providing protection against x-ray-induced damage.

Publications:

Parshad R, Sanford KK, Gantt R. G_2 chromatid radiosensitivity in relation to DNA repair and cancer susceptibility. In: Obe G, Sobti RC, eds. The eukaryotic chromosome. Structural and functional aspects. Berlin-Heidelberg: Springer-Verlag, 1990;173-81.

Sanford KK, Parshad R. Detection of cancer-prone individuals using cytogenetic response to x-rays. In: Obe G, Natarajan AT, eds. Chromosomal aberrations: basic and applied aspects. Berlin-Heidelberg: Springer-Verlag, 1989;113-20.

Sanford KK, Parshad R, Price FM, Jones GM, Tarone RE, Eierman L, Hale P, Waldmann TA. Enhanced chromatid damage in blood lymphocytes after G_2 phase x-irradiation, a marker of the ataxia-telangiectasia gene. JNCI 1990;82:1050-4.

Takai S, Price FM, Sanford KK, Tarone RE, Parshad R. Persistence of chromatid damage after G_2 phase x-irradiation in lymphoblastoid cells from Gardner's syndrome. Carcinogenesis 1990;1425-8.

Takai S, Sanford K, Tarone RE. A procedure for carrier detection in ataxia-telangiectasia. Cancer Genet Cytogenet 1990;46:139-40.

Patents:

Sanford KK, Parshad R, Jones GM. US Patent No. 4,933,274 (Pending): Process for Detecting Genetic Susceptibility to Cancer.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05060-13 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Mechanisms of Carcinogenesis: Neoplastic Transformation of Human Cells

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

PI:	J. S. Rhim	Microbiologist	LCMB	NCI
Others:	P. Arnstein	Veterinary Director	LCMB	NCI
	J. H. Yang	Special Volunteer	LCMB	NCI
	I. H. Lee	Special Volunteer	LCMB	NCI
	M. S. Lee	Special Volunteer	LCMB	NCI

COOPERATING UNITS *(If any)*

Karolinska Institute, Stockholm, Sweden (G. Klein); Georgetown University, Washington, DC (A. Dritschilo)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	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.)*

Objectives 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; and (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, hormones and x-irradiation; and (3) to study roles of activated and suppressor oncogenes in neoplastic transformation.

We have demonstrated (1) neoplastic transformation of immortalized human epidermal keratinocytes by exposure to x-ray irradiation; (2) morphological transformation of human keratinocytes expressing the latent membrane protein (LMP) gene of Epstein-Barr virus; (3) demonstration of CR-2, a cell surface membrane glycoprotein in an immortalized human epidermal keratinocyte line; and (4) establishment of a cystic fibrosis bronchial epithelial cell line by adenovirus 12-SV40 (Ad12-SV40) virus infection.

PROJECT DESCRIPTION

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

J. S. Rhim	Microbiologist	LCMB	NCI
P. Arnstein	Veterinary Director	LCMB	NCI
J. H. Yang	Special Volunteer	LCMB	NCI
I. H. Lee	Special Volunteer	LCMB	NCI
M. S. Lee	Special Volunteer	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 study roles of activated and suppressor oncogenes in neoplastic transformation.

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:

Evidence for the multistep nature of in vitro human epithelial cell carcinogenesis. In keeping with the multistep development of human cancer in vivo, a stepwise approach to neoplastic transformation in vitro presents a reasonable strategy. We have recently developed an in vitro multistep model suitable for the study of human epithelial cell carcinogenesis. Upon infection with the adenovirus 12-simian virus 40 (Ad12-SV_{40})hybrid virus, primary human epidermal keratinocytes acquired an indefinite lifespan in culture but did not undergo malignant conversion. Subsequent addition of Kirsten murine sarcoma virus and human *ras* oncogene or chemical carcinogens (N-methyl-N'nitro'N'nitrosoguanidine or 4'nitroquinoline 1'oxide) to these cells induced morphological alterations and the acquisition of neoplastic properties. Subsequently it was found that this line could be transformed neoplastically by a variety of retrovirus-containing oncogenes, H-*ras*, *bas*, *fes*, *fms*, *erb*B, and *src*. In addition, we found that the immortalized human epidermal keratinocyte (RHEK-1) line can be transformed neoplastically by exposure to ionizing radiation. Thus, this in vitro system may be useful in studying the interaction of a variety of carcinogenic agents and human epithelial cells. These findings demonstrate the malignant transformation of human primary epithelial cells in culture by the combined action of viruses, oncogenes, chemical carcinogens, or x-ray irradiation and support a multistep process for neoplastic conversion.

Morphological transformation of human keratinocytes expressing the LMP gene of Epstein-Barr virus (EBV). The association of EBV with nasopharyngeal carcinoma (NPC) has been known for some time, but the precise role of EBV in this cancer is poorly understood, due partly to the lack of an in vitro system for studying NPC cells and the effect of EBV on epithelial cells. Biopsies of NPC tumors have revealed expression of the EBV latent membrane protein (LMP) in 65% of cases, suggesting that in at least some NPC tumors LMP may contribute to cell transformation. We addressed the question of the effect of LMP expression on epithelial cells. Transfection of an immortalized, nontumorigenic keratinocyte cell line (RHEK-1) with the LMP gene causes a striking morphological transformation: the originally flat, polygonal colonies change to bundles of spindle-shaped cells that form multilayer foci, and cytokeratin expression is downregulated. Our results suggest that LMP expression may be an important causal factor in the development of NPC.

Demonstration of CR-2 complement receptor in an immortalized human keratinocyte line. CR-2, a membrane glycoprotein, is one of a number of cell surface proteins which bind activation and processing fragments of the complement system. CR-2, which is found on normal B lymphocytes, follicular dendritic cells in lymphoid organs, and epithelial cells, interacts preferentially with C3dg, the terminal activation/processing fragment of the third complement component. Attachment of C3dg to CR2 brings complement activators, bearing covalently bound C3dg into direct membrane contact with CR-2-bearing cells. EBV, a human herpesvirus, also binds to CR-2 on B lymphocytes. Attachment of EBV is followed by infection.

A cystic fibrosis bronchial epithelial cell line: immortilization by adenovirus 12-SV_{40} (Ad12-SV_{40}) infection. An immortalized cell line was created from a primary culture of bronchial epithelia isolated from a patient with cystic fibrosis (CF). The culture was transformed with Ad12-SV_{40}, which has been used successfully on a number of different human epithelial tissues. The transformed bronchial epithelial cells have the following characteristics: 1) cAMP is stimulated by beta adrenergic agonists; 2) outwardly rectifying Cl^- channels are present on the apical cell membrane. The channels can be activated by depolarizing voltages but not by protein kinase A or C; 3) keratin is present by immunofluorescence, and this is consistent with the epithelial origin of the cells; 4) the SV_{40} large T antigen is present as demonstrated by immunofluorescence; 5) multiple karyotype analyses show modal chromosome number to be 80-90. There are an average of 4 chromosomes #7 per cell; and 6) the Phe_{508} deletion in the gene coding for CFTR is present on at least one chromosome. The cells can be grown in multiple passages, contain the abnormal regulation of the secretory Cl^- channel, and should be an appropriate substrate for studies of the mutant cystic fibrosis transmembrane regulatory (CFTR) protein and its interaction with the Cl^- channel.

Publications:

Cooper NR, Bradt BM, Rhim JS, Nemerow GR. CR2 complement receptor. J Invest Dermatol 1990;94:112S-7S.

Fahraes R, Rymo L, Rhim JS, Klein G. Morphological transformation of human keratinocytes expressing the LMP gene of Epstein-Barr virus. Nature 1990;345:447-9.

Rhim JS, Arnstein P, Fahraeus R, Rymo L, Klein G, Gradoville L, Miller G, Wang F, Kieff E. Interaction of EBV genes with human epithelial cells. In: Ablashi DV, Hwang A, Pagano J, Pearson G, Yang C, eds. Epstein-Barr virus and human disease. Clifton NJ: Humana Press (In Press).

Rhim JS, Yoo JH, Park JH, Thraves P, Salehi Z, Dritschilo A. Evidence for the multistep nature of in vitro human epithelial cell carcinogenesis. Cancer Res 1990;50:5653s-7s.

Zeithin PL, Lu L, Rhim JS, Cutting G, Stetten G, Kieffer KA, Craig R, Guggino WB. A cystic fibrosis bronchial epithelial cell line: immortalization by AD12-SV_{40} infection. Am J Respir Cell Mol Biol 1991;4:313-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05062-13 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Transforming Genes of Naturally Occuring 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
	D. Ron	Visiting Associate	LCMB	NCI

COOPERATING UNITS *(if any)*

USUHS (S. Srivastava); Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases; (A. Nebreda). National Institute of Child Health and Human Development (G. Guroff).

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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.)*

We have constructed a recombinant retrovirus carrying the dbl transforming gene and infected monolayers of PC12 cells, a rat pheochromocytoma-derived cell line able to differentiate into neuron-like cells upon treatment with nerve growth factor (NGF). Clonal populations of cells infected with the dbl recombinant virus do not differentiate in the presence of NGF. Fibroblast growth factor (FGF) or cAMP, two other inducers of neuronal differentiation of uninfected PC12 cells, also failed to induce PC12 cells infected with the dbl recombinant virus to differentiate. Analysis of these clones has shown that cells carrying the transforming dbl gene no longer express the NGF receptor. Moreover, these cells have also lost TH and CAT activity, two of the neuronal markers characteristic of PC12 cells. PC12 cells expressing the dbl oncogene product also have an altered expression of integrins.

We have expressed at high levels the dbl protein in SF9 insect cells utilizing a baculovirus expression vector system. When the soluble fraction of the infected insect cells was microinjected into Xenopus oocytes, we observed germinal vesicles breakdown in about 50% of the treated oocytes. The same results were obtained when the oocytes were microinjected with the in vitro transcribed dbl mRNA. Moreover, extracts of oocytes microinjected with dbl-encoding RNA showed activation of H1 kinase activity.

PROJECT DESCRIPTION

Names, 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

Objectives:

Studies are directed to determine the transforming activity of the *dbl* oncogene, its cellular targets, and its normal allele function.

Methods employed:

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

Major findings:

We have generated a highly transmissible recombinant retrovirus carrying the *dbl* oncogene coding sequences and used it to infect a rat pheochromocytoma cell line, PC12. Several single cell, colony-derived cell lines have been developed. Each of these cell lines produced the *dbl* recombinant retrovirus, expressed the transforming *dbl* protein and displayed a dramatic change in morphology and surface adherence characteristics. The *dbl*-transformed PC12 cell lines were no longer able to differentiate into neuron-like cells upon treatment with nerve growth factor (NGF), cyclic AMP, or fibroblast growth factor (FGF). Each of these *dbl*-transformed cell lines was analyzed for expression of several markers characteristic of PC12 cells. By immunoprecipitation analysis they were found to lack the NGF receptor. Biochemical assays have revealed the absence of both tyrosine hydroxylase as well as choline acetyl transferase activity. Analysis by radioimmunoprecipitation for surface receptors has shown that the α1 integrin subunit, whose expansion is characteristic of PC12 cells, is absent in these *dbl*-transformed cell lines while they express a novel integrin subunit molecule, α5, more typical of stromal cells.

We have recently discovered that a region of *dbl* essential for its transforming activity shows significant similarity to a yeast cell cycle gene, *CDC24*. In addition, the transcript of the *dbl* proto-oncogene is specifically detected in human gonadal tissues, suggesting a possible role for the *dbl* protein in germ cell development. To investigate whether this gene influences the cell cycle by inducing cell division, we analyzed *dbl* protein for its ability to induce oocyte maturation. In order to obtain high levels of *dbl* protein we have expressed the *dbl* oncogene product using the baculovirus expression system. We observed germinal vesicle breakdown when *Xenopus* oocytes were microinjected with the soluble fraction of insect cells infected with a *dbl* recombinant baculovirus as well as with in vitro transcribed *dbl* mRNA. Moreover, extracts of oocytes microinjected with the *dbl*-encoding RNA showed histone H1 activity.

Publications:

Eva A, Graziani G, Zannini M, Merin LM, Khillan JS, Overbeek PA. Dominant dysplasia of the lens in transgenic mice expressing the *dbl* oncogene. New Biol 1989;3:158-68.

Graziani G, Ron D, Srivastava S, Eva A. Expression of the human dbl oncogene and proto-oncogene products in insect cells using a baculovirus vector. In: Asherson J, Colizzi V, Marini S, Pugliese O, eds. Immunology and biotechnology. Rome: Annuali dell' Istituto Superiore di Sanità (In Press)

Ron D, Zannini M, Lewis M, Wichner RB, Hunt LT, Graziani G, Tronick SR, Aaronso SA,Eva A. A region of proto-*dbl* essential for the transforming activity shows sequence similarity to a yeast cell cycle gene *CDC24* and the human breakpoint cluster gene, *bcr*. New Biol 1991;3:372-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05063-13 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Human Herpesvirus-6 (HHV-6), Epstein-Barr Virus (EBV) and HIV Studies

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

PI:	D. V. Ablashi	Microbiologist	LCMB	NCI
Others:	R. C. Gallo	Chief	LTCB	NCI

COOPERATING UNITS *(if any)*

Harvard U. Med. Sch., Boston, MA (A. Komaroff); U. Kansas, Kansas City, KA (N. Balachandran); U. Cologne, Germany (G. R. F. Krueger); Georgetown U. Sch. Med., Washington, DC (G. Pearson); U. Montreal, Canada (J. Menezes)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Gene Structure Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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 infection of HHV-6 occurs very early in childhood (under one year) and the virus remains for life in a latent state. Using the peripheral mononuclear cells in culture from HIV-1-positive AIDS, chronic fatigue syndrome (CFS), systemic lupus erythematosus (SLE) patients and bone marrow transplant recipients, a high frequency of virus activation (40%) compared to normal donors (12%) was detected indicating active virus replication. HHV-6 was found to induce IL-1β and TNF-α in human peripheral blood mononuclear cells. In contradistinction, HHV-6 has no effect on IL-6 induction. Cytokine induction appeared to be mediated by early viral protein. Cytokine production by HHV-6 in cells suggests how HHV-6 may contribute to disease manifestation. HHV-6 antigen-positive cells were detected by in situ hybridization and by monoclonal antibody assays in Hodgkin's disease, (HD), AIDS, SLE and Sjogren's syndrome patients' cells. The virus DNA-positive cells expressed CD38+, CD4+, and to a lower degree CD8+ and CD21+ cell receptors. Using ELISA and HHV-6 early protein (P41/38), sera from African Burkitt's lymphoma (ABL), HD and American Burkitt's lymphoma (AmBL) showed high frequency of antibody to P41/38 (ABL, 96.5%; HD, 62.5%) compared to normals (12%), whereas ELISA to HHV-6 late antigen (gp110) failed to detect these differences. These findings indicated active HHV-6 replication and suggested a possible role of this virus in the pathogenesis of these malignancies. Thus, this assay may be a good marker for active and chronic HHV-6 infections.

PROJECT DESCRIPTION

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

D. V. Ablashi	Microbiologist	LCMB NCI
R. C. Gallo	Chief	LTCB NCI

Objectives:

To assess the role of human herpesvirus-6 (HHV-6) in human disease.

Methods Employed:

Primary cell tissues were established from peripheral blood lymphocytes and tumor tissues from AIDS and other patients with tumors, lymphoproliferative disorders, and autoimmune diseases, and were used for virus detection and infection with (HHV-6). Continuous human T-cell lines were also used for propagation of HHV-6. Human cord blood mononuclear cells were used for infecting virus stock and for use in immunologic assays. Monoclonal and polyclonal antibodies to human herpesviruses were used in immunologic assays. Biochemical and molecular studies were performed to detect HHV-6 genome.

Major Findings:

1. The data shows that the prevalence rate of HHV-6 in the general population is >80%. By one year of age, most children (<90%) are antibody-positive. This suggests that HHV-6 infection occurs very early in life and persists in latent form. Peripheral blood mononuclear cells (PBMC) from patients with HIV-1-positive AIDS, chronic fatigue syndrome (CFS), systemic lupus erythematosus (SLE) and bone marrow transplants were mitogen stimulated and examined by IFA. HHV-6 antigen-positive cells were detected between 3-14 days postculture in AIDS (<65%), SLE (72%), CFS (48%) and bone marrow transplant (80%) patients compared to normal donors (12%). Moreover, the number of antigen-positive cells in these diseases ranged between 7-30% compared to <2% in healthy donors. These data suggest that the frequency of HHV-6 reactivation is significantly higher in these diseases and that virus reactivation could be contributing to the pathogenesis of these diseases. Further studies are needed to show whether there is a correlation between the onset of disease manifestations and HHV-6 reactivation.

2. The role of HHV-6 in human disease could include induction of cytokines which may stimulate the overgrowth of certain malignant and nonmalignant cells. This possibility was investigated. Adult PBMCs were infected with HHV-6 (GS isolate), or treated with heat-inactivated HHV-6 (56°C for 1hr.) or with the antiherpesvirus agent phosphoacetic acid (PAA) or treated with UV-irradiated virus and were tested for production of IL-1β, TNF-α and IL-6 monokines which are potent immune regulatory proteins secreted by monocyte/macrophages. Peak induction of IL-1β and TNF-α in cells were detected by ELISA at 24 hours postinfection. Using PCR, a threefold increase in IL-1β and TNF-α in the mRNA of virus-infected cells was observed compared to uninfected cells. Heat-inactivated virus failed to induce these cytokines. However, cytokines were induced in the presence of PAA and UV-treated virus, suggesting that an early viral protein may be responsible for the induction and that viral replication is not required. HHV-6 failed to induce IL-6 in PBMC. The induction of

TNF-α HHV-6 protein responsible for induction of IL-1β and TNF-α. Moreover, induction of other cytokines are yet to be evaluated in order to further assess the role of HHV-6 in pathogenesis.

3. Using the monoclonal antibody (9A5D12) to P41 of HHV-6 (GS strain), we examined the biopsy tissues obtained from Hodgkin's disease (HD), HIV-1 positive AIDS, SLE and Sjögren's syndrome patients. Cells were also identified for HHV-6 DNA. The cells carrying HHV-6 DNA were also tested for cellular receptors. The highest number of HHV-6 DNA-positive cells was in HD (10^2 to 10^3), using the HHV-6 ZVH-14 probe. HHV-6 antigen expression in cells was detected by monoclonal antibodies to P41 using the immunoperoxidase method indicating latent infection. The cells that carried HHV-6 DNA or antigen were predominantly $CD38^+$ and $CD4^+$. A small percentage of HHV-6-positive cells was also $CD8^+$ but rarely $CD21^+$ (B-cell receptor for EBV). These data did not indicate whether the cells in HD are tumor cells or infiltrating lymphocytes. Since $CD4^+$ is the receptor for HIV-1, it would be important to find in vivo cells that are doubly infected with HIV and HHV-6 to assess the cofactor role of HHV-6 in AIDS.

4. HHV-6 early protein (P41/38) appeared 10 hours postinfection of HSB2 cells and was also expressed in the presence of PAA which blocks viral replication, suggesting that it is an early event in the virus life cycle. The monoclonal antibody to this protein (SC-EA) showed that all HHV-6 isolates express this protein in the nucleus of infected cells. Using purified P41/38 antigen, the sera from African Burkitt's lymphoma (ABL), HD, chronic fatigue syndrome (CFS), American Burkitt's lymphoma (AmBL) and normal healthy donors showed variable reactivity in the distribution of antibody in the above diseases with highest positivity in Af-BL (96.5%) and HD (62.5%) compared to controls (12%). ELISA to gp110 of HHV-6 failed to detect differences in antibody to this protein in sera from controls and patients with the above diseases. These data indicate that P41/38 of HHV-6 may be of diagnostic value for virus reactivation or primary infection as it has been observed for EBV early antigen in BL and nasopharyngeal carcinoma (NPC). Further studies are in progress to detect the antibody to P41/38 in acute and chronic infections as well as in various stages of HD. Moreover, studies are also in progress to identify if it is an immediate early protein or a late early protein.

Publications:

Ablashi DV, Salahuddin SZ, Josephs SF, Balachandran N, Krueger GRF, Gallo RC. Human herpesvirus-6. In Vivo (In Press)

Ablashi DV, Zompetta CV, Lease C, Josephs SF, Balachandran N, Komaroff AL, Krueger GRF, Henry B, Luka J, Salahuddin SZ. Human herpesvirus-6 (HHV-6) and chronic fatigue syndrome (CFS). In: Rozee K, ed. Proceedings, chronic fatigue syndrome workshop. Toronto, Canada: Canada Diseases Weekly 1990;171/51E:33-40.

Balachandran N, Tirawatnapong S, Pfeiffer B, Ablashi DV, Salahuddin SZ. Electrophoretic analysis of human herpesvirus-6 polypeptides immunoprecipitated from infected cells with human sera. J Infect Dis 1990;163:29-34.

Bertram G, Dreiner N, Krueger GRF, Ablashi DV, Salahuddin SZ, Balachandran N. Frequent double active infection with Epstein-Barr virus and human herpesvirus-6 in patients with active infectious mononucleosis. In Vivo (In Press)

Buchwald D, Freedman AS, Ablashi DV, Sullivan JL, Calligiuri M, Weinberg DS, Hall CG, Bashir R, Singer RM, Ashley RL, Saxinger C, Balachandran N, Ritz J, Nadler LM, Komaroff AL. A chronic "post-infectious" fatigue associated with benign lymphoproliferation, B cell proliferation and active replication of human herpesvirus-6. J Clin Immunol 1990;10:335-44.

Flamand L, Gosselin J, D'addario M, Hiscott J, Ablashi DV, Gallo RC, Menezes J. Human herpesvirus-6 induces interleukin-1β and tumor necrosis factor-α, but not interlukin-6 in peripheral blood mononuclear cell cultures. J Virol (In Press)

Goldblum N, Llana T, Ablashi DV, Josephs SF, Salahuddin SZ. Susceptibility to HIV-1 infection of a human B-lymphoblastoid cell line, DG75, transfected with subgenomic DNA fragments of Epstein-Barr virus. Develop Biol Standard 1990;72:309-13.

Hoffmann A, Kirn E, Kuerten A, Sander C, Krueger GRF, Ablashi DV. Active human herpesvirus-6 infection associated with KiKuchi-Fujimoto disease and systemic lupus erythematosis (SLE). In Vivo (In Press)

Krueger GRF, Ablashi DV, Josephs SF, Salahuddin SZ, Lembke U, Ramon A, Hoffmann A, Bertram G. Clinical indication and current techniques for the demonstration of infection with human herpesvirus-6 (HHV-6). Klin Wschr (In Press)

Krueger GRF, Ablashi DV, Lusso P, Josephs SF. Immunological dysregulation of lymph nodes in AIDS patients. In: Grundmann E, Volmer E (eds). Current topics in pathology. Berlin/Heidelberg: Springer-Verlag Publishers 1990;157-88.

Krueger GRF, Ablashi DV, Ramon A, Lembke U. Frequency and pathogenic implication of reactivated lymphotropic herpesviruses in autoimmune and immune deficiency disorders. In: Heiniger HJ, Imbach P, Morell A, Nydegger U, Studer C, eds. Intravenous immunoglobulin symposium. London: Academic Press (In Press)

Krueger, GRF, Ablashi DV, Salahuddin SZ, Lembke, U, Ramon A, Bertram G. Clinical indication and diagnostic techniques for human herpesvirus-6 (HHV-6) infections. In Vivo (In Press)

Krueger GRF, Wassermann K, DuClerk LS, Stevens WJ, Bourgeosis N, Ablashi DV, Josephs SF, Balachandran N. Latent human herpesvirus-6 in salivary and bronchial glands. Lancet 1990;336:1255-6.

Madea B, Roervert HJ, Krueger GRF, Abashi DV, Josephs SF. Search for early lesions following HIV-1 infection. Study in six individuals with forceful death after seroconversion. Arch Path Lab Med 1990;114:379-82.

Neeqaye J, Viza D, Pizza G, Levine PH, DeVinci C, Ablashi DV, Bigger R, Nkrumah, FK. Specific transfer factor with activity against Epstein-Barr virus reduces late response to endemic Burkitt's lymphoma. Anticancer 1990;10:1183-8.

Popescu NC, Dahlberg JE, Ablashi DV, Monastier M, Bona C, DiPaolo JA, Hooper WC, Swan D. Oncogene expression and immunoglobulin synthesis in a North American Burkitt (NAB-2) lymphoma cell line with a 8:22 chromosome translocation. Oncogene Res 1990;5:292-303.

Steeper TA, Horwitz CA, Ablashi DV, Salahuddin SZ, Saxinger C, Saltzman, Schwartz B. The spectrum of clinical and laboratory findings due to Human Herpesvirus-6 (HHV-6) in patients with mononucleosis-like illness not due to EBV or CMV. Am J Clin Pathol 1990;93:776-83.

Yadav M, Ablashi DV. Nasopharyngeal carcinoma: lack of elevated antibody to human herpesvirus-6 in these patients. Asian J Clin Sci 1990;10:81-4.

Yadav M, Umamaheswarti S, Ablashi DV. Low prevalence of antibody to human herpesvirus-6 (HHV-6) in Kadazans. Southeast Asian J Trop Med Public Health 1990;21:259-63.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05164-11 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Oncogenes, Growth Factor Pathways and Hematopoietic Cell Signal Transduction

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

PI:	J. H. Pierce	Research Microbiologist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	M. A. Heidaran	Staff Fellow	LCMB	NCI
	W. J. La Rochelle	Staff Fellow	LCMB	NCI
	J.-C. Yu	Visiting Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

University of Texas at Austin, TX (A. Sackaguchi); University of Massachusetts Medical School, Worcester, MA (J. Greenberger)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Genetics 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.)*

(1) The IL-3-dependent myeloid progenitor cell line, 32D, was transfected with expression vector containing human cDNAs for epidermal growth factor (EGF), platelet-derived growth factor (PDGF) or colony-stimulating factor-1 (CSF-1) receptor tyrosine kinases. Exposure of the 32D transfectants to their respective ligands triggered proliferation coupled with monocytic differentiation. Expression of receptors lacking tyrosine kinase domains in 32D cells induced long-term proliferation without monocytic differentiation. These receptors included those for erythropoietin (Epo), IL-2 and GM-CSF. Our results suggest that the four hematopoietic receptors analyzed may utilize the same signal transduction pathway in 32D cells and that the four tyrosine kinase-containing receptors may evoke separate or additional pathways for signal transduction in this system.

(2) We have demonstrated that phosphatidylinositol-3 kinase associates with tyrosine-phosphorylated cellular substrates and hematopoietin receptors after ligand-induced stimulation of a variety of receptors including those for IL-2, IL-3, IL-4, Epo and GM-CSF, suggesting that activation of this enzyme may play an integral role in mitogenesis of cells within the hematopoietic lineage.

(3) We have generated a murine monoclonal antibody (MAb) directed against the human α PDGF receptor (PDGFR). This MAb recognizes the human α PDGFR by immunoprecipitation, immunoblot analysis and immunofluorescence on live cells expressing the α PDGFR. The MAb was shown to neutralize PDGF-induced biological responses. Conversely, PDGF blocks the ability of the MAb to recognize the receptor. These results suggest that the epitope recognized by this MAb is in the extracellular PDGF binding domain and may be useful in the diagnosis or treatment of diseases involving overexpression of PDGF or the α PDGFR.

PROJECT DESCRIPTION

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

J. H. Pierce	Research Microbiologist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
M. A. Heidaran	Staff Fellow	LCMB	NCI
W. J. La Rochelle	Staff Fellow	LCMB	NCI
J.-C. Yu	Visiting Fellow	LCMB	NCI

Objectives:

A) To determine mechanisms by which oncogenes abrogate growth factor dependence and induce transformation of factor-dependent hematopoietic cells; (B) to determine the role of growth factors and growth factor receptor expresssion on the signal transduction pathway controlling either growth or differentiation in cells of connective tissue or hematopoietic origin; and (C) to determine the oncogenic potential of growth factor receptor-like oncogenes in mammary carcinoma.

Methods Employed:

Standard hematopoietic culture techniques, including an in vitro hematopoietic colony-forming assay developed to detect transformation of hematopoietic cells by oncogenes and cloning of established cell lines in soft agar. Other procedures include generation of growth factors and differentiation of factor-dependent cell lines.

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

DNA transfection by the electroporation method was utilized to express growth factor receptor and related oncogenes. Analysis of transformants was performed by Southern and northern hybridization analysis, western immunoblotting and growth factor binding and mitogenic assays.

Major Findings:

1. Introduction of tyrosine kinase-containing receptors with closely related tyrosine kinase domains into the clonal myeloid progenitor line, 32D, confers ligand-induced coupling with mitogenic, chemotactic and monocytic differentiation pathways inherently present in these cells. Activation of these receptors also leads to tyrosine phosphorylation of multiple and overlapping substrates and induces either phosphoinositol or phosphocholine metabolism. Whether components of either of these intracellular biochemical pathways play critical roles in modulating the biological responses observed in these cells remains to be determined. However, it is clear that when the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptor pathways are reconstituted in this cell line, they behave in a very similar manner to that of the CSF-1 receptor pathway.

   In contrast to the biological effects induced by triggering of tyrosine kinase-containing receptors, activation of four hematopoietin receptors in 32D cells all induced long-term proliferation without monocytic differentiation. A common set of tyrosine-phosphorylated substrates with molecular weights of 70

and 93 kDa was observed in response to all four cytokines. These results suggest that the hematopoietin receptors analyzed couple through an identical tyrosine kinase in the 32D cells and that tyrosine kinase-containing receptors may trigger additional or separate pathways in these myeloid cells.

2. Activation of several tyrosine kinase-containing receptors, including those for PDGF, CSF-1 and EGF, has previously been shown to induce association with and possible tyrosine phosphorylation of phosphatidylinositol-3 (PI-3) kinase. In order to determine whether PI-3 kinase could be activated by a variety of ligands which do not possess intracellular tyrosine kinase domains, we analyzed six receptors within the hematopoietin family. Ligand stimulation by five of the six receptors induced PI-3 kinase activation. In addition, we were able to demonstrate that PI-3 kinase activity could be found in a complex with the IL-4 receptor and that this association correlated with mitogenicity. These results suggest that PI-3 kinase may play a crucial role in signal transduction through multiple growth factor regulatory networks.

3. We utilized a murine 32D cell line transfected with the human α PDGF receptor (PDGFR) to generate a MAb specific to the human α PDGFR. This MAb should prove to be a valuable tool for biochemical analysis of α PDGFR protein, particularly for screening human tumors for overexpression of α PDGFR by immunohistochemical technique. Moreover, its ability to neutralize PDGF AA-, AB- and BB-induced mitogenic responses could make this MAb a potentially important tool for combating certain pathogenic diseases such as atherosclerosis, and certain malignancies involving α PDGF receptor overexpression.

Publications:

Aaronson SA, Pierce JH. Molecular mimicry of growth factors by products of tumor viruses. Cancer Cells 1990;2:212-4.

Anklesaria P, Teixido J, Laiho M, Pierce JH, Greenberger JS, Massague J. Cell-cell adhesion mediated by membrane TGFα binding to EGF receptor promotes cell proliferation. Proc Natl Acad Sci USA 1990;87:3289-93.

Choudhury GG, Sylvia VL, Wang L-M, Pierce J, Sakaguchi AY. The kinase insert domain of colony-stimulating factor-1 receptor is dispensable for CSF-1-induced phosphatidylcholine hydrolysis. FEBS Lett (In Press)

Choudhury GG, Wang L-M, Pierce J, Harvey S, Sakaguchi AY. A mutational analysis of phosphatidylinositol-3'-kinase activation by human colony-stimulating factor receptor. J Biol Chem (In Press)

Di Fiore PP, Segatto O, Lonardo F, Fazioli F, Pierce JH, Aaronson SA. The carboxy-terminal domains of *erb*B-2 and epidermal growth factor receptor exert different regulatory effects on intrinsic receptor tyrosine kinase function and transforming activity. Mol Cell Biol 1990;10:2749-56.

Di Marco E, Pierce JH, Knicley CL, Di Fiore PP. Transformation of NIH/3T3 cells by overexpression of the normal coding sequence of the rat *neu* gene. Mol Cell Biol 1990;10:3247-52.

Di Marco E, Pierce JH, Aaronson SA, Di Fiore PP. Mechanisms by which EGF receptor and TGFα contribute to transformation. Nat Immun Cell Growth Regul 1990;9:209-21.

Finzi E, Fleming T, Pierce JH. Retroviral expression of transforming growth factor does not transform fibroblasts or keratinocytes. J Invest Dermatol 1990;4:382-7.

Fitzgerald TJ, Henault S, Sakakeeny M, Santucci MA, Pierce JH, Anklesaria P, Kase K, Das I, Greenberger JS. Expression of transfected recombinant oncogenes increases radiation resistance of clonal hematopoietic and fibroblast cell lines selectively at clinical low dose rate. Radiation Res 1990;122:44-52.

Heidaran MA, Pierce JH, Jensen RA, Matsui T, Aaronson SA. Chimeric α- and β-platelet-derived growth factor (PDGF) receptors define three immunoglobulin-like domains of the α-PDGF receptor that determine PDGF-AA binding specificity. J Biol Chem 1990;265:18741-4.

Heidaran MA, Pierce JH, Lombardi D, Ruggiero M, Gutkind JS, Matsui T, Aaronson SA. Deletion or substitution within the α platelet-derived growth factor receptor kinase insert domain: effects on functional coupling with intracellular signalling pathways. Mol Cell Biol 1991;11:134-42.

Kreider BL, Phillips PD, Prystowsky MB, Shirsat N, Pierce JH, Tushinski R, Rovera G. Induction of the granulocyte-macrophage colony-stimulating factor (CSF) receptor by granulocyte CSF increases the differentiative options of a murine hematopoietic progenitor cell. Mol Cell Biol 1990;10:4846-53.

Lonardo F, Di Marco E, King CR, Pierce JH, Segatto O, Aaronson SA, Di Fiore PP. The normal *erb*B-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. New Biol 1990;2:992-1003.

Pierce JH. Signal transduction through foreign growth factor receptors. In: Mond J, Weiss A, Cambier J, eds. Genetic approaches to understanding cell activation. New York: Raven Press (In Press)

Pierce JH, Arnstein P, Di Marco E, Artrip J, Kraus MH, Lonardo F, Di Fiore PP, Aaronson SA. Oncogenic potential of *erb*B-2 in human mammary epithelial cells. Oncogene (In Press)

Pierce JH, Di Marco E, Cox GW, Lombardi D, Ruggiero M, Varesio L, Wang LM, Choudhury GG, Sakaguchi AY, Di Fiore PP, Aaronson SA. Macrophage-colony-stimulating factor (CSF-1) induces proliferation, chemotaxis and reversible monocytic differentiation in myeloid progenitor cells transfected with the human c-fms/CSF-1 receptor cDNA. Proc Natl Acad Sci USA 1990;87:5613-7.

Principato M, Cleveland JL, Rapp UR, Holmes KL, Pierce JH, Morse III HC, Klinken SP. Transformation of murine bone marrow cells with combined v-*raf*/v-*myc* oncogenes yields clonally related mature B cells and macrophages. Mol Cell Biol 1990;10:3562-8.

Puri J, Pierce JH, Hoffman T. Selective inhibition of PGE2 production in cells transfected with c-*fms* encoded CSF-1 receptor genes by the tyrosine kinase inhibitor ST638. Agents Actions (In Press)

Smith EA, Seldin MF, Martinez L, Watson ML, Choudhury GG, Lalley PA, Pierce J, Aaronson S, Barkers J, Naylor SL, Sakaguchi AY. The mouse W19H chromosome 5 deletion encompasses the platelet-derived growth factor receptor alpha gene. Proc Natl Acad Sci USA (In Press)

Yu J-C, Heidaran MA, Pierce JH, Gutkind JS, Lombardi D, Ruggiero M, Aaronson SA. Tyrosine mutations within the αPDGFR kinase insert domain abrogate receptor-associated PI-3 kinase activity without affecting mitogenic or chemotactic signal transduction. Mol Cell Biol (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05366-08 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

The erbB-3 Protein Reveals Structural Features of a Transmembrane Tyrosine Kinase

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

PI:	Matthias H. Kraus	Visiting Scientist	LCMB	NCI
Others:	Paolo Fedi	Special Volunteer	LCMB	NCI
	Stuart A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*

Istituto Patologia Generale, Rome, Italy (R. Muraro)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	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.)*

We have previously identified and characterized by cDNA cloning a third functional gene, designated erbB-3, in the human erbB/epidermal growth factor (EGF) subfamily of tyrosine kinases. Its predicted coding structure revealed features characteristic of a growth factor receptor tyrosine kinase with significantly greater overall similarities to both epidermal growth factor receptor (EGFR) and erbB-2 products than to any other tyrosine kinase. For high level in vitro expression of the encoded erbB-3 gene product, its complete coding sequence was introduced in LTR-based eukaryotic expression vectors and transfected in the presence of a selectable marker gene into NIH/3T3 fibroblasts. Utilizing polyclonal antisera raised against epitope-specific peptides in the predicted erbB-3 coding sequence, the mature erbB-3 product was identified as a 180-kDa protein in marker-selected transfectants containing the recombinant expression vector and was absent from control NIH/3T3 cells. Biochemical characterization of the erbB-3 protein showed its biosynthesis as a 145-kDa precursor polypeptide which becomes posttranslationally modified by N-linked glycosylation into a 180-kDa mature glycoprotein. Immunohistochemical analysis with epitope-specific antisera identified a transmembrane topology of the mature erbB-3 protein. Its ability to autophosphorylate on tyrosine residues demonstrated an intrinsic tyrosine kinase activity associated with the cytoplasmic portion of the gp180erbB-3 protein. Preliminary binding and triggering studies did not reveal direct interaction of EGF or transforming growth factor alpha (TGFα), the two prototype ligands for the closely related EGF receptor, with the mature erbB-3 protein for signal transduction. This suggests that within the erbB/EGFR family, the erbB-3 protein may function as a distinct growth factor receptor for an as yet unidentified ligand.

PROJECT DESCRIPTION

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

Matthias H. Kraus	Visiting Scientist	LCMB	NCI
Paolo Fedi	Special Volunteer	LCMB	NCI
Stuart A. Aaronson	Chief	LCMB	NCI

Objectives:

1. Identification and characterization of novel proto-oncogenes in growth factor receptor tyrosine kinase families.

2. Characterization of their biological function in normal and neoplastic cell proliferation including identification of novel growth factor-mediated signalling pathways.

3. Investigation of mechanisms that lead to the activation of these molecules in the neoplastic process and their role in human neoplasia.

4. Reversion of the neoplastic process in vitro by targeting specific growth factor-mediated signalling pathways known to be involved in human malignancies at the cell membrane receptor level.

Methods Employed:

Southern blotting, northern blotting, dot blot analysis, RNAse protection, recombinant DNA technology including genomic and cDNA cloning, nucleotide sequence analysis, generation of polyclonal peptide antisera and monoclonal antibodies, gene product analysis by immunoblotting and immunoprecipitation, in vivo phosphorylation, autokinase assays, DNA transfection, cell culture, ligand-binding and mitogenic assays.

Major Findings:

1. High level in vitro expression of the *erb*B-3 protein utilizing LTR-based eukaryotic expression vectors and generation of epitope-specific *erb*B-3 polyclonal antisera.

2. The *erb*B-3 protein is synthesized in the cell as a 145-kDa polypeptide precursor which becomes modified by N-linked glycosylation into a 180-kDa mature glycoprotein with transmembrane topology.

3. $gp180^{erbB\text{-}3}$ possesses intrinsic tyrosine kinase activity associated with the cytoplasmic portion of the transmembrane protein.

4. The *erb*B-3 protein represents a distinct receptor tyrosine kinase for an as yet unidentified ligand.

Publications:

Aaronson SA, Rubin JS, Finch PW, Wong J, Marchese C, Falco J, Taylor WG, Kraus MH. Growth factor-regulated pathways in epithelial cell proliferation. Am Rev Respir Dis 1990;142:S7-10.

Iglehart JD, Kraus MH, Langton BC, Huper G, Kerns BJ, Marks JR. Increased *erb*B-2 gene copies and expression in multiple stages of breast cancer. Cancer Res 1990;50:6701-7.

Kraus MH, Aaronson SA. Detection and isolation of novel protein-tyrosine kinase genes employing reduced stringency hybridization. In: Hunter T, Sefton BM, eds. Methods in enzymology: tyrosine kinases. New York, Cold Spring Harbor Press (In Press)

Pierce JH, Arnstein P, DiMarco E, Artrip J, Kraus MH, Lonardo F, Di Fiore PP, Aaronson SA. Oncogenic potential of *erb*B-2 in human mammary epithelial cells. Oncogene (In Press)

Patents:

King CR, Kraus MH, Aaronson SA. US Patent Application Serial No. 7,110,791 (Pending): A Human Gene Related to but Distinct from EGF Receptor Gene.

Kraus MH, Aaronson SA. US Patent Application Serial No. 7,444,406 (Pending): DNA Segment Encoding a Gene for a Receptor Related to the Epidermal Growth Factor Receptor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05457-07 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Growth Factor Receptors and Mitogenic Pathways in Transformation

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

PI:	P. P. Di Fiore	Visiting Scientist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. H. Pierce	Research Microbiologist	LCMB	NCI
	F. Fazioli	Visiting Fellow	LCMB	NCI
	D. P. Bottaro	Senior Staff Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

Molecular Oncology, Inc. Gaithersburg, MD (C. R. King); National Heart, Lung and Blood Institute, Bethesda, MD (S. G. Rhee); Microbiololgy Institute, University of Copenhagen, Denmark (K. Helin); Joslyn Diabetes Center, Harvard University, Boston, MA (M. F. White)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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.)*

1. The role of autophosphorylation in mediating erbB-2-specific signal transduction was analyzed. Results indicate the tyrosine-phosphorylated COOH-terminal of the erbB-2 moiety, influence substrate binding (and henceforth their phosphorylation) by increasing the affinity of erbB-2 for its intracellular substrates. A model was proposed for the role of the phosphorylated COOH-terminus as a stabilizer of the receptor substrate(s) multimolecular complex.

2. Studies on the inhibition of erbB-2-induced transformed phenotype were undertaken with monoclonal antibodies (MAbs) directed against the erbB-2 extracellular domain. Several classes of MAbs were identified which acted as positive or negative modulators of erbB-2 action. In particular, one MAb (#23) was able to cause dramatic reduction of tumorigenic growth in nude mice of NIH/3T3 cells transformed by erbB-2 or human tumor cells with erbB-2 amplification.

3. Based on our previous results, characterization and isolation of novel substrates for the epidermal growth factor receptor (EGFR) was performed. We have at present identified and cloned genes for seven new proteins which are phosphorylated on tyrosine by an active EGFR kinase.

PROJECT DESCRIPTION

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

P. P. Di Fiore	Visiting Scientist	LCMB	NCI
F. Fazioli	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. H. Pierce	Research Microbiologist	LCMB	NCI
D. P. Bottaro	Senior Staff 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.

2. To study how the above "activated" genes alter the growth properties and the differentiated program of certain cell cytotypes, like hematopoietic and epithelial cells.

3. To study the mechanisms of mitogenic signal transduction and isolate intracellular substrates for the tyrosine kinase activity of growth factor receptors.

Methods Employed:

Generation of eukaryotic expression vectors; gene transfer by DNA transfection (Ca++ precipitate method or electroporation); standard recombinant DNA and protein analysis methods; affinity purification of putative growth factor receptor substrates by chromatography on immobilized antiphosphotyrosine antibodies.

Major Findings:

1. In our previous work we showed that autophosphorylation of the *erb*B-2 is required for its optimal transforming activity. However, the nature of the bioassay employed (transformation, which requires *erb*B-2 overexpression at a level of ~2 x 10^6 receptors/cell) and the constitutive kinase activity of the *erb*B-2 molecule prevented the analysis of the role of autophosphorylation under physiological conditions. To overcome this problem, we utilized a chimeric epidermal growth factor receptor (EGFR)/*erb*B-2 molecule which transduces an *erb*B-2-specific signal upon EGF triggering. When expressed at physiological levels (~10^5 receptors/cell), this molecule stimulated DNA synthesis in an EGF-dependent manner and associated and phosphorylated a prototype substrate, PLC-γ. An autophosphorylation-negative mutant in which all tyrosine phosphorylation sites were mutagenized to phenylalanine (EGFR/*erb*B-2 5P) was unable to trigger DNA synthesis and to associate with PLC-γ. Thus, autophosphorylation is absolutely required for *erb*B-2 action and influences its mitogenic action by dictating the affinity of interaction with intracellular substrates.

2. A panel of monoclonal antibodies (MAbs) was generated (in collaboration with C. R. King) against the extracellular domain of *erb*B-2. They could be classified according to their agonist or antagonist action on *erb*B-2 function. One of these MAbs, #23, exerted strong inhibition on DNA synthesis by NIH/3T3 cells transformed by *erb*B-2 and was able to revert the morphology of these cells to a flat phenotype.

It was also able (in combination with other MAbs) to inhibit NIH-*erb*B-2 tumorigenic potential in nude mice and the tumorigenicity of human cell lines with *erb*B-2 overexpression as well. The mechanism of action of MAb #23 is under investigation. It seems to be acting through a novel mechanism since it does not reduce *erb*B-2 activity (as estimated by autophosphorylation), nor induce downregulation.

3. We also continued our studies on the intracellular substrates of the EGFR kinase. After purification of these substrates by affinity chromatogaphy on antiphosphotyrosine antibodies, we utilized them to generate polyclonal sera directed against them. The sera identified seven novel substrates which did not correspond to any of the known ones (like PLC-γ, GAP, *raf* or p85). The sera were used to screen λgt11 expression libraries which led to the molecular isolation of several phages corresponding to seven new genes. After subcloning into bluescript, partial sequence was performed which revealed no homology to known proteins. We have, therefore, cloned novel EGFR substrates. Further characterization is now in progress which involves cloning full length cDNAs and generation of polyclonal and monoclonal antibodies directed against the individual proteins expressed in bacteria as trpE fusion products.

Publications:

Di Fiore PP, Segatto O, Aaronson SA. Cloning, expression and biological effects of the *erb*B-2/*neu* gene in mammalian cells. In: Barnes D, Mathr P, Sato GH, eds. Peptide growth factors. Methods in enzymology: peptide growth factors, Part C. New York: Academic Press (In Press)

Di Fiore PP, Segatto O, Lonardo F, Fazioli F, Pierce JH, Aaronson SA. The carboxy-terminal domains of *erb*B-2 and epidermal growth factor receptor exert different regulatory effects on intrinsic receptor tyrosine kinase function and transforming activity. Mol Cell Biol 1990;10:2749-56.

Di Marco E, Pierce JH, Aaronson SA, Di Fiore PP. Mechanisms by which EGF-receptor and TGFα contribute to malignant transformation. Nat Immun Cell Growth Regul 1990;9:209-21.

Di Marco E., Pierce JH, Di Fiore PP. Transformation of NIH/3T3 cells by overexpression of the normal coding sequence of the rat *neu* gene. Mol Cell Biol 1990;10:3247-52.

Fazioli F, Kim U-H, Rhee SG, Molloy CJ, Segatto O, Di Fiore PP. The *erb*B-2 mitogenic signalling pathways: tyrosine phosphorylation of phospholipase C-γ and GAP does not correlate with *erb*B-2 mitogenic potency. Mol Cell Biol (In Press)

Fleming TP, Pech MW, Di Marco E, Di Fiore PP, Falco JP, Aaronson SA. Gene-encoding growth factors as oncogenes. Current communications in molecular biology. New York: Cold Spring Harbor (In Press)

Heidaran MA, Fleming TP, Bottaro DP, Bell GI, Di Fiore PP, Aaronson SA. Transformation of NIH/3T3 fibroblasts by an expression vector for the human epidermal growth factor precursor. Oncogene 1990;5:1265-70.

Lonardo F, Di Marco E, King CR, Pierce JH, Segatto O, Aaronson SA, Di Fiore PP. The normal erbB-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. New Biol 1990;2:992-1003.

Pierce JH, Arnstein P, Di Marco E, Artrip J, Kraus MH, Lonardo F, Di Fiore PP, Aaronson SA. Oncogenic potential of *erb*B-2 in human mammary epithelial cells. Oncogene (In Press)

Pierce JH, Di Marco E, Cox GW, Lombardi D, Ruggiero M, Varesio L, Wang LM, Choudhury GG, Sakaguchi AY, Di Fiore PP, Aaronson SA. Macrophage-colony-stimulating factor (CSF-1) induces proliferation, chemotaxis and reversible monocytic differentiation in myeloid progenitor cells transfected with the human c-*fms*/CSF-1 receptor cDNA. Proc Natl Acad Sci USA 1990;87:5613-7.

Segatto O, Lonardo F, Wexler D, Fazioli F, Pierce JH, Bottaro DP, White MF, Di Fiore PP. The juxtamembrane regions of the EGF receptor and gp185^{erbB-2} determine the specificity of signal transduction. Mol Cell Biol (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05469-06 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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. Kruh	Senior Staff Fellow	LCMB	NCI
Others:	J. H. Pierce	Research Microbiologist	LCMB	NCI
	S. A. Aaronson	Chief	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:	PROFESSIONAL:	OTHER:
1.0	1.0	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.)*

We have isolated and characterized the coding sequence of a novel human gene, termed arg, which is the second member of the Abelson subfamily of nonreceptor tyrosine kinases. The coding sequence and activated forms of it have been expressed in various systems to characterize its biological properties.

To characterize the arg protein product, the arg coding sequence was expressed in bacteria using an IPTG inducible system. The recombinant protein was detected in bacterial lysates by immunoblotting and exhibited a molecular mass of 145 kDa. Phosphoamino acid analysis of the protein following an in vitro autokinase reaction revealed tyrosine phosphorylation and established that the arg protein possesses tyrosine kinase activity. High titer antibody capable of detecting the cellular arg gene product was generated by expressing a carboxy terminal segment of the protein in bacteria and using the recombinant protein as an immunogen. The arg gene product was identified in cultured human cells as a 145-kDa protein that exhibited autokinase activity. Analysis of arg expression in murine tissues revealed that arg, like c-abl, is widely expressed, further extending the similarities between the two genes.

A chimeric gag-arg molecule, similar to the gag-abl protein of v-abl, has been assembled and placed in a eukaryotic expression vector. Analysis of NIH/3T3 cells transfected with this vector revealed that, unlike gag-abl, it does not transform these cells. Phosphotyrosine analysis of the gag-arg protein revealed that it is less phosphorylated than the gag-abl counterpart. Further studies are underway to determine how the structure of these two related molecules affects their markedly different abilities to transform NIH/3T3 cells. In addition, the ability of the gag-arg molecule to transform hematopoietic cells is being tested.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

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

G. Kruh	Senior Staff Fellow	LCMB NCI
J. H. Pierce	Research Microbiologist	LCMB NCI
S. A. Aaronson	Chief	LCMG NCI

Objectives:

To characterize the biological properties of *arg*, the second member of the Abelson subfamily of nonreceptor tyrosine kinases.

Methods Employed:

Standard cloning techniques were used to construct the arg expression vectors. Protein identification was done by standard immunoprecipitation and immunoblotting. Transfection of eukaryotic cells was done by the calcium precipitation method.

Major Findings:

1. The product of the arg gene has protein tyrosine kinase activity and has been identified as a 145-kDa protein in human cells.

2. Arg is widely expressed and, therefore, likely functions in signalling pathways fundamental to many cells types.

3. An active gag-arg molecule, although similar to the transforming protein of v-abl, does not transform NIH/3T3 cells.

Publications:

Kruh GD, Perego R, Miki T, Aaronson SA. The complete coding sequence of abl-2 defines the Abelson subfamily of cytoplasmic tyrosine kinases. Proc Natl Acad Sci USA 1990;87:5802-6.

Perego R, Ron D, Kruh GD. *Arg* encodes a 145-kDa protein tyrosine kinase. Oncogene (In Press)

Patents:

Kruh G, Perego R, Miki T, Aaronson SA. US Patent Application Serial No. 7,135,280 (Pending): The Complete Coding Sequences of arg Defines the Abelson Subfamily of Cytoplasmic Tyrosine Kinases.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05546-04 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Structural and Functional Characterization of v-sis Gene Product

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

PI:	W. J. LaRochelle	Staff Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	R. A. Jensen	Biotechnology Fellow	LCMB	NCI
	J. Beeler	IRTA 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:	PROFESSIONAL:	OTHER:
2.0	1.0	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.)*

Platelet-derived growth factor (PDGF) is a disulfide-linked dimer consisting of two related polypeptide chains, designated PDGF A and PDGF B, that are the products of distinct genes. The gene encoding the human PDGF B chain is the normal counterpart of the v-sis oncogene.

Since PDGF A and PDGF B have distinct transforming phenotypes and cellular locations, we constructed twelve chimeric PDGF molecules to investigate structural regions of PDGF A or PDGF B associated with PDGF functions. The mechanistic basis of potent PDGF B transformation segregated with the activation of both α and β PDGF receptors. Furthermore, a 40 amino acid domain of PDGF B was identified as responsible for receptor binding properties and the potent transforming phenotype. Two subdomains (106-114 and 135-144) within these 40 PDGF B amino acids were identified as critical for β receptor interaction. Additional studies of this region identified a 13-amino acid subdomain sensitive to deletions, all of which abrogated PDGF receptor functional activation. Further studies will be directed at identification of competitive antagonists to be used for blocking PDGF-mediated responses.

PDGF B's potent transforming phenotype was also dissociated from its membrane-associative property. The differences in PDGF A and B secretory properties was due to differences in the proteolytic processing of analogous functional domains. We further demonstrated that a 15-amino acid domain within PDGF B's final thirty amino acids was responsible for cell surface retention.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

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

S. A. Aaronson	Chief	LCMB	NCI
W. J. LaRochelle	Staff Fellow	LCMB	NCI
R. A. Jensen	Biotechnology Fellow	LCMB	NCI
J. Beeler	IRTA Fellow	LCMB	NCI

Objectives:

The structural/functional characterization of platelet-derived growth factor (PDGF) and PDGF receptors.

Methods Employed:

Standard recombinant DNA techniques, DNA sequencing, PCR, oligonucleotide-directed mutagenesis, transfection assay, tissue culture, immunoprecipitation and SDS-PAGE analysis of proteins, metallothionein in vector or baculovirus vector system for protein overexpression and analysis of PDGF receptor activation, enzyme-linked immunoabsorbent assay, and immunoaffinity purification.

Major Findings:

1. The mechanistic basis of potent PDGF B transformation correlated with the ability to activate both the α and β PDGF receptors and was independent of its membrane association.

2. PDGF B amino acid residues 105-144 were responsible for β PDGF receptor activation. Moreover, two subdomains (106-114 and 135-144) within these 40 PDGF B amino acids were identified as critical for β receptor interaction. Introduction of mutations throughout this domain of v-*sis*/PDGF B also identified a 13-amino acid region of structural and functional importance.

3. Differential proteolytic processing of analogous retention domains explained PDGF A and B secretory differences. A 15 amino acid subdomain of PDGF B was identified as responsible for its membrane-associative properties.

4. In studies of expression of α and β PDGF receptors using a baculovirus expression system, PDGF receptors were functionally characterized and immunoaffinity purified. Purified receptors were used to identify receptor substrates such as GAP.

Publications:

Giese N, LaRochelle WJ, May-Siroff M, Robbins KC, Aaronson SA. A small v-*sis*/PDGF B domain in which subtle conformational changes abrogate PDGF receptor interaction and transforming activity. Mol Cell Biol 1990;10:5496-501.

LaRochelle WJ, Giese N, May-Siroff M, Robbins KC, Aaronson SA. Chimeric molecules map and dissociate the potent transforming and secretory properties of PDGF A and PDGF B. J Cell Sci 1990;13:31-42.

LaRochelle WJ, Giese N, May-Siroff M, Robbins KC, Aaronson SA. Molecular localization of the transforming and secretory properties of PDGF A and PDGF B. Science 1990;248:1541-4.

LaRochelle WJ, Giese, N, Robbins KC, Aaronson SA. Variant PDGF ligands and receptors; structure/function relationships. News Physiol Sci (In Press)

LaRochelle, WJ., May-Siroff, M., Robbins, KC, and Aaronson, SA. Identification of a platelet-derived growth factor B chain retention sequence responsible for its stable cell surface association. Genes Dev (In Press)

Patents:

LaRochelle WJ, Robbins KC, Aaronson SA. US Patent Application Serial No. 7,365,715 (Pending): Neutralizing Monoclonal Antibody to Human Platelet-derived Growth Factor Hetero- and Homodimers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05548-04 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Development of Expression Cloning System for Oncogene cDNAs

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

PI:	T. Miki	Visiting Scientist	LCMB	NCI
Others:	T. P. Fleming	Senior Staff Fellow	LCMB	NCI
	D. P. Bottaro	Senior Staff Fellow	LCMB	NCI
	S. A. Aaronson	Chief	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:	PROFESSIONAL:	OTHER:
2.0	1.0	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.)*

An expression cloning strategy was devised to isolate the keratinocyte growth factor (KGF) receptor cDNA by creation of an autocrine transforming loop. NIH/3T3 fibroblasts, which secrete this epithelial cell-specific mitogen, were transfected with a keratinocyte expression cDNA library. Among several transformed foci identified, one demonstrated the acquisition of specific high affinity KGF binding sites. The pattern of binding competition by related fibroblast growth factors (FGFs) indicated that this receptor had high affinity for acidic FGF (aFGF) as well as KGF. The rescued 4/2-kb cDNA was shown to encode a predicted membrane-spanning tyrosine kinase related to but distinct from the basic FGF (bFGF) receptor. This expression cloning approach may be generally applicable to the isolation of genes that constitute limiting steps in mitogenic signalling pathways.

PROJECT DESCRIPTION

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

T. Miki	Visiting Scientist	LCMB NCI
T. P. Fleming	Senior Staff Fellow	LCMB NCI
D. P. Bottaro	Senior Staff Fellow	LCMB NCI
S. A. Aaronson	Chief	LCMB NCI

Objectives:

We have developed a highly efficient expression cDNA cloning system. As an application of this system, we devised a new approach to clone cDNAs for critical components of signal transduction pathways. As the first target of this approach, we attempted to isolate the keratinocyte growth factor receptor (KGFR) by complementation for autocrine transformation.

Methods Employed:

Standard molecular cloning methods were used to engineer recombinant molecules. DNA-mediated gene transfer (DNA transfection) was used to introduce cloned DNA into NIH/3T3 cells. RNA extraction and cDNA synthesis methods were used to construct cDNA libraries. Growth factor binding assays and cross-linking were used to analyze receptor-ligand binding. Immunoblot analysis was used to analyze protein products.

Major Findings:

An expression cloning strategy was devised to isolate the KGFR cDNA by creation of an autocrine transforming loop. Introduction of an appropriate expression cDNA library prepared from the mRNA of one cell type into another results in the forced expression of genes which might normally be silent in recipient cells. KGF is expressed in stromal fibroblasts and has the properties of a paracrine mediator of epithelial cell proliferation. Thus, we reasoned that introduction of an epithelial cell cDNA library into fibroblasts might create an autocrine loop by ectopic expression of the KGFR cDNA and result in morphological transformation of the recipient cells. The foci that express the KGFR can be identified by specific KGF binding, and the cDNA can be rescued from the cells.

We prepared a cDNA library from BALB/MK epidermal keratinocytes in an improved expression vector, λpCEV27 by the method previously reported, and transfected NIH/3T3 mouse embryo fibroblasts. We detected 15 transformed foci and each was shown to be resistant to G418 indicating that it contained integrated vector sequences. Three representative transformants were chosen for more detailed characterization based upon differences in their morphologies. Several plasmids were isolated from each transformant after plasmid rescue. A single cDNA clone rescued from each transformant was found to possess high titered transforming activity. Transfectants induced by the individual plasmids containing these epithelial cell-transforming cDNAs, designated *ect*1, *ect*2 and *ect*3, were utilized in subsequent analyses.

To investigate the possibility that any of the three genes might encode the KGFR, we performed binding studies with recombinant [^{125}I]-KGF. BALB/MK cells demonstrated specific high affinity binding of [^{125}I]-KGF which was not observed when NIH/3T3 cells were used. Expression of the *ect*1 gene by NIH/3T3 cells resulted

in the acquisition of 3.5-fold more [^{125}I]-KGF binding sites than BALB/MK cells. Under the same conditions, control NIH/3T3 as well as transfectants containing the *ect2* or *ect3* did not bind the labeled growth factor. These results suggested that *ect1* encoded KGFR, whose introduction into NIH/3T3 cells had completed an autocrine transforming loop.

To characterize *ect1*, the transforming 4.2-kb cDNA released by *Sal* I digestion was used as a molecular probe to hybridize *Sal* I-digested genomic DNAs. Since *Sal* I is an infrequent cutter, the large genomic DNA fragments migrated near the origin of the gel. While the expected 4.2-kb DNA fragment was detected in the *ect1* transformant, neither NIH/3T3 nor the other transfectants showed evidence of a *Sal* I fragment hybridized by the cDNA insert. *Ect1*-related sequences were also observed in the DNAs of other species analyzed, including human, indicating its high degree of conservation in vertebrate evolution. A single *ect1* transcript of around 4.2 kb was observed in BALB/MK cells. Thus, our cDNA clone represented essentially the complete *ect1* transcript.

Structural analysis of the 4.2-kb *ect1* cDNA insert of the plasmid revealed that it encoded a predicted membrane-spanning tyrosine kinase closely related to the mouse basic fibroblast growth factor receptor (bFGFR). The extracellular portion of the KGFR contained two immunoglobulin (Ig)-like domains. Both chicken and mouse bFGFRs contain a stretch of eight consecutive acidic amino acid residues between the first and second Ig-like domains. The KGFR lacked such an acidic amino acid domain. The central core of the catalytic domain was flanked by a relatively long juxtamembrane sequence, and the tyrosine kinase domain was split by a short insert of 14 residues, similar to that observed in mouse, chicken, and human bFGF receptors.

Scatchard analysis of [^{125}I]-KGF binding to the NIH/*ect1* transfectant revealed expression of two similar high affinity receptor populations. Out of a total of ~3.8 x 10^5 sites/cell, 40% displayed a Kd of 180 pM, while the remaining 60% showed a Kd of 480 pM. The pattern of KGF and FGF competition for [^{125}I]-labeled KGF binding to NIH/*ect1* cells was also very similar to that observed with BALB/MK cells. Both cells showed 15-fold less efficient competition by bFGF than KGF and acidic FGF (aFGF) for bound [^{125}I]-labeled KGF. Thus, the cloned KGFR exhibited the characteristic pattern of KGF and FGF competition displayed by BALB/MK cells and implied that the KGFR is a high affinity receptor for aFGF as well as KGF.

When [^{125}I]-labeled KGF cross-linking was performed with NIH/*ect1* cells, we observed a single species corresponding in size to the smaller 137-kDa complex in BALB/MK cells. Moreover, detection of this band was specifically and efficiently blocked by unlabeled KGF. When glycosylation is considered, the size of the KGFR predicted by sequence analysis corresponds reasonably well with the corrected size (115 kDa) of the cross-linked KGFR in the *ect1* transfectant.

NIH/*ect1* cells contained several tyrosine phosphorylated proteins that were not detectable in control or KGF-stimulated NIH/3T3 cells. Addition of KGF to NIH/*ect1* cells resulted in the detection of increased tyrosine phosphorylation of several putative substrates. These findings established that the KGFR was enzymatically activated in response to KGF.

Publications:

Gutkind JS, Link DC, Katamine S, Lacal P, Miki T, Ley TJ, Robbins KC. A novel c-*fgr* exon utilized in Epstein-Barr virus infected B-lymphocytes but not normal monocytes. Mol Cell Biol 1991;11:1500-7.

Kruh GD, Perego R, Miki T, Aaronson SA. The complete coding sequence of *arg* defines the Abelson subfamily of cytoplasmic tyrosine kinases. Proc Natl Acad Sci USA 1990;87:5802-6.

Miki T, Fleming TP, Bottaro DP, Rubin JS, Ron D, Aaronson SA. Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. Science 1991;251:72-5.

Miki T, Fleming TP, Crescenzi M, Molloy CJ, Blam SB, Reynolds SH, Aaronson SA. Development of a highly efficient expression cDNA cloning system: application to oncogene isolation. Proc Natl Acad Sci USA (In Press)

Miki T, Matsui T, Heidaran MA, Aaronson SA. Automatic directional cloning: an efficient method to construct eukaryotic expression cDNA libraries. In: Alitalo KK, Huhtala ML, Knowles J, Vaheri A. eds. Recombinant systems in protein expression. Amsterdam: Elsevier Science Publishers BV 1990;125-36.

Noiman S, Gazit A, Tori O, Sherman L, Miki T, Tronick SR, Yaniv A. Identification of sequences encoding the equine infectious anemia virus *tat* gene. Virology 1990;176:280-8.

Rubin JS, Chan AM-L, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirschfield DW, Wong J, Miki T, Finch PW, Aaronson, SA. A broad spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc Natl Acad Sci USA 1991;88:415-9.

Takahashi R, Hashimoto T, Xu HJ, Hu SX, Matsui T, Miki T, Bigo-Marshall H, Aaronson SA, Benedict WF. The retinoblastoma gene functions as a growth and tumor suppressor in human bladder carcinoma cells. Proc Natl Acad Sci USA (In Press)

Tronick SR, Noiman LS, Sherman L, Miki T, Tori O, Lichtman-Pleban H, Dahlberg JE, Yaniv A, Gazit A. Regulation of expression of the equine infectious anemia virus genome. In: Papas TS, ed. Gene regulation and AIDS: transcriptional activation, retroviruses, and pathogenesis. Houston: Portfolio Publishing 1989;339-53.

Patent:

Miki T., Aaronson SA, Fleming TP. US Patent Application Serial No. 7,386,053 (Pending): Efficient Directional Genetic Cloning System to Construct cDNA Libraries Containing Full-length Inserts at a High Frequency.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05596-03 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Characterization of Epithelial Cell Mitogens and Their Receptors

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

PI:	J. S. Rubin	Senior Staff Fellow	LCMB	NCI
Others:	D. P. Bottaro	Senior Staff Fellow	LCMB	NCI
	A. Chan	Visiting Fellow	LCMB	NCI
	M. Chedid	Visiting Fellow	LCMB	NCI
	W. G. Taylor	Biologist	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:	PROFESSIONAL:	OTHER:
2.0	1.0	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 continued to characterize the biological properties and mechanisms of action of two fibroblast-derived mitogens which we previously had purified and cloned: keratinocyte growth factor (KGF, a member of the fiboblast growth factor [FGF] family) and a variant of hepatocyte growth factor (HGF). The former is specific for epithelial-cell targets, while the latter is a broad-spectrum mitogen with activity on endothelial cells and melanocytes as well as epithelial cells.

Molecular cloning of the KGF receptor established that it is a transmembrane tyrosine kinase with alternative extracellular domains comprised of either two or three immunoglobulin-like loops. These forms arise from alternatively-spliced transcripts of the bek gene, which recently had been reported to encode a receptor for other members of the FGF family. Of particular note, the alternative exon encoding the carboxy-terminal half of the immunoglobulin loop closest to the transmembrane domain is crucial in determining the KGF-binding capacity of the receptor molecule. A synthetic peptide corresponding to this region of the receptor specifically antagonized the mitogenic activity of KGF.

An HGF receptor has been identified as the c-met proto-oncogene product, another transmembrane tyrosine kinase. An alternative HGF transcript which contains the sequence for the amino-terminal and first two kringle domains encodes a protein that also binds the met protein but functions as a specific, competitive HGF antagonist.

PROJECT DESCRIPTION

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

J. S. Rubin	Senior Staff Fellow	LCMB	NCI
D. P. Bottaro	Senior Staff Fellow	LCMB	NCI
A. Chan	Visiting Fellow	LCMB	NCI
M. Chedid	Visiting Fellow	LCMB	NCI
W. G. Taylor	Biologist	LCMB	NCI

Objectives:

To purify and study growth regulatory factors that stimulate or inhibit proliferation, especially of epithelial cells; to characterize the cell receptors for these molecules and the mechanism(s) of signal transduction; to determine whether the production of such factors is involved in the pathophysiology of human malignancy or other diseases characterized by abnormal patterns of proliferation.

Methods Employed:

Conventional column chromatography; high performance liquid chromatography; protein microsequencing; [^{3}H]-thymidine mitogenesis bioassay; metabolic labeling with [^{35}S]-methionine and -cysteine, as well as [^{32}P]-orthophosphate; iodination of proteins; polymerase chain reaction; immunocytochemistry; *in situ* hybridization.

Major Findings:

1. The keratinocyte growth factor (KGF) receptor, cloned from a BALB/MK cDNA library, is derived from an alternatively-spliced transcript of the *bek* gene.

2. The alternative exon encoding the carboxy-terminal half of the immunoglobulin loop closest to the transmembrane domain of the KGF receptor is crucial in determining the KGF binding capacity of the receptor protein.

3. A synthetic peptide corresponding to the carboxy-terminal half of the aforementioned immunoglobulin loop of the KGF receptor specifically antagonizes the mitogenic activity of KGF.

4. An hepatocyte growth factor (HGF) receptor has been identified as the c-met proto-oncogene product.

5. HGF and the cell motility agent termed 'scatter factor' are indistinguishable.

6. A competitive HGF antagonist is encoded by an alternative transcript which contains the sequence for the amino-terminal and first two kringle domains.

7. Baculovirus expression of HGF provides an abundant source of biologically-active growth factor.

Publications:

Aaronson SA, Rubin JS, Finch PW, Wong J, Marchese C, Falco J, Taylor WG, Kraus MA. Growth factor-regulated pathways in epithelial cell proliferation. Am Rev Respir Dis 1990;142:S7-10.

Bottaro DP, Rubin JS, Faletto DL, Chan A, Kmiecik TE, Vande Woude GF, Aaronson SA. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 1991;251:802-4.

Bottaro DP, Rubin JS, Ron D, Finch PW, Florio C, Aaronson SA. Characterization of the receptor for keratinocyte growth factor: evidence for multiple fibroblast growth factor receptors. J Biol Chem 1990;265:12767-70.

Marchese C, Rubin J, Ron D, Faggioni A, Torrisi MR, Messina A, Frati L, Aaronson SA. Human keratinocyte growth factor activity on proliferation and differentiation response distinguishes KGF from EGF family. J Cell Physiol 1990;144:326-32.

Miki T, Fleming TP, Bottaro DP, Rubin JS, Ron D, Aaronson SA. Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. Science 1991;251:72-5.

Rubin JS, Chan A, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirschfield DW, Wong J, Miki T, Finch PW, Aaronson SA. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc Natl Acad Sci USA 1991;88:415-9.

Patents:

Bottaro DP, Rubin JS, Faletto D, Chan A, Vande Woude GF, Aaronson SA. US Patent CIP (Pending): Hepatic Growth Factor Receptor is the *met* Proto-Oncogene.

Chan A, Rubin JS, Bottaro DP, Aaronson SA. US Patent CIP (Pending): A Non-Mitogenic Competitive HGF Antagonist.

Rubin JS, Chan A M-L, Bottaro DP, Aaronson SA, US Patent Application Serial No. 7,582,063 (Pending): A Novel Broad Spectrum, Human Lung Fiboblast-derived Mitogen.

Rubin JS, Finch PW, Aaronson Sa. US Patent Application Serial No. 7,304,281 (Pending) and Foreign Patent Application No. PCT/US90/00418 (Pending): DNA Encoding a Growth Factor Specific for Epithelial Cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05626-02 LCMB

PERIOD COVERED

September 1, 1990 to October 30, 1991

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

Characterization of the Hepatocyte Growth Factor (AGF) Signal Transduction Pathway

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

PI:	D. P. Bottaro	Senior Staff Fellow	LCMB	NCI
Others:	J. S. Rubin	Senior Staff Fellow	LCMB	NCI
	A. Chan	Visiting Fellow	LCMB	NCI
	M. Chedid	Visiting Fellow	LCMB	NCI
	S. A. Aaronson	Chief	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:	PROFESSIONAL:	OTHER:
1.0	1.0	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 hepatocyte growth factor (HGF) is thought to be a humoral mediator of liver regeneration. A 145-kD tyrosyl phosphoprotein observed in rapid response to HGF treatment of intact target cells was identified by immunoblot analysis as the beta subunit of the c-met protooncogene product, a membrane-spanning tyrosine kinase. Covalent cross-linking of radiolabeled ligand to cellular proteins of appropriate size that were recognized by c-met antibodies directly established the c-met product as the cell-surface receptor for HGF.

HGF is structurally related to the family of serine proteases that includes plasminogen, prothrombin, urokinase, and tissue plasminogen activator. Urokinase and thrombin associate with a specific cell-surface receptor which bears no homology to the c-met protein or other tyrosine kinase receptors. The direct interaction of HGF with the c-met receptor tyrosine kinase suggests a biochemical mechanism of mitogenic signal transduction similar to that of other peptide growth factors, which diverges significantly from the signal transduction schemes typical of other serine protease family members.

The met oncogene was originally identified in a chemical carcinogen-treated human osteogenic sarcoma cell line by transfection analysis in NIH/3T3 cells. The protooncogene product is a transmembrane receptor-like protein whose transcript is expressed in many tissues. A high proportion of spontaneous NIH/3T3 transformants overexpress c-met. Since this cell line produces HGF, an autocrine mechanism may provide the basis for transformation. Tyrosine phosphorylation of apparently normal c-met protein has been observed in certain human gastric carcinoma cell lines; whether autocrine stimulation is responsible for the constitutive activation of c-met kinase in these cell lines remains to be determined. Knowledge that the HGF receptor is the c-met kinase will provide the opportunity to explore the role of this ligand-receptor system in normal as well as disease states.

PROJECT DESCRIPTION

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

D. P. Bottaro	Senior Staff Fellow	LCMB	NCI
J. S. Rubin	Senior Staff Fellow	LCMB	NCI
A. Chan	Visiting Fellow	LCMB	NCI
M. Chedid	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

Characterization of hepatocyte growth factor (HGF) signal transduction pathways.

Methods Employed:

SDS-PAGE, 2D gel electrophoresis and immunoblotting techniques; protein radiolabeling and radioreceptor assays; protein purification and analytical biochemistry techniques including affinity, ion exchange and reverse-phase chromatography; recombinant DNA techniques including transfection of cultured cells with growth factor and/or receptor cDNA, site-directed mutagenesis; standard tissue culture techniques.

Major Findings:

1. Identification and characterization of the HGF receptors on B5/589 human mammary epithelial cells.

2. Preliminary characterization of putative early substrates of HGF receptors.

Publications:

Bottaro DP, Rubin JS, Faletto DL, Chan A M-L, Kmiecik TE, Vande Woude G, Aaronson, SA. Identification of the hepatocyte growth factor receptor as the *c-met* protooncogene product. Science 1991;251:802-4.

Bottaro DP, Rubin JS, Ron D, Finch PW, Florio C, Aaronson SA. Characterization of the receptor for keratinocyte growth factor. Evidence for multiple fibroblast growth factor receptors. J Biol Chem. 1990;265:12767-70.

Heidaran MA, Fleming TP, Bottaro DP, Bell GI, Di Fiore PP, Aaronson SA. Transformation of NIH/3T3 fibroblasts by an expression vector for the human epidermal growth factor precursor. Oncogene 1990;5:1265-70.

Miki T, Fleming TP, Bottaro DP, Rubin JS, Ron D, Aaronson SA. Expression cDNA cloning of the keratinocyte growth factor receptor by creation of a transforming autocrine loop. Science 1991;251:72-5.

Molloy CJ, Fleming TP, Bottaro DP, Aaronson SA. PDGF signaling pathways in malignancy. In: Papas T. eds. Advances in applied biotechnology series, volume 6: oncogenesis in signal transduction and cell proliferation. Houston: Gulf Publishing, 1990;13-23.

Molloy CJ, Fleming TP, Bottaro DP, Cuadrado A, Pangelinan M, Aaronson SA. Oncogenes and signal transduction in malignancy. In: Brinkley W, Lechner J, Harris C, eds. Current communications in molecular biology. New York: Cold Spring Harbor Press, 1991;67-81.

Rubin JS, Chan A M-L, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirshfield DW, Wong J, Miki T, Finch PW, Aaronson SA. A broad spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc Natl Acad Sci USA 1991;88:415-9.

Segatto O, Lonardo F, Pierce JH, Bottaro DP, Di Fiore PP. The role of autophosphorylation in the modulation of *erbB*-2 transforming function. New Biol 1990;2:187-95.

Segatto O, Lonardo F, Wexler D, Fazioli F, Pierce JH, Bottaro DP, White MF, Di Fiore PP. The juxtamembrane regions of the EGF receptor and gp185^{erbB-2} determine the specificity of signal transduction. Mol Cell Biol (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05627-02 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular Cloning and Characterization of Plasminogen-like 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:	A. Chan	Visiting Fellow	LCMB	NCI
	J. S. Rubin	Senior Staff 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:	PROFESSIONAL:	OTHER:
1.0	1.0	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.)*

Hepatocyte growth factor (HGF) is a broad spectrum mitogen for a variety of cells and is ubiquitously expressed by stromal fibroblasts. Structurally, it resembles plasminogen and consists of four N-terminal kringle domains and a C-terminal protease-like domain. HGF is synthesized as an 87-kDa protein and can be cleaved into a heterodimeric form consisting of a heavy and light chain held together by disulphide bonds.

To provide working quantities of this growth factor for our ongoing studies, we expressed HGF using the baculovirus system with an average yield of approximately 0.5 mg per liter of conditioned medium. Recombinant HGF retained all the biological activities of the naturally purified growth factor.

Northern analysis revealed at least four HGF-related transcripts (6.0, 3.0, 2.2, 1.3 kb) in human fibroblast cell line M426. cDNA cloning and sequencing showed at least three classes of HGF cDNA clones that were generated by an alternative splicing mechanism. They represented HGF molecules with sequences of (1) the full coding region, (2) the first two kringle domains (HGF/NK2), and (3) the first kringle domain (HGF/NK1). We purified to homogeneity the HGF/NK2 protein and demonstrated that it is nonmitogenic but that it could act as an HGF antagonist by competitive binding to the HGF receptor. Its role in HGF-mediated processes will be the focus for future studies.

PROJECT DESCRIPTION

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

S. A. Aaronson	Chief	LCMB	NCI
A. Chan	Visiting Fellow	LCMB	NCI
J. S. Rubin	Senior Staff Fellow	LCMB	NCI

Objectives:

Molecular characterization of the hepatocyte growth factor (HGF) gene.

Methods Employed:

Standard recombinant DNA techniques, DNA sequencing, transfection assay, tissue culture, insect cell system, immunoprecipitation and SDS-PAGE analysis of proteins, and COS cell transient expression system.

Major Findings:

1. Expression of recombinant HGF in baculovirus system.

2. Cloning of multiple forms of HGF transcripts.

3. Identification and purification of an HGF competitive antagonist.

Publications:

Rubin JS, Chan AM-L, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirschfield DW, Wong J, Miki T, Finch PW, Aaronson SA. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc Natl Acad Sci USA 1991;88:415-9.

Patents:

Bottaro DP, Rubin JS, Faletto D, Chan A, Vande Woude GF, Aaronson SA. US Patent CIP (Pending): Hepatic Growth Factor Receptor is the *met* Proto-Oncogene.

Chan A, Rubin JS, Bottaro DP, Aaronson SA. US Patent CIP (Pending): A Non-Mitogenic Competitive HGF Antagonist.

Rubin JS, Chan A M-L, Bottaro DP, Aaronson SA. US Patent No. 7,582,063 (Pending): A Novel Broad Spectrum Human Lung Fibroblast-derived Mitogen.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05630-02 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

The gp185erbB-2 and Epidermal Growth Factor Receptor (EGFR) Signalling Pathways

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

PI:	P. P. Di Fiore	Visiting Scientist	LCMB	NCI
Others:	F. Fazioli	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:	PROFESSIONAL:	OTHER:
1.0	1.0	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 epidermal growth factor receptor (EGFR) and gp185erbB-2 receptor are closely related tyrosine kinases. Despite extensive sequence and structural homology, they display quantitative and qualitative differences in their ability to couple with mitogenic signalling pathways. Utilizing a chimeric EGFR/erbB-2 molecule, a direct comparison of the biological and biochemical events triggered by activation of the erbB-2 and EGFR kinases in NIH/3T3 cells was possible. Under conditions of comparable expression levels in NIH/3T3 cells, the EGFR/erbB-2 chimera conferred increased responsiveness to EGF compared with EGFR, perhaps due to the different intrinsic ability of the two kinases to phosphorylate intracellular substrates. Biochemical analysis of known mitogenic transduction pathways, however, failed to reveal any major difference in the ability of EGFR or gp185erbB-2 to induce tyrosine phosphorylation of PLC-γ and GAP. Other second messengers, such as PI-3 kinase and c-raf, implicated as substrates for receptor tyrosine kinases, do not efficiently couple with either erbB-2 or EGFR kinase. Results suggest that other, unknown, signal transduction pathways responsible for the different biological effects of EGFR and gp185erbB-2 might exist. To identify and characterize new intracellular substrates, we purified EGF-induced phosphotyrosine proteins from NIH/3T3 fibroblasts overexpressing EGFR. The purified proteins were then used to immunize animals for the production of polyclonal sera. With this antisera we were able to identify six molecular species which phosphorylated all tyrosine residues upon EGF stimulation. V8 protease digestions showed that none were degradation products of the EGFR. Furthermore, the newly identified species do not correspond to any of the known substrates for tyrosine kinase receptors. The polyclonal antisera were also used for screening bacterial expression libraries with which we characterized a number of cDNAs.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

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

P. P. Di Fiore	Visiting Scientist	LCMB	NCI
F. Fazioli	Visiting Fellow	LCMB	NCI

Objectives:

To characterize the molecular events triggered by the activation of gp185$^{erbB\text{-}2}$ and epidermal growth factor receptor (EGFR) tyrosine kinases.

Methods Employed:

Recombinant DNA techniques, DNA sequencing, oligonucleotide-directed mutagenesis, transfection assay, tissue culture, standard protein analysis methods, immunoprecipitation and western blot, immunoaffinity chromatography, mitogenesis assay, immunocomplex tyrosine kinase assay in vitro, phosphoamino acid analysis, V8 digestion profile analysis.

Major Findings:

1. The gp185$^{erbB\text{-}2}$ and EGFR kinases couple with at least partially different mitogenic signalling pathways.

2. Phospholipase C-γ (PLC-γ) and GTPase-activating protein (GAP) are both substrates for the kinase activity of gp185$^{erbB\text{-}2}$ and EGFR.

3. Identification of new phosphotyrosine-containing proteins as substrates of EGFR kinase.

4. PI-3 kinase and c-*raf* do not couple efficiently with gp185$^{erbB\text{-}2}$ and EGFR kinases.

5. Identification of six new molecular species as substrates of EGFR kinase.

6. Biochemical characterization of the new substrates.

7. Identification of cDNAs coding for the molecular species characterized.

Publications:

Fazioli F, Kim UH, Rhee SG, Mouoy CJ, Segatto O, Di Fiore PP. The *erb*B-2 mitogenic signalling pathway: tyrosine phosphorylation of phospholipase C-γ and GTPase-activating protein does not correlate with *erb*B-2 mitogenic potency. Mol Cell Biol 1991;11:2040-8.

Segatto O, Lonardo F, Wexler D, Fazioli F, Pierce JH, Bottaro DP, White HF, Di Fiore PP. The juxtamembrane regions of the epidermal growth factor receptor and gp185$^{erbB\text{-}2}$ determine the specificity of signal transduction. Mol Cell Biol (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05631-02 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Isolation of the B-raf Oncogene

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

PI:	T. P. Fleming	Senior Staff Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	T. Miki	Visiting Scientist	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:	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.)*

We utilized a focus assay on NIH/3T3 cells as a method to isolate genes relevant to growth factor signal transduction. The methodology is based on creating an autocrine transforming loop which results in the growth of a focus that is easily identified and isolated. Genomic DNA from these foci are used to specifically "rescue" the relevant cDNAs. This protocol was successfully utilized to isolate the keratinocyte growth factor receptor (KGFR). It was known from binding data that a mouse keratinocyte cell line (BALB/MK cells) possessed high affinity binding sites for KGF and that this high affinity receptor was absent from NIH/3T3 cells. NIH/3T3 cells secrete KGF; therefore, the expression of the appropriate cDNA should result in cellular transformation. The introduction of the BALB/MK cDNA expression library into NIH/3T3 cells resulted in the identification of several foci. The KGFR cDNA was "rescued" from genomic DNA of one of these foci and sequence analysis revealed sequence identity to bek, a member of the fibroblast growth factor receptor (FGFR) gene family. Additionally, NIH/3T3 cells expressing the KGFR cDNA had acquired KGF high affinity binding. This expression cloning strategy should be applicable for isolating other genes critical in growth factor-mediated growth processes.

PROJECT DESCRIPTION

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

T. P. Fleming	Senior Staff Fellow	LCMB NCI
S. A. Aaronson	Chief	LCMB NCI
T. Miki	Visiting Scientist	LCMB NCI

Objectives:

1. To isolate the gene for the keratinocyte growth factor receptor (KGFR).

2. To define the methodologies necessary for cDNA expression cloning which result in cell transformation.

3. To optimize the protocols to "rescue" relevant cDNAs from genomic DNA and to characterize such cDNAs.

Methods Employed:

Eukaryotic cell transfection assay, plasmid DNA isolation from mammalian cell genomic DNA, bacterial transformation protocols, DNA sequencing analysis, RNA northern analysis, DNA Southern analysis, western blot protein analysis, cDNA synthesis from tumor tissue, and standard cloning techniques.

Major Findings:

Growth factors have been implicated in neoplasia and a wide variety of human diseases. The number of novel growth factors has increased dramatically in the past several years. The identification of the relevant growth factor receptor for a given growth factor generally is an arduous process requiring the purification of the protein from an appropriate cell line, deducing the amino acid sequence of this receptor, and, finally, cloning the receptor from a cDNA library based on the amino acid sequence. We sought to identify the receptor for the keratinocyte growth factor (KGF) by a novel cDNA expression vector previously engineered in this laboratory.

The cDNA expression library was constructed from a mouse keratinocyte cell line (BALB/MK) and transfected into NIH/3T3 cells, a cell line that secretes the mitogen KGF but does not bind this growth factor. Several transforming foci were isolated and a cDNA rescue protocol was used to isolate the cDNA clone. Introducing these isolated cDNAs into naive NIH/3T3 cells resulted in transforming activity. These transformed NIH/3T3 cells exhibited high affinity binding to KGF and the tyrosine phosphorylated substrates in the transformed cells were identical to those in the mouse keratinocyte cell line exposed to exogenous KGF. Molecular characterization of the transforming cDNAs revealed sequence identity to a protein called *bek*, a tyrosine kinase. Further analysis demonstrated that the KGF receptor is a member of the fibroblast growth factor (FGF) receptor family of tyrosine kinases.

These studies demonstrate that genes critical to growth factor-mediated signal transduction can be cloned directly using this autocrine transformation protocol. This technology should be widely applicable to defining genes important to cancer and disease processes due to growth factor pathway abnormalities.

Publications:

Crescenzi M, Fleming TP, Lassar AB, Weintraub H, Aaronson SA. MyoD induces growth arrest independent of differentiation in normal and transformed cells. Proc Natl Acad Sci USA 1990;87:8442-6.

Heidaran MA, Fleming TP, Bottaro DP, Bell GI, Di Fiore PP, Aaronson SA. Transformation of NIH/3T3 fibroblasts by an expression vector for the human epidermal growth factor precursor. Oncogene 1990;5:1265-70.

Miki T, Fleming TP, Bottaro DP, Rubin JS, Ron D, Aaronson SA. Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. Science 1991;251:72-5.

Miki T, Fleming TP, Crescenzi M, Molloy CJ, Blam SB, Reynolds SH, Aaronson SA. Isolation of a mouse hepatoma oncogene cDNA using a novel phenotypic expression cloning system. Proc Natl Acad Sci USA (In Press)

Molloy CJ, Fleming TP, Bottaro DP, Cuadrado A, Pangelinan MJ, Aaronson SA. Oncogenes and signal transduction in malignancy. In: Brinkley W, Lechner J, Harris C, eds. Current communications in molecular biology. New York: Cold Spring Harbor Press, 1991;67-81.

Patents:

Miki T, Aaronson SA, Fleming TP. US Patent Application Serial No. 7,386,053 (Pending): Efficient Directional Genetic Cloning System to Construct cDNA Libraries Containing Full-length Inserts at a High Frequency.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05633-02 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Structural and Functional Characterization of αPDGFR Gene Product

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

PI:	M. A. Heidaran	Staff Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. H. Pierce	Research Microbiologist	LCMB	NCI
	J.-C. Yu	Visiting Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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.)*

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesenchymal origin. It consists of homo-E heterodimers of PDGF A and PDGF B chains. All of the isoforms of PDGF have been identified (AA, BB, AB) and they have been shown to bind to two different but related receptor molecules, designated α PDGFR and β PDGFR. The α PDGFR binds to all three isoforms of PDGF, while the β PDGFR exhibits high affinity binding only for PDGF BB. We utilized these different ligand-binding specificities to investigate the PDGF AA binding site in the human α PDGFR by constructing chimeric molecules between the human α PDGFR and β PDGFR. Our results demonstrate that amino acids 1 to 340 of the α PDGFR comprise the region of the extracellular domain at the αPDGFR that confers PDGF AA binding specificities. Dissection within the first three domains of the α PDGFR suggests that amino acids 150-190 are required for high affinity binding of PDGF AA but not PDGF BB. Thus, our findings suggest that binding sites for PDGF AA and PDGF BB in the α PDGFR extracellular domain are not coincident. We have also investigated the
role of α PDGFR's kinase insert. Our results indicate that deletions of the kinase insert region alters the gross structure of kinase domain in a manner that affects multiple functions of the receptor molecules and that its primary importance may be in maintaining the structural features of the kinase domain. Mutagenesis of putative autophosphorylation sites within the α PDGFR kinase insert suggests that PDGF-induced autophosphorylation of these residues is required for the ability of the mutant receptor to associate with the PI-3 kinase. However, the ligand-dependent association of α PDGFR with the PI-3 kinase is not required for its major biological responses.

PROJECT DESCRIPTION

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

M. A. Heidaran	Staff Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. H. Pierce	Research Microbiologist	LCMB	NCI
J.-C. Yu	Visiting Fellow	LCMB	NCI

Objectives:

The structural and functional characterization of the platelet-derived growth factor receptor (PDGFR).

Methods Employed:

Standard recombinant DNA techniques, DNA sequencing, oligonucleotide-directed mutagenesis, transfection assay, tissue culture, immunoprecipitation and SDS-PAGE analysis of proteins, COS cell assay for transient overexpression of proteins, baculovirus vector system for protein overexpression, and analysis of PDGF receptor activation.

Major Findings:

1. Deletion of amino acids 150-190 of α PDGFR leads to the loss of high affinity binding toward PDGF AA without altering its affinity toward PDGF BB.

2. Differential effect of deletion in the second domain of α PDGFR provides direct evidence that binding sites for PDGF AA and PDGF BB are not coincident.

3. Tyrosine phosphorylation of residues 731 and 741 is required for ligand-dependent receptor affinity toward PI-3 kinase.

4. PDGF-induced PI-3 kinase association with α PDGFR is not required for major biological responses of activated α PDGFR.

5. The GTPase activating protein (GAP), a known substrate of PDGFR kinase domain, serves as a poor substrate for α PDGFR as compared to β PDGFR.

6. Activation of the CSF-1 receptor (CSF-1R) leads to the rapid tyrosine phosphorylation of GAP and activation of cellular $p21^{ras}$.

Publications:

Heidaran MA, Fleming TP, Bottaro DP, Bell GI, Di Fiore PP, Aaronson SA. Transformation of NIH/3T3 fibroblasts by an expression vector for the human epidermal growth factor precursor. Oncogene 1990;5:1265-70.

Heidaran MA, Pierce JH, Jensen RE, Aaronson SA. Chimeric α and β PDGF receptors define three immunoglobulin-like domains of the α PDGF receptor that determine PDGF AA binding specificity. J Biol Chem 1990;265:18741-4.

Heidaran MA, Pierce JH, Lombardi D, Ruggiero M, Gutkind JS, Matsui T, Aaronson SA. Deletion or substitution within the αPDGF receptor kinase insert domain: effects on functional coupling with intracellular signalling pathways. Mol Cell Biol 1991;11:134-42.

Miki T, Matsui T, Heidaran MA, Aaronson SA. Automatic directional cloning: an efficient method to construct eukaryotic expression cDNA libraries. In: Alitalo KK, Huhtala M-L, Knowles J, Vaheri A, eds. Proceedings of the international symposium on recombinant systems in protein expression. Amsterdam: Elsevier Science Publishers BV, 1990;125-36.

Yu J-C, Heidaran MA, Pierce JH, Gutkind JS, Lombardi D, Ruggiero M, Aaronson SA. Tyrosine mutations within the αPDGFR kinase insert domain abrogate receptor-associated PI-3 kinase activity without affecting mitogenic or chemotactic signal transduction. Mol Cell Biol (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05634-02 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Functional and Structural Characterization of Human PDGF Receptors

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

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	R. A. Jensen	Biotechnology Fellow	LCMB	NCI
	M. A. Heidaran	Staff Fellow	LCMB	NCI
	J. H. Pierce	Research Microbiologist	LCMB	NCI
	W. J. LaRochelle	Staff 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:	PROFESSIONAL:	OTHER:
1.0	1.0	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.)*

Binding of platelet-derived growth factor (PDGF) to its cell surface receptors stimulates a variety of biochemical and biological responses. Two distinct PDGF receptors have been identified and each exhibits different binding affinities for the variant PDGF isoforms. The α PDGF receptor binds PDGF AA and PDGF BB, whereas the β PDGF receptor binds only PDGF BB. To determine the region of the α PDGF receptor responsible for PDGF AA binding, chimeric molecules between the α and β PDGF receptors were constructed and transfected into the PDGF receptor-negative cell line, 32D. Binding analysis and mitogenic assays revealed that amino acids 1-340 of the α PDGF receptor comprised the region of the receptor responsible for PDGF AA binding specificity. This region corresponds to immunoglobulin-like subdomains 1, 2 and 3 of the extracellular portion of the receptor.

The mechanism of receptor activation was also studied utilizing recombinant PDGF receptors in the baculovirus expression system. In these experiments, intact Sf9 cells expressing β PDGF receptors were stimulated with PDGF ligand, treated with a cross-linking agent, lysed, and submitted to western blot analysis. These experiments revealed that at early time points of infection, the receptor is phosphorylated and forms receptor dimers with ligand stimulation. In contrast, at late time points of infection, receptor phosphorylation was not ligand-dependent and appeared to be a function of increasing receptor concentration.

Baculovirus-produced recombinant PDGF receptors were also utilized to screen hybridoma supernatants for antireceptor monoclonal antibodies, and a monoclonal antibody specific for human α PDGF receptor was identified. This antibody is reactive in ELISA assays, immunoprecipitations, and in western blotting of nonreduced receptor.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

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

S. A. Aaronson	Chief	LCMB	DCE
R. A. Jensen	Biotechnology Fellow	LCMB	NCI
M. A. Heidaran	Staff Fellow	LCMB	NCI
J. H. Pierce	Research Microbiologist	LCMB	NCI
W. J. LaRochelle	Staff Fellow	LCMB	NCI

Objectives:

Functional and structural characterization of human platelet-derived growth factor (PDGF) receptors.

Methods Employed:

Standard recombinant DNA techniques, DNA sequencing, oligonucleotide-directed mutagenesis, tissue culture, SDS-PAGE and western blot analysis of proteins, receptor-binding assays, baculovirus expression system.

Major Findings:

1. The construction of chimeric α and β PDGF receptors has revealed that the region of the α PDGF receptor responsible for the binding of PDGF AA is localized to amino acids 1-340 of the extracellular domain.

2. This region of the α PDGF receptor corresponds to immunoglobulin-like subdomains 1, 2 and 3 of the extracellular portion of the receptor.

3. Cross-linking studies on ligand-stimulated whole Sf9 cells reveals that PDGF receptor activation appears to be dependent on receptor dimerization.

4. Baculovirus-produced recombinant PDGF receptors have been utilized to identify a monoclonal antibody specific for human α PDGF receptor. The antibody is reactive in ELISA assays, immunoprecipitations, and in western blotting of nonreduced receptor.

Publications:

Heidaran MA, Pierce JH, Jensen RA, Matsui T, Aaronson SA. Chimeric α and β PDGF receptors define three immunoglobulin-like domains of the α PDGF receptor that determine PDGF AA binding specificity. J Biol Chem 1990;265:18741-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05644-02 LCMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Functional Characterization of Cytoplasmic Domain of αPDGF Receptor

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. Yu	IRTA Fellow	LCMB	NCI
	M. Heidaran	IRTA 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:	PROFESSIONAL:	OTHER:
1.0	1.0	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.)*

Platelet-derived growth factor (PDGF) is a potent mitogen for mesenchymal cells. Two types (α and β) of PDGF receptor have been cloned and sequenced. The ligand-binding region of the PDGF receptor is in the extracellular domain, which consists of five immunoglobulin-like regions. The cytoplasmic region of the PDGF receptor contains sequences homologous to other tyrosine kinases. The kinase sequences are interrupted by the kinase insert (KI) domain.

The interaction of PDGF with its receptor causes some rapid changes in the cell, which include the activation of the receptor kinase activity, stimulation of several second messenger pathways and initiation of DNA synthesis. Both of the major in vivo and in vitro phosphorylation sites of the βPDGF receptor have been identified. We are studying the role of these phosphorylation sites of the αPDGF receptor in signal transduction. Several point mutations of the αPDGF receptor have been generated by site-directed mutagenesis. Mutation of tyrosine 731 or 742 in the KI domain does not impair PDGF-induced tyrosine phosphorylation of the receptor or of an in vivo substrate, PLCγ. However, both lesions markedly impair receptor association with PI-3 kinase. Anti-P-Tyr antibody recoverable PI-3 kinase was also dramatically reduced in PDGF-stimulated cells expressing either mutant receptor. Since neither mutation abolished PDGF-induced mitogenesis or chemotaxis, we conclude that αPDGF receptor-associated PI-3 kinase activity is not required for either of these major PDGF signalling functions.

PROJECT DESCRIPTION

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

S. A. Aaronson	Chief	LCMB	NCI
J-C. Yu	IRTA Fellow	LCMB	NCI
M. Heidaran	IRTA Fellow	LCMB	NCI

Objectives:

The functional characterization of cytoplasmic domain of the alpha platelet-derived growth factor (αPDGF) receptor.

Methods Employed:

Standard recombinant DNA techniques, DNA sequencing, oligonucleotide-directed mutagenesis, transfection assay, tissue culture, mitogenicity assay, binding assay, immunoprecipitation and SDS-PAGE analysis of protein.

Major Findings:

Mutation of tyrosine 731 or tyrosine 742 in the kinase insert domain of αPDGFR does not impair PDGF-induced receptor autophosphorylation or tyrosine phosphorylation of PLC-γ. The receptor-mediated mitogensis or chemotaxis was not much reduced by these mutations. However, both lesions markedly impair receptor-associated PI-3 kinase activity after PDGF triggering. Therefore, the αPDGFR-associated PI-3 kinase activity is not required for PDGF chemotactic or mitogenic signalling pathways.

Publications:

Yu J-C, Heidaran MA, Pierce JH, Gutkind JS, Lombardi D, Ruggiero M, Aaronson SA. Tyrosine mutations within the αPDGFR kinase insert domain abrogate receptor-associated PI-3 kinase activity without affecting mitogenic or chemotactic signal transduction. Mol Cell Biol (In Press)

CONTRACT IN SUPPORT OF ALL LABORATORY PROJECTS:

STATE OF CALIFORNIA DEPARTMENT OF HEALTH SERVICES (NO1-CP-95618-36)

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

Current Annual Level: $157,937

Man Years: 3.25

Objectives: To provide in vivo support for four major research efforts within the LCMB: (1) viral and cellular genes involved in malignant transformation; direct effect of specific oncogenes introduced into appropriate animals by viral recombinants; (2) analysis of genetically altered target cells by grafting into immunodeficient athymic nude host mice; (3) the role of host immune response in oncogene-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, 1989 through September 30, 1993.

ANNUAL REPORT OF

LABORATORY OF MOLECULAR ONCOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

The Laboratory of Molecular Oncology (LMO) 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 retroviral and cellular transforming oncogenes from malignant and normal cells and tissues, as well as to characterize the encoded products of these genes; (2) identifies, isolates, characterizes and determines the function(s) of these oncogenes 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 oncogene 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-oncogenes) of the acute transforming viral oncogenes by expression in normal eukaryotic and prokaryotic cellular systems; (5) examines the molecular elements and mechanisms which regulate these eukaryotic genes, aiming to adapt, modify and apply this understanding to the expression and control of the neoplastic processes; (6) studies the cellular biology and biochemistry of the oncogene products and their normal cellular counterparts, particularly with respect to their role in cellular proliferation and differentiation; and (7) uses recombinant differential and subtractive gene selection techniques to identify the expressed genes and their products that are involved in the growth and proliferation process of specific neoplastic tissues at various stages of disease. 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 and normal gene expression and the conversion of cells from the normal to the malignant state. Specifically, this association will be accomplished by examining at the molecular level, by recombinant DNA techniques, the organization and pattern of gene expression and their products, of both normal essential cellular genes and those of malignantly transformed cells and tissues. This information will be used to understand the mechanism(s) by which genes and their products are subverted to initiate and maintain the neoplastic state.

The Microbiology Section investigates the mechanism of oncogenic transformation of normal cells and characterizes their mechanisms of action at a molecular level. These studies are accomplished by using a variety of gene transfer techniques to identify such genes present in human tumors and tumor-derived cell lines, and to introduce such recombinant DNA-cloned genes or their retroviral vector homologues into well-characterized cells in vitro in order to study their effects. Model cell culture systems are employed to determine how specific transforming genes can alter normal cell functions, and to identify those normal functions which may serve as primary targets of oncogene action. Since oncogene-induced changes often involve alterations in the structure and/or expression of cellular genes, presumably at the level of DNA-gene structure, the effects of oncogenes and their

products are an area of particular interest. Such investigations should lead to a more complete understanding of the mechanisms by which such altered genes are able to transform normal cells to their malignant phenotype.

The Cellular Biology and Biophysics Section designs and carries out an integrated molecular- and cellular-level research program using the latest recombinant DNA and immunocytochemical methodologies in conjunction with state-of-the-art confocal laser scanning microscopy and image capture and computer processing technologies to detect and elucidate the functional elements involved in the expression and manifestation of the products encoded by the human retroviral genes and the oncogenes, as well as their normal cellular homologs, the proto-oncogenes, particularly as they relate to the maintenance of the retroviral infected state or the malignant transforming phenotype; uses expression vector systems to generate specific antigen products for monoclonal and polyclonal antibodies and other reagents as probes, evaluates the subcellular distribution and functional relationships of the oncogenes and other retroviral-encoded genes, comparing them to their normal cellular proto-oncogene counterparts.

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 and their targets, including the ras family of oncogenes and the p21 ras onco-protein(s), as well as its pleiotropic effects on other oncogenes. 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. Further, this section investigates the malignant transformation of cells by virus-coded onco-protein(s), as well as its reversion by suppressor gene(s) and their products, especially for those oncogenes that are directly responsible for transformation; these studies necessitate comprehending the primary physiological effects of the functioning protein(s), the sequence of molecular changes resulting in the altered transforming profile characteristic of malignant cells, and the molecular and genetic changes necessary for the maintenance of the malignant state.

A primary emphasis of this Laboratory involves molecular investigations entailing the identification, isolation and analyses of potentially neoplastic sequences or their genetic regulatory elements by employment of recombinant cloning techniques and molecular immunological methodologies. These studies extend, as well, to their gene-encoded protein products in order to evaluate their functional relationship to malignant transformation in vitro and neoplasia in vivo. We have pursued these avenues of study into several major areas, where we are analyzing the structural and biological properties of specific retroviral-encoded oncogenes present in both avian and mammalian acute-transforming retroviruses. We are also identifying, isolating and characterizing their normal cellular homologs (the proto-oncogenes) from their species of origin and other metazoans, including, most importantly, from the human genome, in order to understand their functional role(s) in maintaining the non-malignant state. Through the 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 actively pursued investigations to define the regulation of the varied patterns of tissue expression of the normal cellular genes relative to those of comparable pre-neoplastic tissues and cells at various stages of malignancy and metastasis. Finally, we are addressing by comparative molecular genetic methods, the nature of gene expression in matched normal, benign, and malignant tissue to comprehend the differences in their encoded products that

would be responsible for maintenence of their respective states. This study includes the functional, as well as molecular, characterization of these genes and their products by utilization of a battery of state-of-the-art methodologies, such as construction of transgenic murine model systems, transfectants of embryonic stem cell, gene "knock-out" recombinants, employment of polymerase chain reaction (PCR) and confocal laser scanning microscopy (CSLM) systems to deduce and understand the myriad of molecular alterations that are associated with the etiology of malignancy.

In particular, the Carcinogenesis Regulation Section (CRS) has found that the functional relationship between the ets gene of transforming leukemia virus, E26, and its cellular prototypes has been facilitated by structural comparisons at the nucleic acid and predicted protein levels.

We have determined the complete nucleotide sequence of the chicken, mouse and human ets-1 and ets-2 genes and compared them to each other and to the ets gene of E26. The predicted ets-1 and ets-2 proteins possess three distinct domains when compared to v-ets. The domain closest to the carboxyl-termini (C domain) is highly conserved (>90%) and this conservation is seen to be widely preserved throughout evolution, including Drosophila. The domain located at the amino-terminal end of ets-2 (A domain) is less homologous to the virus/chicken proto-oncogene and, thus far, this region has not been identified in lower eukaryotes. The third domain (B domain), which is located centrally, is diverged in ets-2 genes, but is conserved in the ets-1 gene. The highly conserved C domain is the essential criterion for assignment to the ets gene family. Interestingly, the C region is required for DNA-specific binding, thus defining an important functional motif, and the fact that ets represents a family of genes whose members are diverging at variable rates.

The human ETS1 gene consists of eight exons distributed over 65 kb. Sequence analysis of the promoter region upstream from the first exon (which contains the translation start codon) revealed no TATA or CAAT elements. Potential binding sites for transcription factors (SP1, AP1, AP2) are present in the 700 bp region required for functional promoter activity, as measured by chloramphenicol acetyl transferase (CAT) activity. Potential positive and negative elements have been defined for further analysis. Consistent with the absence of TATA and CAAT elements, the ETS1 gene transcripts initiate from multiple sites which span a region of 140 bp.

The mouse ets-1 gene is similar in organization to the human ETS1 gene. The positions of the introns (point of disruption in the predicted open reading frame) are identical. Sequence analysis of the promoter region of the mouse ets-1 gene and alignment with that of the human ETS1 gene reveals several highly-conserved domains, suggesting functional significance. At least two of these conserved sequences can be shown to interact with nuclear proteins by mobility gel shift analyses. These interactions possibly define a new class of regulatory proteins, as analyses of the DNA sequence does not reveal any consensus sites for protein binding. Also, we have not found similar sequences in the promoters of genes (GenBank); thus, they are specific for ets-1 regulation. Tissue-specific distribution of the protein factor(s) responsible for the observed DNA mobility shift is being investigated.

We have previously identified the DNA binding sites of the ETS2 promoter that are involved in the regulation of ETS2. We used these binding motifs to screen cDNA expression libraries to detect factors that are involved in ETS2 transcriptional regulation. We have isolated a cDNA clone that encodes a protein that specifically interacts with the ETS2 promoter sequences. The clone encodes a protein of 328 amino acids, containing a helix-loop-helix, a leucine zipper, an

amphiphatic region, and two acidic domains. Several putative phosphorylation sites, can be detected at the C-terminus of the protein. The gene encoding the protein locates on chromosome Xp11.2-p11.3 near a region implicated with Wiskott-Aldrich syndrome, a sex-linked recessive immunodeficiency of T- and B-cell functions associated with increased malignancies. Northern transfer analyses indicate that this gene is transcribed as a single 3 kb species, which can be detected in a variety of tissues and cell lines. The gene locates in the nucleus and binds to DNA as a dimer, which is consistent with its role as a functional transcriptional regulator. Most interestingly, this gene, following transfection, could morphologically transform cells and subsequently elicit tumors in athymic mice, thus fulfilling the criteria of being a putative novel nuclear oncogene.

The human genome contains more than six ets-related genes (ETS1, ETS2, ERG, ERGB, ELK1, ELK2). In addition to having unique chromosomal locations, members of this ever-expanding gene family have unique patterns of expression. To understand the function(s) and mechanisms responsible for the functional differences between these genes, we are identifying genes whose products are able to interact with ETS proteins. To achieve this goal, constructs containing defined segments of the open reading frames (ORF) from the human ETS1 and ETS2 genes have been placed into prokaryotic vectors that allow for overexpression and rapid purification of the fusion protein produced by each construct. This methodology is also being applied to other genes and will allow us to begin to dissect the role(s) of the ETS genes in the complex network of gene regulation and cellular signal transduction.

Three novel Drosophila genes with cellular sequences related to the ets region of the avian leukemogenic retrovirus, E26, have been isolated in our Laboratory. The characterized portion of the most related gene, D-ets, shows over 90% identity with the last two exons of the chicken c-ets-1 gene at the predicted amino acid level--the highest level of homology thus far shown for any oncogenic sequences so distantly related by evolution. The region of D-ets (a genomic clone) 5′ to this region has been shown to be present in a cDNA clone, and this sequence is divergent from the chicken c-ets-1 gene. D-ets is expressed as a 4.7 kb transcript throughout development. D-ets is localized to chromosome 3R at position 58A/B. D-elg (Drosphila ets-like gene) is a related gene that has been localized to chromosomal position 97D on chromosome 3R. This gene is represented by a clone isolated from a pupae cDNA library. Its sequence shows 60% amino acid similarity with D-ets. D-elg is expressed as a single transcript of 2.0 kb that is expressed throughout embryogenesis, as well as pupae and adult stages, but absent from larvae. D-elg is expressed in all embryonic cells prior to transcriptional activation and has no regional localization. A third gene, E13B, was identified by hybridization with v-ets, and a genomic clone has been partially sequenced. A cDNA clone that hybridized to E13B shows a long Poly A+ tail and is also being sequenced. The E13B gene is expressed as a 1.6 kb RNA species in pupae and adults only. It is located on chromosome 3L at position 66A. P-element vectors have been constructed with both the rosy and the white genes as markers. A human-Drosophila chimeric construct has been made and will be microinjected into the appropriate strain of flies.

The frog, Xenopus laevis, contains two forms of the ets-2 gene (ets-2a and ets-2b), each encoding proteins of 472 amino acids, a number close to that of the human, mouse, and chicken homologs. The percent identity between the Xenopus ets-2a and ets-2b products is 94.1, while that between ets-2a and the other vertebrates is about 68%. The Xenopus sequences are homologous to the other vertebrate ets-2 genes throughout their entire length. Because of the ease of manipulating oocytes and embryos, the Xenopus system has provided a useful means of studying the biological function of ets-2, and results of studies performed with the Xenopus homolog shed some light on the function of this proto-oncogene, which may have relevance to humans. Microinjection of antisense oligonucleotides

homologous to the ets-2 sequence into frog oocytes led to degradation of the mRNA and blocked hormone-induced germinal vesicle (GVBD) breakdown, and hence, subsequent embryonic development. This inhibition of development by ets-2 antisense oligos, could be reversed by competition with authentic human ETS2 mRNA, thus showing that this proto-oncogene effect was specific and essential for further egg development, but even more importantly, the human product could functionally replace and effect the Xenopus' requirement for the meiotic maturation of its oocytes. Preliminary data also indicate that the inhibition of GVBD is due to lack of MPF. In addition, oocytes injected with antisense oligos to Xenopus ets-1 show inhibition of GVBD, indicating that the product of this gene also might be required for oocyte maturation.

The LMO has discovered a molecular clone containing sea urchin DNA that hybridized to a v-ets probe; this was shown to contain an exon of the erg gene. This sea urchin sequence codes for 173 amino acid residues beginning at a 5' splice site. The first 85 residues share homology with all members of the ets gene family (including the erg, elk, Drosophila E74, mouse PU.1 and ets-1 and ets-2 genes), while the remaining 88 amino acids are homologous only to the human ERG gene. This latter region, designated R, represents a highly-conserved, erg-specific domain.

Phylogenetic analysis of ets sequences indicates that the family may be divided into three major groups: (1) the ets genes proper, including the vertebrate ets-1 and ets-2 genes, and the sea urchin and Drosophila ets genes; (2) the human and sea urchin erg genes; (3) the human ELK, the murine PU.1, and Drosophila ecdysone-induced puff 74E genes. Since each of the groups contain representatives of both vertebrate and invertebrate phyla, it is possible to estimate that the gene duplication events from which they originated occurred at least 500 million years ago.

A major role for the proto-myc oncogene in cellular proliferation has long been postulated. The c-myc gene, we have shown, appears to modulate the expression of a number of other cellular genes whose products directly control proliferation. Permanent cell lines (in which the endogenous c-myc gene is tightly regulated by growth factors and cell/cell-contact) have been constructed in which expression of an exogenously-transfected c-myc gene is controlled by the Drosophila heat-shock 70 promoter. Transcription and subsequent translation of the exogenous c-myc gene was specifically induced by heat-shock, and the endogenous c-myc gene was not expressed under these conditions. In order to isolate other genes specifically induced or repressed in conjunction with c-myc expression, cDNA libraries have been constructed from which hybridization subtraction will be performed and subtractive libraries made. Directional cDNA libraries in λZAPII have been constructed so as to produce single-stranded DNA for hybridization subtraction and the production of subtractive libraries. Directional libraries will allow for only interlibrary hybridization (as opposed to intralibrary hybridization), thus increasing the efficiency of the subtraction, which should also allow enrichment for specifically-induced or specifically-repressed sequences.

More than 100 human genomic regions relevant to cancer have been identified by combining genetic, cytogenetic and molecular analysis of both inherited and non-inherited tumors. Our investigations have been focusing on two genomic regions of chromosomes 11 and 21 relevant to genomic rearrange- ments of childhood tumors and associated to inherited and constitutional disorders. Both regions also contain genes of the ETS family actively studied in this Laboratory. Thus, the CRS has discovered that the 21q22 region, as well as the 11q23 region, contains the loci for conditions predisposing to both benign and malignant cancers. The human ETS1 gene has been located in the 11q23 band, and the other ets family gene members, ETS2 and ERG, are associated with

the 21q23 region. The constitutional aneuploidy, Trisomy 21, is associated with leukemia and with a curious transient myeloproliferative disorder of newborns. The two genes of the ETS family, ETS2 and ERG, are being used as probes to look at relative expression patterns in these specific leukemic disorders; these studies are being expanded to include subtractive hybridization methods to detect singular gene changes at the level of transcription.

Colon cancer claims the lives of more than 65,000 people each year in the United States, second only to lung cancer. Surgery remains the only effective means of treatment, and even this is usually only effective when the cancer is discovered early. Patients with an inherited predisposition for developing numerous premalignant polyps (familial polyposis) are at nearly 100% risk of colon cancer by age 40, and early scrutiny of such patients often leads to prophylactic surgery. However, the great majority of patients who will present with colon cancer do not have a prehistory of polyposis that might lead to early detection. Recently, some oncogenes (ras) and candidate tumor suppressor genes (p53, DCC, MCC) have been implicated in playing a role at various stages of premalignant and malignant progression in the colon adenoma-carcinoma sequence. Therefore, it appears that several, if not many, genes can contribute to progression. These genes that have been implicated thus far have been linked to cytogenetically-observable abnormalities or to fortuitously-located restriction fragment length polymorphisms (RFLPs). In order to directly investigate the major alterations in gene expression in polyposis and colon carcinoma, the CRS has constructed cDNA libraries from matched tumor/normal tissues. Subtractive cDNA libraries enriched for either tumor-specific or normal-specific cDNAs were then made and used to isolate clones differentially expressed in tumor and normal tissues. A number of such clones have been isolated. Some of these correspond to already identified genes whose possible role in colon cancer progression has not been previously suspected (e.g., collagen type VI, laminin-binding protein), while other clones do not correspond to any sequences thus far deposited in various sequence databases. One such clone that we have isolated, IH12, is growth-regulated and is also found to be overexpressed in breast carcinoma.

Ovarian carcinoma is the most frequent cause of death from gynecological malignancies in the Western world. It ranks fifth overall as a cause of death from cancer in women, with over 12,000 deaths per year, and an estimated 20,000 new cases will be diagnosed in 1991. Most cases are diagnosed at an advanced stage due to a paucity of clinical symptoms until late in disease progression. Prognosis is poor, with an overall five-year survival of about 30%. Recently, tumor-specific allele losses of chromosomes 6, 11, and 17 have been reported, as well as abnormalities on chromosomes 1 and 3. Amplification of the erbB2 oncogene on chromosome 17q occurs in 30% of cases, and other oncogenes, including K-ras and myc, have been discovered to be amplified in a few ovarian tumors and cell lines. The loss of tumor-suppressor genes at certain chromosomal loci may also be important in the pathogenesis of ovarian carcinoma, as it has been shown to be in other human tumors. In order to directly investigate the molecular genetic aberrations leading to altered gene expression in ovarian neoplasia compared to normal tissue, the CRS has constructed a number of cDNA libraries from matched tumor/normal tissues. Tumor minus normal and normal minus tumor libraries were constructed to enrich for tumor-specific and normal-specific cDNAs, respectively, with the aim of identifying genes critical in the process of carcinogenesis (tumor promoter genes), or for maintaining the normal state (tumor suppressor genes). In addition, ovarian carcinoma samples are being analyzed for the presence of altered known tumor-suppressor genes, such as p53 and the retinoblastoma gene, Rb. Direct sequencing of PCR-amplified segments may show sequence abnormalities contributing to the inactivation or malfunction of these genes.

Members of the Laboratory have recently developed a number of molecular procedures, including the subtractive hybridization procedure, in which low abundance mRNA species that are differentially expressed can be isolated. Also, other procedures, such as PCR and S1-expression protection assays in combination, will enable us to detect molecular alterations (e.g., mutations, deletions and other rearrangements) in specific genes like oncogenes and tumor suppressor genes, as well as in unique genes or their gene products. We propose to use this approach to identify molecular markers for prostatic cancer. Initial investigations have employed these gene methods to screen a number of pancreatic cell lines to identify expressed cancer-specific genes that are present, but perhaps altered or mutated. These analyses are underway using cDNA libraries obtained from highly-metastatic pancreatic cells. Other initial investigations will focus upon isolation of cDNA clones from metastatic human prostatic cell lines (DU 145 and PC-3); these may enable us to distinguish genes expressed in prostate cancers from those expressed in benign hypertrophic tissue and in the normal prostate. Additionally, we will examine these expressed sequences for point mutations and other altered forms of expression. Gene amplification of known oncogenes will be sought, as well as for novel genes. Similarly, molecular examination of genes and their products, particularly between early-stage tumor tissues (A-B) and late-stage samples (C-D), may also enable us to identify important differentially-expressed genes that are altered compared to their normal cellular counterparts. Distinctive clones identified in this phase of the investigation can be used to screen and molecularly categorize potentially metastatic prostatatic and pancreatic cancers, by in situ and by confocal laser scanning microscopic (CLSM) studies. Improved techniques of this type will make available a variety of new molecular probes that will afford increased sensitivity for early neoplasia, especially when used in conjunction with the polymerase chain reaction (PCR) technique.

The CRS has an active AIDS-related research program on the expression of HIV-1 and -2 gene products. Recently, members of this section have made a recombinant HIV-2 (NIH-Z) Nef protein synthesized in E. coli, and purified by the detergent/chaotrope extraction technique and preparative SDS-PAGE electrophoresis. The recombinant product is: immunologically reactive on Western blots with anti-Nef antibodies; an excellent substrate in vitro for phosphorylation, both by purified protein kinase C (PKC) and the maturation promoting factor (MPF) kinase purified from Xenopus oocytes; exhibits an intrinsic low-level autokinase activity, and forms stable homodimers and homotetrameric complexes in vitro, which are significantly increased in the absence of sulfhydryl-reducing agents. Preliminary results of in vivo phosphorylation and oligomerization experiments suggest that the native 25 kD HIV-2 (NIH-Z) Nef protein in infected T-lymphocytes in culture becomes highly phosphorylated following stimulation with calcium ionophores, and the dimeric (50 kD) and tetrameric (100 kD) forms of this protein can be radioimmuno-precipitated with specific anti-HIV-2 Nef monoclonal antibodies. In addition, evidence of a putative Nef-cellular protein complex was also obtained. Structural studies have also revealed the presence of a leucine zipper-like repeat structure at the conserved central "core" region of the Nef proteins, with a characteristic 4,3 repeat similar to the heptad leucine repeat motif of the bZIP factors. Moreover, at the C-terminus of the Nef proteins is a highly acidic sequence (net charge of -5 to -8) stretched over 40 amino acids, and it contains two predicted α-helices separated by a predicted β-turn structure, with homology to known acidic activation domains of transcriptional activation factors. Biochemical characterization of individual biological Nef clones expressing only p27nef (clone 3B-3) or p25nef (clone 3B-5), isolated by limiting dilution of HTLV-IIIB-infected H9 cells, are continuing. In addition, a baculovirus vector-expressed HIV-1 Vpu protein, which contains a

potential calcium binding or calcium channel-like sequence similar to one of the S-IV sequences of the dihydropyridine (DHP)-receptor, is being purified for use in calcium channel drug-binding studies.

Also, the CRS has isolated mRNA from HIV-1-infected and uninfected H9 cells by combining the RNAzol and oligo dT methods. These mRNAs are being used to make directional cDNA libraries in lambda gt22A. Additionally, cDNA libraries are being constructed in lambda Uni-Zap XR and phagmid libraries and produced by in vivo excision. Differentially-expressed clones will be isolated from the Uni-Zap libraries, as described by Schweinfest et al., in our Laboratory. The lambda gt22A libraries will be screened using a subtracted probe enriched for infection-specific sequences produced by enzymatic amplification of subtracted cDNAs. Clones that strongly hybridize to the amplified subtracted probe will be tested for differential expression by Southern blot hybridization using cDNA probes produced from mRNAs from infected and uninfected cells. Differential expression will be confirmed by RNA gel (Northern) blot assays using filters containing RNA from both infected and uninfected cells and radiolabeled probes from clones that appear promising on the Southern blot assay. Confirmed infection-specific genes will be sequenced and the sequences will be compared to DNA databases in order to determine if they correspond to known genes. Genes that are induced upon viral infection should provide useful insight into the mechanism of viral replication and pathogenesis. Two highly-immunogenic recombinant Env polypeptides of the HIV-1 gp41 (protein 566) and HIV-2 gp35 (protein 996) transmembrane glycoproteins were expressed in E. coli in quantity, purified in milligram quantities to near homogeneity, and used in immunoassays (Western and dot blots) against sera from Central and West Africa, Mexico, and the United States to distinguish between HIV-1 and HIV-2 infections. In addition, recombinant HTLV-I (rpBI) and HTLV-II (rpBII) gp46 Env polypeptides, and HTLV-I p21E Env polypeptides (protein 400), previously expressed in E. coli, were also used in immunoassays (Western blot) to distinguish between HTLV-I and HTLV-II infections. Antibodies developed to the HTLV-I (rpBI) and HTLV-II (rpBII) Env polypeptides were used in antibody-dependent cellular cytotoxicity (ADCC) assays to delineate the type-specific epitopes residing on the surfaces of the HTLV-I and HTLV-II Env glycoproteins. Currently available serological tests do not distinguish between HIV-1 and HIV-2 or HTLV-I and HTLV-II infections because of significant antigen-antibody cross-reactivity due to the close genetic relatedness between HIV-1/HIV-2 and HTLV-I/HTLV-II virus groups. Thus, the application of these highly-immunogenic HIV-1 (protein 566), HIV-2 (protein 996), HTLV-I (rpBI and protein 400) and HTLV-II (rpBII) candidate antigens in development of immunoassays for differentiating single, as well as dual viral infections, and as potential subunit vaccines requires further serological and biological characterization in well-defined animal systems for their full characterization. Following this, they may prove to be useful as diagnostic reagents, targets for immunotherapy, or targets for intracellular vaccination.

Research studies in the Cellular Biology and Biophysics Section (CBBS) are designed to carry out an integrated molecular and cellular biology research approach that uses the latest recombinant DNA in conjunction with transgenic and immunocytochemical methodologies to study the functions of cancer genes, oncogenes and their normal cellular counterparts. By employment of monoclonal antibodies raised against specific prokaryotic and eukaryotic vector-expressed proteins, in conjunction with confocal laser scanning microscopy (CLSM) and in situ hybridization (ISH) investigations, the CBBS will define the biology and functional role of these genes and their products. One of the more important developments in the revelation of the functional importance of the ets family of genes has come from CBBS studies showing that the human ETS1 gene expression correlated well with the T-cell receptor (TCR-α gene)

expression during thymic development in different thymic and T-cell subsets and during T-cell activation, indicating that ETS1 regulates TCR-α genes. The expression of the TCR-α gene is regulated by a T-cell-specific enhancer located downstream of the Cα locus, and a cDNA clone, upon sequencing, was found to code for the entire ETS1 protein. The vector expressed ETS1 fusion protein produced in E. coli bound to the Tα2 motif, while mutation in the Tα2 region abolished the ETS1 binding and T-cell enhancer function, indicating strongly that the ETS1 nuclear protein binds to DNA in a sequence-specific manner. Comparison of DNA sequences bound by ETS1 and subsequent site-specific mutational analyses indicate that centrally-located, purine-rich sequences (ETS Responsive Element) are involved in this regulation. Other nuclear proteins bind to ERE, and different sized ERE protein complexes are seen in T-cell and other hematopoietic and non-hematopoietic cells. In T-cells, three different complexes are seen with ERE. Complex I contains ETS1, and complex III contains an additional ETS-related protein. Since ETS1 expression and regulation are different in T and other cell types, characterization of these transacting factors are in progress. Jurkat cells have been transfected with ETS1 expression vectors in both sense and antisense orientations. Several clones have been obtained expressing antisense ETS1 mRNA, which blocks ETS1 protein formation. Characterization of these clones with respect to their growth property, mitogenic requirements and other target candidate gene expression are under investigation.

A novel member of the human ETS oncogene family, ERG, has recently been identified. A specific antibody was produced in rabbits with erg protein expressed and purified from E. coli. With the antibody, a 52 kD nuclear protein, with a half-life of 21 hours, encoded by the ERG gene was identified in human cell lines. This protein was detected in very limited types of cells; it was phosphorylated after PMA treatment, and bound specifically to PEA3 oligonucleotides. The results suggested that the ERG protein is a new ETS-gene family member of the specific DNA binding protein which may mediate signals transmitted from the membrane. Also the ERG gene is known to encode a nuclear phosphoprotein that is turned over slower than ETS2, and characterization of hematopoietic stem cells expressing the ERG gene is in progress.

Since we have found that the ets proteins can bind DNA in a sequence-specific manner to purine-rich sequences found in the promoter/enhancer elements of a variety of genes, we have come to understand that members of the ets family of proteins can function as transcriptional activators, and induce transcription via binding to a purine-rich ets-consensus sequence [AGGAA]. We have also studied the function and regulation of the ets family of genes, and have shown that ets-1 is autoregulated and the overexpression of ets-1 and ets-2 proto-oncogenes transforms mouse fibroblasts. The ets-1 promoter contains sequences identical to the PEA3 motif; to examine whether the ets-1 protein is able to bind to the PEA3 motif localized in the ets-1 promoter, we synthesized oligonucleotides corresponding to the ets recognition site, as well as unrelated regions from the ets-1 promoter, and tested their binding activity. The v-ets/ets-1 protein we synthesized in E. coli binds to sequences derived from the ets-1 promoter, as well as MSV LTR and polyoma enhancer (PEA3)-derived sequences, but not to unrelated sequences that lack the core elements of the ets recognition site. Thus, it is likely that the ets-1 protein binds to its own promoter in order to autoregulate its expression. In order to investigate whether the ets genes regulate the expression of other gene promoters, we have started testing the activation of MHC class I promoters linked to the CAT reporter gene as a model system after cotransfection with the β-actin promoter-linked ets-1 and ets-2 gene expression vectors. The ets family members (ets-1, ets-2 and erg) have some homology with the helix-loop-helix protein. To delineate the transactivation domain in ets proteins, as

well as to investigate whether it is similar to other helix-loop-helix family proteins (E12, E47, myoD), and also to determine if the helix-loop-helix homology region in ets plays a role in the transactivation process, we have linked the ets-1 and ets-2 genes in frame with the GAL4 DNA binding domain in the expression vector, pSG424. We then tested for the transactivation ability, using these vectors, after cotransfection with reporter plasmids containing the CAT-gene linked to the GAL4 promoter and DNA binding site.

The human ETS1 proto-oncogene proteins have been isolated and purified by CBBS members from the T-cell line, CEM, by immunoaffinity chromatography and their identity confirmed by N-terminal amino acid sequencing. The p51 and p42 ETS1 isoforms react with monoclonal antibodies directed against a bacterially-expressed ETS1 protein and to an oligopeptide directed to the carboxyl-terminal 13 amino acids of the human ETS1 protein. The p42 human ETS1 does not react with an antibody directed to exon VII of the human ETS1, indicating that it is the product of alternatively-spliced mRNA lacking exon VII. The p48 and the p39 isoforms of the human ETS1 are shown to be derived from the p51 and p42 isoforms of the human ETS1 by the covalent modification of -SH groups by the protease inhibitor Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK). The renatured human ETS1 was shown to have DNA sequence-specific binding to the PEA-3 (AGGAAGT) motif; this complex can be observed by electrophoretic mobility shift assays (EMSA). The purified ETS1 retards a complex which is exactly the same size complex as is retarded from nuclear extracts prepared from the T-cell leukemia cell line, CEM. Reduced ETS1 is required to form the ETS1•PEA-3 complex, but modification of the ETS1 -SH groups by N-ethylmaleiamide or by TLCK does not inhibit formation of the ETS1•PEA-3 complex. The ETS1•PEA-3 complex formed with TLCK-modified ETS1 has a slower mobility than does the complex formed with unmodified ETS1. Zone sedimentation analysis of the purified ETS1 indicates that it is the monomer of ETS1 which binds to PEA-3 oligonucleotide.

Six monoclonal antibodies were prepared from mice immunized with a bacterially-expressed human ETS2 protein. These antibodies specifically recognize the two human ETS2-encoded proteins, p56 and p54, but failed to react with chicken, mouse, rat, bovine, or monkey proteins, suggesting that the antibodies recognize epitopes specific to the human ETS2 protein. Differential reactivities of these monoclonal antibodies with the peptide fragments generated by partial proteolytic digestion of the bacterially-expressed ETS2 protein indicated that the six antibodies recognize at least three distinct epitopes in the B domain of the ETS2 protein. Immuno-precipitation experiments comparing native and denaturing conditions suggested that the ETS2 domain detected by the monoclonal antibodies is masked in the native condition by either protein folding or interacting proteins. The biochemical analysis of the ETS2 protein will be facilitated by the development of these monoclonal antibodies, which may be useful as both domain-specific probes and tools for specifically detecting the human ETS2 protein in heterologous expression systems.

Expression of the ETS proteins during the cell cycle has been examined in synchronized cells, or in cells blocked in mitosis using a specific inhibitor of microtubules. Both methodologies have revealed that there is a hyper-phosphorylated species of ETS1 present in the early phase of mitosis. This isoform of ETS1 is present in four different cell lines examined (three human T-cell lines and one human astrocytoma line) that express this gene. Moreover, treatment of unsynchronized cells with okadaic acid (a phosphatase inhibitor) can cause a shift in ETS1 to its hyperphosphorylated form, suggesting that the normal cellular phosphatases may play an important

functional role in the post-translational processing and regulation of ETS1. The action of carbachol, a potentiator of nerve cells, causes a marked increase of ETS1 phosphorylation in astrocytoma cells. The relationship of intracellular calcium and ETS1 phosphorylation observed during this stimulation is being compared to that occurring in mitosis.

Recombinant DNA technology can now be applied to monoclonal antibody (Mab) production. Monoclonal antibodies facilitate biochemical analysis of proteins by their highly-specific recognition of a single epitope. Conventional methods for generating Mabs are not capable of efficiently surveying the induced antibody response to a given antigen. For example, an individual animal has 5-10,000 different B-cell clones, each capable of producing unique antibodies to an antigen. However, with the current cell-fusion technique, only a few hundred different antibodies can be produced. Recombinant DNA technology allows the immunoglobin variable region genes to be amplified, which provides for the generation of a large cDNA library using the bacteriophage lambda-immuno-zap expression vectors. This library is much easier to access than hybridomas produced from cell fusion. Screening is also greatly enhanced, considering immunoglobin gene products of at least 50,000 clones or 10^6-10^7 antibodies can be readily examined in one day, compared to screening hybridomas, which is labor-intensive, time-consuming and expensive. Once produced, these expression vectors can be transfected into mammalian cells or used for making transgenic mice.

Currently, the CBBS members have cloned the heavy and light chains of the pan-*ets* antibody directed against the human ETS2 oncoprotein. The light chain has been successfully inserted into the lambda-Lcl expression vector and a library is under construction. Work continues with progress to construct the heavy chain library.

Analysis of the *ets*-1 and *ets*-2 genes has revealed much molecular and biochemical information. Functions of these proto-oncogenes during development, differentiation and transformation remains to be determined. The transgenic mouse system will be utilized to study specific functions of the murine *ets*-1 and *ets*-2 genes. Four approaches are being studied: first, 5′ regulatory regions for the *ets* genes will be defined using varying lengths of the 5′ regulatory regions controlling expression of the *lacZ* reporter gene; second, mice will be made incapable of producing *ets*-1 by mutating this gene through the use of homologous recombination in embryonic stem cells; third, the specificity of developmental and differentiating functions of the *ets* genes will be analyzed by altering the control of their expression through the use of chimeric genes in which the cDNA of one gene is expressed using the regulatory control elements of the other gene. Finally, further functional information will be obtained by studying the effects of the overexpression of *ets*-1 and *ets*-2 in various tissues of transgenic animals by misdirecting the expression of these genes through the use of heterologous promoters.

Previously reported results with the transgenic model system have shown that the different transgenes can be regulated and expressed appropriately. In an *in vitro* system using NIH3T3 cells, we have shown that the *ets*-2 gene has mitogenic and transforming activity. To study the role of the *ets* gene family in normal developmental processes and tumorigenesis *in vivo*, we have generated transgenic mice with the *ets*-2 proto-oncogene linked to a heterologous promoter. The transgenic mice were generated by microinjection of *ets*-2 cDNA linked to the mouse metallothionein promoter into the pronuclei of one cell embryo. The injected embryo was implanted into the oviduct of pseudopregnant mothers and brought to term. One of the 32 pups was shown to contain the

ets transgene by Southern blot analysis of the DNA prepared from the tails. The founder mouse (female) was bred again to produce offspring that contain the ets-2 transgene. The ets-2 heterozygous mice were inbred to produce homozygous mice. The ets-2 homozygous mice appear to develop hydrocephalus and die within two weeks of birth.

The members of CBBS have undertaken research to evaluate a number of genes, like p53 and the ETS family members that are expressed in cells derived from normal breast and malignant breast tissue, for point mutations and gene rearrangements that would result in their inactivation. Since early detection is very critical to the treatment and survival of individuals with this cancer, the CBBS has initiated a program to clone novel breast tumor specific genes as probes, as well as study the expresssion of other known genes to develop a battery of potential markers that would detect asymptomatic neoplasms.

A unique approach to treating HIV and HTLV-I infections has been initiated by members of the CBBS, to develop a potential method using a system of gene therapy which provides "intracellular vaccination." The herpes thymidine kinase (HSV-tk) gene has been placed under the regulatory control of the HIV-LTR which includes the TAR regulatory sequence in an MSV packaging-defective viral vector containing a selectable marker for neomycin resistance. Stable 3T3-derived cell lines containing this construct and packaging-defective virus have been produced. Studies will determine whether the HSV-tk enzyme will be synthesized in response to the HIV Tat trans-acting protein. Cells containing the HSV-tk enzyme will selectively metabolize specific drug analogues (such as acyclovir) to a toxic metabolite, which will specifically kill those dividing cells. Thus, any Tat-producing cell infected by HIV can be selectively destroyed, while leaving a normal, uninfected stem cell population to replenish the lost T-cells. Studies will be conducted to establish cell lines stably transformed with the HIV-LTR-HSV-tk construct. The HIV-LTR-HSV-tk T-cell lines will be infected with live HIV virus. Toxicity response to acyclovir will be established for live virus infection. Gene therapy utilizing human cells will be tested in SCID mice. The HIV-LTR-tk construct in the defective retroviral vector will infect and integrate into human bone marrow cells. Resistance to HIV infection with acyclovir administration will be tested in vivo. Similar studies for HTLV-I will be conducted.

Work on transgenic mice containing the HTLV-I tax gene will continue in the CBBS since it is known that they develop tumors of peripheral nerve sheaths, iris, adrenal medulla and salivary gland, myopathy and localized lymph node hyperplasias. The mechanisms leading to these abnormalities remain unclear, but may involve the deregulation of cellular genes important for cell growth and differentiation. The expression of tax in these transgenic mice has been correlated with the induction of the genes for the interleukin-2 receptor (IL-2R), the granulocyte-macrophage colony stimulating factor (GM-CSF), and more recently, nerve growth factor (NGF). Cell lines from the peripheral nerve and salivary tumors appear to secrete significant quantities of IL-6 and perhaps other B-cell-related growth factors, which may account for the lymphadenopathy associated with these tumors. This transgenic mouse system provides an important in vivo model for HTLV-I-induced transformation and tax activation of growth-regulating genes.

The Microbiology Section has developed an approach, using three different systems, to understand the mechanisms by which retroviruses cause erythro-leukemia in mice and identify the viral and host genes that are crucial for the biological effects observed. Studies on the acute erythroleukemia-inducing Friend spleen focus-forming virus have concentrated on understanding

how the viral envelope protein abrogates the erythropoietin (Epo) requirement of erythroid cells. The protein appears to interact with and trigger the Epo receptor, and studies are in progress to determine if this interaction results in a mitogenic signal like that initiated by Epo.

We have also been studying the effects of another erythroleukemia-inducing virus, the gag-myb-ets-containing ME26 virus, on hematopoietic cell growth. Our results indicate that this virus, which encodes a DNA-binding protein, may be activating the Epo receptor in an immature hematopoietic cell that does not normally express it. The virus, however, may not be directly transactivating the Epo receptor, but may be working through another erythroid-specific gene, GATA-1. Studies on the third erythroleukemia-inducing virus, Friend MuLV, have been twofold. We have molecularly cloned a candidate for a host gene, Rmcf-r, that is involved in resistance to early erythroleukemia induced by the virus, and are now testing its biological activity. We have also molecularly cloned a variant of Friend MuLV, PVC-211, which no longer causes erythroleukemia in mice, but which induces a progressive neurodegenerative disease. We are now generating recombinants between PVC-211 and wild-type Friend MuLV in order to localize the regions of the viral genome responsible for inducing either leukemia or neurological disease.

The avian acute leukemia virus, E26, expresses oncogenic fusion proteins containing portions of the myb and ets-1 genes derived from the avian genome. In order to study both the normal and oncogenic functions of these genes, particularly ets, we have developed a murine model for myb-ets oncogenesis by introducing the gag-myb-ets coding sequences from E26 into a murine retroviral vector. This defective murine viral construct (ME26) induces leukemia in newborn mice and abrogates the serum dependence for growth of NIH3T3 murine fibroblasts. We have now analyzed the expression of serum-response genes in NIH3T3 fibroblasts which have lost their dependence on serum or growth following infection of ME26. No increase in fos, jun or myc expression was seen in ME26-infected cells in comparison to uninfected 3T3 cells, and expression in ME26-infected hematopoietic cells was also not altered. Likewise, c-ets-1 and c-ets-2 endogenous expression was not increased in these cells. These results suggest that ME26 may induce serum-independent growth in fibroblasts by a mechanism that does not involve the serum-response pathway. We have also detected a novel 3.7 kb subgenomic ME26 message in both infected fibroblasts and hematopoietic cells in culture. The subgenomic message contains both myb and v-ets specific sequences and is not incorporated into viral particles. The coding potential of this subgenomic message remains to be determined.

While the detailed mechanisms of tumorigenesis are unknown, increasing evidence suggests that genetic alterations of cellular oncogenes are, in part, responsible for the neoplastic transformation of cells. Some studies in rodent models have implicated the direct chemical activation of oncogenes by carcinogen exposure. The reproducible detection of specific transforming genes in animal model systems strongly suggests that these genes have a significant role in the development of certain tumors. Data from our preliminary transfection experiments with DNA from carcinogen-induced medaka tumors supported this hypothesis and suggested that an unknown gene may be activated in the DEN-induced cholangiocarcinoma. It does not appear to have homology to any known oncogene sequence based on hybridization of Southern blots. Recently, studies have been initiated to examine DNA from gonadal tumors in two species of clams (mya arcnaria and mercenatia mercenara) exposed to herbicides. We have demonstrated transformation of NIH3T3 cells and have produced one tumor in nude mice using DNA from an advanced tumor. The identification and characterization of this apparently novel oncogene is now

in progress. Also, transgenic fish (medaka) have been produced by the introduction of the E. coli lacZ gene under the control of the mouse Mt-1 metallothionein promoter. Fish which contain the lacZ gene have been identified by PCR analysis. This construct has also been introduced into fish cell lines to form the basis of in vitro toxicity assays. We have not yet been able to demonstrate expression of this construct in either fry or the fish cell lines.

Understanding cellular mechanisms of viral control may be useful in our attempts to develop antiviral agents which affect human retroviruses, such as HIV and HTLV-I. The goal of this project is to characterize the role of cellular factors in the growth and restriction of retrovirus infection. Previously, we had reported that RD114 restriction in feline cells does not occur at the level of the proviral LTR, since reporter genes linked to the LTR function efficiently in both restrictive and permissive cells. We have extended our analysis of the mechanism of restriction of growth of RD114 virus in feline fibroblasts, and can show that the expression of endogenous RD114-related mRNAs correlates with the restricted phenotype. Additionally, restricted cells express RD114 glycoprotein molecules of ~gp85 kD instead of the expected gp70 following RD114 transfection or infection. Interaction between the RD114 receptor and the modified gp70 molecule is probably responsible for the restricted ability of RD114 virus to spread in feline fibroblasts. We have also studied the growth of feline immunodeficiency virus (FIV) in the cat brain-derived cell line, G355, and have shown that certain strains of the virus are fusigenic and cytotoxic for these cells. Interference analysis indicates that FIV utilizes a receptor on G355 cells which is different from the ones used by feline endogenous and exogenous viruses. Cell fusion is enhanced by the presence of replicating C-type viruses and is further enhanced in cells transformed by the mos-containing Mo-MuSV. The fusion of MSV-transformed G355 cells provides an easily quantifiable biological assay for virus infectivity in an adherent cell line.

Topoisomerases are enzymes which modify the topological state of DNA and have been shown to be involved in DNA replication and transcription. Since the process of retroviral replication, integration and expression are likely to require topological changes in either the integrated or unintegrated provirus, we have begun to determine if topoisomerases play a critical role in retrovirus growth or infection, or if topoisomerases might be a target for the control of retroviral infections. We had previously described a novel topoisomerase I (topo I) activity associated with various retrovirus and lentivirus particles, and showed that the specific topo I inhibitor, camptothecin (CPT), could block HIV infection of H9 cells in tissue culture. Using spleen focus-forming virus (SFFV) induction of splenomegaly as an in vivo model, we have shown that CPT can block SFFV-induced diseases when coinjected with the virus into susceptible mice. To determine the ability of CPT to affect chronic virus production by infected cells, we treated CF2Th (dog thymus) cells producing equine infectious anemia virus with CPT. Continuous exposure to low levels of the drug resulted in 85-92% inhibition of virus production as measured by RT released into the culture media, and similar levels of reduction in viral protein production by immunofluorescence and radioimmunoprecipitation analysis.

Members of the Office of the Chief (OC) have found that genetic mutagenesis is a powerful tool to study the roles of ras oncogenes and proto-oncogenes in cell transformation and normal cellular function. A very potent dominant negative ras mutant (asparagine-116 to tyrosine mutation of the v-H-ras) has been identified and characterized. This 116Y mutant strongly inhibits the c-H-ras proto-oncogene function. It suppresses transformation induced by

overexpression of c-H-ras in NIH3T3 cells. NIH3T3 cells transfected with the 116Y mutant are resistant to transformation by a group of retroviruses carrying a variety of protein-tyrosine kinase oncogenes, including v-abl, v-fes/flp, v-src and v-fms. Surprisingly, transformation by retroviruses carrying the homologous ras oncogenes, including v-H-ras, v-K-ras and v-bas, is not affected. These results suggest blocking of a c-ras proto-oncogene function upstream to transformation induced by the activated ras oncogenes. Since oncogenes are an activated version of cellular proto-oncogenes that control cell growth and differentiation, it is believed that these genes participate in signaling pathways. We have identified a dominant, negative ras mutant capable of inhibiting c-ras proto-oncogene function (N116Y mutation of v-H-ras). This mutation is present in one of the consensus sequences conserved in the GTP-binding proteins and is critical in the interaction of ras proteins with the GTP or GDP nucleotides. The N116Y mutant blocks transformation by retroviruses carrying protein tyrosine kinase oncogenes, including v-abl, v-fes/flp and v-fms, but did not affect v-H-ras, v-K-ras and v-bas. We have established a series of NIH3T3 cell lines to investigate the biochemical target and signaling pathways mediated by ras. In this study we have found that phosphorylation of a protein kinase C (PKC) substrate (80 kD protein) was lower in cells transfected with the N116Y ras mutant than in normal NIH3T3 cells. Expression of the N116Y ras mutant completely blocked DNA synthesis induced by EGF, but not by other growth factors or serum. Sequestration or inhibition of ras targets could be an explanation for these differences. Members of the OC have identified a new class of dominant negative ras mutants which inhibit transformation induced by overexpression of c-H-ras proto-oncogenes in NIH3T3 cells. These transformation-defective mutants were constructed by site-directed mutagenesis of the conserved sequence motif, NKXD, of the ras GTP-binding site.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04963-15 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Toward a Molecular Description of Malignant Transformation by 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. Gutierrez	Visiting Fellow	LMO	NCI
	L. S. Wrathall	Microbiologist	LMO	NCI
	R. H. Bassin	Research Microbiologist	LTIB	NCI

COOPERATING UNITS *(if any)* NY State Inst. for Basic Res. in Devel. Disability, Staten Island, NY (Y. Hwang); Cancer Inst., Hokkaido U. School of Med., Sapporo, Japan (Y. Ogiso, N. Kuzumaki); U. Kentucky Medical Center, Lexington, KY (S. Zimmer)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.00	0.70	0.3

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.)*

Genetic mutagenesis has been used to study the roles of ras oncogenes and proto-oncogenes in cell transformation and normal cellular function. A very potent dominant negative ras mutant (asparagine-116 to tyrosine mutation of the v-H-ras) has been identified and characterized. This 116Y mutant strongly inhibits the c-H-ras proto-oncogene function. It suppresses transformation induced by over-expression of c-H-ras in NIH3T3 cells. NIH3T3 cells transfected with the 116Y mutant are resistant to transformation by a group of retroviruses carrying a variety of protein-tyrosine kinase oncogenes, including v-abl, v-fes/flp, v-src and v-fms. Surprisingly, transformation by retroviruses carrying the homologous ras oncogenes, including v-H-ras, v-K-ras and v-bas, is not affected. These results suggest blocking of a c-ras proto-oncogene function upstream to transformation induced by the activated ras oncogenes.

PROJECT DESCRIPTION

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

T. Y. Shih	Research Chemist	LMO	NCI
L. Gutierrez	Visiting Fellow	LMO	NCI
L. S. Wrathall	Microbiologist	LMO	NCI
R. H. Bassin	Research Microbiologist	LTIB	NCI

Objectives:

The major objective of the present project is to determine the molecular mechanisms by which ras oncogenes transform cells to malignant phenotypes, and to elucidate the normal cellular function of ras proto-oncogenes. Human oncogenesis is a multistep and multifactorial process that progressively converts normal cells into malignant cancer cells. Approximately 50 oncogenes that accelerate development of cancer, as well as an emerging number of onco-suppressor genes that retard tumor development, have been identified. We hypothesize that these genes participate in a network of cellular signaling pathways controlling these cellular processes. The task of elucidating the signaling network resembles the task of delineating the biochemical pathways of intermediate metabolism. The present project employs both genetic mutagenesis and biochemical methods to investigate the functional relationship of oncogenes and their interactions with cellular signaling mechanisms, with particular emphasis on the roles of ras oncogenes.

Methods Employed:

1. Site-specific mutagenesis. The H-ras oncogene of the proviral pH-1 DNA was cloned into the single-stranded M13 phage. The specific amino acid residue of p21 ras proteins was altered by oligonucleotide-directed mutagenesis of the M13 template DNA. The mutant ras was reconstructed into the pH-1 DNA and a chimeric plasmid containing the neoR gene of pSVneo was constructed. Similar sets of mutants were constructed for the K-ras (4B) oncogene to study its function.

2. Transfection of NIH3T3 cells. Transforming activities of mutant ras genes were evaluated by transfection into NIH3T3 cells. Transformation-suppressor activities of mutant ras were studied by transfection into cells transformed by ras and various other oncogenes. Phenotypic reversion of transfected cells selected by the neo^R gene in the ras mutant construct was studied. A series of NIH3T3 cells transfected with the dominant negative mutant ras was also established.

3. Isolation and characterization of revertants. Flat revertants, as well as transformed clones, were isolated from NIH3T3 transformed by overexpression of normal c-H-ras, following transfection with ras mutant DNA and selection with G418. The cell lines were twice single-cell cloned. The doubling time, colony-forming efficiency in soft agar, and the expression of mutant ras were examined. Susceptibility of a revertant cell line for retransformation was assayed by infection with Harvey sarcoma virus.

Major Findings:

A general approach to elucidate the cellular function of a molecularly-cloned, regulatory gene is to construct dominant negative mutants capable of functional inactivation of the resident wild-type gene when introduced into a recipient cell. We employed a strategy to construct a new class of dominant negative ras mutants by altering the highly conserved sequence motif, NKXD, at the ras GTP binding site. We have studied the trans-dominant suppressor activity of the asparagine-116 to tyrosine (N116Y) and to isoleucine (N116I) mutations of the v-H-ras oncogene in detail. The 116Y ras mutant suppresses very efficiently the transforming activity of the c-H-ras proto-oncogene when overexpressed in NIH3T3 cells. The suppressor activity is so strong that in a selected revertant cell line (F33), the cells appear to compensate for the suppressor effect by producing a large quantity of c-H-ras p21, several hundred-fold higher than that in normal cells.

We also constructed a series of NIH3T3 cells transfected with the 116Y ras mutant to study the functional relationship of oncogenes. These cells were then challenged with a series of transforming retroviruses carrying a variety of viral oncogenes. We found that transformation by a group of retroviruses carrying protein-tyrosine kinase oncogenes, including v-abl, v-fes/flp, v-src and v-fms, was greatly inhibited. To our surprise, transformation by retroviruses carrying the homologous ras oncogenes, including v-H-ras, v-K-ras and v-bas, was not significantly affected. It appears that the dominant negative 116Y ras mutant inhibits the function of normal resident cellular ras which is required for transformation by these protein-tyrosine kinase oncogenes, but the target of its inhibition lies upstream to transformation induced by the activated ras oncogenes. We are investigating the precise site of this inhibition. Biochemical studies on the ras-mediated pathways employing the 116Y mutant are described in Project No. Z01CP05658-02 LMO.

We have employed a cloned rat embryo fibroblast cell line (CREF) to study the roles of wt ras p21 and various GTP-binding deficient mutants in the stepwise increases of its malignant properties, i.e., morphological transformation, growth in soft agar, tumorigenicity in nude mice or syngeneic Fischer rats, and lung metastasis following tail vein injection. The main conclusions are as follows: (a) The v-ras oncogene can both morphologically transform and progress untransformed CREF cells and a non-tumorigenic WtAd5 CREF cell (a CREF cell transformed by Ad5 adenovirus) to an oncogenic and metastatic phenotype; (b) the 116K ras mutant (N116K mutation of v-H-ras), which is defective in GTP-binding, cannot induce morphological changes in either CREF or WtAd5 CREF, but it can induce oncogenic potential in WtAd5 CREF cells; (c) although oncogenic in both nude mice and syngeneic rats, WtAd5-116K transformed CREF cells are non-metastatic when injected into the tail veins of rats. These studies indicate that the tumorigenic properties and the metastatic properties of ras p21 can be dissociated, and they are cell-type specific and related to the GTP-binding properties of this protein.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05120-12 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Structural, Biochemical, and Biological Characterization of HIV Nef and Vpu

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

PI:	J. A. Lautenberger	Research Chemist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	D. R. Hodge	Biologist	LMO	NCI
	Y-M. A. Chen	Visiting Fellow	LMO	NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (K. P. Samuel, G. K. Pei)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.40	0.70	0.7

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.)*

A recombinant HIV-2 (NIH-Z) Nef protein synthesized in E. coli, and purified by the detergent/chaotrope extraction technique and preparative SDS-PAGE is: immunologically reactive on Western blots with anti-Nef antibodies; an excellent substrate in vitro for phosphorylation both by purified protein kinase C (PKC) and the maturation promoting factor (MPF) kinase purified from Xenopus oocytes; exhibits an intrinsic low-level autokinase activity, and forms stable homodimers and homotetrameric complexes in vitro, which are significantly increased in the absence of sulfhydryl-reducing agents. Preliminary results of in vivo phosphorylation and oligomerization experiments suggest that the native 25 kD HIV-2 (NIH-Z) Nef protein in infected T-lymphocytes in culture becomes highly phosphorylated following stimulation with calcium ionophores, and the dimeric (50 kD) and tetrameric (100 kD) forms of this protein can be radioimmunoprecipitated with specific anti-HIV-2 Nef monoclonal antibodies. In addition, evidence of a putative Nef cellular protein complex was also obtained. Structural studies have also revealed the presence of a leucine zipper-like repeat structure at the conserved central "core" region of the Nef proteins, with a characteristic 4,3 repeat similar to the heptad leucine repeat motif of the bZIP factors. Moreover, at the C-terminus of the Nef proteins is a highly acidic sequence (net charge of -5 to -8) stretched over 40 amino acids, and it contains two predicted α-helices separated by a predicted β-turn structure, with homology to known acidic activation domains of transcriptional activation factors. Biochemical characterization of individual biological Nef clones expressing only p27nef (clone 3B-3) or p25nef (clone 3B-5), isolated by limiting dilution of HTLV-IIIB-infected H9 cells, are continuing. In addition, a baculovirus vector-expressed HIV-1 Vpu protein, which contains a potential calcium binding or calcium channel-like sequence similar to one of the S-IV sequences of the dihydropyridine (DHP)-receptor, is being purified for use in calcium channel drug-binding studies.

PROJECT DESCRIPTION

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

J. A. Lautenberger	Research Chemist	LMO NCI
T. S. Papas	Chief	LMO NCI
D. R. Hodge	Biologist	LMO NCI
Y-M. A. Chen	Visiting Fellow	LMO NCI

Objectives:

Regulation of the replication and expression of HIV is controlled by an elaborate and complex network of several viral regulatory genes (vif, rev, tat, vpu, vpr, and nef) whose protein products function by mechanisms not yet well enough understood to exert either positive or negative regulatory effects. One of these regulatory gene products, Nef, is reported to be a negative regulator of HIV expression, while Vpu is believed to play a positive regulatory role in viral pathogenesis. Because of the important regulatory roles that the Nef and Vpu proteins play at specific stages in the pathophysiology of HIV, we are investigating both their structural and biochemical properties in order to delineate their function and understand their mechanism of action, so as to develop strategies for subsequent therapeutic intervention. To achieve this goal, we have utilized both the prokaryotic (E. coli) and eukaryotic (baculovirus) expression vector systems, as well as mammalian (HeLa and T-lymphocytic) cell lines in transient expression assays, to produce sufficient quantities of these scarce viral proteins in bacteria, insect cells, and mammalian cells. We also aim to identify host-gene products that interact with or bind these viral proteins to control HIV expression, and to gain insight into their physiological function.

Methods Employed:

1. Expression of the HIV-1 Vpu Protein in Insect Cells. A 603 bp EcoRI/Asp718 DNA fragment containing the entire 243 bp open reading frame (ORF) of the HIV-1 (BH10) vpu gene, was subcloned into the pGEM7f plasmid. A 292 bp RsaI restriction fragment, containing the entire coding sequences of vpu, including two extra amino acids preceding the first ATG (+1) codon, was ligated with NheI linkers to the NheI cloning site in the baculovirus vector, pJVP10Z, to give the vpu expression vector, pJVP10Z-vpu.

2. Purification of HIV-2 (NIH-Z) Nef Protein Synthesized in E. coli by Detergent/Chaotrope Extraction and Preparative SDS-PAGE. Induced cell pellets from 1 liter of E. coli (DC1148) cells harboring a recombinant HIV (NIH-Z) nef expression plasmid, pDRH-9, were extracted by a standard detergent/chaotrope protocol to enrich for the 25 kD recombinant Nef protein, which was subsequently purified, without any prior denaturing by boiling, on preparative SDS-PAGE. The 25 kD Nef protein band is eluted from the gel pieces, the SDS is removed by acetone extractions, and the purified protein is solubilized with 8M urea and stored at -30°C. Total yield is 75-100 μg purified HIV-2 Nef per liter induced culture.

3. Development of Murine Anti-Nef (HIV-2) Monoclonal Antibodies. Purified E. coli-synthesized HIV-2 Nef protein was used to raise a panel of mouse monoclonal antibodies (e.g., 583-11S, -15S, and 29S) specific for recognizing both the native and recombinant 25 kD protein. Development of a rabbit anti-Nef (HIV-2) polyclonal antiserum was previously detailed in the 1990 report.

4. Amino Acid Sequence Alignment of Nef Proteins with Transcriptional Activation Factors. Alignment of the predicted amino acid sequences of the Nef proteins with consensus sequences in transcription factors was performed by eye. The predicted Nef amino acid sequences at the highly acidic C-terminal regions were compared with the corresponding predicted amino acid sequence at the activation domains of the acidic class of transcriptional activation factors, such as GCN4, VP16, CPC1 of Neurospora crassa, HBVX protein of hepatitis B virus, and the yeast heat-shock transcription factor (HSTF). In addition, alignment of the Nef protein leucine zipper repeat sequence was made with the corresponding leucine zipper repeats of the bZIP factors, C-Fos, FRA1, mXBP protein of mouse, the human C-Myc and murine L-Myc proteins, the Epstein-Barr virus BZLF1 factor, and the Neurospora crassa CPC1 protein.

5. Secondary Structural Analyses. Secondary structural analyses were conducted on the predicted amino acid sequences of the leucine zipper and acidic C-terminal regions of the Nef proteins by the computer algorithms of Chou and Fasman and Garnier et al., using the VAX computers of the Advanced Scientific Computing Laboratory at the Frederick Cancer Research and Development Center.

Major Findings:

1. Structural Characterization of HIV-1, HIV-2, and SIV Nef Proteins:

A. Nef leucine zipper structure and protein oligomerization. Located at the highly conserved middle region (core) of the HIV-1, HIV-2, and SIV Nef proteins, is a heptad repeat structure of 4 consecutive leucine residues or analogous conservative (hydrophobic) amino acid substitutions lying on one face of a putative α-helix. This structure also has the characteristic 4,3 arrangement of predominantly hydrophobic amino acids which have previously been reported for the coiled-coil structure within the dimerization domain of such proteins.

Evidence for the formation of Nef homodimers (~50 kD) and homotetrameric complex (~100 kD) have previously been demonstrated (1990 report) with a recombinant HIV-2 Nef protein, and these structures are enhanced and stabilized by the absence of sulfhydryl-reducing agents and chemical cross-linking with glutaraldehyde, respectively. Moreover, both HIV-2 Nef complexes are detected by specific anti-Nef monoclonal antibodies in ^{35}S-labeled cell extracts of H9-infected HIV-2 (NIH-Z) cells.

In addition to a leucine zipper structure, the Nef protein sequences of the HIV-1, HIV-2, and SIV isolates contain a conserved, highly acidic sequence at their C-terminus, with a net charge of between -5 and -8 stretched over about 40 amino acid residues. Alignment and secondary structural analysis of this region of the Nef proteins with sequences within the minimal activation regions of the acid transcriptional activation factors, reveal significant amino acid homology and conservation of two or more predicted α-helices separated by β-turns.

B. Association of specific cellular protein(s) with HIV-1 and HIV-2 Nef. Current studies to identify specific cellular protein factors that associate with the HIV-1 and HIV-2 Nef proteins are being conducted using mixing experiments and RIP analysis with the anti-Nef specific monoclonal antibodies. In preliminary results with the HIV-2 (NIH-Z) Nef, a 41-43 kD cellular protein was specifically immunoprecipitated from ^{35}S-labeled H9 cell extracts in mixing experiments only when unlabeled baculovirus-expressed HIV-2 Nef is added to the radioactively-labeled T-cell extract.

C. Isolation of two different Nef-expressing cell lines by limiting dilution cloning. Human T-lymphocytic cell line H9 infected with the HTLV-IIIB isolate of human immunodeficiency virus type 1 (HIV-1) synthesizes two forms of the Nef protein (p25 and p27) that differ both in molecular weight and charge. We have used the limiting dilution method to clone out the p25 and p27 nef genes from the HTLV-IIIB stock which induces expression of only p25 or p27. In addition, cells infected with HIV-1 derived from the HXB3 clone of the HTLV-IIIB isolate made only the p25 species, whereas the 8E5/LAV cell line which harbors a single defective LAV provirus produces only the p27 species. These findings are consistent with the notion that the HTLV-IIIB isolate consists of at least two distinct variants with different nef genes, one specifying p25 and the other encoding p27. After a considerable number of passages in culture, H9 cells chronically infected with the HTLV-IIIB isolate produced high levels of p25 and lower levels of p27. Passages in culture appear to select for a subpopulation of virus variants that specify high levels of p25 Nef expression.

2. Biochemical Characterization of HIV-1 and HIV-2 Nef Proteins:

A. Phosphorylation of HIV-2 Nef protein. In the sequence and structural analysis of the predicted Nef protein sequences, consensus phosphorylation sequences for several cellular target kinases, including PKC, MPF, casein kinase II, calmodulin kinase II, and autophosphorylation, were identified. In vitro phosphorylation studies have confirmed that a recombinant HIV-2 Nef protein serves as a good substrate for phosphorylation by purified PKC and MPF kinase. The autokinase activity of the purified protein is labile, and the activity can also be demonstrated by in vivo ^{32}P-orthophosphoric acid-labeling of the induced recombinant protein in E. coli. Phosphoamino acid mapping analyses have identified a serine and threonine residue, respectively, as the phosphorylation target sites for PKC and MPF kinases in the in vitro phosphorylated HIV-2 Nef protein.

Further, preliminary in vivo phosphorylation results show that the HIV-2 (NIH-Z) Nef protein expressed in H9-infected cells become hyperphosphorylated following stimulation with calcium ionophores.

B. Structural and biochemical characterization of HIV-1 Vpu protein: The full-length HIV-1 (BH10) Vpu protein of 17 kD was expressed to relatively high levels in insect cells (SF9) with a baculovirus vector. The 17 kD Vpu protein was used in cell crude extracts on immunoblot (Western) assays to screen a panel of sera from HIV-1-infected individuals and normal human sera. This study showed that between 37-40% of HIV-1 sera were immunoreactive with the full-length recombinant Vpu protein.

Because the bacterially-expressed, full-length Vpu protein previously described (1990 report) is expressed at lower levels and is technically more difficult to purify from insoluble E. coli inclusion bodies, the baculovirus Vpu protein is being used for further biochemical characterizations. As was previously reported, we have identified a stretch of 20 amino acids within the predicted 82 amino acid sequence of the HIV-1 Vpu protein, which shows a 55% identity with the positively-charged III-S4 region of the dihydropyridine (DHP) calcium channel receptor; and preliminary Vpu-binding studies, using a membrane filtration method and CHAPS-solubilized baculovirus-expressed Vpu, suggest a dose-response binding of ^{3}H-Nitrendipine (a dihydropyridine calcium channel antagonist). In addition, $^{45}Ca^{2+}$-binding studies conducted on the crude E. coli-synthesized Vpu protein did not reveal any binding. These studies are continuing.

Publications:

Zweig M, Samuel KP, Showalter SD, Bladen SV, DuBois GC, Lautenberger JA, Hodge DR, Papas TS. Heterogeneity of Nef proteins in cells infected with human immunodeficiency virus type 1. Virology 1990;179:504-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05238-10 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

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	Research Microbiologist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	A. K. Seth	Visiting Scientist	LMO	NCI
	G. J. Mavrothalassitis	Visiting Associate	LMO	NCI
	C. L. Jorcyk	Biologist	LMO	NCI
	M. C. Venanzoni	Visiting Fellow	LMO	NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (F. Smyth); Centre for Early Human Development, Clayton, Victoria, Australia (I. Kola)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.15	1.15	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 functional relationship between the ets gene of transforming leukemia virus, E26, and its cellular prototypes has been facilitated by structural comparisons at the nucleic acid and predicted protein levels. The nucleotide sequences of the chicken, mouse and human ets-1 genes are over 95% identical to one another. The mammalian ets-2 genes from man and mouse encode for nearly identical amino acids and are over 90% conserved relative to the chicken ets-1 gene. Alignment of the predicted ets proteins suggests that three domains exist. The domain closest to the carboxyl-termini is highly conserved in the predicted gene products from species ranging from human to Drosophila. The domain located at the amino-terminal end of the ets proteins is more divergent, being highly conserved only between the same gene isolated from different species (e.g., chicken ets-1 vs. human ETS1; mouse ets-2 vs. human ETS2). The central domain encoding the ets proteins is found to be most divergent, even between ets family genes of the same species. Thus, ets represents a family of genes whose members are diverging at variable rates. We have characterized the genomic structure of the human ETS1 and ETS2 genes and find that while the ETS1 locus consists of eight exons over 65 kb, the ETS2 gene contains 10 exons spanning 25 kb. The ETS1 and ETS2 genes have transcript complexity due to multiple initiation sites, alternate splicing and multiple polyadenylation sites. They represent members of an increasingly large family of genes lacking TATA/CAAT promoter elements. ETS1 reveals several highly conserved (near 100% identical) nucleic acid sequences. These may represent unique regulatory domains, binding a novel class of nuclear factors. Genes in addition to ETS1, ETS2, ERG, ELK1 and ELK2 are present in the human genome. Thus far, we have defined two loci that appear to represent ETS2 pseudogenes and a new gene designated ERGB that, although related to ERG at the nucleotide level, has unique properties, including novel chromosome location and a more ubiquitous pattern of gene expression. Additional ETS-related genes are currently under analysis.

PROJECT DESCRIPTION

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

D. K. Watson	Research Microbiologist	LMO NCI
T. S. Papas	Chief	LMO NCI
A. K. Seth	Visiting Scientist	LMO NCI
G. J. Mavrothalassitis	Visiting Associate	LMO NCI
C. L. Jorcyk	Biologist	LMO NCI
M. C. Venanzoni	Visiting Fellow	LMO NCI

Objectives:

The purpose of this investigation is to determine the relationship between v-ets and its 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-ets 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. Dephosphorylation by incubation with CIP (calf intestinal phosphatase).

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Specific DNA fragments were obtained by electroelution or by extraction from agarose.

3. Preparation of DNA probes using purified ets-specific DNA by nick-translation using E. coli DNA polymerase and DNaseI. Alternatively, probes were prepared utilizing random primers and DNA polymerase. Strand-specific probes prepared by transcription of defined primers (riboprobes) or by asymmetric polymerase chain reaction.

4. Preparation of nitrocellulose nylon 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 ets-related sequences by hybridization of ets-specific probes to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the procedure of Benton and Davis (Science 1977;196:180-2). Isolation of plasmid DNA from colonies lifted from plates by the method of Grunstein and Hogness (Proc Natl Acad Sci USA 1975;72:3961-5).

7. Subcloning of isolated DNA fragments into appropriate plasmid vectors, as required.

8. DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol 1980;65:499-560) and/or Sanger (Proc Natl Acad Sci USA 1977; 75:5463-7). In addition, uniquely labeled DNA will be sequenced following RNA-directed primer extension. Preparation of nested (sequential) deletions employing exonuclease III and mung bean nuclease.

9. Total cellular RNA from cultured cells or tissues was prepared by either the LiCl/urea, RNasol, or guanidine isothiocyanate 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 gels and Northern analysis.

10. Construction of cDNA library: Double-stranded cDNA was prepared from polyA+ RNA and ligated into λgt10, λgt11, λZapII, or unizap vector DNA for amplification.

11. Cloning of ets-genes into prokaryotic vectors capable of overexpression of inserted DNA. Isolation of proteins to be used as antigens and for functional studies.

12. Controlled expression of ets genes in eukaryotic vectors, using constructions with regulatable promoters. Transfection of eukaryotic vectors in mammalian cell lines for identification of expressed product(s) and for analysis of possible biological activity associated with the construct.

13. Identification of promoter and regulatory sequences present in genomic clones of the cellular homologs of oncogenes. To include nucleotide sequence analysis and functional assays using expression of defined reported genes, such as chloramphenicol acetyltransferase (CAT). DNA:protein binding evaluated by mobility gel shift, methylation interference and DNaseI protection analyses.

14. Nuclease protection assays to define the 5′ and 3′ ends of transcripts. Verification by sequence analysis of cDNA clones and by primer-extension of mRNA.

15. Polymerase chain reaction (PCR) to analyze tissue-specific splicing of the ets genes.

Major Findings:

1. Chicken, mouse and human genomic libraries were screened with ets-specific probes and clones have been isolated. In addition, human, chicken and mouse cDNA clones have been isolated and subjected to nucleotide sequence analysis. Additional ets clones are being isolated and characterized for complete understanding of the molecular structure of the RNA products of the ets and ets-related genes.

2. The predicted ets-1 and ets-2 proteins possess three distinct domains when compared to v-ets. The domain closest to the carboxyl-termini (C domain) is highly conserved (>90%) and this conservation is seen to be widely preserved throughout evolution, including Drosophila. The domain located at the amino-terminal end of ets-2 (A domain) is less homologous to the virus/chicken proto-oncogene and, thus far, this region has not been identified in lower eukaryotes. The third domain (B domain), which is located centrally, is diverged in ets-2 genes, but is conserved in the ets-1 gene. The highly conserved C domain is the essential criterion for assignment to the ets gene family. Interestingly, the C region is required for DNA-specific binding, thus defining an important functional motif.

3. The human ETS1 gene consists of eight exons, distributed over 65 kb. Sequence analysis of the promoter region upstream from the first exon (which contains the translation start codon) revealed no TATA or CAAT elements. Potential binding sites for transcription factors (SP1, AP1, AP2) are present in the 700 bp region required for functional promoter activity as measured by chloramphenicol acetyl transferase (CAT) activity. Potential positive and negative elements have been defined for further analysis. Consistent with the absence of TATA and CAAT elements, the ETS1 gene transcripts initiate from multiple sites which span a region of 140 bp.

4. The mouse ets-1 gene is similar in organization to the human ETS1 gene. The positions of the introns (point of disruption in the predicted open reading frame) are identical. Sequence analysis of the promoter region of the mouse ets-1 gene and alignment with that of the human ETS1 gene reveals several highly conserved domains, suggesting functional significance. At least two of these conserved sequences can be shown to interact with nuclear proteins by mobility gel shift analyses. These interactions possibly define a new class of regulatory proteins, as analyses of the DNA sequence does not reveal any consensus sites for protein binding. Also, we have not found similar sequences in the promoters of genes (GenBank), and thus they are specific for ets-1 regulation. Tissue-specific distribution of the protein factor(s) responsible for the observed DNA mobility shift is being investigated. Oligonucleotides will be used to further define the precise DNA binding site (methylation interference analysis) and as a means to isolate the genes encoding these novel proteins.

5. Polymerase chain reaction (PCR) analyses of human and mouse ets-1 cDNA has identified several amplification products, indicating alternative splicing. In addition to the human ETS1 transcript containing all eight exons, transcripts lacking exon IV, lacking exon VII, and lacking both exons IV and VII have been identified by PCR and hybridization analysis. Quantitative differences exist between different tissues and cell lines in terms of their degree of alternative splicing.

6. The human ETS2 gene is composed of ten exons, nine of which contribute to the open reading frame encoding 469 amino acids. The exons range in size from 72 (exon II) to 264 (exon VIII). The sizes of exons I and X are variable due to multiple transcription initiation sites and to multiple polyadenylation sites, respectively. Introns from 160 bp (intron 2) to 4.2 kb (intron 3) contribute to the size of the ETS2 gene, a locus of 25 kb. Thus, although the human ETS2 gene is smaller than the human ETS1 gene, it is composed of more exons.

7. Northern analysis of RNA reveals that the human ETS2 gene directs the synthesis of three unique transcripts of 4.7, 3.2 and 2.8 kb, the origin of which can be attributed to differential use of unique polyadenylation signals. These signals have been identified by sequence and by S1 nuclease protection analyses.

8. PCR amplification of ETS2 messages and Southern analysis reveals a single band unique to each primer combination (all within the predicted open reading frame). The presence of a single-sized amplified band indicates that differential splicing of the ETS2 gene does not occur. This observation is valid using cell lines of different origin [e.g., CEM (T-cell), P3HR-1 (B-cell), HeLa (cervical epithelium) and COLO 320 (neuroendocrine)]. Thus, the coding potential for each of the messages are identical.

9. Comparison of the structural organization of the human ETS1 and ETS2 genes reveals that regions that encode for identical or similar amino acids (domains A and C) have the same genomic organization (intron/exon junctions), while regions

that are divergent in protein coding sequence (domain B) have different gene organization. These similarities in structure provide some insight into the possible evolution of the ets-1 and ets-2 genes, suggesting that they were derived from each other. If they represented convergent evolution of independent genes, they probably would not have such a high degree of structural relatedness.

10. In addition to ETS1 and ETS2, other human ets-related genes have been identified (ERG, ELK1, ELK2). All of these genes map near translocation breakpoints characteristic of human malignancies. In addition, ETS2 and ERG map in the minimal region required to be triploid for manifestation of Down's syndrome. We have identified several additional human ETS-related genes by PCR, and several of these are in the process of being characterized.

Two of these (H11, L11) were isolated from a genomic library and appear to be ETS2 pseudogenes, as they contain DNA sequences nearly identical to ETS2 exons VI-X, but without any introns in this region. These genes have unique flanking sequences.

A more intriguing gene has been isolated from a cDNA library. The entire cDNA clone has been isolated and sequence analyses of several different clones have revealed several interesting properties of this gene. It has been designated ERGB because it appears to have sequence identity most closely related to the ERG gene, previously characterized in this laboratory. As revealed by comparison between other members of the ets family, the ERGB and ERG genes are most closely related in the C domain and less in the A domain. The ERGB gene transcription pattern is quite distinct from ERG, in that it is expressed in more cell lines and tissues. While the ERG gene is expressed primarily in the thymus, the ERGB gene is expressed in thymus, and PBL and RNA transcripts can be detected by Northern analyses of ovary, bone marrow, spleen and heart. Analyses of RNA isolated from human cell lines also shows a more restrictive pattern of expression by the ERG gene. While ERG is expressed in COLO320 and MOLT4 cells, the ERG gene is expressed in CEM, HL60, P3HR1 and MOLT4, with no RNA detected in COLO320.

Genomic clones homologous to ERGB have been isolated and we have determined that this gene is located on chromosome 11, the same chromosome as the ETS1 gene.

11. Viral ets fragments, and human ets exons and cDNA have been placed in appropriate systems for protein overexpression and these proteins have been utilized to elicit antibody response and to study DNA:protein interactions.

15. Genomic clones containing the mouse ets-2 gene have been isolated. Genomic organization, promoter function and comparison with the human ETS2 gene are being carried out. In collaboration with I. Kola, gene knock-out experiments using a neo/TK vector containing the last two exons are underway. Several hundred transfected ES cells have been selected and are being analyzed for homologous recombination into the endogenous ets-2 gene.

Publications:

Jorcyk CL, Watson DK, Mavrothalassitis GJ, Papas TS. The human ETS1 gene: genomic structure, promoter characterization and alternate splicing. Oncogene (In Press)

Jorcyk CL, Watson DK, Mavrothalassitis GJ, Papas TS. Regulation and processing of the human ETS1 gene. Miami Short Rep 1991;1:78.

Mavrothalassitis GJ, Watson DK, Papas TS. The human ETS-2 gene promoter: molecular dissection and nuclease hypersensitivity. Oncogene 1990;5:1337-42.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. In: Patterson D, Epstein CJ, eds. Molecular genetics of chromosome 21 and Down syndrome. New York: Wiley-Liss, 1990;137-68.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. Prog Clin Biol Res 1990;360:137-68.

Papas TS, Watson DK, Sacchi N, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Kottaridis SD, Ascione R. The ETS family of genes in leukemia and Down syndrome. Am J Med Genet 1990; 7(Suppl):251-61.

Pribyl LJ, Watson DK, Schulz RA, Papas TS. D-elg, a member of the Drosophila ets gene family: sequence, expression and evolutionary comparison. Oncogene (In Press)

Seth A, Hodge DR, Kottaridis SD, Thompson DM, Panayiotakis A, Watson DK, Papas TS. DNA binding activity of the ets proto-oncogene family proteins. Miami Short Rep 1991;1:83.

Watson DK, Mavrothalassitis GJ, Jorcyk CL, Smyth FE, Papas TS. Molecular organization and differential polyadenylation sites of the human ets-2 gene. Oncogene 1990;5:1521-7.

Watson DK, Papas TS. Oncogene file: ets-1 and ets-2. Cancer Cells 1990;2:32-3.

Watson DK, Pribyl LJ, Smyth FE, Ascione R, Papas TS. Members of the ETS gene family share a unique domain. Miami Short Rep 1991;1:85.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05295-10 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Activation of Oncogenes 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:	K. J. Dunn	Microbiologist	LMO	NCI
	X. Wang	Visiting Fellow	LMO	NCI
	N. Sacchi	Visiting Scientist	LMO	NCI

COOPERATING UNITS *(if any)* Basic Res. Prog., ABL, Frederick, MD (G. Vande Woude, N. Schultz); Lab. Cell. Biochem., PRI, Frederick, MD (D. Halverson); Recomb. DNA Lab., PRI, Frederick, MD (T. Wood); Fox Chase Cancer Ctr., Phila., PA (J. Testa)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Microbiology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.20	0.95	0.25

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 isolated an NIH3T3 fibroblast cell line which grows in defined media in the absence of protein growth factors. The cells appear to release soluble factors which are capable of inducing serum-free growth of normal NIH3T3 cells, as well as several normal human diploid fibroblasts. These spontaneously-selected 3T3 cell lines, which appear to arise at very low frequency in 3T3 cell lines, express a phenotype clearly distinguishable from that induced by the ets-1 and ets-2 genes and the myb-ets fusion oncogene, suggesting that multiple mechanisms are available for abrogation of serum dependence in 3T3 fibroblasts.

We have tested DNAs from human leukemias containing 4:11 and 8:21 translocations for transfectable sequences which can dominantly alter the morphology, tumorigenicity, or serum dependence of 3T3 fibroblasts. The majority of these DNAs contain no detectable activity in multiple assays, suggesting that these translocations do not generate dominant oncogenes detectable by DNA transfection.

We have generated a series of CHO cell lines carrying single human chromosomes which contain drug-selectable markers. Such cell lines will be useful as donors of specific chromosomes to determine the presence of genes capable of suppressing the transformed phenotype of established human tumor cells.

PROJECT DESCRIPTION

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

D. G. Blair	Supv. Research Chemist	LMO	NCI
K. J. Dunn	Microbiologist	LMO	NCI
X. Wang	Visiting Fellow	LMO	NCI
N. Sacchi	Visiting Scientist	LMO	NCI

Objectives:

To understand the mechanism of transformation by murine sarcoma viruses (MSV) and the function of specific viral and cellular gene products in this process.

To identify and isolate human DNA sequences which act as dominant initiators or dominant suppressors of cell transformation. To identify, isolate and characterize such sequences and their gene products. To characterize the normal cellular homologs of dominant oncogenes 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, microcell and somatic cell fusion mediated gene transfer immunoprecipitation and protein gel analysis to detect the expression of specific cellular proteins.

Major Findings:

1) NIH3T3 fibroblasts can be adapted for growth in defined media in the absence of protein growth factors, and media from these cells promote the growth of human diploid fibroblasts. We and others have utilized reduced serum and serum-free growth conditions to identify and characterize dominant oncogenes following transfection into NIH3T3 fibroblasts. We have observed in such assays normal 3T3 cells from colonies in defined serum-free media with a frequency of ~10^{-6}-10^{-7}. We have succeeded in establishing a cell line from one of these colonies which can be maintained in serum-free culture. The cells grow on tissue culture dishes, initially as flat monolayers, but tend to form round balls or spheroids as the cell density increases. Pre-treatment of plates with cell attachment factors reduces this tendency and increases growth.

Media from these cells (NIH3T3 SF) will complement normal NIH3T3 cells for growth in the absence of added bovine serum. Media from NIH3T3 SF also complements the growth of several human diploid fibroblast cell lines in serum-free media. The presence of growth factors in the media of NIH3T3 SF cells suggests that the mechanism of growth induction differs from that seen after the introduction of ets-containing plasmids into 3T3 fibroblasts since these cell lines, while they have acquired serum independent growth properties, release no factors into the culture media. The spontaneous NIH3T3 SF cell variant will be useful for comparison to serum independent cell lines induced by oncogenes such as ets, as well as providing a potential source of novel growth factors capable of promoting the serum-free growth of human cells.

2) Dominant oncogene sequences are detectable in a minority of human leukemia DNA samples containing 4:11 and 8:21 translocations. In order to examine the possibility that 4:11 and 8:21 translocations, which occur in a number of human childhood leukemias, might result in the activation of oncogene or growth factor-related genetic sequences, we analyzed DNAs from several leukemic samples containing well-characterized 4:11 translocations, as well as human hamster hybrid cell lines containing $21q^{+}$ and $8q^{-}$ portions of an 8:21 translocation by DNA transfection into NIH3T3 fibroblasts. DNA was retransfected with plasmids carrying drug-selectable markers and pools of drug-selected cells were analyzed in tissue culture for morphologically-altered foci, increased growth potential in low serum (0.1%) and serum-free conditions and tumorigenesis in nude mice. Although several transfections yielded cells with altered morphology or growth potential in the first transfection cycle, only two primary foci and tumors gave clearly distinguishable second cycle foci of morphologically-altered cells. The low frequency of transfection events which are stable through two cycles of transfection indicate that if dominant oncogenes are associated with 4:11 and 8:14 transfections, they are not detectable in either morphological, growth, or tumor induction assays in 3T3 fibroblasts.

3) Generation of a library of CHO cells carrying human chromosomes marked with a drug-resistant marker for use in the analysis of tumor suppressor activity. The detection of genetic sequences containing tumor suppressors has always been difficult in cell culture because of the difficulty in detecting a few morphological revertants in the population of transformed or tumorigenic cells. Using microcell-mediated gene transfer, we have transformed human chromosomes from normal human fibroblasts previously infected with defective retroviruses carrying a drug-selectable marker and generated a series of Chinese hamster cell lines resistant to the drug, G418, and carrying a small number of human chromosomes. The human chromosomes can be distinguished by banding techniques, and, initially, we have identified lines carrying human chromosomes 3, 8 and 10. These cell lines can be used as clones in microcell transfers into human mesothelioma cell lines and others to determine if specific human chromosomes will act as suppressors of the transformed phenotype of these types of human tumors.

Publications:

Lu Y, Blair DG. Stable oncogenic transformation induced by microcell-mediated gene transfer. Miami Short Rep 1991;1:80.

Lu Y, Blair DG, Segal S, Shih TY, Clanton DJ. Tumorigenicity, metastasis and suppression of MHC class-I expression in murine fibroblasts transformed by mutant v-ras deficient in GTP binding. Int J Cancer (In Press)

Testa JR, Park M, Blair DG, Kalbakji A, Arden K, Vande Woude GF. Analysis by pulsed field gel electrophoresis reveals complex rearrangements in two MET alleles in a chemically-treated human cell line, MNNG-HOS. Oncogene 1990;5:1565-71.

Van Beneden RJ, Henderson KW, Blair DG, Papas TS, Gardner HS. Oncogenes in hematopoietic and hepatic fish neoplasms. Cancer Res 1990;50(Suppl):5671s-4s.

Yew N, Oskarsson M, Daar I, Blair DG, Vande Woude GF. Mos gene transforming efficiencies correlate with oocyte maturation and cytostatic factor activities. Mol Cell Biol 1991;11:604-10.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05441-07 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of the Gene Products of the ETS Locus

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

PI:	T. S. Papas	Chief	LMO	NCI
Others:	L. F. Fleischman	IRTA Fellow	LMO	NCI

COOPERATING UNITS *(if any)*
Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (R. J. Fisher, N. K. Bhat, K. Midelfort)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.60	0.60	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 human ETS1 proto-oncogene proteins have been isolated from the T-cell line, CEM, by immunoaffinity chromatography and their identity confirmed by N-terminal amino acid sequencing. The p51 and p42 ETS1 isoforms react with monoclonal antibodies directed against a bacterially-expressed ETS1 protein and to an oligopeptide directed to the carboxyl-terminal 13 amino acids of the human ETS1 protein. The p42 human ETS1 does not react with an antibody directed to exon VII of the human ETS1, indicating that it is the product of alternatively-spliced mRNA lacking exon VII. The p48 and the p39 isoforms of the human ETS1 are shown to be derived from the p51 and p42 isoforms of the human ETS1 by the covalent modification of -SH groups by the protease inhibitor N_{α}-p-tosyl-L-lysine chloromethyl ketone (TLCK). The renatured human ETS1 was shown to have DNA sequence-specific binding to the PEA-3 (AGGAAGT) motif; this complex can be observed by electrophoretic mobility shift assays (EMSA). The purified ETS1 retards a complex which is exactly the same size complex as is retarded from nuclear extracts prepared from the T-cell leukemia cell line, CEM. Reduced ETS1 is required to form the ETS1•PEA-3 complex, but modification of the ETS1 -SH groups by N-ethylmaleiamide or by TLCK does not inhibit formation of the ETS1•PEA-3 complex. The ETS1•PEA-3 complex formed with TLCK-modified ETS1 has a slower mobility than does the complex formed with unmodified ETS1.

Six monoclonal antibodies were prepared from mice immunized with a bacterially-expressed human ETS2 protein. These antibodies specifically recognize the two human ETS2-encoded proteins, p56 and p54, but failed to react with chicken, mouse, rat, bovine, or monkey proteins, suggesting that the antibodies recognize epitopes specific to the human ETS2 protein. The biochemical analysis of the ETS2 protein will be facilitated by the development of these monoclonal antibodies, which may be useful as both domain-specific probes and tools for specifically detecting the human ETS2 protein in heterologous expression systems.

PROJECT DESCRIPTION

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

T. S. Papas	Chief	LMO NCI
L. Fleischman	IRTA Fellow	LMO NCI

Objectives:

The objective of this is to characterize the protein products of the human proto-oncogenes. The characterization includes identification, subcellular localization, and determination of function for the normal gene products.

Methods Employed:

Biological Materials. Cell lines are used for enriched sources for the ETS and ETS-related proteins, including the human adenocarcinoma line, Colo 320, the human T-cell leukemia line, CEM, and the Burkitt's lymphoma line, Daudi. The Colo 320 cells are a rich source of MYC proteins, ETS2 and ERG proteins. Daudi cells are a source of ETS1, and CEM cells produce both ETS1 and ETS2. The human T-cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml of streptomycin. Normal tissues, such as mouse thymus or calf thymus, are used as an enriched source of c-ETS proteins.

Oligonucleotides. The oligonucleotides below were synthesized on an automated Applied Biosystems Synthesizer Type 300 and were obtained from a commercial source. They were purified by gel electrophoresis or by HPLC according to the vendors' protocols. The oligonucleotides were dissolved in 10mM Tris-Cl, 0.5mM EDTA, pH 7.4 (TE buffer) and concentrations were determined by UV spectroscopy. Equimolar amounts of two complementary oligonucleotides were mixed, heated to 100° for 5 minutes, and allowed to slowly (6 hours) cool to room temperature. The annealed PEA-3 probe was end-labelled by the Klenow procedure and used without any further purification.

1.	5'-GATCTCGAGCAGGAAGTTCGA-3'	
2.	3'-AGCTCGTCCTTCAAGCTCTAG-5'	PEA3
3.	5'-GATCTCGAGCAAGAAGTTCGA-3'	
4.	3'-AGCTCGTTCTTCAAGCTCTAG-5'	PEA3M
5.	5'-GATCGGGAGGCGTGGCCTGGGCGGGACT-3'	
6.	3'-CCCTCCGCACCGGACCCGCCCTGACTAG-5'	SP1

Antibodies. MAbs specific for the ETS1 protein were developed as described below. The expression vector, pTSP-8, was constructed by inserting the PstI fragment (amino acids 43-266) from the v-ets gene at the PstI site of pAJH-9. pTSP-8 was expressed in Escherichia coli and purified protein (TSP-8) was injected intraperitoneally into BALB/c mice. The anti-L13E (αL13E) has been prepared against a synthetic oligopeptide derived from the predicted amino acids 429-441 (LHAMLDVKPDADE), corresponding to the carboxyl-terminal 13 amino acids from exon IX of the human ETS1 gene. After the mice had been immunized several times, spleen cells were fused with NS-1 mouse myeloma cells using polyethylene glycol. The hybridomas were screened by enzyme-linked immunosorbent assay.

A single clone was isolated after two subclonings by limiting dilution. The ETS1 MAb and the αL13E MAb were purified from culture supernatant by protein A-Sepharose affinity column chromatography and were characterized as IgG1 antibodies, kappa subclass. The anti-Y12K (αY12K) antibody has been prepared against a synthetic oligopeptide derived from the predicted amino acids 307-318 (YVRDRADLNKDK), corresponding to exon VII of the human ETS1 gene. These antibodies were prepared in rabbits and purified by antigen affinity chromatography.

Electrophoretic Mobility Shift Assay. DNA sequence specific binding by nuclear extracts from CEM cells or by purified renatured ETS1 protein was assessed by electrophoretic mobility shift assay (EMSA). The nuclear extracts or purified ETS1 were incubated with ^{32}P-labelled PEA-3 probe for 60 minutes at room temperature in 4% glycerol, 1 mM EDTA, 5.0 mM DTT, 10 mM tris-Cl, pH 7.5, and 1.0 mg/ml BSA. Experiments using CEM nuclear extracts had 1300 ng/20 μl poly dI-dC and experiments using purified ETS1 had 270 ng/20 μl poly dI-dC as non-specific competitor to reduce the background signal in the lanes. After incubation, 20-60 μl were loaded onto 4.5% or 6% native polyacrylamide gels. These gels had 0.25X TBE (89 mM Tris, 89 mM boric acid and 1 mM EDTA), 5% glycerol, 50 μl/50 ml TEMED, and 0.7 ml/50 ml of 5% ammonium persulfate and the electrode buffer was 0.25X TBE. The EMSA gels were prerun for 30 minutes at 250 mV and then 20 μl to 60 μl of the sample was added and electrophoresis was continued for an additional 1.5 hours. The EMSA gel was then dried for 30 minutes and exposed to X-ray film for an appropriate length of time.

ETS1 Purification. The cellular ETS1 was purified by immunoaffinity chromatography by two different techniques, depending upon the end use of the ETS1.

Method 1: The ETS1 used for N-terminal amino acid sequencing was purified from 1.2 X 10^{10} CEM cells. Forty ml of packed CEM cells were extracted with 1000 ml of RIPA buffer [50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), 1.0 mM EDTA, 0.1 mM EGTA (ethylene glycol-bis-[β-aminoethyl ether]-N,N,N′N′-tetraacetic acid), 0.4 mM phenylmethylsulfonyl fluoride, 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone, 0.01 mM N-α-β-tosyl-L-lysine chloromethyl ketone, and 10 μg/ml aprotinin], and the crude extract was centrifuged at 165,000 g for 18 hours. The supernatant fluid (1.5 g protein) was passed through a 0.22-micron filter, diluted to 2000 ml with low salt HTP buffer (10 mM sodium phosphate, pH 6.8, 1% Triton-X-100, 0.5% DOC, 0.1% SDS, and 20 μM $CaCl_2$), and loaded onto an open column containing 100 g of hydroxylapatite (Biogel HTP). This column was washed with 500 ml of low salt HTP buffer, and the ETS1-enriched fractions were eluted with 500 ml of HTP buffer containing 200 mM sodium phosphate (300 ml collected between 150 and 450 ml contain essentially all of the ETS1 isoforms). The ETS1-enriched HTP batch fraction was incubated with 1.5 g of protein A-Sepharose 4B to which 50 mg of an ETS1 MAb (3) had been attached by cross-linking with dimethyl-pimelimidate (5). After 18 hours of gentle mixing at 0°C, the immunomatrix was made into a column and washed with 50 ml of RIPA buffer, 50 ml of RIPA buffer containing 500 mM NaCl, and 50 ml of H_2O. The ETS1 isoforms were then eluted with successive additions of 50 mM triethylamine, pH 11.5. The column fractions (1.0 ml) containing the ETS1 were taken to dryness in a SPEED-VAC and stored at -20°C until used.

Method 2: Ets1 used for DNA binding was prepared from 10 g CEM cells which were lysed in 24 ml of RIPA buffer and incubated at 4°C for 5 minutes before centrifugation at 100,000 g for 30 minutes and the equivalent 0.3 ml of packed ETS1 agarose beads are added. A stock suspension of 7.5 ml of packed ETS1 agarose

beads was made to 50 ml in TBS, plus 0.1% NaN_3, and two ml of this mixture is used per 10 g of CEM cells. The extract is incubated at 4°C for 18 hours and then is centrifuged at 3000 g to collect the ETS1 agarose beads. The beads are washed 3 times with 10 ml of RIPA buffer, transferred to a 1.5 ml microcentrifuge tube and washed twice with 1.0 ml of PBS. The ETS1 is released from the agarose by incubation twice with 300 μl of 6M guanidine-HCl containing 0.1% β-mercaptoethanol. After removal of the agarose beads by centrifugation, the two 6M guanidine supernatants are combined and dialyzed against 200 ml of 0.1M KCl nuclear extraction buffer containing 0.1% aprotinin and 0.1% NaN_3 for 18 hours. The buffer is changed one time and then the renatured ETS1 is distributed in small quantities for storage at -70°C. ETS1 stored in this way remained active for up to six months without loss of sequence-specific DNA binding activity. Comparison of the total amount of ETS1 estimated by Western blot analysis with the amount of ETS1 found in a PEA-3 retarded complex allows calculation of active ETS1 which we found to be about 1% in these preparations. Part of the reason for this low activity is due to the presence of aggregated ETS1 which is inactive for PEA-3 binding.

Zone Sedimentation of Renatured ETS1. The ETS1 monomers were separated from the ETS1 oligomers by zone sedimentation through a 10%-30% linear gradient of glycerol containing 20 mM Tris-Cl, pH 7.5, 5.0 mM DTT, 1 mM EDTA and 0.1 M KCl. Up to 150 μl of the renatured ETS1 was loaded onto the 4.8 ml glycerol gradients and centrifugation at 150,000 g was done at 4°C for 42 hours in a SW50 Beckman swinging bucket rotor. The gradients were unloaded from the top, collecting about 34 fractions of 150 μl each. In some experiments ovalbumin and bovine serum albumin were used as internal standards to mark 3.5S and 4.2S positions in the gradient. The fractions corresponding to the PEA-3 binding activity were pooled and stored in small fractions at -70°C until needed.

N-Terminal Amino Acid Sequence. The human ETS1 isoform proteins (p51, p48, p41, p39) were separated by SDS-PAGE (10% polyacrylamide, Novex) and electroblotted onto PVDF membranes. The Coomassie blue-stained bands were excised from the membrane and subjected to amino-terminal sequence analysis using an Applied Biosystems model 470A protein sequencer equipped with a model 120A PTH analyzer and a 900A control/data analysis module. The membrane containing the blotted protein was cut into small pieces (2 x 6 mm) and placed on top of a polybrene-conditioned, glass fiber filter; Edman degradation was performed for 25 cycles using the standard RUN470-L program. A readable sequence was obtained for 15 cycles, and the initial yield was calculated by extrapolating the regression line of these cycles to the 0 cycle.

Amino Acid Analysis. The PVDF membrane containing the protein was cut into small pieces (2 x 6 mm), placed in the bottom of a pyrolyzed pyrex tube (6 x 50 mm), and hydrolyzed in the gas phase with 0.3 ml of 6 N HCl containing 4% thioglycolic acid and 0.5% phenol at 120°C for 24 hours using a Waters Pico-Tag Workstation apparatus. Amino acids were extracted from the membrane using 0.1 N HCl/30% methanol (3 x 200 μl). The extracts were transferred to clean Eppendorf polypropylene tubes and evaporated to dryness in a Speed-Vac concentrator. Amino acid compositions were determined with a model 6300 Beckman high-performance amino acid analyzer equipped with a single ion-exchange column and a dual channel data system.

Protein Sequence Analysis. The ASCL VAX and CRAY supercomputer facility is used to analyze amino sequences of the ETS and ETS-related proteins.

Major Findings:

Characterization of the ETS1 protein. Our new monoclonal antibody to ETS1 identified several more nuclear acidic proteins distinct from ETS2. These proteins are more abundant than the ETS2 proteins and show both nuclear and cytoplasmic localization. Pulse-chase experiments show that there is no precursor product relationship between the non-phosphorylated forms of the ETS1 proteins. We have found a p51 predominantly cytoplasmic protein which can be phosphorylated to a pp52 protein. This protein corresponds to the ETS1 protein previously identified in our laboratory using an antipeptide antibody specific for ETS1. Additionally, a nuclear p48 which can be phosphorylated to a pp49 and two non-phosphorylated p42 and p39 nuclear proteins were found. Peptide mapping of these proteins indicated that they are all related but that the p42 and p39 lacked several peptides shown to be phosphorylated in the p48 and p51 proteins. Examination of purified ETS1 proteins with exon-specific antipeptide antibodies demonstrated that, while the p51 and p48 reacted with all of the ETS1 exon-specific antibodies, the p39 and p42 did not react with an ETS1 exon 7-specific antibody. This data suggested to us that the p42 and p39 ETS1 proteins may have come from alternatively-spliced mRNA which are lacking exon 7. Examination of the exon 7 amino acid sequence reveals a putative calcium-dependent protein kinase phosphorylation site (RXXS/T). These results are consistent with the calcium-dependent phosphorylation of the ETS proteins and suggest that the p39 and p42 are not subject to the same regulation as the p48 and p51.

Thus far, the identification of the ETS1 isoforms has relied primarily on immunological methods because of the technical problems associated with the structural analysis of proteins present at only low levels in cells. Recent advances in direct amino acid sequencing of proteins first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then electroblotted onto polyvinylidene difluoride (PVDF) membranes have increased the sensitivity to the picomole level. Using our ETS1 MAb to purify picomole quantities of the ETS1 isoforms, in combination with gas-phase amino acid sequencing, we were able to determine the sequence of the N-terminal amino acids of all four isoforms of ETS1 isolated from the human T-cell leukemia line, CEM.

The ETS1 isoforms were extracted with RIPA buffer from 1.2×10^{10} CEM cells and fractionated by hydroxylapatite chromatography and ETS1 MAb immunoaffinity chromatography. The ETS1 proteins were eluted from the immunoaffinity matrix with 50 mM triethylamine and these fractions were further characterized by immunoblot analysis. As expected, all of the four isoforms of ETS1 (p51, p48, p42, and p39) react with the ETS1 MAb used to isolate the proteins. Some lower molecular weight proteins that also reacted with this antibody were evidently minor contaminants, because they are not visible on the stained gels and they do not react with the pan-ETS antibody, αF13W. The ETS1 isoforms do not react with an ETS2 MAb or with anti-mouse IgG. The ETS1 isoform found at p42 sometimes resolves into two bands, and one of these bands reacts with anti-actin. We do not know whether the presence of the actin in our ETS1 isoforms is fortuitous, but we have often noted that actin contaminates immunoprecipitates from cultured cells.

A major difficulty in the analysis of the ETS1 isoforms is their limited abundance in cells and tissues. Direct amino acid analysis of the electroblotted p51 hydrolyzed *in situ* tells us that one lane of p51 contains approximately 0.3 μg or 6.3 pmol of protein. The total p51 recovered is about 1.5 μg or 31.5 pmol protein. This was sufficient protein for determining the N-terminal amino acid sequence from p51, as well as from the p48, p42, and p39. The human ETS1 isoforms

isolated by immunoaffinity chromatography were separated by SDS-PAGE (10% polyacrylamide gels) and electroblotted onto PVDF membranes, as described previously. Coomassie blue-stained bands were excised from the membrane and subjected to amino-terminal sequence analysis. We were able to determine the initial 15 amino acids, which correspond exactly to the first 15 amino acids of the amino acid sequence derived from the human ETS1 cDNA. The initial yield corresponded to 3.6 pmol and the repetitive yield was 95%. The p48, p42, and p39 isoforms of ETS1 were also sequenced and yielded results the same as those obtained in our studies with p51 (data not shown). Thus, all of the ETS1 isoforms have the same N-terminal amino acid sequence. This rules out the possibility that N-terminal processing explains the size difference between the p48 and p51 isoforms and the p39 and p42 isoforms. We think that this size difference may be due to some post-translational modification, such as glycosylation or proteolytic modification, at the carboxyl termini of the isoforms. Studies are now underway to examine these possibilities. Phosphorylation can be ruled out as a mechanism, because we have already shown that the p39 and p42 isoforms are not phosphorylated.

The human ETS1 proto-oncogene protein was isolated by immunoaffinity chromatography and four isoforms of the ETS1 were obtained. Our previous work indicated that no precursor-product existed between the isoforms and that the p42 and the p39 were products of alternatively spliced mRNA which lacked exon VII. It was possible that the differences in the Mr observed between the p51 and p48 or the p42 and the p39 were due to differences at either the N-termini or C-termini of these isoforms. The N-terminal amino acid sequencing was sufficient to show that the p51, p48, p42 and p39 all began at the ATG defined in exon A for the human ETS1. Amino acid analysis of the isolated p51 was consistent with the theoretical amino acids for the ETS1 p51 open reading frame and also with the relatively low abundance of the ETS1 protein (about 1 μg of isoforms/gram weight wet CEM cells).

Because of the difficulties of C-terminal amino acid sequencing, we instead chose to prepare an antibody against the unique 13 amino acids (LHAMLDVKPADADE) predicted to be found at the carboxyl-terminus of the human ETS1 protein. This MAb reacted with all of the ETS1 isoforms, demonstrating that these proteins are intact at the carboxyl terminus. Thus, the human cellular ETS1 proteins isolated by immunoaffinity techniques were complete both at the N-terminal and at the C-terminal.

The p48 and the p39 ETS1 isoforms were generated by covalent modification of -SH groups by the protease inhibitor, TLCK. It has been observed by others that TLCK can alter the electrophoretic mobility of some viral proteins (either increasing or decreasing the mobility in SDS-PAGE) by some undefined mechanism unrelated to the ability of TLCK to inhibit proteases. The chloromethyl ketones from lysine and phenylalanine (TLCK and TPCK) were originally used to label the histidine group at the active sites of trypsin and chymotrypsin. These compounds, as well as antipain and leupeptin, were used to interfere with complex biological processes, such as tumorigenesis, growth and fertilization, inferring that proteases were important in these processes. In addition, TLCK and TPCK can also covalently modify -SH groups and their ability to inhibit SH-dependent proteases, such as ficin or papain, because of this property. TLCK, a water soluble compound, was useful in reversing the transformed phenotype in certain cell lines, but because of the pleiotropic effects of this compound, it is not clear how TLCK is exerting its effect. It is very interesting that TLCK can modify the trans-acting factor, ETS1, because it demonstrates that some of the pleiotropic effects of TLCK could come from directly modifying proteins involved in genetic reprogramming.

We make use of the observation that TLCK alters the mobility of the p51 and p42 ETS1 isoforms to determine conformational properties of the ETS1 isoforms in cells by differential modification of SH groups. The ETS1-enriched nuclear extracts from CEM cells were treated with TLCK and then copper orthrophenanthroline to oxidize remaining SH groups in the ETS1 to corresponding disulfides. The ETS1 in these extracts was examined by SDS-PAGE in the presence or absence of β-mercaptoethanol. The TLCK reacted with half of the ETS1 present in these extracts, indicating that this population of ETS1 had an SH group(s) which was modifiable. However, the ETS1 not modified in this nuclear extract had SH groups which were oxidized to disulfide or were protected from the TLCK modification. Reduction of the extracts allowed the protected ETS1 to be visualized. Thus, the TLCK modification of ETS1 helps to define two populations of ETS1 in CEM cells. The reduced form of ETS1 reacts with TLCK and the oxidized form of the ETS1 does not. Among the ETS proteins, only the ETS1 isoforms exhibit altered mobility on SDS-PAGE after TLCK treatment and, thus, the ETS2 proteins are insensitve to TLCK modification. Inspection of the ETS1-derived amino acid sequence for unique cysteines which are not in common with the ETS2 derived amino acid sequence and are not in exon VII reveal that cysteines at positions 99, 112 and 169 are the likely candidates for the TLCK covalent modification. The location of these cysteine groups is outside of the putative DNA binding domain, so it was unlikely that their modification would interfere with specific DNA binding. We could show that modification of ETS1 with TLCK or NEM had no effect on the specific binding of PEA-3 to ETS1. Oxidation of the ETS1 causes it to oligomerize and, thus, no specific DNA binding is observed when the ETS1 has been oxidized. The same SH groups which are involved in TLCK binding are involved in the oxidation reactions because TLCK modified ETS1 does not oligomerize. It is possible that this region is important for interaction with other proteins or for transactivation. These results provide the first direct evidence for the proteins related to the cellular homologues of the v-ETS oncogene. We have shown that all four ETS1 isoforms begin at the initiator methionine that was defined from the ETS1 cDNA cloned and sequenced in our laboratory.

Characterization of the ETS2 protein. We have shown that the nuclear ETS2 protein is phosphorylated, has a 20-minute half-life, is an acidic protein, and responds to the mitogenic activation of the protein kinase C by increasing its half-life from 20 minutes to greater than 2 hours. Stimulation of the T-cell antigen receptor by antibodies to the antigen receptor or with calcium ionophore allowed a rapid phosphorylation of the ETS2 protein. Thus, ETS2 responds to two cellular signalling systems, one by a calcium dependent phosphorylation, and the other by increasing the amount of ETS2 by a post-translational mechanism. The interaction of the mitogenic signal transduction pathway suggests that the ETS2 protein is a nuclear regulatory protein with properties similar to those of fos, myc, myb, and p53.

Antibodies. We have isolated six mouse hybridoma clones producing MAbs against the human ETS2 protein. The results of immunopeptide mapping indicate that these MAbs recognize at least three distinct epitopes on the ETS2 protein. Based on these results, we divided the six MAbs into three different groups: Group a, O-55, P-78, and U-244; group b, O-137 and T-7; and group c, P-96. The MAbs within each group detect an epitope(s) located on the same small peptide of several thousand daltons generated by the staphylococcal V8 protease. However, it is not clear if the MAbs within each group react with an identical epitope or recognize different, but closely spaced, epitopes on the same peptide fragment. Competition analysis will be required to address this question. Isolation and amino acid sequencing of the peptide fragments detected by each MAb will show the exact locations of the epitopes in the primary structure of the human ETS2 protein.

These MAbs appear to be specific to the human ETS2 protein. They did not react with the proteins from chicken, mouse, rat, cow, or monkey cells, suggesting that their epitopes reside on less conserved regions of the ETS2 protein. Since the MAbs were isolated from mice immunized with the human ETS2 protein, it is reasonable to expect that the most significant antibody responses were directed to the domain where the mouse and human ETS2 proteins are highly diverged. A comparison of the deduced amino acid sequences of the mouse and human ETS2 proteins showed that the middle portion of the protein (domain B) contains most of the amino acid divergence, whereas the carboxy terminal C domain is completely conserved. The ETS2 sequence in the p35 protein, which was used as an immunogen in this study, starts with ^{190}Trp and consists basically of the B and C domains. We therefore suggest that the MAbs prepared in this study are directed to the epitopes located in the B domain of the ETS2 protein. This hypothesis is consistent with the results of immunopeptide mapping, which indicated that the epitopes detected by the MAbs are located upstream of the F-13-W sequence. Immunoprecipitation studies comparing native and denaturing conditions indicated that the MAb epitopes in the B domain are not exposed in a relatively native condition with low concentrations of salt and non-ionic detergents. The high level of background reactivity observed in immunoprecipitation in this condition is consistent with the hypothesis that some of these background proteins are associated with the ETS2 protein and are masking the epitopes.

Our previous work showed that the ETS2 protein has an extremely short (20 minutes) half-life and can be stabilized quickly by activating protein kinase C. The ETS2 protein level rises rapidly after protein kinase C activation with no requirement for protein synthesis. These findings suggest that the cellular level of the protein is regulated very precisely, and this is of particular interest in view of the intriguing localization (21q22.3) of the human ETS2 gene to the small chromosomal region implicated in Down's syndrome. Partial trisomy of this region gives rise to a full spectrum of Down's syndrome manifestations. A gene dosage effect on the ETS2 locus conferred by the trisomy 21 may thus significantly disturb the regulation of the level of the ETS2 protein, and this impaired regulation of the ETS2 expression might have some role in the pathogenesis of Down's syndrome at molecular levels. A careful comparison of ETS2 protein levels in normal cells and cells carrying trisomy 21 will be the first step in testing this hypothesis. The MAbs against the ETS2 protein will provide a tool for quantitating this protein. Finally, the specific reactivity of the MAbs with the human ETS2 protein will provide a unique advantage of detecting only the human protein in heterologous expression experiments such as transfection of the mouse NIH3T3 cells.

Quantitation of the ets proteins. The rapid turnover of the ETS2 protein allowed us to quantitate the ETS2 protein by metabolic labelling with S^{35}-methionine. Quantitative immunoprecipitations were carried out and we found that there were about 5000 molecules of ETS2 per CEM cell. We are now able to use Western blotting methods for quantification of the ETS1 and ETS2 in CEM and Jurkat cells. As expected, the level of ETS2 was about 5000 molecules per cell (CEM) and the level of ETS1 was about 27,000 molecules per cell.

Publications:

Bhat NK, Willette-Brown J, Garrett LJ, Thompson C, Lindsten T, Leiden JM, Tan TH, Fisher RJ, Papas TS. Role of ETS1 proto-oncogene products in lymphoid and non-lymphoid cells. Miami Short Rep 1991;1:74.

Fisher RJ, Koizumi S, Kondoh A, Bhat NK, Papas TS. Modification of the T-cell trans-acting factor ETS1 by the tumor inhibitor 1-Chloro-3-Tosylamido-7-Amino-L-2-Heptanone (TLCK). Miami Short Rep 1991;1:76.

Fujiwara S, Koizumi S, Fisher RJ, Bhat NK, Papas TS. Monoclonal antibodies specific to human ETS-2 oncoprotein: recognition of epitopes clustered on the B domain. Hybridoma 1990;9:559-71.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05443-07 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
C-ets Gene Expression During Cell Proliferation and Differentiation

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

PI:	T. S. Papas	Chief	LMO NCI
Others:	H. Suzuki	Visiting Fellow	LMO NCI
	V. Romano-Spica	Visiting Fellow	LMO NCI
	R. Ascione	Research Chemist	LMO NCI
	H. Young	Microbiologist	BRMP NCI

COOPERATING UNITS *(if any)* LCB, PRI, Frederick, MD (N. Bhat, R. Fisher, K. Midelfort); BCDP, PRI, Frederick, MD (K. Komschlies, T-H. Tan); HHMS, U. Mich., Ann Arbor, MI (C. Thompson, T. Lindsten, J. Leiden); Naval Med. Inst., Bethesda, MD (C. June)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.55	0.55	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 ETS1 gene expression co-related well with the T-cell receptor (TCR) α gene expression during thymic development, in different thymic and T-cell subsets and during T-cell activation, indicating that ETS1 may regulate TCR α genes. The expression of the TCR α gene is regulated by a T-cell-specific enhancer located downstream of the Cα locus. One of the enhancer elements (Tα2) was used for screening the λgt11 expression library made from Jurkat T-cell mRNA to identify the cDNA that programs the expression of the Tα2 binding proteins. One clone, upon DNA sequencing, is found to code for the entire ETS1 protein. The βgal-ETS1 fusion protein produced in E. coli binds to the Tα2 motif. Mutation in the Tα2 abolishes ETS1 binding and T-cell enhancer function, indicating ETS1 nuclear protein binds to DNA in a sequence-specific manner. Comparison of DNA seqeunces bound by ETS1 and subsequent site-specific mutational analyses indicate that centrally-located, purine-rich sequences (ETS Responsive Element) are involved in binding. Many nuclear proteins bind to ERE. Different size ERE protein complexes are seen in T and other hematopoietic and non-hematopoietic cells. In T-cells, three different complexes are seen with ERE. Complex I contains ETS1, and complex III contains an additional ETS-related protein. Since ETS1 expression and regulation are different in T and other cell types, characterization of these transacting factors are in progress. Jurkat cells have been transfected with ETS1 expression vectors in both sense and antisense orientations. Several clones have been obtained expressing antisense ETS1 mRNA, which blocks ETS1 protein formation. Characterization of these clones with respect to their growth property, mitogenic requirements and other target candidate gene expression are under investigation. The ERG gene encodes a nuclear phosphoprotein. Characterization of hematopoietic stem cells expressing the ERG gene is in progress.

PROJECT DESCRIPTION

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

T. S. Papas	Chief	LMO NCI
H. Suzuki	Visiting Fellow	LMO NCI
V. Romano-Spica	Visiting Fellow	LMO NCI
R. Ascione	Research Chemist	LMO NCI
H. Young	Microbiologist	BRMP NCI

Objectives:

To determine the role of c-ets gene products in cell proliferation and differentiation and to understand the molecular mechanisms involved in the regulation of their expression in different cell types. To identify the "target DNA sequences" for c-ets gene products in different cells. To use probes of the c-ets genes as potential diagnostic markers for a particular type of leukemia.

Methods Employed:

1. Isolation of nucleic acids, and RNA and DNA blot transfer analysis. Isolation of high molecular weight DNA, total poly A+ RNA, nucleic acid fractionation on agarose gels, transfer of nucleic acids to membranes, probe preparation, and hybridization washing of filters were done as described by Sambrook, Fritsch and Maniatis (Molecular Cloning, A Laboratory Manual, 1989).

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 in the Molecular Cloning Manual. Appropriate viral and cellular ets DNA fragments were subcloned in Gemini vectors to get a higher yield of plasmids and to prepare labeled riboprobes. Human ETS1 and m-ets-2 cDNA were cloned in PCMD vector systems in both orientations.

3. Preparation of ETS1 cDNA expression plasmids and transfection into Jurkat T-cell lines. ETS1 cDNA coding for 51 kD protein was cloned into HindIII sites of the PCDM8 vector using linkers. Expression of the ETS1 protein was checked by transcribing RNA and translating in vitro in the rabbit reticulocyte lysate system. ETS1 cDNA expression plasmid DNA was cotransfected with pSV2neo DNA into Jurkat cells by electroporation. Neomycin-resistant colonies were selected, subcloned and expanded for further analyses.

4. Cell labeling, immunoprecipitation and protein blot analyses. These experiments were carried out as described (Fujiwara et al., Mol Cell Biol 1988;8:4700-6).

5. Preparation of nuclear extracts and identification and characterization of DNA binding proteins. Nuclear extracts were prepared as described by Dignam et al. (Nucleic Acids Res. 1983;11:1475-89). Proteins were estimated by micro-BCA assay using a kit obtained from Pierce Chemicals. Ten micrograms of nuclear extracts were used for DNA binding studies. Oligonucleotides were annealed and end-labeled with ^{32}P-GTP using Klenow enzyme. One nanogram of DNA was used for protein-binding studies. The protein DNA complexes were separated by electrophoretic mobility shift assay as described in Current Protocols in Molecular Biology (Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds., 1987).

In competition experiments, different amounts of annealed double-stranded oligonucleotides were incubated with the nuclear extracts before addition of the probes.

Major Findings:

1. T-cell receptor (TCR) α gene is one of the target genes for ETS1. During murine thymic development, expression of ETS1 and TCR α begins two days before birth. Their expression co-relates well in different thymic and T-cell subsets. Both genes are expressed at high levels in resting T-cells rather than in activated T-cells. ETS1, a nuclear protein, may regulate the transcription of the TCR α gene. TCR α gene expression is regulated by an enhancer located downstream of the Cα gene segment. The core enhancer contains two nuclear protein binding sites, Tα1 and Tα2, which are essential for full enhancer activity. The cyclic adenosine monophosphate response element binding proteins binds to the Tα1 site. To identify proteins binding to the Tα2 site, a λgt11 Jurkat cell cDNA expression library was screened and positive clones were further analyzed. One clone expressing ETS1 β-galactosidase fusion protein bound to Tα2 specifically, and contained the entire ETS1 protein. ETS1 protein binds at the 3′ end of the Tα2 enhancer segment. Mutations in the ETS1 binding site abolish both binding and the enhancer activity in T-cells, indicating proteins binding to the Tα2 site are important for the regulation of the TCR α gene.

2. Comparison of DNA sequences bound by ETS1 proteins (ETS Responsive Elements) and subsequent mutational analyses indicate that centrally-located, purine-rich sequences (5′AGGAAGT3′ or 5′CGGAAGC3′) are important for binding. Substitution of the guanine residues with adenine or cystosine residues abolishes the binding of ETS1.

3. The search for the ERE in the promoter regions of other genes indicate that many genes expressed during T-cell activation (e.g., lymphokines, FOS, ETS2, and other genes) contain the site, indicating that the ETS1 may act as a repressor for some of these genes in T-cells.

4. Different transacting factors bind to ERE in T and other cell types. In T-cells, three protein-ERE complexes are observed on electrophoretic mobility shift assays. Complex I contains ETS1 proteins, and its formation co-relates well with the expression of the ETS1 gene. Complex III is expressed in most cell types, and its formation is not co-related well with the known expression pattern, ETS1, ETS2, ERG, ELK and PU.1/spi1 gene. In T-cells, complexes II and III may contain another ETS-related protein. Its complex formation is inhibited by pan-ets antibodies, but not by ETS1 and ETS2 antibodies. Pan-ets antibodies are raised against an oligopeptide adjacent to DNA binding domains of ETS1 which is highly conserved among many ETS-related proteins. In the presence of pan-ets antibodies, new faster migrating ERE-protein complexes appear, indicating that complex III contains heterodimers. In B, erythroid, myeloid and other non-hematopoietic cells, additional complexes are seen. These results suggest that different transacting factors interacting with ERE may modulate ETS1 function differently in T versus non-T cells. Characterization of these complexes is in progress.

5. ERG-specific antisera have been prepared using purified ERG proteins expressed in bacteria. In KG1 cells, the ERG gene encodes a 55 kD nuclear phosphoprotein.

6. Permanent Jurkat cell lines expressing antisense ETS1 have been made. Characterization of these cell lines are in progress.

Publications:

Bhat NK, Patel R, Showalter SD, Komschlies K, Bristol L, Watson DK, Fujiwara S, Koizumi S, Fisher R, Papas TS. Characterization and regulation of ets gene products in murine thymocytes and T-cells. In: Streilein JW, Ahmad F, Bialy H, Black S, Blomberg B, Chin YH, Lopez D, Malek T, Podack ER, Rabin MB, Stein-Streilein J, Van Brunt J, Whelan WJ, eds. Advances in gene technology: the molecular biology of immune diseases and the immune response. Oxford: IRL Press, 1990;149.

Bhat NK, Willette-Brown J, Garrett LJ, Thompson C, Lindsten T, Leiden JM, Tan TH, Fisher RJ, Papas TS. Role of ETS1 proto-oncogene products in lymphoid and non-lymphoid cells. Miami Short Rep 1991;1:74.

Bristol LA, Smith MR, Bhat NK, Durum SK. IL-1 induces ornithine decarboxylase in normal T-lymphocytes. J Immunol 1991;146:1509-15.

Fisher RJ, Koizumi S, Kondoh A, Bhat NK, Papas, TS. Modification of the T-cell transacting factor ETS1 by the tumor inhibitor 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK). Miami Short Rep 1991;1:76.

Fujiwara S, Koizumi S, Fisher RJ, Bhat NK, Papas TS. Monoclonal antibodies specific to human ETS2 oncoprotein: recognition of epitopes clustered on the B domain. Hybridoma 1990;9:559-71.

Ho IC, Bhat NK, Gottschalk LR, Lindsten T, Thompson CB, Papas TS, Leiden JM. Sequence specific binding of human ETS1 to the T-cell receptor alpha gene enhancer. Science 1990;250:814-8.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. In: Patterson D, Epstein CJ, eds. Molecular genetics of chromosome 21 and Down syndrome. New York: Wiley-Liss, 1990; 137-68.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. Prog Clin Biol Res 1990;360:137-68.

Papas TS, Watson DK, Sacchi N, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Kottaridis SD, Ascione R. The ETS family of genes in leukemia and Down syndrome. Am J Med Genet 1990; 7(Suppl):251-61.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05564-04 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Analysis of HIV Gene Expression

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

PI:	J. A. Lautenberger	Research Chemist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	S. Qi	Visiting Fellow	LMO	NCI
	C. W. Schweinfest	Sr. Staff Fellow	LMO	NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (K. P. Samuel); Walter Reed Army Institute of Research, Washington, DC (H. E. Gendelman)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.55	0.55	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.)*

Total cellular RNA was isolated from HIV-1-infected and uninfected H9 cells by the RNAzol method. Polyadenylated mRNA was purified by centrifugation of the total RNA through oligo(dT) cellulose spin columns. These mRNAs are being used to make directional cDNA libraries in lambda gt22A. Additionally, cDNA libraries will be made in lambda Uni-Zap XR and phagmid libraries will be produced by in vivo excision. Differentially-expressed clones will be isolated from the Uni-Zap libraries, as described by Schweinfest et al. (Gene Anal Tech Appl 1990;7:64-70). The lambda gt22A libraries will be screened using a subtracted probe enriched for infection-specific sequences produced by enzymatic amplification of subtracted cDNAs, as described by Hla and Maciag (Biochem Biophys Res Comm 1990;167:637-43). Clones that strongly hybridize to the amplified subtracted probe will be tested for differential expression by Southern blot hybridization using cDNA probes produced from mRNAs from infected and uninfected cells. Differential expression will be confirmed by RNA gel (Northern) blot assays using filters containing RNA from both infected and uninfected cells and radiolabeled probes from clones that appear promising on the Southern blot assay. Confirmed infection-specific genes will be sequenced and the sequences will be compared to DNA databases in order to determine if they correspond to known genes. Genes that are induced upon viral infection should provide useful insight into the mechanism of viral replication and pathogenesis. Furthermore, they may be useful as diagnostic reagents, targets for immunotherapy, or targets for intracellular vaccination.

PROJECT DESCRIPTION

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

J. A. Lautenberger	Research Chemist	LMO NCI
T. S. Papas	Chief	LMO NCI
S. Qi	Visiting Fellow	LMO NCI
C. W. Schweinfest	Sr. Staff Fellow	LMO NCI

Objectives:

We wish to use the techniques of molecular biology, protein chemistry, and nucleic acids chemistry to study the mechanism of viral replication and the regulation of the expression of viral gene products. We hope to understand how host gene products control viral development, and in this way gain insight into their physiological function. In particular, we are interested in comparing the biology of HIV infection of T lymphocytes with that of cells of the monocyte/macrophage lineage. While it is known that each of these cell types may harbor virus in infected individuals, their relative roles in AIDS pathogenesis is poorly understood.

Methods Employed:

1. RNA Gel Blot Analysis. Total cellular RNA was prepared by the RNAzol method using the procedure of the supplier (Cinna/Biotecx), an adaptation of the method of Chomczynski and Sacchi (Anal Biochem 1987;162:156-9). The total RNA was transferred by capillary blotting to NYTRAN membranes that were then hybridized to radiolabeled HIV sequences in 40% formamide/5 X SSC/5 X Denhardt's solution/0.5% M sodium citrate, pH 7.0; 1 X Denhardt's solution = 0.02% each BSA, polyvinyl-pyrrolidone, and Ficoll) and extensively washed. HIV hybridizing sequences were visualized by autoradiography. The radiolabeled HIV probes were generated by the random sequence oligonucleotide primer method of Feinberg and Vogelstein (Anal Biochem 1984;137:266-7).

2. Isolation of Polyadenylated RNA. An oligo(dT) cellulose spin column (Pharmacia) is equilibrated with TE-0.5 M NaCl [TE = 10 mM Tris-HCl, pH 7.4, 1 mM EDTA] and a preparation of total RNA in TE-0.5 M NaCl is applied to the column. After the RNA solution has run into the column at 1 x g, the column is centrifuged for 2 min at 350 x g. The column is then washed with TE-0.5 M NaCl followed by TE-0.1 M NaCl. Bound polyadenylated RNA (mRNA) is eluted with TE equilibrated at 65°C. This process is repeated one more time in order to minimize the level of non-polyadenylated RNAs, such as ribosomal RNA.

3. cDNA Library Construction. The first strand of cDNA is synthesized by the use of the SuperScript RT modification of Moloney murine leukemia virus (MMLV) reverse transcriptase (BRL) which lacks RNAseH activity. In order to allow the library to be directional, synthesis is primed from a primer-adapter oligonucleotide containing sequences recognized by the rare-cutting restriction enzyme, NotI, located 5' to the oligo(dT) sequences. The second strand is synthesized by nick-translation of the RNA template by the action of E. coli DNA polymerase I, E. coli RNase H, and E. coli DNA ligase. Following second-strand synthesis, adapter oligonucleotide molecules containing a SalI site are ligated to the double-stranded cDNA and asymmetric ends are created by NotI digestion. This DNA is ligated into the SalI-NotI arms of vector lambda, gt22A, and infectious phage particles are produced by in vitro packaging.

4. Subtractive Hybridization of Phagmid cDNA Libraries. cDNA libraries will be made in lambda Uni-Zap XR in a manner similar to that described above, but using the adapters and primers provided by the vendor (Stratagene, Inc.). Single-stranded phagmid libraries will be made by in vivo excision by the use of R408 helper-phage. Differentially-expressed clones will be isolated from these libraries, as described by Schweinfest et al. (Gene Anal Tech Appl 1990;7:64-70). In this process, DNA from the library of uninfected cells, as well as viral DNA, is photobiotinylated and used to remove homologous sequences in the infected cell library by subtractive hybridization. The remaining sequences are enzymatically converted to a double-stranded form and used to transform E. coli to produce a plasmid library that is enriched for sequences that are differentially expressed in infected cells.

5. Enzymatic Amplification of Subtracted cDNA. A subtracted probe is isolated by a modification of the method of Hla and Maciag (Biochem Biophys Res Comm 1990;167:637-43). Polyadenylated RNA from HIV-1-infected cells is reverse transcribed into cDNA using MMLV reverse transcriptase and the dT-17-Adapter1 primer oligonucleotide. RNA template is hydrolyzed by treatment with NaOH and single-stranded cDNA is hybridized to biotinylated polyadenylated RNA from uninfected cells. The biotinylation is performed by the method of Sive and St. John (Nucleic Acids Res 1988;16:1037) by exposure of a mixture of polyadenylated RNA and photoactivatable biotin to a high-intensity sunlamp. Optionally, biotinylated viral DNA is included in the hybridization mixture. Duplex molecules are removed by treatment with streptavidin followed by phenol extraction. The DNA that is not removed by phenol extraction, consisting of single-stranded cDNA molecules enriched for nonviral sequences that are absent in the mRNA of uninfected cells, is tailed with poly(dC) by terminal transferase. This cDNA is then made double-stranded by Taq DNA polymerase with the dG-17-Adapter2 primer oligonucleotide and amplified by polymerase chain reaction (PCR) using Adapter1 and Adapter2 primer oligonucleotides. The resulting amplified DNA is used as a probe to screen the lambda gt22A cDNA library prepared from the RNA from infected cells.

Major Findings:

Total cellular RNA was isolated from HIV-1-infected and uninfected H9 cells, and mRNA was purified by centrifugation through oligo(dT) cellulose spin columns. This mRNA is being used to make directional cDNA libraries in lambda gt22A. Additionally, cDNA libraries will be made in lambda Uni-Zap XR and phagmid libraries will be made by in vivo excision. Differentially-expressed clones will be isolated from these libraries, as described by Schweinfest et al. (Gene Anal Tech Appl 1990;7:64-70). This involves the production of single-stranded phagmid libraries by in vivo excision induced by helper-phage, followed by subtractive hybridization using biotinylated DNA. The unique DNA sequences will be converted to double-stranded form (pBluescript vector) and introduced into E. coli by transformation. Differentially-expressed clones in the resulting library will be identified by hybridization to cDNA probes.

The lambda gt22 libraries will be screened using a subtracted probe enriched for infection-specific sequences produced by enzymatic amplification of subtracted cDNAs. Bacteriophage clones that hybridize to the amplified subtracted probe will be plaque-purified and inserts will be amplified by PCR. Clones from mRNAs that are preferentially expressed in infected cells will be identified by Southern blot hybridization using cDNA probes produced from mRNAs from infected and uninfected cells. Differential expression will be confirmed by the RNA gel (Northern) blot assay. The filters for this assay will contain separate lanes of RNA from

infected and uninfected cells and the radiolabeled probes will be prepared from clones that appear promising on the Southern blot assay.

If differentially-expressed sequences are isolated from infected H9 cells, we will use the same methodology to isolate differentially-expressed genes from HIV-1-infected macrophages. RNA from infected and uninfected macrophages has been supplied to us by Howard Gendelman of the Walter Reed Army Institute of Medical Research.

Sequences confirmed to be differentially expressed will be sequenced, and the sequences will be compared to the GenBank and EMBL databases to determine if they represent known sequences. RNA gel blot and PCR methods will be used to determine if the infection specificity is general by surveying a variety of infected and uninfected cells. Genes that are induced upon viral infection should provide useful insight into the mechanism of viral replication and pathogenesis. Furthermore, they may be useful as diagnostic reagents, targets for immunotherapy, or targets for intracellular vaccination. Intracellular vaccination could be performed if an infection-specific gene is found to be required for viral replication. Vectors for the production of the products of dominant negative mutant genes or for the production of antisense RNA would be introduced into hematopoietic stem cells. The progeny of such cells could repopulate the immune system of the infected individual and disease manifestations could be eliminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05565-04 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Study of the Biochemical and Functional Properties of the ets Proto-oncogenes

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

PI:	A. K. Seth	Visiting Scientist	LMO	NCI
Others:	D. M. Thompson	Biologist	LMO	NCI
	A. Panayiotakis	Special Volunteer	LMO	NCI
	D. R. Hodge	Biologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Hellenic Anticancer Institute, Athens, Greece (S. D. Kottaridis)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.37	0.82	0.55

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.)*

Ets is a superfamily of genes and its members (ets-1, ets-2, ERG, ELK, PU.1) share amino acid homology with v-ets oncogenes of the E26 virus. The ETS1 and ETS2 genes have been involved in translocations associated with leukemias and lymphomas. The ETS2 gene is located on chromosome 21q22, suggesting that it may be one of several genes whose amplification is associated with Down's syndrome. Recently, we have demonstrated that the ets-1 gene is autoregulated and the overexpression of Ets-1 and Ets-2 proto-oncogenes transforms mouse fibroblasts and induces tumors in nude mice. The members of the ets family of proteins are transcriptional activators and induce transcription via a purine-rich consensus sequence (AGGAA). We have been interested in studying the role of DNA binding activity of the ets proteins in cell proliferation and transformation; to address that aspect we expressed the ets proteins in the in vitro expression system in E. coli and insect cells. Similar to the ets-1 product, the recombinant ets-2 protein shows binding activity with the purine-rich oligonucleotides derived from MSV-LTR and the PEA3 enhancer element.

PROJECT DESCRIPTION

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

A. K. Seth	Visiting Scientist	LMO	NCI
D. M. Thompson	Biologist	LMO	NCI
A. Panayiotakis	Special Volunteer	LMO	NCI
D. R. Hodge	Biologist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

To produce recombinant ets family products in E. coli, insect cells and in vitro expression systems to study the nature and specificity of interaction of ets family proteins with cellular targets, such as proteins and DNA.

Methods Employed:

All the recombinant DNA techniques, such as digestion of DNA, bacterial transformation, labeling of DNA with ^{32}P, preparation of small- and large-scale DNA, DNA and protein gel electrophoresis, extraction and purification of proteins, immunoprecipitation, Western and Southern blotting were according to the published procedures.

Major Findings:

1. The ets-1 protein binds DNA in a sequence specific manner to purine-rich sequences (AGGAA). We have prepared the nuclear extracts from the CEMS cells that produce large amounts of ets-1 and tested the binding with several different probes derived from the MSV-LTR, T-cell receptor, ets-1 promoter, polyoma enhancer element and MHC class I enhancer. The results showed that all the probes that contain the AGGAA core sequence bind to the ets-1 protein, whereas the mutant oligos with substitutions in the core sequence did not show detectable binding. Moreover, the ets-1 specific monoclonals also block the same band in the gel shift assay.

2. The ets-2 protein also binds to the purine sequences (AGGAA). The DNA binding activity of ets-2 expressed in SF-9 cells and E. coli was assayed by Southwestern blots using the MSV-LTR probe. Both the ets-2 proteins (expressed in E. coli and insect cells) showed specific binding; the control extracts did not show such protein:DNA interaction. The purified ets-2 expressed in E. coli also showed binding with the MSV-LTR probe in an electrophoretic mobility shift assay.

3. Localization of the ets DNA binding domain. The ets family of proteins possesses a unique DNA binding domain which is found only in ets-related proteins. The ets DNA binding domain contains three tryptophan residues with a spacing of 17-18 amino acids and two basic regions that are rich in arginine and lysine residues. We expressed a polypeptide (141 aa) spanning the DNA binding domain only, and showed that this domain is sufficient to bind to ets-specific sequences.

By contrast, the polypeptide derived from the N-terminal portion of the ets did not show detectable binding. Thus, this data suggests that the N-terminal region is not required for DNA binding, but is probably needed for transactivation.

Publications:

Chen ZQ, Burdett LA, Seth A, Lautenberger JA, Papas TS. Requirement of ets-2 expression for Xenopus oocyte maturation. Science 1990;250:1416-8.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. In: Patterson D, Epstein CJ, eds. Molecular genetics of chromosome 21 and Down syndrome. New York: Wiley-Liss, 1990;137-68.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. Prog Clin Biol Res 1990;360:137-68.

Papas TS, Watson DK, Sacchi N, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Kottaridis SD, Ascione R. ETS family of genes in leukemia and Down syndrome. Am J Med Genet 1990; 7(Suppl):251-61.

Samuel KP, Ascione R, Kottaridis SD, Seth AK, Lautenberger JA, Zuber M, Strouboulis J, Papas TS. Expression of animal and human retroviral gene products in Escherichia coli with the λP_L promoter pJL6 vector system. Gene Anal Tech Appl 1990;7:178-208.

Seth A, Papas TS. The c-ets-1 protooncogene has oncogenic activity and is positively autoregulated. Oncogene 1990;5:1761-8.

Seth AK, Hodge DR, Kottaridis SD, Thompson DM, Panayiotakis A, Watson DK, Papas TS. DNA binding activity of the ets proto-oncogene family proteins. Miami Short Rep 1991;1:83.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05566-04 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of the Biological and Biochemical Functions of the ets Proto-oncogenes

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

PI:	A. K. Seth	Visiting Scientist	LMO	NCI
Others:	D. M. Thompson	Biologist	LMO	NCI
	A. Panayiotakis	Special Volunteer	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*
Laboratory of Developmental and Molecular Immunity, NICHD, NIH (K. Ozato)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.80	0.55	0.25

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 ets proteins bind DNA, in a sequence-specific manner, to purine-rich sequences found in the promoter/enhancer elements of a variety of genes. The members of the ets family of proteins are transcriptional activators, and induce transcription via binding to a purine-rich consensus sequence (AGGAA). We studied the function and regulation of the ets family of genes, and have shown that ets-1 is autoregulated and the overexpression of ets-1 and ets-2 proto-oncogenes transforms mouse fibroblasts. The ets-1 promoter contains sequences identical to the PEA3 motif; therefore, to examine whether the ets-1 protein is able to bind to the PEA3 motif localized in the ets-1 promoter, we synthesized oligonucleotides corresponding to the ets recognition site, as well as unrelated regions from the ets-1 promoter, and tested their binding activity. The v-ets/ets-1 protein we synthesized in E. coli binds to sequences derived from the ets-1 promoter, as well as MSV LTR and polyoma enhancer (PEA3) derived sequences, but not to unrelated sequences that lack the core elements of the ets recognition site. Thus, it is likely that the ets-1 protein binds to its own promoter in order to autoregulate its expression. In order to investigate whether the ets genes regulate the expression of other gene promoters, we have started testing the activation of MHC class I promoters linked to the CAT reporter gene as a model system after cotransfection with the β-actin promoter linked ets-1 and ets-2 gene expression vectors. The ets family members (ets-1, ets-2 and erg) have weak homology with the helix-loop-helix protein. To delineate the transactivation domain in ets proteins, and to investigate whether the ets gene is similar to other helix-loop-helix family proteins (E12, E47, myoD), as well as determine if the helix-loop-helix homology region in ets plays a role in the transactivation process, we have linked the ets-1 and ets-2 genes in frame with the GAL4 DNA binding domain. Using the ets expression vector, pSG424, we tested for transactivation ability after cotransfection with a reporter plasmid containing the CAT gene linked to the GAL4 promoter and DNA binding site.

PROJECT DESCRIPTION

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

A. K. Seth	Visiting Scientist	LMO NCI
D. M. Thompson	Biologist	LMO NCI
A. Panayiotakis	Special Volunteer	LMO NCI
T. S. Papas	Chief	LMO NCI

Objective:

To study the function and regulation of the ets family of genes; to understand the autoregulation mechanism of the ets-1 proto-oncogene.

Methods Employed:

Recombinant DNA techniques, such as isolation of DNA fragments, ligation, in situ colony hybridization, DNA sequencing, labeling the DNA by nick translation, gel electrophoresis, Northern and Southern blotting, transfection of NIH3T3 cells, CAT assays, electrophoretic mobility shift assay and Southwestern blots were all according to the published procedures.

Major Findings:

1. Ets-1 protein binds to its promoter for autoregulation. Previously, we have shown that ets-1 is autoregulated by its product. Autoregulation could occur either by nuclear factors that are induced by the ets-1 product or, similar to jun and myoD, could also occur by binding to ets-1 promoter/enhancer sequences and inducing its expression directly. Ets-1 binds to purine-rich core sequences (AGGAA) that are located in MSV LTR, PEA3 and many other promoters. We searched for similar sequences in the ets-1 promoter and found that this sequence is present at multiple sites. Oligos corresponding to one of the sites showed specific binding with the ets-1 protein made in E. coli, suggesting that ets-1 may bind to its promoter directly in order to autoregulate its expression.

2. Ets-2 proteins have homology with the Id protein. The ets-1, ets-2 and erg have homology with members of the HLH family of proteins in the helix I and helix II region. The ets family of proteins lacks the basic domain located upstream of the helix I; however, a basic domain is localized at the C-terminal end. Interestingly, the Id and a Drosophila gene regulatory protein, emc, also lack this basic domain; however, the Id and ets-2 proteins share 45% homology in this region. Similar to the HLH family of proteins, the HLH homology regions in ets-1, ets-2 and erg may also be involved in protein:protein interaction, such as noted with other transcriptional regulators.

3. Construction of β-actin ets-1 expression vector. The I.7 kb ThaI-FspI ets-1 DNA fragment that contains the entire human gene was inserted at SmaI of pWI2. pWI2 is a vector that contains the SV40 poly A and β-actin promoter. Two positive vectors with the ets-1 gene in correct and opposite orientation with the β-actin promoter were isolated and used for CAT assays.

4. Construction of the GAL4-ets-1 fusion expression vectors. The GAL4-ets-1 fusion vectors were constructed by inserting a complete human ETS1 gene fragment or a fragment lacking the N-terminal 65 amino acids at the SmaI site of pSG424. The positive clones containing the insert in correct orientation were further confirmed for the appropriate frame by DNA sequencing. The correct constructs were utilized for transactivation assays.

Publications:

Chen ZQ, Burdett LA, Seth A, Lautenberger JA, Papas TS. Requirement of ets-2 expression for Xenopus oocyte maturation. Science 1990;250:1416-8.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. In: Patterson D, Epstein CJ, eds. Molecular genetics of chromosome 21 and Down syndrome. New York: Wiley-Liss, 1990;137-68.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. Prog Clin Biol Res 1990;360:137-68.

Papas TS, Watson DK, Sacchi N, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Kottaridis SD, Ascione R. ETS family of genes in leukemia and Down syndrome. Am J Med Genet 1990; 7(Suppl):251-61.

Seth A, Papas TS. The c-ets-1 protooncogene has oncogenic activity and is positively autoregulated. Oncogene 1990;5:1761-8.

Seth AK, Hodge DR, Kottaridis SD, Thompson DM, Panayiotakis A, Watson DK, Papas TS. DNA binding activity of the ets proto-oncogene family proteins. Miami Short Rep 1991;1:83.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05569-04 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Effect of c-myc on Cellular Gene Expression

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

PI:	C. W. Schweinfest	Sr. Staff Fellow	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.12	0.12	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.)*

C-myc plays an important role in cellular proliferation. Its membership in the helix-loop-helix family of genes, as well as the recent discovery of its ability to bind as a heterodimer with Max, suggests that this dimer may act as a transcription factor. Such a dimeric transcription factor complex may impart to c-myc a specificity for DNA binding to the sequence CACGTG. In addition to acting as a transcription factor, c-myc appears to regulate genes expressed in the GO/G1 transition. We have previously demonstrated this effect with two such GO/G1 genes.

In order to identify additional genes whose expression is regulated by c-myc, we have constructed orientation-specific cDNA libraries for cell lines which differ only in their ability to express an exogenously transfected c-myc construct in response to heat shock. A method of cDNA subtraction which we developed for colon carcinoma (see Project Z01CP05585-03 LMO) will be used here to enrich for cDNAs uniquely co-expressed with c-myc, as well as cDNAs whose expression may be down-regulated by c-myc. Co-expressed genes are expected to include proliferation-specific genes, as well as some genes that may repress differentiation. Down-regulated genes may include certain key differentiation-inducing genes of the homeobox class.

PROJECT DESCRIPTION

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

C. W. Schweinfest	Sr. Staff Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

A large body of circumstantial evidence has implicated c-myc as having a role in cellular proliferation in both normal and neoplastic cells. Like most oncogenes, a specific biological function for c-myc has yet to be defined. Since c-myc is one of a class of nuclear oncoproteins and has been demonstrated to have DNA binding capability (albeit nonspecific), c-myc's biological function may be to regulate the expression of other genes. Therefore, the objective of this work is to study the effect of c-myc on the regulation of other genes and, by understanding the function of these target genes, be able to define the role of c-myc.

Methods Employed:

1. A set of cell lines has been constructed in which the human c-myc gene (exons 2 and 3 only and exons 1, 2 and 3) can be expressed under control of the Drosophila heat shock 70 promoter. Permanent cell lines were constructed in Balb/c 3T3 cells by co-transfection of the myc-containing plasmids, along with the selectable G418-resistance plasmid, pSV2neo, followed by selection in 400 μg/ml G418. Resistant clones are screened for heat shock-inducible expression of c-myc. A control cell line was transfected with a construct lacking the c-myc gene. Under conditions of serum arrest, the endogenous c-myc gene is not expressed.

2. Directional cDNA libraries are constructed from these cell lines.

3. Hybridization subtraction will be performed to make subtraction libraries enriched for specifically-induced or specifically-repressed sequences.

Major Findings:

1. Exogenously transfected human c-myc can be expressed under control of the Drosophila heat shock 70 promoter.

2. Directional cDNA libraries in λZAPII have been constructed so as to produce single-stranded DNA for hybridization subtraction and the production of subtractive libraries. Directional libraries allow for only interlibrary hybridization (as opposed to intralibrary hybridization), thus increasing the efficiency of the subtraction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05570-04 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Scale-up Purification of HIV-1 and HIV-2 Recombinant Env Polypeptides

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

PI:	J. A. Lautenberger	Research Chemist	LMO	NCI
Others:	Y-M. A. Chen	Visiting Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (K. P. Samuel, G. K. Pei); Harvard University, School of Public Health, Boston, MA (P. J. Kanki)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.62	0.62	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.)*

Two highly-immunogenic recombinant Env polypeptides of the HIV-1 gp41 (protein 566) and HIV-2 gp35 (protein 996) transmembrane glycoproteins were expressed in E. coli in quantity, purified in milligram quantities to near homogeneity, and used in immunoassays (Western and dot blots) against sera from Central and West Africa, Mexico, and the United States to distinguish between HIV-1 and HIV-2 infections.

In addition, recombinant HTLV-I (rpBI) and HTLV-II (rpBII) gp46 Env polypeptides, and HTLV-I p21E Env polypeptides (protein 400), previously expressed in E. coli, were also used in immunoassays (Western blot) to distinguish between HTLV-I and HTLV-II infections. Moreover, antibodies developed to the HTLV-I (rpBI) and HTLV-II (rpBII) Env polypeptides were used in antibody-dependent cellular cytotoxicity (ADCC) assays to delineate the type-specific epitopes residing on the surfaces of the HTLV-I and HTLV-II Env glycoproteins.

Currently available serological tests do not distinguish between HIV-1 and HIV-2 or HTLV-I and HTLV-II infections because of significant antigen-antibody cross-reactivity due to the close genetic relatedness between HIV-1/HIV-2 and HTLV-I/HTLV-II virus groups. Thus, the application of these highly-immunogenic HIV-1 (protein 566), HIV-2 (protein 996), HTLV-I (rpBI and protein 400) and HTLV-II (rpBII) candidate antigens in development of immunoassays for differentiating single, as well as dual viral infections, and as potential subunit vaccines, requires further serological and biological characterization.

PROJECT DESCRIPTION

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

J. A. Lautenberger	Research Chemist	LMO NCI
Y-M. A. Chen	Visiting Fellow	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

We have previously utilized bacterial expression technology to produce several different Env polypeptides of the HIV-1, HIV-2, and HTLV-I envelope glycoproteins for use as diagnostic antigens and to develop subunit vaccines. In view of the continuing serious public health hazard posed by HIV and HTLV infections, and in response to the need for more highly-specific and sensitive immunoassays to distinguish between the various HIV and HTLV infections, including dual HIV and/or HTLV infections, our objectives are to evaluate the recombinant Env polypeptides for their sensitivity, specificity, diagnostic, and seroepidemiological usefulness in immunoassays for screening blood.

Methods Employed:

1. Scale-up fermentation and expression of recombinant Env polypeptides. Cultures of E. coli cells harboring the recombinant expression plasmids were grown at 32°C in 350-liter fermenters, and protein production was induced by shifting culture to 42°C for 1 hour. The induced cell pellets (500-750 grams cell paste) were used as the starting source of recombinant Env polypeptides.

2. Isolation of inclusion bodies. A sequential detergent and salt extraction procedure, which removes the bulk of contaminating bacterial proteins from the recombinant HIV-1 gp41 (polypeptide 566) and HIV-2 gp35 (polypeptide 996) Env glycoproteins, was utilized. The isolated inclusion bodies were solubilized by extraction with a 2-5% unbuffered solution of sodium dodecyl sulfate (SDS).

3. Large-scale purification of recombinant Env glycoproteins 996 and 566. Preparative gel filtration and SDS-PAGE procedures were performed on the 3% SDS-solubilized 22 kD recombinant Env polypeptide 566 and the 20 kD Env polypeptide, 966. Each antigen was fractionated by preparative gel filtration on a Bio Gel-P60 column (10 cm x 35 cm) in the presence of 3% unbuffered SDS, followed by chromatography on a second Bio Gel-P60 column (5 cm x 40 cm) in 3% SDS. The peak protein fractions of interest were pooled, extracted with acetone, and further purified by preparative SDS-polyacrylamide gel electrophoresis. The final gel-eluted proteins were further extracted with acetone, the pellets resuspended in 0.5% SDS, and the concentration determined spectrophotometrically (1.0 A_{280}/ml = 1 mg protein).

4. Recombinant Env polypeptide serology.

(A) **Western blot assay**: Aliquots containing different amounts of the purified HIV-1 and HIV-2 recombinant Env polypeptides, 566 and 996, respectively, were

resolved on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. To block protein-binding sites, the membranes were incubated in 5% nonfat dry milk in TBS (TBS = 50 mM TrisHCl, pH 7.5/0.5 M NaCl) and reacted with different panels of diluted human sera for 18 hours at 23°C. After several washes with TBS, the filters were incubated with peroxidase-conjugated goat antibodies to human IgG. Immune complexes were then detected by staining with 4-chloro-naphthol and hydrogen peroxide.

(B) **Modified Western blots for distinguishing HTLV-I and HTLV-II**: Recombinant HTLV-I and HTLV-II Env polypeptides, PBI and PBII, respectively, were partially purified from small-scale production by the detergent/chaotrope extraction method, and the urea-solubilized inclusion bodies containing the proteins were resolved by SDS-PAGE and blotted onto nitrocellulose filter paper. Filter strips were reacted with HTLV-I, HTLV-II and normal human sera.

A modified HTLV-I/HTLV-II immunoblot assay was performed with viral lysates from the MT2 and MT4 cell lines, and with purified HTLV-I recombinant Env polypeptide P400 (P21E).

(C) **Mapping of HTLV type-specific epitopes on native gp61 by antibody-dependent cellular cytotoxicity (ADCC) assay**: Rabbit antisera to recombinant HTLV-I Env polypeptides and nine HTLV-I (SP-1 to SP-9) and one HTLV-II synthetic peptides were used for ADCC studies. ADCC assays were performed by standard procedures, using the HTLV-I cell line (C91/PL) and HTLV-II cell line (729pH6neo) labeled with ^{51}Cr as target cells.

(D) **Enzyme immunoassay (EIA)**: This assay was used to analyze the antibody response of rabbits immunized with the different HTLV-I and HTLV-II synthetic Env peptides. The sera were used at 1:400 dilutions and the peptides were pre-absorbed to wells of flat-bottomed EIA polystyrene plates. The optical density was read spectrophotometrically on a Titertek Multiskan spectrophotometer.

Major Findings:

1. Protein yield and purity. The final SDS-PAGE purified Env polypeptides were extracted twice with acetone and lyophilized. A typical yield of apparently homogeneously pure 566 polypeptide and 996 range from 15-20 mg per 500-700 gm wet cell paste per 350-liter induced cell culture. The proteins were judged to be homogeneous by obtaining a single band on 12% or 15% SDS-polyacrylamide gels as visualized by Coomassie blue staining, and Western blot analysis with the appropriate monoclonal antibodies and human HIV-positive test sera. One A_{280} unit of absorption was taken as representing 1 mg of purified protein.

2. Comparative HIV-1 and HIV-2 recombinant Env serology. In collaboration with Dr. Phyllis Kanki, we have extensively evaluated the highly immunoreactive HIV-2 gp35 recombinant polypeptide 996 in immunoassay (Western blot) procedures with the corresponding HIV-1 gp41 recombinant polypeptide 566 for their ability to distinguish HIV-1 from HIV-2 and HIV-1/HIV-2 dual infections. Our results from this study, which is still ongoing, with HIV-positive sera from Central and West Africa, Mexico, and the United States conclude that, when optimized for specificity and sensitivity by several hundred-fold dilution of the sera, the

purified antigens accurately identify the respective viruses in the infected sera. Without optimization, our preliminary studies showed that antibodies in HIV-2-positive sera from West African individuals infected with HIV-2 cross-react with or recognize an epitope on the 566 gp41 polypeptide. Of 31 dually-infected HIV-1 and HIV-2 human sera from West Africa, obtained through the World Health Organization, both the HIV-1 (peptide 556) and HIV-2 (peptide 996) recombinant Env polypeptides recognized 100% of the HIV-1 and HIV-2 infections, respectively. These studies are still continuing.

In an attempt to use these antigens as sensitive and specific antigens to distinguish HIV-1 from HIV-2 infections, large-scale seroepidemiological approaches are being developed with our collaborator, Dr. Phyllis Kanki, to identify singly- or dually-infected HIV-1/HIV-2 individuals in West Africa by a modified dot blot assay.

3. Comparative HTLV-I and HTLV-II Env serology. The recombinant HTLV-II Env polypeptide, rpBII, was expressed in E. coli with a PCR-cloned env gene fragment (residues 96-235) of the HTLV-II exterior glycoprotein, gp52. When used with the HTLV-I recombinant Env polypeptide, rpBI, containing residues 166-201 of the exterior glycoprotein, gp46, in Western blot studies on PCR-confirmed HTLV-I and HTLV-II sera, the results showed that all PCR-confirmed HTLV-I-positive sera reacted specifically with the rpBI protein. Similarly, all HTLV-II-positive sera reacted specifically with the rpBII protein, while 65% of the HTLV-I-positive sera also cross-reacted with the HTLV-II rpBII protein, but at lower intensity.

In addition, the recombinant HTLV-I polypeptide, p400, containing amino acids 307-440 of gp21E, was used in a modified Western blot assay in a cocktail with HTLV-I viral lysate from the MT-2 cell line and a recombinant HTLV-I protein, MTA-4, containing a unique epitope reactive only with HTLV-I-positive sera. In this modified Western blot, differentiation of HTLV-I from HTLV-II-positive sera was determined by reactivity to the MTA-4 protein, which is recognized by HTLV-I, but not HTLV-II sera. Moreover, all type I and type II sera were confirmed and differentiated from each other by reactivity with the cocktail antigen containing the combined HTLV-I (MT-2 cell line) viral lysate, and recombinant p21E (p400) and MTA-4 protein. However, this assay does not identify coinfections with HTLV-I and HTLV-II viruses.

4. Mapping of type-specific HTLV-I and HTLV-II epitopes. The Western blot results on rpBII and rpBI showed that these recombinant polypeptides are specific for distinguishing HTLV-II from HTLV-I infections, respectively. Antibodies to synthetic Env peptides and recombinant polypeptides of HTLV-I gp46 and gp21E raised in rabbits were used in the ADCC ^{51}Cr release assay. The results concluded that rabbit antibodies to the rpBI polypeptide had 2.5-fold higher specific ADCC activity on HTLV-I cells than on HTLV-II cells. The region on rpBII containing this type-specific epitope mapped to the middle (amino acids 157-225), and is bounded by two conserved cysteines. These are ongoing studies.

Publications:

Samuel KP, Ascione R, Kottaridis SD, Seth, AK, Lautenberger, JA, Zuber M, Strouboulis J, Papas TS. Expression of animal and human retroviral gene products in Escherichia coli with the λP_L promoter pJL6 vector system. Gene Anal Tech Appl 1990;7:178-208.

Patents:

Papas TS, Samuel KP, Lautenberger JA, Wong-Staal FL. Serial No. 6-664,972 (Pending): Production of Human T-cell Leukemia (Lymphotropic) Retrovirus (HTLV-I) Envelope Protein Fragments in Bacteria and Use in Seroepidemiological Survey of Human Lymphoid Malignancies.

Papas TS, Samuel KP, Lautenberger JA, Wong-Staal FL. Serial No. 6-824,426 (Pending): Production of Human T-cell Lymphotropic Virus Transactivating Protein (p42-LOR).

Papas TS, Zuber M, Samuel KP. Serial No. 7-407,317 (Pending): Antigen and Immunoassay For Human Immunodeficiency Virus Type 2 (HIV-2).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05571-04 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Studies of E26 Avian v-ets and its Cellular Homologue in Mouse Cells

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:	X. Wang	Visiting Fellow	LMO	NCI
	R. E. Aurigemma	IRTA Fellow	LMO	NCI
	S. K. Ruscetti	Microbiologist	LMO	NCI
	A. K. Seth	Visiting Scientist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	D. K. Watson	Research Microbiologist	LMO	NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (C. Hanson)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.59	1.59	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 acute leukemia virus, E26, expresses oncogenic fusion proteins containing portions of the myb and ets-1 genes derived from the avian genome. In order to study both the normal and oncogenic functions of these genes, particularly ets, we have developed a murine model for myb-ets oncogenesis by introducing the gag-myb-ets coding sequences from E26 into a murine retroviral vector. This defective murine viral construct (ME26) induces leukemia in newborn mice and abrogates the serum dependence for growth of NIH3T3 murine fibroblasts.

We have now analyzed the expression of serum-response genes in NIH3T3 fibroblasts which have lost their dependence on serum for growth following infection of ME26. No increase in fos, jun or myc expression was seen in ME26-infected cells in comparison to uninfected 3T3 cells, and expression in ME26-infected hematopoietic cells was also not altered. Likewise, c-ets-1 and c-ets-2 endogenous expression was not increased in these cells. These results suggest that ME26 may induce serum-independent growth in fibroblasts by a mechanism that does not involve the serum-response pathway.

We have also detected a novel 3.7 kb subgenomic ME26 message in both infected fibroblasts and hematopoietic cells in culture. The subgenomic message contains both myb and v-ets specific sequences and is not incorporated into viral particles. The coding potential of this subgenomic message remains to be determined.

PROJECT DESCRIPTION

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

D. G. Blair	Supv. Research Chemist	LMO	NCI
X. Wang	Visiting Fellow	LMO	NCI
R. E. Aurigemma	IRTA Fellow	LMO	NCI
S. K. Ruscetti	Microbiologist	LMO	NCI
A. K. Seth	Visiting Scientist	LMO	NCI
T. S. Papas	Chief	LMO	NCI
D. K. Watson	Research Microbiologist	LMO	NCI

Objectives:

To study the mechanism and cooperative role of the myb and ets oncogenes of the avian erythroleukemia virus, E26, in oncogenesis.

To determine the biological function of v-ets and its cellular homologue in altering fibroblast cell growth and hematopoietic development, and to develop biological assays to characterize these functions.

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 specific cellular proteins.

Major Findings:

1) NIH3T3 fibroblasts expressing the gag-myb-ets or ets oncogenes, and inhibiting increased growth potential in low serum or serum-free media, express normal levels of serum-response genes. NIH3T3 fibroblasts acquire the ability to proliferate in defined media containing 0.1% calf serum upon infection or transfection with the ME26 retroviral construct expressing the gag-myb-ets fusion oncogene. The addition of serum to serum-starved 3T3 fibroblasts induces the expression of a number of genes such as c-fos, c-myc and c-jun, in a defined temporal pattern. We have examined whether ME26 infection alters the expression of any members of this gene family in order to investigate possible targets for ME26 action. No increased expression of fos, jun or myc was observed in ME26 infected fibroblasts grown in 5% serum and normal media. Similarly, erythropoietin-dependent or independent hematopoietic cell lines also showed only low levels of expression of these genes. Analysis of these and other potential growth-related genes under low-serum growth conditions is currently being determined. These results suggest that the target for the ME26 oncogene in NIH3T3 fibroblasts still remains to be determined.

2) A novel spliced v-et containing mRNA is expressed in murine cells infected with ME26. The avian progenitor of the murine recombinant retrovirus, ME26, is known to express only a single genome length RNA in avian cells. However, RNA from ME26-infected fibroblasts and hematopoietic cells, as well as transfected fibroblasts, contain two species of RNA hybridizing to a v-ets probe, a 5.2 kb genomic sized message and a second band of 3.7 kb. Both bands also hybridize to

a myb-specific probe. Viral particles, however, contain only the 5.2 kb species, consistent with the hypothesis that the 3.7 kb RNA represents a spliced, subgenomic form of the virus genome. The exact structure of this 3.7 kb species remains to be determined, and computer analysis does not indicate potential splice acceptors which would be likely to generate a 3.7 kb message. It is also unknown if this fragment is capable of expressing a truncated protein, although no major protein fragments smaller than the p135 whole genome product have been observed.

Publications:

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti, SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. Prog Clin Biol Res 1990;360:137-68.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05572-04 LMO

PERIOD COVERED: October 1, 1990 to March 31, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Isolation of Potential Oncogenes from Teleost 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:	R. J. Van Beneden	Guest Researcher	LMO	NCI
	D. K. Watson	Research Microbiologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)* USABRDL, Ft. Detrick, Frederick, MD (W. van der Schalie, H. Gardner); Duke U., Beaufort, NC (R. Winn, M. Roberts, Z. Fan); Gulf Coast Res. Lab., Ocean Sprgs, MS (W. Hawkins, W. Walker); EPA, Narragansett, MA (G. Gardner)

LAB/BRANCH: Laboratory of Molecular Oncology

SECTION: Microbiology Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.62	0.62	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.)*

While the detailed mechanisms of tumorigenesis are unknown, increasing evidence suggests that genetic alterations of cellular oncogenes are, in part, responsible for the neoplastic transformation of cells. Some studies in rodent models have implicated the direct chemical activation of oncogenes by carcinogen exposure. The reproducible detection of specific transforming genes in animal model systems strongly suggests that these genes have a significant role in the development of certain tumors. Data from our preliminary transfection experiments with DNA from carcinogen-induced medaka tumors supported this hypothesis and suggested that an unknown gene may be activated in the DEN-induced cholangiocarcinoma. It does not appear to have homology to any known oncogene sequence based on hybridization of Southern blots. Recently, studies have been initiated to examine DNA from gonadal tumors in two species of clams (mya arcnaria and mercenatia mercenara) exposed to herbicides. We have demonstrated transformation of NIH3T3 cells and have produced one tumor in nude mice using DNA from an advanced tumor. The identification and characterization of this apparently novel oncogene is now in progress.

Transgenic medaka were produced by the introduction of the E. coli lacZ gene under the control of the mouse Mt-1 metallothionein promoter. Fish which contain the lacZ gene have been identified by PCR analysis. This construct has also been introduced into fish cell lines to form the basis of in vitro toxicity assays. We have not yet been able to demonstrate expression of this construct in either fry or the fish cell lines.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on the Project:

D. G. Blair	Supv. Research Chemist	LMO	NCI
R. J. Van Beneden	Guest Researcher	LMO	NCI
D. K. Watson	Research Microbiologist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The purpose of this investigation is to examine DNA isolated from tumors of aquatic organisms for the presence of transforming genes. These genes, identified by transfection assays, will be cloned and characterized. This will allow us to determine whether they are cellular homologs of known oncogenes or if they represent a new oncogene sequence. Characterization of transforming genes isolated from chemically-induced lesions will also enable us to examine the mechanism of chemical carcinogenesis. Further studies involve the development of transgenic fish and fish cell lines as models for biomonitors of specific aquatic contaminants and the development of stably transfected fish cell lines for *in vitro* toxicity studies. Studies have been initiated using DNA from ovarian tumors in hardshell and softshell clams that had been exposed to herbicides.

Methods Employed:

Standard techniques of cell culture, DNA isolation and purification, Southern and Northern blot analysis, and DNA transfection using $CaPO_4$ were used. Transfected cells were analyzed for the appearance of morphologically-transformed foci, for the ability to grow in defined media at low levels of serum and for the ability to form tumors upon injection into athymic (nu/nu) mice. Studies on chemical induction of tumors were done in collaboration with Dr. William van der Schalie and Mr. Henry S. Gardner at the U. S. Army Biomedical Research and Development Laboratory (BRDL), Fort Detrick, Frederick, MD. Japanese medaka were treated with either (1) diethylnitrosamine (DEN), 200 mg/l, 48 hr exposure to 14-day-old fry or (2) methylazoxymethanol acetate (MAMAc), 50 mg/l, 4 hour exposure to 8-day-old embryos. Additional studies were done at the Gulf Coast Research Laboratory in collaboration with Drs. W. Hawkins and W. Walker.

To examine the effect of toxic metals on fish cell lines, rainbow trout cell lines derived from gonad tissue (RTG-2) and a mesothelioma tumor (RTM) were exposed to copper and zinc at concentrations of 0 to 100 μM for a period of one to two weeks. Surviving cells were then fixed and stained with Giemsa stain and quantitated using a JVA video analyzer program (Jandel Corp.).

For microinjection into medaka embryos, a linearized portion of a construct containing the *MT-1* promoter and the *lacZ* gene was microinjected into fertilized eggs using a Pico-injection (Medical Systems) microinjection apparatus.

Major Findings:

1. Twelve positive clones were identified from the library made from NIH 3T3 cells transfected with DNA from a medaka cholangiocarcinoma, using pSV2neo as a probe. Southern blots prepared from mini-prep DNA isolated from these clones were hybridized to both pSV2neo and medaka DNA. Our results indicate that a single clone (C7) contains *EcoRI* fragments that hybridize to both of these probes. Further characterization of this clone by sequence analysis is in progress.

2. Transfection of clam DNA into NIH 3T3 cells was accomplished at lower efficiency than for fish DNA. DNA from a single individual hardshell clam (#881) induced significant focus formation, enhanced colony growth (in QBSF media supplemented with 0.1% serum) and to date (3 weeks post-injection) has induced one tumor in a nude mouse. DNA isolated from an early stage tumor was positive (to date) in the standard focus assay only. DNAs isolated from a reference (normal) animal and an animal with a very early stage tumor were negative in our assays.

3. Studies of the effect of heavy metals on the growth of fish cell lines indicate that, in general, fish RTM cells are less tolerant than mammalian NIH 3T3 cells to exposure to high levels of zinc and copper. All cell lines appeared more tolerant of exposure to zinc than to copper at the same concentrations. RTM cells exhibited 50% mortality at 50 μM copper after a 14-day exposure to a range of 0 to 100 μM of the metal. The cells were more tolerant of zinc treatment in the same concentration range, with 50% of the cells dying after exposure to 70 μM zinc following 14 days of treatment. NIH 3T3 cells exhibited some mortality only at very high levels of both metals (80-100 μM).

4. Microinjection of medaka embryos has been successfully accomplished, with a 40% survival rate to hatch. DNA has been isolated from fin clips of the fry for analysis of gene integration using PCR. These results were positive; however, we have not yet conclusively demonstrated gene expression in these animals.

5. Development of stable fish cell lines transfected with the MT/lacZ construct are still in progress. RTM and RTG-2 cells which had been transfected with this construct via calcium-phosphate precipitation were fixed and stained using X-gal. No blue color, indicative of lacZ gene expression, was observed. DNA isolated from transfected cells will be assayed by PCR analysis for the presence of the lacZ gene.

This project was terminated as an independent project because Dr. Van Beneden has moved to the Duke Marine Laboratory in Beaufort, NC. Aspects of it will continue as a collaboration between the P.I. and Dr. Van Beneden within project Z01CP05295.

Publications:

Van Beneden RJ, Henderson KW, Gardner HS, Blair DG, Papas TS. New models for oncogene isolation in the study of carcinogenesis. In: Compendium of FY '88 and FY '89 research reviews for the Research Methods Branch, U. S. Army Technical Report. Rockville: Technical Resources, Inc. (In Press).

Van Beneden RJ, Henderson KW, Roberts MA, Gardner HS, Papas TS. Role of oncogenes in chemical carcinogenesis. In: Compendium of FY '88 and FY '89 research reviews for the Research Methods Branch, U. S. Army Technical Report. Rockville: Technical Resources, Inc. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05574-04 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of Drosophila melanogaster ets and ets-like Genes

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

PI:	D. K. Watson	Research Microbiologist	LMO	NCI
Others:	L. J. Pribyl	IRTA Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*
Department of Biochemistry & Molecular Biology, The University of Texas, M. D. Anderson Cancer Center, Houston, TX (R. A. Schulz)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.42	0.42	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.)*

Three Drosophila genes with cellular sequences related to the ets region of the chicken retrovirus, E26, have been isolated in this laboratory. The characterized portion of the most related gene, D-ets, shows over 90% identity with the last two exons of the chicken c-ets-1 gene at the predicted amino acid level. The region of D-ets (a genomic clone) 5′ to this region has been shown to be present in a cDNA clone, and this sequence is divergent from the chicken c-ets-1 gene. D-ets is expressed as a 4.7 kb transcript throughout development. It is localized to chromosome 3R at position 58A/B. D-elg (Drosophila ets-like gene) is a related gene that has been localized to chromosomal position 97D on chromosome 3R. This gene is represented by a clone isolated from a pupae cDNA library. Its sequence shows 60% amino acid similarity with D-ets. D-elg is expressed as a single transcript of 2.0 kb that is expressed throughout embryogenesis, as well as pupae and adult stages, but absent from larvae. D-elg is expressed in all embryonic cells prior to transcriptional activation and has no regional localization. A third gene, E13B, was identified by hybridization with v-ets, and a genomic clone has been partially sequenced. A cDNA clone that hybridized to E13B shows a long Poly A+ tail and is also being sequenced. The E13B gene is expressed as a 1.6 kb RNA species in pupae and adults only. It is located on chromosome 3L at position 66A.

PROJECT DESCRIPTION

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

D. K. Watson	Research Microbiologist	LMO	NCI
L. J. Pribyl	IRTA Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

To determine if there are sequences present in the Drosophila genome that are homologous to the v-ets sequences of the avian retrovirus, E26. If such sequences do exist, it will then be necessary to characterize this gene or gene family to understand the structural evolution of these genes. This would then allow one to develop a model for the domains along the ets protein which may have functional implications and may provide a functional assay.

Methods Employed:

1. Preparation of high molecular weight DNA from Drosophila collected during specific developmental stages and 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 genomic and clonal DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Nitrocellulose filters containing immobilized restriction fragments were prepared by the Southern blot technique and hybridized with specific ets probes. Specific DNA fragments were purified by electroelution or by extraction from low-melting agarose and were used either to prepare ets-specific DNA probes by nick-translation using E. coli DNA polymerase and DNase I or in the construction of a partial recombinant phage library by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging.

3. Isolation of phage from the libraries containing virus-related sequences by hybridization of ets-specific probes to nitrocellulose filters containing phage DNA prepared from plaques by the procedure of Benton and Davis (Science 1977;196:180-2). Isolation of plasmid DNAs containing Drosophila cDNAs from colonies lifted from plates by the method of N. Brown (personal communication).

4. Subcloning of isolated Drosophila DNA fragments into appropriate plasmid vectors, as required.

5. DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol 1980;65:499-560). Also, dideoxy sequencing using the method of Sanger (Proc Natl Acad Sci USA 1977;74:5463-7) is employed.

6. Total cellular RNA from cultured cells or flies collected from defined developmental stages was prepared by the urea method (Biochemistry 1973;12:2330-8), or the SDS-proteinase K method described by Schulz et al. (Dev Biol 1989;131:515-23). Separation of polyA+ and polyA- RNA by one cycle of purification through oligo (dT) cellulose. Characterization of RNA by electrophoresis on formaldehyde-agarose gels and Northern analysis.

7. Chromosomal in situ hybridization of Drosophila third instar larvae, using the method of Pardue and Gall (Methods Cell Biol 1975;10:1-16).

8. Total protein extractions from Schneider cells and flies of defined developmental stages were prepared, with the resultant proteins electrophoresed on polyacrylamide gels and then transferred by electroblotting to nitrocellulose paper.

9. Western analysis using antibodies created against viral peptides were applied. The peroxidase conjugated antibody technique was used to detect reactivity.

10. Construction of P-element vectors, as designed by Rubin and Spradling (Science 1982;218:341-7 & 348-53), containing a human-Drosophila ets chimera. This chimera was produced by cloning the 5′ portion of the human ets-2 cDNA with the 3′ portion of the genomic Drosophila ets gene.

11. Microinjection of P-element vectors into embryos that have been prepared using techniques described by Dr. F. Kafatos.

12. Preparation of sections of Drosophila embryos and in situ hybridization with D-elg and D-ets probes to determine tissue RNA expression patterns according to the procedures of Ingham et al. (Nature 1985;318:439-45) and Angerer et al., (Methods Enzymol 1987;152:649-61).

13. Preparation of strand-specific (sense and antisense) probes by a modification of the Stratagene protocol.

14. cDNA cloning was carried out according to the method of Gubler and Hoffman (Gene 1983;25:263-9), as modified by Canaani (Nature 1985;315:55-7). Asymmetric PCR (polymerase chain reaction) to obtain full-length cDNA clones of D-ets.

15. Primer extension and S1 nuclease mapping experiments by standard procedures as described in Sambrook et al. (Molecular Cloning, Second Edition, Book 1, 1989).

16. Cloning of Drosophila ets and ets-related genes into prokaryotic vectors for overexpression of the inserted DNA. Pure proteins isolated from these constructs will be utilized as antigens, for functional analyses and for microinjection studies.

17. Isolation of the promoter region of D-ets genes and functional characterization by functional assays using expression of chloramphenicol acetyl transferase (CAT). Once functionally localized, promoter regions will be placed upstream from reporter genes (e.g., LacZ) and injected into the embryo to evaluate promoter activity by in situ analysis.

18. The 5′ and 3′ ends of D-elg transcripts by S1 and nuclease protection analyses. Mobility gel shift, methylation interference and DNaseI protection analyses to evaluate DNA:protein interactions.

Major Findings:

1. As with chicken and mammalian species, multiple ets genes are present in Drosophila. Five ets/ets-related genes have been identified and we have been characterizing three of these--D-ets, D-elg and E13B.

2. D-ets was originally isolated in a genomic clone of 10 kb. Restriction enzyme, Southern blot and sequence analyses identified the region most related to the ets gene. This locates to the carboxyl terminal end (last two exons) of the

ets-1 and ets-2 genes (C domain). D-ets is nearly 90% identical to chicken c-ets-1 over this region of 110 amino acids. D-ets encodes a single transcript of 4.7 kb, which is expressed in all developmental stages with higher levels in embryos and pupae. D-ets has been localized to chromosome 2R at position 58A/B using in situ hybridization of polytene chromosome. Several cDNA clones have been isolated by hybridization to D-ets. Sequence analysis of one of these clones has verified that it represents a D-ets transcript; in addition, alignment of the D-ets genomic and cDNA sequence data defines the 5' boundary of a novel D-ets exon. The sequences present in this region predict an open reading frame that contains an amino acid sequence that is not present in any predicted product of any other ets or ets-related gene. Additional cDNA clones have been isolated by PCR utilizing a D-ets specific 3' primer and will be analyzed.

3. D-elg (the Drosophila ets-like gene) was isolated as a 1.4 kb cDNA clone and sequenced. D-elg cDNA encodes a predicted protein of 15.4 kD (134 amino acids) that has a region of 86 amino acids with 72% similarity to the carboxy terminal region of the Drosophila ets-2 gene. Thus, the sequence of elg serves to further define the minimal ets domain. Alignment between elg and all the other characterized ets genes reveals two clusters of highly conserved (invariant) amino acids. These may thus serve to localize those amino acids required for DNA binding. Northern analysis using the D-elg probe detects two transcripts (2.0 and 2.8 kb). The clone was digested with the RsaI restriction enzyme and three fragments corresponding to the 5' and 3' untranslated regions; and the coding ORFs were used as probes. The 2.0 kb transcript hybridized to all three probes, while only the 3' untranslated probe hybridized to the 2.8 kb message. Thus, the 2.0 kb message appears to be the true D-elg transcript. Strand-specific riboprobes confirm this conclusion and demonstrated that the two messages are derived from different DNA strands. Collaborative work with Dr. R. Schulz has shown D-elg to be a maternal transcript that is present at constant levels throughout embryogenesis. D-elg has been localized to 97D on the right arm of chromosome 3.

4. Genomic clones of the D-elg gene have been isolated and are being characterized by restriction enzyme mapping and by sequence analysis. We have subcloned the fragments containing the 5' end of the gene for functional promoter analysis. Southern blot analysis of genomic DNA and λ clones suggest that the D-elg gene is quite small, and sequence analysis has identified a single intron. S1, primer extension, and RNA protection analyses are being carried out to further characterize the 5' end of D-elg.

5. Defined segments of the open reading frame (ORF) of D-elg have been placed into two prokaryotic vectors, pMAL (New England Biolabs) and pGEX-2T (Pharmacia). Successful production of fusion products has been accomplished. Purified D-elg will be utilized as antigen to generate antibodies which will be used in in situ analyses.

6. The third Drosophila gene (E13B) was isolated by hybridization to v-ets. A cDNA that hybridized to this clone has also been isolated. A single E13B message of 2.6 kb is expressed in pupae and adult organisms. Its chromosomal position is on the third chromosome (left arm) at position 66A. Hybridization and sequence analyses are underway to identify the region with homology to ets.

7. P-element vectors have been constructed with both the rosy and the white genes as markers. A human-Drosophila chimeric construct has been made and will be microinjected into the appropriate strain of flies.

Publications:

Pribyl LJ, Watson DK, Schulz RA, Papas TS. D-elg, a member of the Drosophila ets gene family: sequence, expression and evolutionary comparison. Oncogene (In Press)

Watson DK, Pribyl LJ, Smyth FE, Ascione R, Papas TS. Members of the ETS gene family share a unique domain. Miami Short Rep 1991;1:85.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05585-03 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Expression in Colon Carcinoma and Polyposis

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

PI:	C. W. Schweinfest	Sr. Staff Fellow	LMO	NCI
Others:	N. Kondoh	Visiting Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*
Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (K. Henderson); Hellenic Anticancer Institute, Athens, Greece (S. D. Kottaridis)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	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.)*

Colon cancer claims the lives of more than 65,000 people each year in the United States, second only to lung cancer. Surgery remains the only effective means of treatment, and even this is usually only effective when the cancer is discovered early. Patients with an inherited predisposition for developing numerous premalignant polyps (familial polyposis) are at nearly 100% risk of colon cancer by age 40, and early scrutiny of such patients often leads to prophylactic surgery. However, the great majority of patients who will present with colon cancer do not have a prehistory of polyposis that might lead to early detection.

Recently, some oncogenes (ras) and candidate tumor suppressor genes (p53, DCC, MCC) have been implicated in playing a role at various stages of premalignant and malignant progression in the colon adenoma-carcinoma sequence. Therefore, it appears that several, if not many, genes can contribute to progression. The genes that have been implicated thus far have been linked to cytogenetically-observable abnormalities or to fortuitously located restriction fragment length polymorphisms (RFLPs). In order to directly investigate the major alterations in gene expression in polyposis and colon carcinoma, cDNA libraries from matched tumor/normal tissues were constructed. Subtractive cDNA libraries enriched for either tumor-specific or normal-specific cDNAs were then constructed and used to isolate clones differentially expressed in tumor and normal tissues. A number of such clones have been isolated. Some of these correspond to already identified genes whose possible role in colon cancer progression has not been previously suspected (e.g., collagen type VI, laminin-binding protein), while other clones do not correspond to any sequences thus far deposited in various sequence databases. One such clone, IH12, is growth-regulated and is also found to be overexpressed in breast carcinoma.

PROJECT DESCRIPTION

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

C. W. Schweinfest	Sr. Staff Fellow	LMO	NCI
N. Kondoh	Visiting Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

Current effective treatment of colon cancer is limited to surgical intervention at an early stage of progression. Early detection remains a key to effective treatment. Recently, some combinations of chemotherapy have yielded positive results; however, identifying patients which are most likely to respond is difficult. Subtractive hybridization can identify differentially-expressed genes which may play a causal role in neoplastic progression and/or which may be valuable clinical markers for early detection and response to treatment. Subtractive hybridization can also identify potentially important genes for which there is no a priori information (e.g., located near a RFLP, involved in an observable cytogenetic alteration, or a known oncogene). We have used subtractive hybridization to identify such genes.

Methods Employed:

1. RNA is isolated from matched tumor/normal tissues taken from patients with polyps or colon cancer. Cell line RNA is also isolated. mRNA is isolated by two successive rounds of binding and elution prior to use in cDNA synthesis.

2. cDNA libraries are constructed in λZAPII by unidirectional insertion into the vector, such that libraries to be subtracted are inserted in opposite orientations from each other. λZAPII is capable of producing single-stranded phage bearing the cloned DNA--it is used directly in the subtractive hybridization. Subtraction is performed essentially as described in Schweinfest et al. (Genet Anal Tech Appl 1990;7:64-70). Rate enhancing techniques will also be tried.

3. Cell lines from primary colon cancer and a subsequent metastatic tumor from the same patient have also undergone directional cDNA cloning in order to isolate metastatic-specific genes.

4. Differentially-hybridizing cDNA clones from the subtractive library are identified by Southern blot analysis using total cDNA probes from normal and tumor cell RNA.

5. Subtractive library clones are used to probe Northern blots of RNA from matched tissues and well-characterized colon carcinoma cell lines (see below) to allow for positive identification of differentially-expressed genes.

6. Development of sodium butyrate-induced, well-differentiated subclones of colon carcinoma cell line, HT29, as a model for expression of tumor markers.

7. Sequencing of differentially-expressed cDNAs for identification or characterization.

8. Chromosome mapping of previously uncharacterized genes.

9. Transfection of sense and antisense clones of differentially-expressed clones into HT29 and its differentiated subclones.

10. Use of PCR to a) generate large quantities of tumor or normal enriched probes for differential plaque hybridization, and b) quickly clone cDNA ends to generate full-length clones.

Major Findings:

1. Subtractive library formation has enabled us to detect mRNA transcripts whose abundance is less than 0.01%. This has been accomplished using PCR amplified subtractive probes. It is estimated that one or two orders of magnitude of increased sensitivity will be attained when the subtractive technique is utilized in conjunction with rate-enhancement techniques and/or repeated cycles of hybridization and subtraction. At these increased sensitivities, mRNAs present at 1-10 copies per cell should be detectable.

2. Several genes have been found to be differentially expressed in colon carcinoma whose identity has been previously determined, but whose possible role and/or marker status for colon carcinoma has not been previously delineated. The laminin-binding protein and the ribosomal S19 protein genes are more highly expressed in carcinoma than normal. Recently, laminin binding protein expression has been found to be correlated with increased metastasis in some malignant cells.

3. Laminin-binding protein and S19 expression are also correlated with the tumorigenic phenotype (e.g., anchorage-independent growth) in HT29 cells and its differentiated subclones. They are also more highly expressed in the poorly differentiated subclones.

4. A normal-specific clone, the histocompatibility class I gene HLA BW62, has been identified as correlated with well-differentiated HT29 subclones and poor anchorage-independent growth. Class I gene expression has been previously shown to be lower in colon tumors compared to normal tissue. It is also demonstrated to be induced upon contact inhibition.

5. Laminin-binding protein, S19 ribosomal protein, and histocompatibility class I gene, HLA BW62, may be useful tumor markers.

6. Clone IH12 is a tumor-specific clone which hybridizes with a 2.7 kb mRNA. Its sequence does not match anything in the databases. Its expression is tightly growth-regulated and it is also overexpressed in breast carcinoma, but not in bladder carcinoma.

7. Clone 611 is down-regulated in tumor cells and its sequence also does not correspond to any sequence in the databases.

Publications:

Schweinfest CW, Henderson KW, Kondoh N, Nelson P, Papas TS. Alterations in colorectal carcinoma: isolation and identification by subtractive hybridization. Miami Short Rep 1991;1:83.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05587-03 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Structural Analysis of ets-related Genes in Lower Eukaryotes

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

PI:	J. A. Lautenberger	Research Chemist	LMO	NCI
Others:	S. Qi	Visiting Fellow	LMO	NCI
	L. A. Burdett	Staff Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	S. J. O'Brien	Chief	LVC	NCI

COOPERATING UNITS *(if any)*

Department of Chemistry, Duke University, Durham, NC (B. R. Shaw)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.12	2.12	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 frog Xenopus laevis contains two forms of the ets-2 gene (ets-2a and ets-2b), each encoding proteins of 472 amino acids, a number close to that of the human, mouse, and chicken homologs. The percent identity between the Xenopus ets-2a and ets-2b products is 94.1, while that between ets-2a and the other vertebrates is about 68%. The Xenopus sequences are homologous to the other vertebrate ets-2 genes throughout their entire length. Because of the ease of manipulating oocytes and embryos, the Xenopus system should provide a useful means of studying ets-2 function, and results of studies performed with the Xenopus homolog should shed light on the function of this proto-oncogene in humans.

A molecular clone containing sea urchin DNA that hybridized to a v-ets probe was shown to contain an exon of the erg gene. This segment codes for 173 amino acid residues beginning at a 5′ splice site. The first 85 residues share homology with all members of the ets gene family (including the erg, elk, Drosophila E74, mouse PU.1 and ets-1 and ets-2 genes), while the remaining 88 amino acids are homologous only to the human ERG gene. This latter region, designated R, represents a highly conserved erg-specific domain.

Phylogenetic analysis of ets sequences indicates that the family may be divided into three major groups: (1) the ets genes proper including the vertebrate ets-1 and ets-2 genes, and the sea urchin and Drosophila ets genes; (2) the human and sea urchin erg genes; (3) the human ELK, the murine PU.1, and Drosophila ecdysone-induced puff 74E genes. Since each of the groups contain representatives of both vertebrate and invertebrate phyla, it is possible to estimate that the gene duplication events from which they originated occurred at least 500 million years ago.

PROJECT DESCRIPTION

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

J. A. Lautenberger	Research Chemist	LMO	NCI
S. Qi	Visiting Fellow	LMO	NCI
L. A. Burdett	Staff Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI
S. J. O'Brien	Chief	LVC	NCI

Objectives:

The major objective of this study is to use developmental systems to determine the function of the various members of the ets gene family. Two experimental systems, the sea urchin and Xenopus, were chosen because of the ease of experimental manipulation of oocytes and early embryos. These animals fill in a gap between the studies on Drosophila and the higher vertebrates. Both of these models have been extensively characterized by developmental biologists. They offer the possibility of determining the spacial and temporal localization of gene expression in the maturing oocyte and the developing embryo.

Another aspect of this study is the use of phylogenetic analysis to systematically analyze the relationships between the canonical ets genes and the ets-related genes (ERG, ELK, elg, and ecdysone-induced puff 74E). This can be used to establish the evolutionary history of the ets gene family. This might shed insight into two questions, in particular. These are: (1) why do vertebrates have separate ets-1 and ets-2 genes, and when did these genes diverge? and (2) when did the ets-related genes diverge from the canonical ets genes? In particular, did this divergence precede phylogenesis?

This additional knowledge on ets developmental biology and phylogenetic history may provide insight into the mechanisms by which the E26 v-ets sequences influence leukemogenesis and the possible relationships between human ETS genes and human cancer.

Methods Employed:

1. DNA Gel Blot (Southern) Hybridization. DNA was digested with restriction enzymes, electrophoresed on a 1% agarose gel, and transferred to nitrocellulose filters (Southern, J Mol Biol 1975;98:503-17). Hybridizations were performed in 40% formamide/5 X SSC/32.5 mM sodium phosphate, pH 7.5/20 mM EDTA/5 X Denhardt's solution/100 μg/ml salmon sperm DNA. Filters were extensively washed and sequences that hybridized to ets-related probes were visualized by autoradiography. The radiolabeled probes were generated by the random sequence oligonucleotide primer method (Feinberg and Vogelstein, Anal Biochem 1984;137:266-7).

2. DNA Sequence Analysis. Nucleic acid sequences were determined by the dideoxynucleotide chain termination method (Sanger et al., Proc Natl Acad Sci USA 1977;74:5463-7) from templates derived from m13mp18 or Bluescript (Stratagene,

Inc.) vectors. For Bluescript plasmid vectors, single-stranded DNA template was prepared by rescue with the m13 helper phage R408. The polymerase used was Sequenase (US Biochemical), a derivative of the T7 DNA polymerase.

3. Determination of Molecular Phylogeny. The ets sequences were analyzed by several phylogenetic programs including FITCH (Felsenstein, Phylogeny Inference Package (PHYLIP), Seattle, WA: University of Washington, 1984), NJTREE (Saitou and Nei, J Mol Biol 1987;4:406-25), PAUP (Swofford, Phylogenetic Analysis Using Parsimony [PAUP], Champaign, IL: Illinois Natural History Survey, 1984), and Progressive Alignment (Feng and Doolittle, J Mol Evol 1987;25:351-60). FITCH uses the Fitch-Margoliash least squares method (Fitch and Margoliash, Science 1967;155:279-84).

Major Findings:

1. Two distinct molecular clones of the frog Xenopus laevis ets-2 gene were obtained. The nucleotide sequence of the clones revealed that there are two Xenopus ets-2 genes, designated as ets-2A and ets-2B. In contrast to a published report based on cDNA clones that did not contain complete coding regions (Wolff et al., Nucleic Acids Res 1990;18:4603-4), we find that both the ets-2a and ets-2b genes have homology with the amino terminal sequences of the chicken, mouse and human genes. Both Xenopus genes code for proteins of 472 amino acids which is quite close to the length of the chicken (479 aa), mouse (468 aa), and human (469 aa) gene products. The percent identity between the Xenopus ets-2a and ets-2b products is 94.1, while the degree of amino acid identity of ets-2a with the human, mouse and chicken homologs is 68.1, 66.9, and 68.0 percent, respectively. Of the three region domains previously described for the human ETS2 gene, the C region is the most highly conserved between the Xenopus sequence and the other vertebrate sequences, followed by the A region. The B region is the least conserved. However, the homology between the B regions of the Xenopus homologs with the ets-2 B regions from other vertebrates is significant, while there is little homology between these B regions and the ets-1 B regions. Thus, because of the ease of manipulating oocytes and embryos, the Xenopus system should provide a useful means of studying ets-2 function, and results of studies performed with the Xenopus homolog should shed light on the function of this proto-oncogene in humans.

2. A bacteriophage lambda recombinant clone (18EA) containing sea urchin sequences that hybridized to an avian leukemia virus E26 v-ets probe was characterized by restriction mapping and nucleotide sequence analysis. This clone was shown to be distinct from a sea urchin clone, 12E3, previously analyzed in our laboratory (Chen et al., Dev Biol 1988;125:432-40). The nucleotide sequence revealed that clone 18EA was the urchin erg homolog; clone 12E3 had been shown to be the ets homolog. This segment of the sea urchin erg homolog was found to contain an open reading frame containing a coding region beginning at a 5′ splice site that conforms to the consensus pattern observed for vertebrate splice sites. The coding region extends for 173 amino acid residues. The first 85 amino acids share homology with all members of the ets gene family (including the erg, elk, Drosophila E74, mouse PU.1, etc., as well as ets-1 and ets-2 genes), while the remaining 88 amino acids are homologous only with the human ERG gene. This latter region, designated R, represents a highly conserved erg-specific domain.

3. Southern gel blot hybridization was used to investigate the degree of conservation of the sea urchin ets and erg homologs. Both of these genes had been isolated from a library prepared from Lytechinus variegatus sperm DNA from animals collected in Bermuda. We had previously found that the ets homolog hybridized well to RNA from early embryos from the Pacific Ocean sea urchin Strongylocentrotus purpuratus (Chen et al., Dev Biol 1988;125:432-40). Restriction digests were performed on DNA from each of these species, as well as DNA from the sea urchin species Arbacia punctulata (collected on the North Carolina coast). We observed a high degree of hybridization between the L. variegatus ets probe with all three DNAs, while the L. variegatus erg probe hybridized well only with L. variegatus DNA. This suggests that among sea urchins, the ets gene is more highly conserved than the erg gene.

4. The various members of the ets gene family were compared by a variety of phylogenetic programs including FITCH, NJTREE, PAUP, and Progressive Alignment (see Methods Employed for references). The analysis indicated that the family may be divided into three major groups. The first contains the ets genes proper including the ets-1 and ets-2 genes from human, mouse, chicken, and Xenopus, as well as the sea urchin and Drosophila ets genes. The second group includes the human and sea urchin erg genes, and possibly the Drosophila elg gene. The third group contains the human ELK gene, the murine PU.1 gene and the Drosophila ecdysone-induced puff 74E gene. Each of these three groups arose from a common ancestor by at least two gene duplication events. Since each of the groups contain representatives of both vertebrate and invertebrate phyla, it is possible to estimate that these events occurred at least 500 million years ago. The data also suggest that the ets and erg C regions have existed in essentially their present form for at least that amount of time.

5. A computer program was written to assist in performing calculations necessary for the production of oligonucleotide solutions. For an oligonucleotide of a specified sequence, the program will provide all interconversions between spectrophotometric units, mass units, and molar units of either quantity or concentration. The program will determine the factor necessary to dilute a stock solution of a measured absorbance at 260 nm to prepare a working solution of a specified concentration in picomoles/microliter. The program is available in versions for Digital Equipment Corporation VAX computers and IBM-PC compatible personal computers.

Publications:

Chen ZQ, Burdett LA, Seth AK, Lautenberger JA, Papas TS. Requirement of ets-2 expression for Xenopus oocyte maturation. Science 1990;250:1416-8.

Lautenberger JA. A program to assist in the preparation of oligonucleotide solutions. BioTechniques 1991;6:778-80.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05588-03 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Transgenic Mouse Model System for the Ets-1 and Ets-2 Proto-oncogenes Function

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

PI:	A. K. Seth	Visiting Scientist	LMO	NCI
Others:	D. M. Thompson	Biologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Centre for Early Human Development, Clayton, Victoria, Australia (I. Kola)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.52	0.22	0.3

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.)*

Transgenic mice offer a powerful model system for studying the molecular mechanisms of gene regulation during development. Previously reported results with the transgenic model system have shown that the different transgenes can be regulated and expressed appropriately. In an in vitro system using NIH3T3 cells, we have shown that the ets-2 gene has mitogenic and transforming activity. To study the role of the ets gene family in normal developmental processes and tumorigenesis in vivo, we have generated transgenic mice with the ets-2 proto-oncogene linked to a heterologous promoter. The transgenic mice were generated by microinjection of ets-2 cDNA linked to the mouse metallothionein promoter into the pronuclei of one cell embryo. The injected embryo was implanted into the oviduct of pseudopregnant mothers and brought to term. One of the 32 pups was shown to contain the ets transgene by Southern blot analysis of the DNA prepared from the tails. The founder mouse (female) was bred again to produce offspring that contain the ets-2 transgene. The ets-2 heterozygous mice were inbred to produce homozygous mice. The ets-2 homozygous mice appear to develop hydrocephalus and die within two weeks of birth.

PROJECT DESCRIPTION

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

A. K. Seth	Visiting Scientist	LMO NCI
D. M. Thompson	Biologist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

To study regulation of the ets-1 and ets-2 proto-oncogenes under heterologous and normal promoters, to study involvement of the ets-1 and ets-2 in cell growth, development, differentiation and oncogenesis. To obtain better insight into the role of cis-acting ets-1/ets-2/promoter/enhancer elements in the expression and regulation of ets-1/ets-2 proto-oncogenes during embryogenesis.

Methods Employed:

1. Isolation of the ets-2 gene linked to β-actin or mouse metallothionein promoter and analysis of tail DNA. The ets-2 gene linked to the β-actin or metallothionein promoter was isolated from the pGE-1 and pMME-18 vectors by standard techniques. Preparation of high molecular weight DNA from the mouse tails was performed as described previously.

2. Production of ets-2 transgenic mice. Microinjection of fertilized eggs with ets-2 DNA and implantation of fertilized eggs into the oviduct of foster mothers was done in collaboration with Dr. I. Kola of Monash University, Australia.

3. Analysis of tissue RNA. RNA from the transgenic or normal tissues was prepared by grinding directly into the RNAzol solution followed by $CHCl_3$ extraction and ethanol precipitation. The RNA samples were then analyzed by Northern blots using ^{32}P-labeled ets-2 probes.

Major Findings:

1. The ets-2 transgenic mice develop hydrocephalus. We have inbred our ets-2 transgenic mice to develop a homozygous mouse, the homozygous ets-2 mouse appears to have hydrocephalus. In the ets-2 hydrocephalus mice the brain is enlarged, the cranial cavity is fluid-filled, the thymus is atrophic and they have osteomalacia; these mice die within two weeks. Currently, we are testing whether these defects are due to overexpression of the ets-2 gene or due to inactivation of certain mouse genes by integration of the ets-2 DNA fragment in close proximity or directly into the gene.

2. Induction of the ets-2 transgene. Since the ets-2 gene is linked to the metallothionein promoter, we determined the ets-2 RNA expression pattern following induction by heavy metals. Cadmium sulfate was injected subcutaneously into transgenic and control mice and the RNA was extracted after 24 hours and investigated by Northern blots. The transgenic RNA was detected only in testis and not in other tissues. This finding is consistent with earlier results

observed with transgenic mice in which the mouse metallothionein promoter was linked to the IFN gene. It may be possible that liver and other tissues such as brain, intestine and kidney, where the metallothionein promoter has been shown to be expressed constitutively, also express exogenous ets-2 mRNA, but at levels undetectable by Northern blot analysis. Currently, we are investigating the expression of ets-2 mRNA by more sensitive techniques, such as RNase protection and PCR analysis, and are also analyzing the RNA from hydrocephalus mice.

3. Production of transgenic mice with the ets-2 gene linked to the β-actin promoter. Although we were able to generate β-actin ets-2 transgenic mice, they died within 3-7 days. It appears that the overexpression of the ets-2 genes driven by the β-actin promoter is lethal.

4. Construction of the ets-1 promoter β-gal construct. The ets-1 proto-oncogene is expressed mainly in thymus in adult mice. To understand the regulation of the ets-1 gene during embryogenesis, we have constructed a vector that contains the β-gal gene linked to the ets-1 promoter fragment. Production of transgenic mice with this construct will enable us to study the tissue specificity of the ets-1 proto-oncogene cis regulatory elements, as well as allow us to study their expression and regulation during embryogenesis.

Publications:

Chen ZQ, Burdett LA, Seth A, Lautenberger JA, Papas TS. Requirement of ets-2 expression for Xenopus oocyte maturation. Science 1990;250:1416-8.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. In: Patterson D, Epstein CJ, eds. Molecular genetics of chromosome 21 and Down syndrome. New York: Wiley-Liss, 1990;137-68.

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. Prog Clin Biol Res 1990;360:137-68.

Papas TS, Watson DK, Sacchi N, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Kottaridis SD, Ascione R. ETS family of genes in leukemia and Down syndrome. Am J Med Genet 1990; 7(Suppl):251-61.

Seth A, Papas TS. The c-ets-1 protooncogene has oncogenic activity and is positively autoregulated. Oncogene 1990;5:1761-8.

Seth AK, Hodge DR, Kottaridis SD, Thompson DM, Panayiotakis A, Watson DK, Papas TS. DNA binding activity of the ets proto-oncogene family proteins. Miami Short Rep 1991;1:83.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05591-03 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Cellular Function and Regulation of ETS Proteins

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

PI: T. S. Papas Chief LMO NCI

Others: L. F. Fleischman IRTA Fellow LMO NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (R. J. Fisher, K. Midelfort)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.53	0.53	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.)*

Expression of the ETS proteins during the cell cycle has been examined in cells synchronized by centrifugal elutriation or mitotic block using the microtubule-destabilizing agent, nocodazole. Both methods reveal the presence of a hyperphosphorylated isoform of ETS1 during the early mitotic phase of the cell cycle. This isoform exhibits a strong mobility shift in SDS-PAGE and has been observed during mitosis in each of four cell lines (three human T-cell lines and one human astrocytoma line) which express c-ETS1. This isoform is distinguishable from previously described phosphorylated isoforms of ETS1 and appears to arise due to multiple phosphorylations on serine in the domain of the protein encoded by exon 7 of the ETS1 gene. Treatment of unsynchronized cells with the phosphatase inhibitor, okadaic acid, results in a stoichiometric shift of ETS1 to this hyperphosphorylated state, suggesting that cellular phosphatase activity is important for regulation of ETS1. The potential correlation of the different ETS1 phosphorylation states with functional activity of the protein in sequence-specific DNA binding is currently under investigation. Carbachol-induced stimulation of ETS1 phosphorylation in 1321N1 astrocytoma cells is also being studied; the relation to intracellular Ca++ levels and enzymes involved during this stimulation is being compared to the mitotic ETS1 phosphorylation.

PROJECT DESCRIPTION

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

T. S. Papas	Chief	LMO	NCI
L. F. Fleischman	IRTA Fellow	LMO	NCI

Objectives:

The major objective of this project is to study the biological function of the proteins encoded by the c-ETS1 and c-ETS2 proto-oncogenes. Research is focused on the possible role of these proteins during cellular proliferation and signal transduction in T-cells and astrocytes, two cell lineages which express c-ETS1.

Methods Employed:

For studies of ets expression at different phases of the cell cycle, human T-cell lines (CEM, Jurkat and MOLT-4) have been fractionated using centrifugal elutriation. This technique exploits the principle of counterflow centrifugation to rapidly and gently sort cell populations according to size. Since volume increases during passage through the cell cycle, temporally synchronized populations can be obtained in this manner. In other experiments, the microtubule-destabilizing drug, nocadazole, is used to obtain cells blocked specifically in mitosis. In both types of synchronization experiments, cell-cycle stage is confirmed by FACSCAN analysis of DNA content. Ets proteins in staged cells are analyzed by a variety of methods, including metabolic labelling, immunoprecipitation, immunoblotting, peptide mapping and phosphoamino acid analysis. These techniques are also being applied to investigate ets function in cells of astrocytic lineage. Changes in phosphorylation and activity of ETS proteins in response to a variety of neurotransmitters and cytokines are being studied in 1321N1 human astrocytoma and other astrocyte-derived cells. Methods for induction of differentiation in these cells are also under investigation. Changes in intracellular Ca++ (associated with ets phosphorylation) are measured in vivo using fluorescent Ca++-sensitive techniques. Gel retardation assays, which measure binding of ets proteins to specific DNA sequences, are utilized to study this activity under the various conditions and cellular contexts described. Various methods are used to prepare cell extracts for use in the DNA binding assays, including gel chromatography and other protein purification techniques.

Major Findings:

A hyperphosphorylated form of ets-1 has been detected during the mitotic phase of the cell cycle. It is seen in mitotic cells obtained by either method of synchronization used, i.e., elutriation or nocodazole arrest, and has been observed in each of the four cell lines studied which express ETS1 (three human T-cell lines and one human astrocytoma line). This hyperphosphorylated form displays a characteristic strong mobility shift, running at approximately 57 kilodaltons in SDS-PAGE. This isoform is therefore distinguishable from the previously characterized phosphorylated isoforms of ETS1 which migrate at 52 and 53 kD, as well as the nonphosphorylated ETS1 isoforms which migrate at 51 and

42 kD; these are seen at all stages of the cell cycle in elutriated CEM cells, with no significant variation in steady-state level. When cells are released from nocodazole arrest, the hyperphosphorylated isoform disappears within 1-2 hours as the cells complete mitosis and enter the G_1 phase. In elutriated cells, only a small proportion of the ETS1 in cells from the G_2/M fractions is in the hyperphosphorylated form, while in nocodazole-arrested cells, most of the ETS1 is shifted to the 57 kD isoform (as assessed from steady-state level measurements of the protein by Western blotting). This suggests that this isoform is present transiently during early mitosis, before or around the time of the metaphase-anaphase transition. The dramatic shift in mobility observed suggests that a conformational change has occurred in the protein.

We have also found that exposure of (unsynchronized) cells to okadaic acid, a potent, cell-permeant inhibitor of serine/threonine phosphatases, results in a striking shift of virtually all ETS1 in the cell to the hyperphosphorylated state. This finding raises the possibility that some endogenous suppression of phosphatase activity during mitosis may contribute to the ets-1 hyperphosphorylation seen at this stage of the cell cycle. Okadaic acid treatment also results in phosphorylation of the 42 kD isoform, which had not been seen previously. The 42 kD isoform, a product of alternate splicing, has been shown to lack an exon 7-encoded domain which contains the consensus site for Ca++/calmodulin kinase phosphorylation. Peptide mapping of in vivo-labeled CEM cells indicates that the hyperphosphorylation site(s) lies within exon 7, in peptides shared with nocodazole-treated and basally-phosphorylated cells, but not with those found by analysis of the 42 kD phosphorylated product. Phosphoamino acid analysis indicates the presence of phosphoserine in the hyperphosphorylated form. The exon 7-encoded region contains a region of seven serines and one threonine arranged near the Ca++/calmodulin kinase consensus region. Current evidence therefore suggests phosphorylation of some of these serines gives rise to the 52 and 53 kD isoforms, while phosphorylation of additional serine(s) results in the shift to 57 kD. These phosphorylation states differ conformationally and are apparently capable of differential regulation by phosphatase, and possibly also by kinase, activity. This could enable the ets-1 protein to respond to several levels of local Ca++ concentration differentially.

We have found that carbachol stimulates a rapid and dramatic increase in ETS1 phosphorylation in 1321N1 astrocytoma cells; this results in production of mainly the 52 and 53 kD isoforms with some of the 57 kD and contrasts with the predominance of the 57 kD isoform in mitotic 1321N1 cells. Similarly, in T-cell lines, treatment with Ca++ ionophore or other Ca++-mobilizing mitogenic agents results (as shown previously) mainly in production of the 52 and 53 kD isoforms with trace amounts of the 57 kD. We are continuing to investigate the mechanistic basis and functional consequences of the different phosphorylation states observed in ETS1 upon Ca++-mediated stimulation and in the mitotic phase. We are also extending our studies on the role of ETS in astrocytic cells during differentiation and proliferation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05593-03 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Transcriptional Regulation of the Human ETS2 Oncogene

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

PI:	G. J. Mavrothalassitis	Visiting Associate	LMO	NCI
Others:	D. K. Watson	Research Microbiologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD
(G. Beal, R. Patel)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.72	0.72	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.)*

We have previously identified the DNA binding sites of the ETS2 promoter that are involved in the ETS2 transcriptional regulation. We used these motifs to screen cDNA expression libraries, and we were able to isolate a cDNA clone that encodes for a protein which specifically interacts with ETS2 promoter sequences. The 2.4 kb cDNA clone was sequenced and a 328 amino acid open reading frame was determined. The predicted amino acid sequence contains several motifs found in other transcription factors as a leucine zipper, a helix-loop-helix, an amphiphatic region and two acidic domains. In addition, several putative phosphorylation sites can be detected at the carboxy-terminus of the protein. Somatic cell hybrids, Southern analysis and in situ hybridization indicate that the gene is localized on chromosome Xp11.2-p11.3. Northern analysis indicates that the gene is transcribed to a single 3 kb mRNA splice which can be detected in a variety of tissues and cell lines. The protein binds DNA as a dimer and the leucine zipper motif is required for this dimerization. We used protein expressed in insect cells via a baculovirus system to determine the optimal binding sequence by random selection and PCR--CAC/t G/A T/A G. We obtained cell line clones that express the gene under an inducible promoter after transfection of the appropriate constructs and G418 selection. These cell lines were morphologically altered and the transformation was serum and induction dependent. These cell lines could not grow in semisolid media, but did form solid tumors after injection in athymic mice.

PROJECT DESCRIPTION

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

G. J. Mavrothalassitis	Visiting Associate	LMO	NCI
D. K. Watson	Research Microbiologist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The purpose of this investigation is to identify the proteins that interact with the promoter region of the ETS2 oncogene. The identification of such interactions will allow us to characterize the genes that are involved in its transcriptional regulation and that may be associated with abnormalities in human malignancies.

Methods Employed:

1. 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.

2. Subcloning of DNA fragments into appropriate plasmid vectors, as required.

3. Preparation of end-labeled DNA and synthetic oligonucleotide probes corresponding to the ETS2 promoter region using T4-polynucleotide kinase or E. coli DNA polymerase Klenow fragment. Labeled DNAs were purified on acrylamide gels and eluted by diffusion in the appropriate buffer.

5. Detection of DNA protein complexes by mobility shift assays. Labeled DNA fragments from the ETS2 promoter region were incubated with nuclear protein extracts and the complexes were detected by DNA mobility shift on acrylamide electrophoresis.

6. Construction of cDNA libraries in expression vectors (λgt11, λgt28A).

7. Screening of the cDNA libraries with oligonucleotide probes.

8. Deletion analysis and DNA sequencing of cDNA clones.

9. In vitro transcription and translation of the cDNAs and determination of the products by SDS/acrylamide electrophoresis.

10. Northern and Southern DNA and RNA analysis.

11. Cell line transfection and transformation in defined media.

12. In vivo expression of recombinant proteins in insect cells via a baculovirus system.

13. Polymerase chain reaction (PCR) amplification of oligonucleotides that specifically interact with the recombinant protein.

Major Findings:

1. We have identified one cDNA clone that encodes for a protein capable of interacting specifically with the ETS2 promoter regulatory sequences.

2. The 2.4 kb cDNA clone has a 328 amino acid open reading frame which initiates from a methionine surrounded by a translation initiation consensus sequence.

3. The predicted amino acid sequence contains a leucine zipper, a helix-loop-helix, an amphiphatic region, two acidic domains and several phosphorylation consensus sequences.

4. The protein binds DNA as a dimer and the leucine zipper is required for this interaction.

5. The protein can interact with the CAC/t G/A T/A G sequences and has higher affinity for the sequence CACGTG.

6. The gene is transcribed from a single locus on chromosome Xp11.2-p11.3 to a 3 kb mRNA splice. This mRNA can be detected predominantly in brain, ovary, placenta and PBC.

7. The gene was able to transform mouse fibroblasts in low-serum media. The transforming effect was inhibited by serum.

Publications:

Mavrothalassitis G, Beal G, Papas TS. Defining target sequences of DNA-binding proteins by random selection and PCR: determination of GCN4 binding sequence repertoire. DNA Cell Biol 1990;9:783-8.

Mavrothalassitis G, Papas TS. Positive and negative factors regulate the transcription of the ETS2 gene via an oncogene-responsive-like unit within the ETS2 promoter region. Cell Growth Differ (In Press)

Mavrothalassitis G, Watson DK, Papas TS. The human ETS2 gene promoter: molecular dissection and nuclease hypersensitivity. Oncogene 1990;5:1337-42.

Papas TS, Watson DK, Sacchi N, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Kottaridis SD, Ascione R. The ETS family of genes in leukemia and Down syndrome. Am J Med Genet 1990; 7(Suppl):251-61.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05594-03 LMO

PERIOD COVERED

October 1, 1990 to October 25, 1990

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

Suppression of Transformation by Dominant Negative Mutants of H-ras Oncogene

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:	Y. Ogiso	Visiting Fellow	LMO	NCI
	L. Gutierrez	Visiting Fellow	LMO	NCI
	L. S. Wrathall	Microbiologist	LMO	NCI
	R. H. Bassin	Research Microbiologist	LTIB	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.10	0.90	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 project has identified a new class of dominant negative ras mutants which inhibit transformation induced by overexpression of c-H-ras proto-oncogenes in NIH3T3 cells. These transformation-defective mutants were constructed by site-directed mutagenesis of the conserved sequence motif, NKXD, of the ras GTP-binding site.

This project was terminated when Dr. Ogiso left in October 1990.

PROJECT DESCRIPTION

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

T. Y. Shih	Research Chemist	LMO	NCI
Y. Ogiso	Visiting Fellow	LMO	NCI
L. Gutierrez	Visiting Fellow	LMO	NCI
L. S. Wrathall	Microbiologist	LMO	NCI
R. H. Bassin	Research Microbiologist	LTIB	NCI

Objectives:

It is generally believed that ras p21 plays an important role in signaling pathways involved in cellular transformation. Smith et al. demonstrated that microinjection of a neutralizing p21 antibody induced morphological reversion of NIH3T3 cells transformed by activated ras and several tyrosine kinase oncogenes (Nature 1986;320:540-3). However, the effect of microinjected antibody is transient and it is unclear whether morphological reversion is accompanied by loss of other transformation parameters. Therefore, continuous inhibition of ras is necessary for further investigation on the role of ras in cell transformation. For this purpose, we pursued a strategy to construct dominant negative mutations of ras oncogenes in order to obtain cell lines in which the normal ras activities are down-regulated by the mutants. Feig and Cooper reported an H-ras mutant (S17N) which severely inhibited cell growth (Mol Cell Biol 1988;8:3235-43). In this case, however, it is difficult to study the mechanism of growth control using this mutant because it is lethal to NIH3T3 cells. In the course of studying the structure/function relationship of ras proteins, several transformation-defective mutants were constructed in this laboratory which were also deficient in GTP-binding. The major focus of the present study is to identify useful ras mutants which are capable of suppressing ras-related transformation.

Methods Employed:

1. Cells. NIH3T3 cells transformed by an LTR-linked c-H-ras, v-H-ras, v-src, v-fms, v-sis, v-fes, v-mos and a chemical carcinogen (EMS) were grown in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum.

2. Plasmids. Specific amino acid residues of p21 were altered by oligonucleotide-directed mutagenesis. The mutant ras was reconstituted into the pSV_2neo for transfection and G418 selection.

3. Transfection. Transfection of plasmid DNA was performed according to the method of Felgner et al. using a DNA-liposome-mediated gene transfer technique. One day after transfection, cells were trypsinized and split 6-10 times in growth medium containing G418 (400 μg/ml). After 2-3 weeks, G418-resistant colonies were stained by Giemsa and cell morphology was observed under a microscope.

4. Isolation and characterization of revertants. Flat revertants, as well as transformed clones, were isolated from a NIH3T3 cell transformed by a LTR-linked c-H-ras following transfection with ras mutant DNA and selection with G418. The cell lines were twice single-cell cloned. The doubling time, colony-forming

efficiency in soft agar, and the expression of mutant p21 were examined. Susceptibility of a revertant cell line for retransformation was assayed by infection with Harvey sarcoma virus.

Major Findings:

This project was terminated when Dr. Ogiso left in October 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05595-03 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
DNA Topoisomerase I Activity in Retroviruses

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:	K. J. Dunn	Microbiologist	LMO	NCI
	S. K. Ruscetti	Microbiologist	LMO	NCI

COOPERATING UNITS *(if any)* Lab. Cell. Biochem., PRI, Frederick, MD (M. Athanassiou); Ben-Gurion U., Beer Sheva, Israel (E. Priel, S. Segal, M. Aboud); Advanced BioScience Laboratory, Inc., Frederick, MD (M. Roberts, S. Oroszlan)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Microbiology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.00	0.75	0.25

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.)*

Topoisomerases are enzymes which modify the topological state of DNA and have been shown to be involved in DNA replication and transcription. Since the process of retroviral replication, integration and expression are likely to require topological changes in either the integrated or unintegrated provirus, we have begun to determine if topoisomerases play a critical role in retrovirus growth or infection, or if topoisomerases might be a target for the control of retroviral infections.

We had previously described a novel topoisomerase I (topo I) activity associated with various retrovirus and lentivirus particles, and showed that the specific topo I inhibitor, camptothecin (CPT), could block HIV infection of H9 cells in tissue culture. Using spleen focus-forming virus (SFFV) induction of splenomegaly as an in vivo model, we have shown that CPT can block SFFV-induced diseases when coinjected with the virus into susceptible mice. To determine the ability of CPT to affect chronic virus production by infected cells, we treated CF2Th (dog thymus) cells producing equine infectious anemia virus with CPT. Continuous exposure to low levels of the drug resulted in 85-92% inhibition of virus production, as measured by RT released into the culture media, and similar levels of reduction in viral protein production by immunofluorescence and radioimmunoprecipitation analysis.

PROJECT DESCRIPTION

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

D. G. Blair	Supv. Research Chemist	LMO	NCI
K. J. Dunn	Microbiologist	LMO	NCI
S. K. Ruscetti	Microbiologist	LMO	NCI

Objectives:

Retroviruses establish persistent infections as a result of integration of their proviral DNA into the host cell genome. The viral replication cycle from intracellular penetration to final integration and expresson is undoubtedly dependent on numerous topological changes in the viral and host genomes. Since DNA topoisomerases have been shown to be responsible for the induction of topological changes in DNA and to participate in many vital cellular reactions involving DNA, our objective was to investigate whether a topoisomerase activity could be demonstrated in retroviral particles and whether such enzymes are involved in the retroviral life cycle.

Methods Employed:

Standard methods of cell culture, retroviral infection, and reverse transcriptase analysis using synthetic templates were employed. Topoisomerase I was assayed by measuring the relaxation of purified supercoiled plasmids on agarose gels following incubation with viral lysates or viral cores. Standard methods of Northern and Southern analysis, immunoprecipitation, polyacrylamide gel analysis, and Western blot techniques were used.

Major Findings:

1. Coinjection of camptothecin blocks the induction of splenomegaly in SFFV-infected NFS mice. We had shown that CPT blocked the infection of the human T-cell line, H9, in culture at non-cytotoxic doses. Similar experiments showed that infection of NIH3T3 fibroblasts by Mo-MuLV was also inhibited by CPT, although the efficiency of inhibition was not as high as observed for HIV. In order to determine if CPT could also block in vivo infection and subsequent disease induction by retrovirses, we injected 6-week-old NFS mice with SFFV (Friend), together with various concentrations of CPT in DMSO, or with DMSO alone, as controls. Preliminary cytotoxicity experiments indicated that CPT doses of 20 mg/kg or below were not cytotoxic when injected either intraperitoneally (IP) or intravenously. When 6-week-old mice were given IP injections of SFFV, 4/5 showed splenic enlargement at 20 days post-injection (avg. wt. 1.2 g), while mice coinjected with CPT all had normal spleens (0/5) with an average weight of 0.3 g. Similar results were obtained with IV injections. Without drug, 9/10 mice showed enlarged spleens (avg. wt. 1.65 g), while no mice coinjected with virus and CPT showed enlarged spleens (0/10, avg. wt. 0.11 g). These results suggest that CPT may be able to function as an antiviral agent in vivo, as well as in vitro.

2. Camptothecin inhibits the replication of equine infectious anemia virus (EIAV) in chronically-infected CF2Th (dog thymus) cells. We had shown that purified core particles of EIAV, a member of the lentivirinae subfamily of retroviruses, contains a topoisomerase I activity. To further elucidate the potential involvement of topo I and the retroviral life cycle, we examined the effects of

non-cytotoxic doses of CPT on chronically-infected CF2Th/EIAV producer cells. Exposure of producer cells to 0.1-0.01 μM CPT for 1 hr reduced the virus production 32-52% with no effect on cell viability (trypan blue) or cell growth 24-98 hrs post-treatment. When cells were continuously treated with CPT (0.02 M) for 14 days, the virus production was reduced to 10% of control levels. Cell growth was identical to that of control cells treated with DMSO alone. The production of virus particles, as measured by immunoprecipitation of S^{35}-labeled viral particles with an anti-p26 specific antibody, were similarly reduced and immunofluorescent analysis of treated cells indicated that the fraction of cells stainable with the antibody was also reduced to 45% of control levels. These results indicate that EIAV virus production by chronically-infected cells is blocked by CPT by an unknown mechanism.

Publications:

Priel E, Showalter SD, Blair DG. Inhibition of human immunodeficiency virus (HIV-1) replication in vitro by noncytotoxic doses of camptothecin, a topoisomerase I inhibitor. AIDS Res Hum Retroviruses 1991;7:65-72.

Priel E, Showalter SD, Roberts M, Oroszlan S, Segal S, Aboud M, Blair DG. Topoisomerase I activity associated with human immunodeficiency virus (HIV) particles and equine infectious anemia virus cores. EMBO J 1990;9:4167-72.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05607-03 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
HTLV-I Transgenic Mice

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

PI:	J. E. Green	Sr. Research Investigator	LMO	NCI
Others:	L. J. Garrett	Chemist	LMO	NCI
	J. Hyde	Medical Staff Fellow	LG	NCI

COOPERATING UNITS *(if any)*
Johns Hopkins University, Baltimore, MD (J. Schneck)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.55	0.35	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.)*

Transgenic mice containing the HTLV-I tax gene develop tumors of peripheral nerve sheaths, iris, adrenal medulla and salivary gland, myopathy and localized lymph node hyperplasias. The mechanisms leading to these abnormalities remain unclear, but may involve the deregulation of cellular genes important for cell growth and differentiation. The expression of tax in these transgenic mice has been correlated with the induction of the genes for the interleukin-2 receptor (IL-2-R), the granulocyte-macrophage, colony-stimulating factor (GM-CSF), and more recently, nerve growth factor (NGF). Cell lines from the peripheral nerve and salivary tumors appear to secrete significant quantities of IL-6 and perhaps other B-cell-related growth factors, which may account for the lymphadenopathy associated with these tumors. This transgenic mouse system provides an important in vivo model for HTLV-I-induced transformation and tax activation of growth-regulating genes.

PROJECT DESCRIPTION

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

J. E. Green	Sr. Research Investigator	LMO	NCI
L. J. Garrett	Chemist	LMO	NCI
J. Hyde	Medical Staff Fellow	LG	NCI

Objectives:

To study hematologic and neural growth factors and receptors induced in HTLV-I transgenic mice. To determine whether activation of growth factors is involved in the transformation process and other disease processes occurring in these mice.

Methods Employed:

1. Microinjection of mouse embryos, embryo transfer to pseudopregnant female mice and screening of offspring for transgenic incorporation by Southern and PCR analyses.

2. Analysis of transgenic tissues by Northern, Western and in situ hybridization techniques.

3. Histochemical and immunocytochemical analyses of tissue sections.

4. ELISA and functional assays for cytokines; ligand cross-linking studies for receptor assays.

5. Co-transfection of cytokine promoter-CAT constructs with or without tax-producing plasmids into cell lines. Analysis of CAT activity.

Major Findings:

1. HTLV-I tax transgenic mice develop tumors in multiple locations, including peripheral neurofibromas and tumors of the adrenalla medulla, iris and salivary gland.

2. Tax appears to activate multiple endogenous genes in these tumors, including interleukin-2 receptor, granulocyte macrophage colony stimulating factor and nerve growth factor. Activation of these genes may be involved in the transformation process.

3. Tax-induced adrenal tumors appear to alter normal function of neighboring chromaffin cells, such as the down-regulation of neural peptide Y.

4. Tax-transgenic animals develop lymph node hyperplasia localized to regions adjacent to the tumor. This suggests that the tumors may secrete factors which stimulate lymph node hyperplasia. Lymph node hyperplasia is the result of B-cell proliferation.

5. Conditioned medias from tax-transformed transgenic tumor cell lines stimulate B-cell proliferation of normal mouse spleen cells. Tax-induced B-cell growth factors are being characterized.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05657-02 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Basis for the Erythroleukemias Induced by Murine Retroviruses

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

PI:	S. K. Ruscetti	Microbiologist	LMO	NCI
Others:	R. E. Aurigemma	IRTA Fellow	LMO	NCI
	M. Masuda	Visiting Fellow	LMO	NCI
	D. G. Blair	Supv. Research Chemist	LMO	NCI
	N. Vyas	Student Volunteer	LMO	NCI

COOPERATING UNITS *(if any)*
Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (C. Hanson); AIDS Research Center, VA Medical Center, Baltimore, MD (P. Hoffman)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Microbiology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.50	2.00	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.)*

Using three different systems, studies have been aimed at understanding the mechanisms by which retroviruses cause erythroleukemia in mice and identifying the viral and host genes that are crucial for the biological effects observed.

Studies on the acute erythroleukemia-inducing Friend spleen focus-forming virus have concentrated on understanding how the viral envelope protein abrogates the erythropoietin (Epo) requirement of erythroid cells. The protein appears to interact with and trigger the Epo receptor, and studies are in progress to determine if this interaction results in a mitogenic signal like that initiated by Epo.

We have also been studying the effects of another erythroleukemia-inducing virus, the gag-myb-ets-containing ME26 virus, on hematopoietic cell growth. Our results indicate that this virus, which encodes a DNA-binding protein, may be activating the Epo receptor in an immature hematopoietic cell that does not normally express it. The virus, however, may not be directly transactivating the Epo receptor, but may be working through another erythroid-specific gene, GATA-1.

Studies on the third erythroleukemia-inducing virus, Friend MuLV, have been two-fold. We have molecularly cloned a candidate for a host gene, Rmcf-r, that is involved in resistance to early erythroleukemia induced by the virus, and are now testing its biological activity. We have also molecularly cloned a variant of Friend MuLV, PVC-211, which no longer causes erythroleukemia in mice, but which induces a progressive neurodegenerative disease. We are now generating recombinants between PVC-211 and wild-type Friend MuLV in order to localize the regions of the viral genome responsible for inducing either leukemia or neurological disease.

PROJECT DESCRIPTION

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

S. K. Ruscetti	Microbiologist	LMO	NCI
R. E. Aurigemma	IRTA Fellow	LMO	NCI
M. Masuda	Visiting Fellow	LMO	NCI
D. G. Blair	Supv. Research Chemist	LMO	NCI
N. Vyas	Student Volunteer	LMO	NCI

Objectives:

To determine the mechanism by which the envelope protein encoded by the Friend spleen focus-forming virus (SFFV) renders erythroid cells erythropoietin-independent and causes an acute erythroleukemia in mice.

To determine the mechanism(s) by which the ME26 virus alters the growth factor responsiveness of early hematopoietic cells and causes leukemia in mice.

To molecularly clone and characterize the $Rmcf^r$ gene, which confers host resistance to Friend MuLV-induced erythroleukemia.

To determine why a variant of Friend MuLV, which normally induces erythroleukemia in mice, causes an acute neurological disease, but not leukemia, in mice and rats.

Methods Employed:

Molecular cloning of viral genes and construction of viral recombinants; DNA and RNA isolation and Southern and Northern blotting using probes prepared from a variety of cloned DNAs; analysis of viral gene products and other proteins by immune precipitation with specific sera and gel electrophoresis or by Western blotting; receptor binding assays using iodinated ligands; injection of mice with viruses; in vitro culture of leukemic cells from virus-infected mice; in vitro assays to measure the proliferation and differentiation of normal and virus-infected cells to growth factors and hormones; hematopoietic colony assays; transient expression assays to measure transactivation of particular promoter sequences attached to reporter genes.

Major Findings:

1. Studies on Friend spleen focus-forming virus (SFFV). Our studies on the spleen focus-forming virus have concentrated on determining the mechanism by which the viral envelope protein, which is essential for pathogenicity, abrogates erythroid cells from their dependence on the erythroid hormone erythropoietin (Epo). Studies from our laboratory and others have suggested that the SFFV envelope protein may induce Epo-independence of erythroid cells by binding to and triggering the Epo receptor. Li and his colleagues at the Whitehead Institute, Cambridge, MA, reported the co-immune precipitation of the Epo receptor and the SFFV envelope protein, using antisera to either protein, when both were expressed either in NIH3T3 cells or a lymphoid cell line, BaF3. In a more sensitive and

controlled study, we have been able to confirm their results. This was done by immune precipitation of extracts of these cells with an Epo-receptor antibody in the presence or absence of an Epo receptor peptide, followed by Western blotting with a monoclonal antibody prepared against the envelope protein of SFFV. We are currently using the same protocol to study erythroid cells that have been rendered Epo-independent by infection with SFFV. If the SFFV envelope protein is found to interact with the Epo receptor in these cells, we will analyze the importance of such an interaction by studying mutants and recombinants of SFFV that are no longer pathogenic, as well as studying a variant of SFFV that alters the proliferation of erythroid cells without making the cells Epo-independent.

Although SFFV is able to abrogate Epo-dependence in erythroid cells and BaF3 cells expressing the Epo receptor, we have not been able to abrogate other Epo receptor-expressing cell lines with SFFV. These include myeloid cells transfected with the Epo receptor cDNA, as well as myeloid and early hematopoietic cell lines in which the Epo receptor gene is transactivated due to infection with the oncogene-containing ME26 virus. We are currently investigating whether the failure of SFFV to render these particular cells Epo-independent is due to (1) lack of viral expression; (2) differences in the processing of either the Epo receptor or the SFFV envelope protein, resulting in failure of the two proteins to associate, or (3) the absence of a cellular component crucial for initiating the signal transduction pathway activated by SFFV.

We have also initiated studies to determine if SFFV activates the same signal transduction pathway in erythroid cells as that activated by Epo binding to its receptor. Since the pathway activated by Epo is not known, we are comparing Epo- and SFFV-induced erythroid cell proliferation for a variety of potential post-receptor events, including studies to determine if guanine-nucleotide regulatory (G) proteins are involved, if activation of specific protein kinases occurs, and if phosphorylation of specific proteins, including the c-raf encoded protein kinase and casein kinase II, is important. We are also studying whether SFFV, like Epo, can block programmed cell death (apoptosis) in erythroid cells.

2. Studies on the ME26 virus. Amphotropic MuLV pseudotypes of ME26 virus induce a high incidence of leukemia 6-15 weeks after injection into newborn NIH Swiss mice. The leukemic cells in these mice are very immature and resemble stem cells or very early erythroid/myeloid precursor cells. Spleen cells from a high percentage of the leukemic mice can be established in culture as permanent cell lines, but only in the presence of the erythroid hormone, erythropoietin (Epo), suggesting that the ME26 virus is transforming an Epo-dependent erythroid cell or is inducing Epo responsiveness in early hematopoietic cells. The virus does not appear to be activating the Epo receptor in non-erythroid cells by promoter/enhancer insertion because no rearrangement of the Epo receptor gene could be detected using restriction enzymes that cut within 9 kb of the first exon. To test the possibility that ME26 virus, which encodes a DNA-binding protein, is transactivating the Epo receptor in non-erythroid cells, IL-2- and IL-3-dependent hematopoietic cell lines were infected with ME26 virus and grown in medium containing Epo as the only source of growth factor. FDC-P2 cells, an IL-3-dependent myeloid cell line that will not grow in the presence of Epo, became Epo responsive after infection with an amphotropic MuLV pseudotype of ME26 virus, but not after infection with amphotropic MuLV alone. The ME26 virus-infected cells

show a large increase in the amount of Epo receptor mRNA compared to their uninfected counterparts, suggesting that the ME26 virus is activating the Epo receptor in these cells. However, the virus may not be directly activating the Epo receptor gene since ME26 viral DNA cannot activate the Epo receptor promoter after co-transfection into fibroblasts during a transient gene expression assay. Furthermore, GATA-1, an erythroid-specific gene which can activate the Epo receptor promoter, is also activated in the ME26 virus-infected FDC-P2 cells. We are attempting to determine if the ME26 virus can directly transactivate the GATA-1 gene. Our data clearly indicate that ME26 virus can induce Epo responsiveness in mouse hematopoietic cells. We are currently testing a series of mutants and recombinants of ME26 virus, which contain changes in either the myb or ets regions of the genome, to determine the role of particular ME26 viral sequences in this process.

Like most retrovirus-induced leukemias, the leukemia induced by ME26 virus appears to involve multiple steps. While transactivation of the Epo receptor may be responsible for the abnormal proliferation of early hematopoietic cells, other events appear to be necessary to immortalize these cells so that they can be established as permanent cell lines in vitro. We are, therefore, investigating whether the virus integrates into specific sites in the host DNA and activates by promoter/enhancer insertion a gene that is responsible for immortalization. DNAs from the leukemic spleen cells and cell lines show a limited number of viral integrations and we plan to clone virus/host junction fragments in order to identify common sites of ME26 viral integration that may be involved in the immortalization stage of the disease.

3. Studies on the Rmcf gene. The $Rmcf^r$ gene plays a major role in resistance to early Friend MuLV-induced erythroleukemia. One can detect in resistant mice an 80K glycoprotein related to the envelope glycoprotein of mink cell focus-inducing (MCF) viruses that is thought either to be encoded for by this locus or whose expression is influenced by the locus. Susceptible mice carrying the $Rmcf^s$ locus do not express detectable levels of the 80K MCF-related protein. In order to determine if the Rmcf gene is a viral structural gene, we examined Pvu-II-digested DNA from three $Rmcf^r$ strains (DBA/2 and two independent BALB/c x DBA/2 $Rmcf^r$ congenic strains) and three $Rmcf^s$ strains (BALB/c and two independent BALB/c x DBA/2 $Rmcf^s$ congenic strains) for the presence of modified polytropic (mPT) endogenous MuLV envelope gene sequences using a specific oligonucleotide probe. All three $Rmcf^r$ strains examined had a specific band of about 2.9 kb, while the $Rmcf^s$ strains did not, suggesting that these mPT sequences represent a good candidate for the $Rmcf^r$ gene. In order to molecularly clone this gene, we prepared a size-selected genomic library by extracting DNA from the 2.9 kb region of a gel on which Pvu-II-digested DNA from an $Rmcf^r$ congenic mouse was run, and ligated it to lambda gt10 phage vector DNA. A partial clone, which was shown by sequencing to contain the 3′ half of a mPT envelope gene and 3′ flanking mouse genomic sequences, was obtained and, using a probe to the flanking sequences, a DBA/2 ($Rmcf^r$) genomic library was screened. From this library a phage clone with a 16.5 kb insert containing an intact mPT envelope gene was isolated and subcloned into a plasmid vector. Preliminary data indicate that the nucleotide sequence of the envelope region of this clone is almost identical to the reported sequence of the mPT class of envelope genes, except for a few point mutations. Further sequence analysis is in progress. DNA from this clone has been cotransfected with

pSV2hyg^r to Rmcf^s NIH3T3 cells and hydromycin-resistant clones have been isolated. Southern analysis of Pvu-II-digested DNA using the flanking region probe shows the presence of the characteristic 2.9 kb mPT band in these cells. Examination of mPT virus-specific RNA and protein expression is in progress. In order to determine if the cloned DNA, like the Rmcf^r gene, can inhibit MCF virus infection, the transfected cells will be infected with a retrovirus vector bearing the neo^r gene pseudotyped with either Friend MCF virus or Friend MuLV and the efficiency of neo^r gene transduction will be assayed.

4. Studies with the PVC-211 variant of Friend MuLV. Friend MuLV causes an erythroleukemia 4-6 weeks after injection into NIH Swiss or NFS/N mice. When this virus was passaged several times in Fischer rats by Dr. K. Kai in Japan, a variant of the virus emerged which caused a rapidly progressive neurodegenerative disease, but not leukemia, in rats. In collaboration with Dr. Paul Hoffman at the Baltimore VA Medical Center, we have shown that the variant virus, designated PVC 211, also causes a neurodegenerative disease without leukemia in NFS/N mice. The disease is characterized by tremor, weakness, spasticity and a rapidly developing paralysis of the hind limbs. Symptoms develop 17-21 days after intracerebral infection of two-day-old NFS/N mice. Virus replication can be detected in the spleen at seven days post-injection and in brain capillary endothelial cells and pericytes at 14 days post-infection. Glial cell proliferation appears concomitant with endothelial cell infection, but virus cannot be identified within proliferating astrocytes or microglia. Vacuolation and neuronal dropout are apparent in severely-affected animals.

We are very interested in understanding the molecular basis for the differences between wild-type Friend MuLV and the PVC-211 variant that determines the different biological effects of the viruses. Comparison of the viral proteins produced by the two viruses by immune precipitation of pulse-labeled cellular extracts showed no obvious differences. Also, MCF viruses, which are thought to be a crucial intermediate in the development of Friend MuLV-induced erythroleukemia, can be detected in the spleens of mice infected with PVC-211. Thus, the failure of PVC-211 to cause leukemia is not due to the failure of the virus to generate the crucial MCF virus intermediate. In order to compare PVC-211 with wild-type Friend MuLV at the molecular level, we have prepared a molecular clone of PVC-211. Fibroblasts transfected with this clone produce infectious virus, and the virus causes the same rapid neurodegenerative disease, but not leukemia, when injected into newborn NFS/N mice or Fischer rats. The restriction map of the molecularly-cloned PVC-211 is very similar to that of the molecular clone of wild-type Friend MuLV, indicating that no major structural alterations have occurred, such as insertions or deletions. To date, we have sequenced the LTR and part of the env gene of the PVC-211 clone. Sequences in the 3′ end of the env gene were almost identical to that of wild-type Friend MuLV, with the exception that the termination codon is further downstream in PVC-211, which would result in a gene product with an additional amino acid at its carboxyl terminus. The LTR sequences of the two viruses are also very homologous, except for the fact that PVC-211 has a deletion of 74 base pairs overlapping one of the two direct repeat sequences in the enhancer region. In order to determine whether differences in the envelope or LTR sequences of these two viruses are responsible for the ability of PVC-211 to cause neurological disease and for its failure to cause erythroleukemia, we are constructing recombinants between wild-type Friend

MuLV and PVC-211 that will be tested for their ability to cause neurological disease or erythroleukemia.

Publications:

Keller JR, Sing GK, Ellingsworth LR, Ruscetti SK, Ruscetti FW. Two forms of transforming growth factor-β are equally potent selective growth inhibitors of early murine hematopoiesis. Ann NY Acad Sci 1990;593:172-80.

Lu L, Shen RJ, Zhou SZ, Srivastava C, Harrington M, Miyazawa K, Wu B, Lin ZH, Ruscetti S, Broxmeyer HE. Synergistic effect of human lactoferrin and recombinant murine interferon-gamma on disease progression in mice infected with the polycythemia-inducing strain of the Friend virus complex. Int J Hematol 1991;20:126-37.

Lu L, Shen R-J, Zhou SZ, Wu B, Kim YJ, Lin ZH, Ruscetti S, Ralph P, Broxmeyer HE. Efficacy of recombinant human macrophage colony stimulating factor in combination with whole body hyperthermia in the treatment of mice with the polycythemia-inducing strain of the Friend virus complex. Exp Hematol (In Press)

Papas TS, Blair DG, Watson DK, Yuan CC, Ruscetti SK, Fujiwara S, Seth AK, Fisher RJ, Bhat NK, Mavrothalassitis G, Koizumi S, Jorcyk CL, Schweinfest CW, Ascione R. The ETS family of genes: structural analysis, gene products, and involvement in neoplasia and other pathologies. Prog Clin Biol Res 1990;360:137-68.

Ruscetti S. Friend spleen focus-forming virus. In: Young NS, ed. Viruses and the bone marrow. New York: Marcel Dekker (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05658-02 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Role of Ras Oncogenes in Signal Transduction

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. Gutierrez	Visiting Fellow	LMO	NCI
	L. S. Wrathall	Microbiologist	LMO	NCI

COOPERATING UNITS *(if any)*
Laboratory of Molecular Genetics, Cancer Institute, Hokkaido University School of Medicine, Sapporo, Japan (Y. Ogiso)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.50	1.0	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.)*

Oncogenes are an activated version of cellular proto-oncogenes that control cell growth and differentiation. It is believed that these genes participate in signaling pathways. We have identified a dominant, negative ras mutant capable of inhibiting c-ras proto-oncogene function (N116Y mutation of v-H-ras). This mutation is present in one of the consensus sequences conserved in the GTP-binding proteins and is critical in the interaction of ras proteins with the GTP or GDP nucleotides. The N116Y mutant blocks transformation by retroviruses carrying protein tyrosine kinase oncogenes, including v-abl, v-fes/flp and v-fms, but did not affect v-H-ras, v-K-ras and v-bas (for details see Project No. Z01CP05594-03). We have established a series of NIH3T3 cell lines to investigate the biochemical target and signaling pathways mediated by ras. In this study we have found that phosphorylation of a protein kinase C (PKC) substrate (80 kD protein) was lower in cells transfected with the N116Y ras mutant than in normal NIH3T3 cells. Expression of the N116Y ras mutant completely blocked DNA synthesis induced by EGF, but not by other growth factors or serum. Sequestration or inhibition of ras targets could be an explanation for these differences.

PROJECT DESCRIPTION

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

T. Y. Shih	Research Chemist	LMO NCI
L. Gutierrez	Visiting Fellow	LMO NCI
L. S. Wrathall	Microbiologist	LMO NCI

Objectives:

The major emphasis of the current study is directed toward elucidating the signal transduction pathways in which ras protein is involved, as well as the mechanism of transformation by this oncogene. Previous studies, by this and other laboratories, with ras mutants defective in transformation have shown that the mutants can interfere with the normal activity of the ras protein and, in some cases, they can be lethal to the cell system. Based on this information, we have produced a system in which we have been able to block the signal transduction pathway for c-H-ras. Now, we will have to elucidate the molecular mechanism by which ras functions in the signal transduction pathway. Attempts will be made to identify cellular targets for ras, as well as to elucidate the interaction of the ras pathway with other oncogenes.

Methods Employed:

1) Expression of the N116Y ras mutant in the transfected cell lines was analyzed by looking at the protein level by immunoprecipitation with specific anti-ras antibodies, but also by looking at the mRNA by Northern blot analysis.

2) Protein kinase C (PKC) activity was measured in vivo by monitoring the phosphorylation of an 80 kD protein (specific substrate for PKC) after stimulation with the phorbol ester TPA.

3) Total protein kinase C activity in vitro was determined by phosphorylation of a peptide specific for PKC. This was done in an assay where enzyme activity was measured in cytosol and membrane fractions from the different cell lines.

4) [^{3}H]-thymidine incorporation was used to measure DNA synthesis after cell activation by different growth factors.

Major Findings:

We have described a novel ras mutant (v-H-ras N116Y) whose expression in NIH3T3 cells slows down cellular growth and induces morphological reversion of transformed cells overexpressing c-H-ras, but not v-H-ras. Using transfected NIH3T3 cell lines (6Y3 and 6Y6), and a revertant cell line, F33, that expresses the N116Y ras mutant, we have been looking at the biochemical features of the mutant protein and its interference in the signal transduction pathways mediated by ras.

First, we look for the expression of the N116Y ras mutant in the different cell lines. We did this by SDS gels, but we could not differentiate between the N116Y ras mutant and the endogenous c-H-ras, as the molecular weight is the same. Northern blot analysis showed that only the three transfected cell lines (6Y3, 6Y6 and F33) present two extra bands for the N116Y mRNA, with an approximate size of 6.5 kb and 3.5 kb that were not present in the parental cell lines.

The fact that F33 is defective in transformation suggests an interference of the N116Y ras mutant over the normal c-ras activity. This down-regulation could be the result of interference in one of several of the pathways in which ras is involved. To study this effect and to learn when this interference takes place, we have been looking for activation or inhibition of signal transduction pathways by different growth factors. One of these pathways in which ras was shown to be involved is the protein kinase C (PKC) pathway. We have looked at this system by monitoring the phosphorylation in vivo of an 80 kD protein that is a specific substrate for PKC. Looking at this system, we found that phosphorylation of 80 kD protein was lower in the cell lines expressing the N116Y ras mutant (6Y3, 6Y6 and F33) than in the parental cell lines (NIH3T3, 18A that overexpresses c-H-ras) after TPA stimulation. This reduction in the phosphorylation of 80 kD was not due to the absence of the substrate, because when we looked for the 80 kD protein, we found the same level of this protein in the different cell lines. In order to determine if there was a deficiency in the enzyme activity, we measured the PKC activity in an in vitro assay. We found an increment in the total activity of the PKC in the cells expressing the N116Y ras protein, and this was corroborated by protein analysis on a Western blot, where an increment in the amount of PKC protein was observed in the cells carrying the ras mutant. More experiments need to be done to determine if the interference is on the availability of the 80 kD substrate or at the enzyme level.

It has been shown that ras protein is essential for induction of DNA synthesis. Response to mitogenic stimulation was measured by [^{3}H] thymidine incorporation and with this system we could discriminate different pathways stimulated by specific growth factors, such as insulin, IGF-I, PDGF, EGF, CSF-I. In these experiments, we found that 3T3, 6Y3 and F33 cell lines had similar responses to the different growth factors, except for EGF where 6Y3 and F33 cells were not stimulated. Increasing concentrations of EGF did not overcome the inhibitory effect on DNA synthesis observed in 6Y3 and F33 cell lines. It seems like this growth factor pathway is completely blocked by the presence of the N116Y ras mutant. Experiments are in progress to investigate if this interference in the EGF pathway is at the receptor level or at targets leading to ras activation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05664-01 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Expression in Ovarian Neoplasia

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

PI:	C. W. Schweinfest	Sr. Staff Fellow	LMO	NCI
Others:	P. S. Nelson	Biotechnology Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*
New York University Medical Center, New York, NY (R. Demopoulos)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.45	1.45	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.)*

Ovarian carcinoma is the most frequent cause of death from gynecological malignancies in the Western world. It ranks fifth overall as a cause of death from cancer in women, with over 12,000 deaths per year, and an estimated 20,000 new cases will be diagnosed in 1991. Most cases are diagnosed at an advanced stage due to a paucity of clinical symptoms until late in disease progression. Prognosis is poor, with an overall five-year survival of about 30%.

Recently, tumor-specific allele losses of chromosomes 6, 11, and 17 have been reported, as well as abnormalities on chromosomes 1 and 3. Amplification of the erbB2 oncogene on chromosome 17q occurs in 30% of cases, and other oncogenes, including K-ras and myc, have been discovered to be amplified in a few ovarian tumors and cell lines. The loss of tumor-suppressor genes at certain chromosomal loci may also be important in the pathogenesis of ovarian carcinoma, as it has been shown to be in other human tumors.

In order to directly investigate the molecular genetic aberrations leading to altered gene expression in ovarian neoplasia compared to normal tissue, cDNA libraries from matched tumor/normal tissues were constructed. Tumor minus normal and normal minus tumor libraries were constructed to enrich for tumor-specific and normal-specific cDNAs, respectively, with the aim of identifying genes critical in the process of carcinogenesis (tumor promoter genes), or for maintaining the normal state (tumor suppressor genes).

In addition, ovarian carcinoma samples are analyzed for the presence of altered known tumor-suppressor genes, such as p53 and the retinoblastoma gene, Rb. Direct sequencing of PCR-amplified segments may show sequence abnormalities contributing to the inactivation or malfunction of these genes.

PROJECT DESCRIPTION

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

C. W. Schweinfest	Sr. Staff Fellow	LMO	NCI
P. S. Nelson	Biotechnology Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

Isolate and identify genes which are differentially expressed in ovarian carcinoma vs. normal ovary. Some of these genes may play a causal role in tumor formation and progression, whereas others may play a less direct role and serve primarily as useful clinical markers. This is of particular value since ovarian carcinoma is often asymptomatic until later stages when the prognosis is poor. The possible involvement of known anti-oncogenes, p53 and Rb, will also be investigated.

Methods Employed:

1. Directional cDNA library cloning from matched tissues (tumor and normal) will be prepared for subseqeunt subtractive hybridization. As per the related colon cancer project, we expect to be able to detect differentially-expressed mRNAs whose abundance is lower than 0.01%.

2. DNAs from several ovarian tumors and cell lines have been prepared and will be used as templates for PCR analysis of p53 and Rb mutations. Hot spots for mutations exist which should facilitate primer design.

Major Findings:

1. Patient tissues and some cell lines have been analyzed by Northern blot hybridization to see if any genes differentially expressed in colon carcinoma (see Project Z01CP05585-03 LMO) are also differential among ovarian carcinomas. No consistent correlations have been observed.

2. RNA isolations from some tissues have yielded sufficient high-quality mRNA. cDNA library constructions have begun.

3. Some DNA templates for PCR analysis have been prepared.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05665-01 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Modulation of ETS Function by Protein:Protein Interactions

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

PI:	D. K. Watson	Research Microbiologist	LMO	NCI
Others:	G. J. Mavrothalassitis	Visiting Associate	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (R. Patel, F. Smyth)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.40	0.40	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 human genome contains more than six ets-related genes (ETS1, ETS2, ERG, ERGB, ELK1, ELK2). In addition to having unique chromosomal locations, members of this ever-expanding gene family have unique patterns of expression. To understand the function(s) and mechanisms responsible for the functional differences between these genes, we intend to identify genes whose products are able to interact with ETS proteins. To achieve this goal, constructs containing defined segments of the open reading frames (ORF) from the human ETS1 and ETS2 genes have been placed into prokaryotic vectors that allow for overexpression and rapid purification of the fusion protein produced by each construct. These vectors contain defined protein cleavage sites that allow for isolation of the unfused protein. These proteins will be labelled and utilized as probes to identify gene products (and the appropriate genes) with which they are able to interact. This methodology will be applied to other genes and will allow us to begin to dissect the role(s) of the ETS genes in the complex network of gene regulation and cellular signal transduction.

PROJECT DESCRIPTION

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

D. K. Watson	Research Microbiologist	LMO	NCI
G. J. Mavrothalassitis	Visiting Associate	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

To identify genes whose products interact with the ets and ets-related proteins. To characterize these genes and to analyze the functional relationships between them and the ets gene family.

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. Dephosphorylation by incubation with CIP (calf intestinal phosphatase).

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Specific DNA fragments were obtained by electroelution or by extraction from agarose.

3. Preparation of DNA probes using purified DNA by nick-translation using E. coli DNA polymerase and DNaseI. Alternatively, probes were prepared utilizing random primers and DNA polymerase. Strand-specific probes prepared by transcription of defined primers (riboprobes) or by asymmetric polymerase chain reaction.

4. Preparation of nitrocellulose nylon 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 ets-related sequences by hybridization of ets-specific probes to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the procedure of Benton and Davis (Science 1977;196:180-2). Isolation of plasmid DNA from colonies lifted from plates by the method of Grunstein and Hogness (Proc Natl Acad Sci USA 1975;72:3961-5).

7. Subcloning of isolated DNA fragments into appropriate plasmid vectors, as required.

8. DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol 1980;65:499-560) and/or Sanger (Proc Natl Acad Sci USA 1977; 75:5463-7). In addition, uniquely labeled DNA will be sequenced following RNA-directed primer extension. Preparation of nested (sequential) deletions employing exonuclease III and mung bean nuclease.

9. Total cellular RNA from cultured cells or tissues was prepared by either the LiCl/urea, RNasol, or guanidine isothiocyanate 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 gels and Northern analysis.

10. Construction of cDNA library: Double-stranded cDNA was prepared from polyA+ RNA and ligated into λgt10, λgt11, λZapII, or unizap vector DNA for amplification.

11. Cloning of ets-genes into prokaryotic vectors capable of overexpression of inserted DNA. Isolation of proteins to be used as antigens and for functional studies.

12. Controlled expression of ets genes in eukaryotic vectors, using constructions with regulatable promoters. Transfection of eukaryotic vectors in mammalian cell lines for identification of expressed product(s) and for analysis of possible biological activity associated with the construct.

13. Identification of promoter and regulatory sequences present in genomic clones of the cellular homologs of oncogenes. To include nucleotide sequence analysis and functional assays using expression of defined reported genes, such as chloramphenicol acetyltransferase (CAT). DNA:protein binding evaluated by mobility gel shift, methylation interference and DNaseI protection analyses.

14. Nuclease protection assays to define the 5′ and 3′ ends of transcripts. Verification by sequence analysis of cDNA clones and by primer-extension of mRNA.

15. Polymerase chain reaction (PCR) to analyze tissue-specific splicing of the ets genes.

16. S^{35}-methionine cell labelling and preparation of protein extracts. Cells (5×10^5/ml) are starved in methionine-free media for 1 hr, followed by incubation with S^{35} methionine for 1-4 hr. Cells are washed with PBS (plus Ca^{+2} and Mg^{+2}) and cytoplasmic and nuclear proteins are prepared from fractionated cells.

17. Fractionation of proteins by polyacrylamide gel electrophoresis and visualization by staining with CBB/silver. Transfer of proteins by electro-blotting to nitrocellulose paper followed by Western or Southwestern blot analysis.

18. Labelling of proteins by iodination or biotinylation. Purified fusion proteins or unfused proteins (50-100 μg) are labelled to high, specific activity with I^{125} (Iodobeads, Pierce), as described (Markwell, Anal Biochem 1982;125:427). Alternatively, protein can be biotinylated (BRL).

Major Findings:

1. Subclones containing human ETS1 or ETS2 cDNAs were digested with defined restriction enzymes and appropriate fragments were cloned (after filling in/blunting where necessary) into pMAL (New England Biolabs) or pGEX-2T (Pharmacia) prokaryotic expression vectors.

2. Induction of pMAL/ETS1 (D6, D11, D18) and pMAL/ETS2 (R2, R3) constructs with IPTG and purification of fusion products on amylose columns. Cleavage of purified fusion products by the specific protease factor, Xa, and isolation of unfused protein.

Additional constructs in pGEX-2T vector-induced and proteins purified on glutathione-Sepharose. Site-specific cleavage by Thrombin and isolation of purified proteins.

3. Fused proteins bound to appropriate resin were incubated with labelled cell extracts (prepared from CEM, COLO320 and P3HR1 cells). Identification of proteins whose binding was specific (i.e., present only when appropriate constructs were used). This approach is used to define conditions required for specific binding.

4. Unfused or fused expressed proteins will be labelled with I^{125} or biotin. These proteins will be utilized to screen λgt11 expression libraries constructed in the laboratory under conditions determined, as described above. Prior to library analyses, cell extracts from several cell lines will be fractionated on polyacrylamide gels and transferred to nitrocellulose. These blots will be incubated with labelled infusion/fusion proteins to further define conditions required for recognition of protein interaction. If necessary, modifications of protocol (e.g., inclusion of reversible cross-linker prior to washing of blot to stabilize weak interactions) will be employed. The cDNAs encoding the proteins of interest will be isolated and characterized by sequence analysis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05666-01 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Functional Analysis of Ets-related Sequences by Microinjection

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

PI:	L. A. Burdett	Staff Fellow	LMO	NCI
Others:	J. A. Lautenberger	Research Chemist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)* Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (R. J. Fisher); Basic Research Program, Advanced BioScience Laboratories, Inc., Frederick, MD (G. Vande Woude)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.87	0.87	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.)*

We have characterized a molecular clone of the Xenopus laevis ets-2 gene that was isolated from an oocyte cDNA library. We have observed that injection of antisense oligonucleotides homologous to the ets-2 sequence into oocytes led to degradation of the mRNA and blocked hormone-induced germinal vesicle breakdown. The ets-2 product is thus required for the meiotic maturation of Xenopus oocytes. The specificity of this phenomena was demonstrated by the rescue experiment showing that GVBD could occur after injection of ets-2 mRNA into antisense-injected oocytes. Preliminary data indicate that the inhibition of GVBD is due to lack of MPF. In addition, oocytes injected with antisense oligos to Xenopus ets-1 show inhibition of GVBD, indicating that the product of this gene also might be required for oocyte maturation.

PROJECT DESCRIPTION

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

L. A. Burdett	Staff Fellow	LMO	NCI
J. A. Lautenberger	Research Chemist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The major objective of this study is to use the well-characterized developmental system of Xenopus to determine the function of the ets gene family. Xenopus is an excellent system since it is very well characterized and the oocytes are easily manipulated. Microinjection analysis can be done to determine biological function. This can be done with proteins or messenger RNA to observe the effects of overexpression of protein. Alternatively, the effect of loss of function may be determined by the injection of antisense oligonucleotides or antibodies.

Methods Employed:

1. Microinjection of Antisense Oligonucleotides into Xenopus Oocytes. Stage VI oocytes were collected from a Xenopus ovary by dissection and microinjected with 60 nl of a solution containing one or two oligonucleotides (1 mg/ml each) in 88 mM NaCl. The injected oocytes were then incubated at 22°C in 1 X modified Barth solution (MBS; Gurdon and Wilkes, Methods Enzymol 1983;101:370-86). For the analysis of oocyte maturation, the oocytes were treated with 5 μg/ml progesterone in 1 X MBS four hours after microinjection. After incubation overnight at 22°C, they were fixed in 5% trichloroacetic acid and examined for GVBD as described (Sagata et al., Nature 1988;335:519-25).

2. Rescue of Ets-2 Function. In vitro transcribed RNA was synthesized with T7 polymerase (Stratagene). The RNA was analyzed by RNA gel blot (Northern) analysis and, in addition, was in vitro translated (Promega) and analyzed on a polyacrylamide gel (Laemmli). Oocytes injected with antisense ets oligos (as described above) were injected a second time with ets-2 in vitro transcribed RNA. The second injection was done four hours after the antisense injection to insure degradation of native RNA (as observed by RNA gel blot analysis). The oocytes were treated with 5 μg/ml progesterone and analyzed for GVBD after overnight incubation at 22°C.

3. MPF Assay. The presence of MPF was assayed for by homogenizing oocytes in extraction buffer (Yew et al., Mol Cell Biol 1991;11:604-10). After centrifugation the crude extract was injected into oocytes and assayed for GVBD without the addition of progesterone.

4. Monoclonal Antibody Production. A full-length Xenopus ets-2 sequence was cloned into expression vector, pJL16. A full-length Xenopus protein was expressed in E. coli and purified for antigen. Monoclonal antibodies were prepared by R. Fisher. These monoclonal antibodies were assayed by Western blot analyses and immunoprecipitation.

Major Findings:

Microinjection of Xenopus Oocytes with ets-2 Antisense Oligonucleotides. Previous experiments have shown that the injection of Xenopus ets-2 antisense oligonucleotides into immature oocytes induce degradation of ets-2 mRNA. Upon addition of the natural inducer, progesterone, to oocytes injected with antisense oligonucleotides, maturation, as observed by germinal vesicle breakdown (GVBD), was inhibited.

The specificity of the inhibition of GVBD by ets-2 antisense oligos was further established by a rescue experiment. In vitro transcribed ets-2 mRNA was injected into oocytes previously injected with antisense ets-2 oligonucleotides. The in vitro transcribed RNA was injected four hours after injection of the antisense oligonucleotides, allowing adequate time for the degradation of the endogenous ets-2 RNA and subsequent degradation of the excess oligonucleotides. GVBD was found to occur in 35% of the oocytes rescued with human ETS2 RNA. This was specific to the ETS2 RNA, as a control RNA did not exhibit the rescue of GVBD. Interestingly, the use of human mRNA in a Xenopus system shows the highly conserved function of ets-2 through evolution.

Xenopus oocytes injected with ets-2 antisense oligonucleotides that did not undergo GVBD after the addition of progesterone were found to lack the maturation promoting factor (MPF). Crude extracts from antisense injected oocytes did not stimulate GVBD when injected into oocytes, while crude extracts of sense-injected or progesterone-treated oocytes injected into immature oocytes did induce GVBD.

Experiments involving the injection of Xenopus oocytes with antisense ets-1 oligonucleotides indicate that ets-1 in Xenopus may have a similar function to ets-2. Ets-1 antisense oligonucleotides have been found to inhibit GVBD in progesterone-induced oocytes. Ets-1 antisense oligonucleotides from various locations in the gene have been used, but inhibition of GVBD in all cases is not as high as with the ATG spanning ets-2 antisense oligonucleotides. As with antisense ets-2 inhibition of GVBD, antisense ets-1 inhibition of GVBD is also due to lack of MPF.

A monoclonal antibody (MAb) raised against bacterially-expressed Xenopus ets-2 protein is currently being characterized. The MAb has been found to be of the IgM class and it reacts by Western analysis to the E. coli purified protein. Currently, it has not been shown to react with the native Xenopus ets-2 protein by Western analysis or immunoprecipitation. It is possible that this is because the level of ets-2 protein in Xenopus oocytes is extremely low. Purification of the crude MAb preparation is underway.

Experiments are currently underway to determine if ets-1 mRNA or mutant ets-2 can rescue inhibition of GVBD by ets-2 antisense oligonucleotides. It is hoped that the functional domains can be defined using this technique.

Publications:

Chen ZQ, Burdett LA, Seth AK, Lautenberger JA, Papas TS. Requirement of ets-2 expression for Xenopus oocyte maturation. Science 1990;250:1416-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05667-01 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

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

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

PI:	T. S. Papas	Chief	LMO	NCI
Others:	J. M. Mariano	Biologist	LMO	NCI

COOPERATING UNITS *(if any)*
Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (R. J. Fisher)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.02	0.02	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.)*

Recombinant DNA technology has greatly increased our ability to study gene structure and function. This technology can now be applied to monoclonal antibody (Mab) production. Monoclonal antibodies facilitate biochemical analysis of proteins by their highly-specific recognition of a single epitope. Conventional methods for generating Mabs are not capable of efficiently surveying the induced antibody response to a given antigen. For example, an individual animal has 5-10,000 different B-cell clones, each capable of producing unique antibodies to an antigen. However, with the current cell-fusion technique, only a few hundred different antibodies can be produced. Recombinant DNA technology allows the immunoglobin variable region genes to be amplified, which provides for the generation of a large cDNA library using the bacteriophage lambda-immuno-zap expression vectors. This library is much easier to access than hybridomas produced from cell fusion. Screening is also greatly enhanced, considering immunoglobin gene products of at least 50,000 clones or 1-10,000,000 antibodies can be readily examined in one day, compared to screening hybridomas, which is labor-intensive, time-consuming and expensive. Once produced, these expression vectors can be transfected into mammalian cells or used for making transgenic mice.

Currently, we have cloned the heavy and light chain genes of the pan-ets (T-7) antibody directed against the human ETS2 oncoprotein. The light chain has been success-fully inserted into the lambda-Lcl expression vector and a library has been constructed. Work continues with progress to construct the heavy chain library.

PROJECT DESCRIPTION

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

T. S. Papas	Chief	LMO NCI
J. M. Mariano	Biologist	LMO NCI

Objectives:

The first objective of this project was to verify the technique using the well-characterized monoclonal antibody, T-7. Immediate objectives include cloning the antibodies already existing to the proteins encoded by the human proto-oncogenes, ETS1 and ETS2; they include E-44, U-244, A77 and R-57. Second, to generate and clone the antibodies in mice already immunized against specific peptide sequences of the ets protein. Once constructed, these vectors will be transfected into mammalian leukemic cell lines, including CEM and Jurkat, using the bacteriophage lambda Zap vector. They will also be used to produce transgenic mice to characterize the function of ets in vivo.

Methods Employed:

The antigen used in preparing the antibody clones are peptide sequences from the ets protein and were provided by the Midland Peptide Synthesis Laboratory. BALB/c mice were immunized with these peptides. Messenger RNA is extracted from mouse spleen or existing hybridomas and a DNA copy of all transcripts is made via the PCR, according to primers specific for the heavy and light chain variable regions. The cDNA is then ligated into its respective vector and packaged. A library is created for both the heavy and light chain variable regions. A combinatorial library of both chains is constructed from the individual libraries. Positive clones will be identified using the respective peptide sequence as the selector.

Major Findings:

A cDNA library was constructed from the T-7 hybridoma cell line. The T-7 cDNA was translated in vitro and was shown to produce antibody which is active against the human ETS2 oncoprotein. The cDNA was then used with the light chain primers to generate a light chain library. Work has been slow because of the need to optimize PCR conditions for the heavy chain primers. However, recent PCR trials have been successful and the heavy chain library is under construction. Thus, our first objective is nearly complete.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05668-01 LMO

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanism of Suppressor Gene Deletions and Mutations in Breast Cancer

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

PI:	A. K. Seth	Visiting Scientist	LMO	NCI
Others:	H. Li	Visiting Fellow	LMO	NCI
	A. Panayiotakis	Special Volunteer	LMO	NCI
	D. M. Thompson	Biologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*
Hellenic Anticancer Institute, Athens, Greece (S. D. Kottaridis)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.58	1.38	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.)*

One out of every ten women develops breast cancer, and it is the leading cause of death for women between the ages of 35-50 in the United States. In some Asian countries, the incidence of breast cancer is much lower than in the U.S., which may be due to their dietary practice. Dietary fat has been linked with the increased risk of breast, colon and prostate cancers. Epidemiologists have estimated that 75% of all cancer deaths can be avoided in the United States by early detection of neoplasms. Currently, mammography is recommended for the early detection of breast cancer; however, more than 60% of women over 40 have never had a mammogram. Chromosomal deletions at various locations in different types of cancer have been reported and include 17p12-17p13.3 (breast, brain, colon, lung); 5q and 18q (colon); and 11p (Wilms' tumor). Novel genes from a few of these regions have been isolated, which also led to the identification of p53 as a suppressor gene, and a target of deletions or mutations in colon, breast or lung cancer. p53 is a nuclear phosphoprotein that is expressed at very low levels in normal cells, whereas the transformed cells contain mutated p53 at extremely high levels. Inactivation or mutation in p53 protein is associated with a variety of neoplasia. In order to study the role of p53 in breast cancer, we studied a large number of tumors and normal tissue for point mutations and inactivation of the p53 gene. Since early detection is very important in the treatment of cancer, we have initiated a program to clone novel breast tumor-specific markers, as well as study the expression of known markers to detect asymptomatic neoplasms.

PROJECT DESCRIPTION

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

A. K. Seth	Visiting Scientist	LMO NCI
H. Li	Visiting Fellow	LMO NCI
A. Panayiotakis	Special Volunteer	LMO NCI
D. M. Thompson	Biologist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

To identify deletions or mutations in the p53 gene in familial breast cancer, isolate novel breast tumor-specific markers by screening subtraction libraries made from tumor and normal tissue, and study the expression of tumor markers in breast cancers.

Methods Employed:

Isolation of genomic DNA; preparation of mRNA and cDNA; PCR amplifications; purification of DNA fragments from gels; DNA sequencing by dideoxy chain termination technique, gel electrophoresis, Northern blotting, and preparation of DNA probes by nick-translation.

Major Findings:

1. Amplification of p53 gene exons. High molecular weight DNA was prepared by previously described procedures. Oligonucleotides corresponding to intron sequences were synthesized and used to amplify the exon sequences on the PCR machine. The amplified products were run on a gel and the appropriate bands were purified for DNA sequencing.

2. Mutations in the p53 gene in breast cancer. To determine the mechanism of p53 inactivation that occurs in breast cancer, purified DNA fragments corresponding to exons 5, 6 and 8 were subjected to DNA sequencing by the dideoxy chain termination technique. However, the tumor and normal samples we have sequenced so far did not show point mutations or deletions in the p53 gene. Previously, it has been suggested that multiple chromosomes are involved in breast tumors. Therefore, it is likely that suppressor genes other than p53 also play a major role in breast cancer.

3. Expression of the c-erbB-2 and estrogen receptor in primary breast carcinomas. mRNA from 14 tumor tissues and 3 breast tumor cell lines were evaluated for the expression of the c-erbB-2, estrogen receptor and estrogen-responsive gene, pS2. C-erbB-2 amplification was observed in 12% of the tumors, whereas the estrogen receptor was amplified only in 6% of the tumor samples. The pS2 estrogen-induced marker was found to be low in almost every tumor and cell line tested. Presently, we are determining the correlation between the estrogen receptor and c-erbB-2 amplification with a number of clinicopathological parameters and also are analyzing a large number of tumor samples.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05669-01 LMO

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Mechanism of Transcriptional Activation by the Human ETS Family Proteins In Vitro

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

PI: T. S. Papas Chief LMO NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Biochemistry, Program Resources, Inc., Frederick, MD (K. Midelfort, R. J. Fisher)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.08	0.08	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 novel member of the human ETS oncogene family, ERG, has recently been identified. A specific antibody was produced in rabbits with erg protein expressed and purified from E. coli. With the antibody, a 52 kD nuclear protein, with a half-life of 21 hours, encoded by the ERG gene was identified in human cell lines. This protein was detected in very limited types of cells; it was phosphorylated after PMA treatment, and bound specifically to PEA3 oligonucleotides. The results suggested that the erg protein is a new ETS gene family member of the specific DNA binding protein which may mediate signals transmitted from the membrane.

To further characterize ets oncogene products as transcriptional activators, this project will establish an in vitro transcription system, which will allow us to study 1) how ets proteins and basic transcriptional factors interact with each other to promote RNA polymerase II, and 2) how ets protein and other transcriptional activators cooperate to organize cellular response to stimuli, such as growth factors, oncogenes, etc.

PROJECT DESCRIPTION

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

T. S. Papas Chief LMO NCI

Objectives:

To characterize the proteins encoded by the human ETS proto-oncogenes as transcriptional activators, and to study the mechanism of ets protein-mediated trans-activation process in vitro.

Methods Employed:

The accumulating evidence has suggested that the products of ets-family proto-oncogenes are transcriptional activators that activate mRNA transcription through the PEA3 motif.

Gene transcription by RNA polymerase II is a complex process involving multiple components. The basic factors required for promotor-specific transcription have been identified: TFIIA, TFIIB, TFIID, TFIIE, and TFIIF. TFIID is a TATA-binding protein, and the only factor which binds to DNA. These basic factors and RNA polymerase II form on the promoter a pre-initiation complex. There are other factors which are required for initiation only at certain promoters. These are promoter-specific activators which bind to specific DNA sequences in their respective target promoters.

The nuclear fraction from eukaryotic cells contains factors that impart a promoter specificity to RNA polymerase II. With purified factors, it has been revealed how these factors are assembled in an ordered fashion in a complex to initiate specific transcription. Recent studies have further shown that the promoter-specific activator may interact specifically with one of the basic transcription factors in the assembly process of the pre-initiation complex.

The products of ets-family proto-oncogenes appear to represent a transcription activator which may bind the PEA3 motif of different promoters. This project will establish an in vitro-specific transcription system which is activated by proteins of ets-family genes. We will study how ets-family proteins as promoter activators activate basic transcription machinery.

Various promoters, such as interleukins, contain the PEA3 motif. The transfection experiments have revealed that the products of the ets proto-oncogene trans-activate the polyomavirus (Py) enhancer and the long-terminal repeat of HTLV-I. Py enhancer mediates transcriptional activation by serum growth factor, the tumor promoter TPA, or oncogenes. PEA3 and transcriptional factor AP-1 (c-Jun) appear to be responsive elements in these growth stimuli. We will establish in vitro transcription systems under the control of the Py enhancer and others. Cooperative interactions between transcriptional activators and mechanisms of signal transmission from membrane will be analyzed in an in vitro system.

To reconstruct the transcription system in vitro, the nuclear extract from HeLa cells will be used as a source of transcriptional factors, since this system has been successfully used for in vitro assay. The transcriptional factors will be purified with phosphocellulose, FPLC MonoQ cationic exchange, FPCL Superose 12 and

6, and DNA cellulose columns according to the published methods. During purification steps, each factor will be measured by complementary assays using column-fractionated preparations. Calf thymus DNA will be used for non-specific transcription assay of RNA polymerase II. DNA constructs for specific transcription reaction will be constructed in pMClneo (Stratagene), which contains the neomycin-resistant gene preceded by the HSV thymidine kinase (tk) promoter. The HSV-tk promoter has successfully directed expressions of regulated genes in mammalian cells. Monomers and multimers of PEA3 will be inserted at the EcoRI site in the tk promoter. DNA fragments from XhoI and SalI will be used for transcription assay. Erg, ets-1, and ets-2 proteins will be obtained from KG1 and CEM cells by phosphocellulose column purification and immunoprecipitation.

DNA fragments from constructed pMClneo will be immobilized on streptavidin Sepharose. The purified basic factors and ets-family gene products will be assembled on immobilized DNA and isolated. This will establish sequence and specific interaction of factors to form a functional initiation complex.

Major Findings:

Recently, a novel gene named erg was identified in human COLO320 cells (colon tumor-derived). When screened with the human ets-2 cDNA, two transcripts of 5.0 kb (erg1) and 3.2 kb (erg2) were found. Expression of cDNA for the two transcripts resulted in synthesis of polypeptides of approximately 41 and 52 kD. Their nucleotide sequences showed that erg2 differed from erg1 by a coding frameshift at the amino terminus, resulting in an insertion of 99 amino acids at the amino terminus.

We prepared a polyclonal antibody against human erg protein. With erg-specific antibody, this study identified and characterized the protein products of the human ERG oncogene.

1. **ERG-Specific Antibody.** The specific antibody to erg protein was produced in rabbits with purified 7446 protein (^{58}Val - ^{325}Thr of human erg2 protein) expressed in E. coli. After IgG reactive to contaminating bacterial proteins were removed with the proteins from E. coli before induction for 7446 proteins, the erg-specific IgG was purified on protein A-Sepharose.

2. **Identification of Erg-Gene Protein Product in Human Cell Lines.** A major protein was identified with antibody from ^{35}S-methionine-labeled proteins of KG1 (myelogenous leukemic cells) and Molt-4 (immature T-lymphocytes).

a. It has a molecular mass of 52 kD which matched exactly with one calculated from cDNA.

b. It was not immunoprecipitated with antibody when 7446 protein was present.

c. It was also precipitated with Pan-ets monoclonal antibody which was produced against the common peptide for proteins of the ets oncogene family.

d. It was not recognized by monoclonal antibodies which were specific to other members of ets oncogene products--ets-1 (E44) and ets-2 (U244).

e. The erg antibody recognized an in vitro translation product from cDNAs of the erg1 and erg2 gene. When the erg proteins which were immunoprecipitated from either KG1 cell lysates or translation products of erg2 cDNA were hydrolyzed with V8 protease, identical peptides were produced at the equivalent kinetics.

These results proved that a 52 kD protein which was identified with antibody was a product of the human c-erg2 gene.

3. **Characterization of Human Erg Protein.** The half-life of the erg protein was 21 hours in KG1 cells, which was found to be relatively long compared to c-ets-1 ($t_{1/2}$=2 hours) and c-ets-2 ($t_{1/2}$=20 minutes).

With analyses by Western blot and immunoprecipitation, erg protein was localized in the nuclear fraction both from KG1 and Molt-4 cells, and was extracted from the nuclei with a high concentration of salt (>0.2M).

The erg protein was phosphorylated, as shown with c-ets-1 and c-ets-2. PMA-stimulated phosphorylation, suggesting a function of erg protein as a nuclear target for signal transduction. However, unlike ets-1 and ets-2 proteins, Ca^{++} ionophore did not increase phosphorylation of the erg protein. Further study will identify sites of phosphorylation and relate them to DNA binding and transactivating activity of the erg protein.

From the nuclear extract of KG1 cells, the erg protein was first purified in the eluate with 0.3M NaCl on a phosphocellulose column. A subsequent gel filtration column chromatography showed that the erg protein was isolated as a monomer. Thus purified, erg protein bound to PEA3 oligonucleotides by DNA retardation assay. Cold PEA3 oligonucleotides, but not mutant PEA3, competed with ^{32}P-PEA3 for binding of erg protein. The complex was shifted with erg-specific antibody, suggesting that erg protein bound specifically to the PEA3 site of DNA. It has been suggested that the DNA-binding domain is located in the C-terminal part of the protein, which is highly conserved among ets gene family members. Binding of the ets-1 protein to the PEA3 motif is highly specific. Similar binding of the erg protein to the PEA3 site may further support the notion that ets gene products are transcriptional activators which recognize a common DNA sequence.

The highest level of erg2 protein was found in KG1 cells, and 1/3-1/4 of that in Molt-4 cells. These levels paralleled the ones of mRNA found in these cells. Even though mRNA was identified in COLO320 cells (colon tumor-derived), erg protein was not detectable. Erg1 protein was not detected in any of these cells. No erg protein was found in other types of cells, including CEM and H9 (T-lymphocytic), HEL and K562 (erythroid cell line), HL60 (myelomonocytic), or Jurkat (mature T-cell). The restricted expression may suggest that the erg gene is involved in differentiation and maturation of hematopoietic cell lineage.

The KG1 cell is an acute myeloid leukemia cell line that retains its myeloid characteristics and forms granulocytic colonies in response to human CSF. To study functions of the erg gene, we will test expressions of the erg gene during colony formation of KG1 cells after treatment with human CSF in established cell lines for various stages of hematopoietic differentiation or in blood cells from leukemia patients.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05670-01 LMO

PERIOD COVERED

November 5, 1990 to September 30, 1991

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

Functional Analysis of Murine ets-1 and ets-2 Genes in Transgenic Mice

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

PI:	J. E. Green	Sr. Research Investigator	LMO	NCI
Others:	D. K. Watson	Research Microbiologist	LMO	NCI
	L. J. Garrett	Chemist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA (D. Kappes, S. Tonegawa)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.42	0.62	0.8

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.)*

Analysis of the ets-1 and ets-2 genes has revealed much molecular and biochemical information. The functions of these proto-oncogenes during development, differentiation and transformation remains to be determined. The transgenic mouse system will be utilized to study specific functions of the murine ets-1 and ets-2 genes. Four approaches are being studied: first, 5′ regulatory regions for the ets genes will be defined using varying lengths of the 5′ regulatory regions controlling expression of the lacZ reporter gene; second, mice will be made incapable of producing ets-1 by mutating this gene through the use of homologous recombination in embryonic stem cells; third, the specificity of developmental and differentiating functions of the ets genes will be analyzed by altering the control of their expression through the use of chimeric genes in which the cDNA of one gene is expressed using the regulatory control elements of the other gene. Finally, further functional information will be obtained by studying the effects of the overexpression of ets-1 and ets-2 in various tissues of transgenic animals by misdirecting the expression of these genes through the use of heterologous promoters.

PROJECT DESCRIPTION

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

J. E. Green	Sr. Res. Investigator	LMO NCI
D. K. Watson	Research Microbiologist	LMO NCI
L. J. Garrett	Chemist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

To define the regulatory control regions of the ets-1 and ets-2 genes, their pattern of developmental, tissue-specific expression and functional properties through the use of the transgenic mouse approach.

Methods Employed:

1. Isolation of DNA fragments from appropriate λ and plasmid vectors, as required. Insertion of DNA fragments into appropriate plasmid vectors to produce hybrid ETS gene constructs.

2. Isolation of DNA fragments for microinjection into mouse embryos by sucrose gradient centrifugation.

3. Microinjection of DNA fragment into 1-day gestation mouse embryos, reimplantation into pseudopregnant mice and screening of offspring by PCR and Southern analysis to identify founder mice containing the appropriate transgene.

4. Electroporation of embryonic stem (ES) cells and selection of neomycin-resistant clones. Screening of neomycin-resistant clones for homologous recombination by PCR and Southern analysis. Implantation of ES cells into mouse blastocysts and implantation into pseudopregnant mice.

5. Breeding of transgenic mice for propagation of founder series. Breeding of mice to homozygosity.

6. Determination of the tissue-specific and developmental pattern of expression of the transgene by RNA, Western, in situ hybridization, histochemical and immunocytochemical analyses.

7. Analysis of variant phenotypes.

Major Findings:

Various hybrid genes of ets-1 and ets-2 have been made.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05671-01 LMO

PERIOD COVERED

November 5, 1990 to September 30, 1991

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

Gene Therapy for HIV and HTLV-I

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

PI:	J. E. Green	Sr. Research Investigator	LMO	NCI
Others:	J. A. Lautenberger	Research Chemist	LMO	NCI
	D. G. Blair	Supv. Research Chemist	LMO	NCI
	S. Qi	Visiting Fellow	LMO	NCI
	V. Evtushenko	Guest Researcher	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Biology and Biophysics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.27	1.27	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 unique approach to treating HIV and HTLV-I infections has been initiated using a system of gene therapy which provides "intracellular vaccination." The herpes thymidine kinase (HSV-tk) gene has been placed under the regulatory control of the HIV-LTR, which includes the TAR regulatory sequence in an MSV packaging-defective viral vector containing a selectable marker for neomycin resistance. Stable 3T3-derived cell lines containing this construct and packaging-defective virus have been produced. Studies will determine whether the HSV-tk enzyme will be synthesized in response to the HIV Tat trans-acting protein. Cells containing the HSV-tk enzyme will selectively metabolize specific drug analogues (such as acyclovir) to a toxic metabolite, which will specifically kill those dividing cells. Thus, any Tat-producing cell infected by HIV can be selectively destroyed, while leaving a normal, uninfected stem cell population to replenish the lost T-cells. Studies will be conducted to establish cell lines stably transformed with the HIV-LTR-HSV-tk construct. The HIV-LTR-HSV-tk T-cell lines will be infected with live HIV virus. Toxicity response to acyclovir will be established for live virus infection. Gene therapy utilizing human cells will be tested in SCID mice. The HIV-LTR-tk construct in the defective retroviral vector will infect and integrate into human bone marrow cells. Resistance to HIV infection with acyclovir administration will be tested in vivo. Similar studies for HTLV-I will be conducted.

PROJECT DESCRIPTION

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

J. E. Green	Sr. Research Investigator	LMO	NCI
J. A. Lautenberger	Research Chemist	LMO	NCI
D. G. Blair	Supv. Research Chemist	LMO	NCI
S. Qi	Visiting Fellow	LMO	NCI
V. Evtushenko	Guest Researcher	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

To determine whether "intracellular immunization" using an LTR-herpes simplex viral thymidine kinase (HSV-tk) construct is feasible as an approach to antiviral gene therapy for HIV and HTLV-I infections.

Methods Employed:

1. Isolation of appropriate DNA fragments from plasmid DNA. Insertion of DNA fragments into appropriate plasmid vector containing packaging-defective retroviral genomes.

2. Transfection of LTR-HSV-tk vector into 3T3-derived packaging cell lines to make ecotropic and amphotropic packaging-defective virus and cell lines with stable integration of recombinant viral genome. Selection of appropriate cell lines using neomycin.

3. Determination of viral titer production from individual transfectant colonies.

4. Production of T-cell lines containing stably integrated viral vector with LTR HSV-tk using neomycin selection.

5. Confirmation of intact viral integration using PCR and Southern analysis.

6. In vitro analysis of cell death following induction of HSV-tk by cotransfection of a Tat-producing plasmid and reporter gene or live virus infection with the addition of acyclovir.

7. In vivo analysis of therapeutic response to live infection using SCID mice implanted with cells containing the LTR HSV-tk construct.

Major Findings:

The defective viral construct was expressed as an ecotropic pseudotype following transfection into the ecotropic packaging line, ψCRE. These stocks were used to infect the amphotropic packaging cell line, ψCRIP and infected single cell-derived colonies were isolated following selection with the drug, G418. These cells were individually tested for the level of virus production and levels from $10\text{-}10^5$ infectious virus/ml were detected.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05672-01 LMO

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Analysis of Cellular Factors Affecting Retrovirus Infection and Growth

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:	K. J. Dunn	Microbiologist	LMO	NCI

COOPERATING UNITS *(if any)*: Recombinant DNA Laboratory, Program Resources, Inc. (T. G. Wood, D. J. Prusak); Department of Molecular Biology, Scripps Clinic and Research Foundation (J. A. Elder, T. R. Phillips)

LAB/BRANCH: Laboratory of Molecular Oncology

SECTION: Microbiology Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.55	0.05	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.)*

Retrovirus replication has been shown to require both viral and cellular components, and cellular factors or functions are known to restrict retrovirus infection and growth. This occurs most frequently at the level of virus attachment and penetration, but other post-penetration effects have also been described. Understanding cellular mechanisms of viral control may be useful in our attempts to develop antiviral agents which affect human retroviruses, such as HIV and HTLV-I. The goal of this project is to characterize the role of cellular factors in the growth and restriction of retrovirus infection. Previously, we had reported that RD114 restriction in feline cells does not occur at the level of the proviral LTR, since reporter genes linked to the LTR function efficiently in both restrictive and permissive cells. We have extended our analysis of the mechanism of restriction of growth of RD114 virus in feline fibroblasts, and can show that the expression of endogenous RD114-related mRNAs correlates with the restricted phenotype. Additionally, restricted cells express RD114 glycoprotein molecules of ~gp85 kD instead of the expected gp70 following RD114 transfection or infection. Interaction between the RD114 receptor and the modified gp70 molecule is probably responsible for the restricted ability of RD114 virus to spread in feline fibroblasts. We have also studied the growth of feline immunodeficiency virus (FIV) in the cat brain-derived cell line, G355, and have shown that certain strains of the virus are fusigenic and cytotoxic for these cells. Interference analysis indicates that FIV utilizes a receptor on G355 cells which is different from the ones used by feline endogenous and exogenous viruses. Cell fusion is enhanced by the presence of replicating C-type viruses and is further enhanced in cells transformed by the mos-containing Mo-MuSV. The fusion of MSV-transformed G355 cells provides an easily quantifiable biological assay for virus infectivity in an adherent cell line.

PROJECT DESCRIPTION

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

D. G. Blair	Supv. Research Chemist	LMO NCI
K. J. Dunn	Microbiologist	LMO NCI

Objectives:

To determine cellular mechanisms of retroviral regulation and inhibition in the RD114/feline fibroblast system. To study the mechanism of infection and replication of FIV in non-hematopoietic cells. To analyze cell factors involved in virus infection, provirus synthesis and integration as a model for HIV infection of non-hematopoietic cells in man.

Methods Employed:

Standard methods of cell culture and retroviral infection; reverse transcriptase assays of virus release; standard techniques of DNA, RNA and protein analysis; Southern and Northern analysis of DNA and RNA. Protein analysis using metabolic labeling techniques, immunoprecipitation and Western blot analysis of viral and cell proteins; indirect immunofluorescent analysis of viral proteins using monoclonal and polyclonal antisera.

Major Findings:

1) RD114 restriction in feline embryo fibroblasts correlates with the expression of an 85 kD glycosylated form of the envelope glycoprotein. The feline endogenous virus, RD114, in normal cat cells is restricted at the surface by a complex protein competition for the RD114 receptor. We have previously shown, by chloramphenicol acetyl transferase (CAT) constructs, that negative regulation at the LTR level was not responsible for the restriction of RD114 virus in feline cells. Transfection of RD114 proviral DNAs showed that feline cells could properly transcribe, translate, process and package RD114 virus. However, it was found that the envelope glycoprotein was ~gp85 in restricted cells, instead of the expected gp70. Subsequent experiments showed that feline cells could easily be infected by RD114 virions containing gp70 env molecules, but not by virions containing gp85 molecules. These results suggested that RD114 virus failed to spread in feline fibroblasts because of the altered envelope protein. This observation is supported by Hirt extract data and abrogation of restriction in feline cells by pre-treatment with tunicamycin.

2) The feline immunodeficiency virus variant, 34TF10, replicates and is capable of inducing fusion in the cat brain-derived cell line, G355, and fusion is enhanced in mos-transformed derivatives. Feline immunodeficiency virus (FIV), a lentivirus isolated from domestic cats, replicates preferentially in feline T-lymphoblastoid cells and induces a fatal AIDS-like syndrome in cats. Some FIV variants have been shown to replicate in the cat brain-derived cell line, G355. When we compared the effect of FIV (34TF10) on G355 and the Mo-MuSV transformed variant, PG4, we observed higher levels of fusion in PG4 cells than in their G355

parent. Analysis of viral stocks grown on cat PBL or the established cat line, CFRK, indicated that these stocks contained moderate levels of an infectious C-type virus which we identified as RD114. Stocks of RD114-free 34TF10 FIV were prepared by transfecting G355 cells with cloned FIV, and these could be shown to induce fusion of both G355 and PG4 cells, as well, although the size and number of fusions could be increased if RD114 or FeLV were added. Neither pure or mixed FIV virus induced fusion of feline fibroblast or CFRK cells, consistent with previous reports which suggested that the G355 cells express unique properties, perhaps related to its cat brain origin. Interference analysis of pure FIV stocks indicated that production of RD114 or FeLV did not block infection and subsequent fusion by FIV, suggesting that FIV utilizes a different receptor than either of these two viruses.

To further characterize the potential effects of transformation on FIV toxicity and growth, we "supertransformed" PG4 cells and transformed G355 cells using helper-free stocks of Mo-MuSV. These cells showed increased sensitivity to FIV-induced cell fusion, suggesting that transformation and/or mos expression alters the sensitivity of cat brain cells to FIV.

These results indicate that G355 cells provide a tissue culture model and assay system for FIV functions, and provide a means to study cell toxicity, infectivity and other aspects of lentivirus biology in an animal and cell culture model for HIV.

ANNUAL REPORT OF

LABORATORY OF MOLECULAR VIROLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

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) plans and conducts research on transforming proteins to define their properties in normal cells and their potential role in the development of neoplasms; and (4) investigates the mechanisms by which cellular gene expression, viral gene expression, and interactions between viruses may influence the transformation of cells. A summary of some of the major efforts follows:

HTLV-I/HIV

Human T-cell lymphotropic virus type I (HTLV-I) is associated with two human diseases, adult T-cell leukemia (ATL) and tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM). Lymphocytes from both ATL and TSP/HAM patients display abnormal proliferation properties in culture. Purified, soluble Tax1 protein can be taken up by, and stimulate proliferation of, uninfected phytohemagglutinin-treated human peripheral blood lymphocytes (PBLs). Tax1 was 40-70% as active as IL-2 in stimulating proliferation of PBLs. Heat-inactivated or chloroform-extracted Tax1 failed to stimulate PBL proliferation. Tax1 could not stimulate proliferation of PBLs in the absence of PHA. Following an initial round of cell division, Tax1-treated PBLs exhibited prolonged sensitivity to interleukin-2-induced proliferation. These results indicate that Tax1 can stimulate lymphocyte proliferation in culture and imply that extracellular Tax1 may be involved in the spontaneous proliferation of TSP/HAM lymphocytes and the interleukin-2-dependent proliferation of ATL lymphocytes.

Purified HTLV-I Tax1 protein can be taken up by 70Z/3 lymphoid cells and localized in both the nuclear and cytoplasmic compartments. Introduction of the Tax1 protein into the growth medium of 70Z/3 cells resulted in the rapid and transient induction of NF-kB binding activity in the nuclear fraction. Tax1 activation of NF-kB was not sensitive to either staurosporin or prolonged stimulation with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, suggesting that Tax1-dependent NF-kB activation did not require the protein kinase C pathway. Purified Tax1 did not directly increase NF-kB binding activity in 70Z/3 cytoplasmic extracts, suggesting that NF-kB induction may require cellular factors. Western blot and competitive radioimmunoassays demonstrated that Tax1 protein was present in the tissue culture media of HTLV-I-transformed cell lines. These results show that extracellular Tax1 may regulate cellular gene expression in noninfected cells.

Human T-cell lymphotropic virus type I (HTLV-I) encodes a 40-kD nuclear trans-activating phosphoprotein, Tax1. The analysis of Tax1 mutants demonstrates that deletion of amino acids 2 through 59 of Tax1 (Δ58 Tax1) decreased transactivation of the HTLV-I long terminal repeat 10- to 20-fold. S1 nuclease analysis revealed that the decrease in transactivation of the HTLV-I long terminal repeat was associated with a lack of RNA synthesis. In contrast to the nuclear localization

of the wild-type Tax1 protein, indirect immunofluorescence analysis demonstrated that Δ58 Tax1 failed to localize to the nucleus, indicating that the Tax1 nuclear localization sequence is present in amino acids 2 through 59. Cotransfection of wild-type and mutant Tax1 DNAs resulted in the cytoplasmic accumulation of Tax1 and a 25-fold decrease in transactivation. Although several possibilities which may account for this transdominant effect exist, we favor a model in which Δ58 Tax1 interferes with the nuclear localization of wild-type Tax1 protein, perhaps by forming heterodimer complexes.

The c-ets-1 proto-oncogene and the related c-ets-2 gene encode related nuclear chromatin-associated proteins which bind DNA in vitro. To investigate the possibility that Ets1 and Ets2 are transcriptional activators, we analyzed the ability of these proteins to transactivate promoter/enhancer sequences in transient co-transfection experiments. The HTLV-I LTR was found to be transactivated by both Ets1 and Ets2. An ets-responsive sequence between positions -117 and -160 of the LTR was identified by analyses of a series of 5′ deletion mutants of the HTLV-I LTR and of dimerized versions of specific motifs of the LTR enhancer region. Ets1 was found to bind specifically to the -117 to -160 regulatory sequence. These results show that Ets1 and Ets2 are sequence-specific transcriptional activators. In view of the high level expression of Ets1 in lymphoid cells, Ets1 could be part of the transcription complex which mediates the response to Tax1 and the control of HTLV-I replication. More generally, Ets1 and Ets2 could regulate transcription of cellular genes.

The fission yeast Schizosaccharomyces pombe has been utilized as a model system to study transcription factors. Yeast replicating vectors containing HIV-1 LTR-CAT (chloramphenicol acetyl transferase gene) and deletions of the HIV-LTR promoters were transfected in S. pombe cells. In a separate experiment, an SV40 promoter transcribing the TAT gene was used in a cotransfection assay. Our results show HIV-LTR promoter is functional in yeast and RNA synthesis initiates from two sites. One of the 5′ ends of the RNA corresponds to the 5′ end of the HIV-mRNA observed in viral cells. Deletion analysis of the promoter suggests NF-kB binding sequences are required for promoter activity. However, the TAT gene product failed to transactivate transcription.

CMV

Human cytomegalovirus (CMV) infection has been associated with several neoplastic diseases, including prostatic carcinoma, adenocarcinoma of the colon, cervical carcinoma, and Kaposi's sarcoma. The morphological transforming region II (mtrII) of CMV Towne has been localized to a 980 base-pair fragment containing three putative open reading frames (ORFs) of 79, 83, and 34 amino acids (aa). Homologous regions exist in strains Towne, AD169 and Tanaka. However, Tanaka mtrII has reduced transforming activity when compared to Towne and AD169. A 79 aa (ORF) present in both Towne and AD169 is truncated in Tanaka, indicating that it may be crucial for efficient transformation.

The promoter activity of the 980 bp mtrII DNA fragment has been investigated. The 980 bp colinear DNA acts as a promoter when inserted in the sense orientation with reference to the ORF but not in the opposite orientation. In further experiments, two distinct promoter regions were detected by separately testing the left-hand 440 bp and the right-hand 540 bp fragment. Promoter activity was shown to be similar in all three strains, implying that this is not a factor in transformation, emphasizing the importance of the 79 aa ORF.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05254-10 LMV

PERIOD COVERED

October 1, 1990 through September 30, 1991

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

Regulation of HTLV-I Gene Expression

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

P.I.:	J. Brady	Chief, VTBS	LMV NCI
Others:	S. Gitlin	Senior Staff Fellow	LMV NCI
	P. Lindholm	Research Associate (MSF)	LMV NCI
	S. Marriott	Biotechnology Fellow	LMV NCI
	M. Radonovich	Biologist	LMV NCI
	J. Duvall	BioLab Technician	LMV NCI

COOPERATING UNITS *(if any)*

Institute Curie Biologie, Paris, France (Dr. Jacques Ghysdael)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	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 T-cell lymphotropic virus type I (HTLV-I) encodes a 40-kD nuclear trans-activating phosphoprotein, Tax1. The analysis of Tax1 mutants demonstrates that deletion of amino acids 2 through 59 of Tax1 (Δ58 Tax1) decreased transactivation of the HTLV-I long terminal repeat 10- to 20-fold. S1 nuclease analysis revealed that the decrease in transactivation of the HTLV-I long terminal repeat was associated with a lack of RNA synthesis. In contrast to the nuclear localization of the wild-type Tax1 protein, indirect immunofluorescence analysis demonstrated that Δ58 Tax1 failed to localize to the nucleus, indicating that the Tax1 nuclear localization sequence is present in amino acids 2 through 59. Cotransfection of wild-type and mutant Tax1 DNAs resulted in the cytoplasmic accumulation of Tax1 and a 25-fold decrease in transactivation. Although several possibilities which may account for this transdominant effect exist, we favor a model in which Δ58 Tax1 interferes with the nuclear localization of wild-type Tax1 protein, perhaps by forming heterodimer complexes.

The c-ets-1 proto-oncogene and the related c-ets-2 gene encode related nuclear chromatin-associated proteins which bind DNA in vitro. To investigate the possibility that Ets1 and Ets2 are transcriptional activators, we analyzed the ability of these proteins to transactivate promoter/enhancer sequences in transient co-transfection experiments. The HTLV-I LTR was found to be transactivated by both Ets1 and Ets2. An ets-responsive sequence between positions -117 and -160 of the LTR was identified by analyses of a series of 5′ deletion mutants of the HTLV-I LTR and of dimerized versions of specific motifs of the LTR enhancer region. Ets1 was found to bind specifically to the -117 to -160 regulatory sequence. These results show that Ets1 and Ets2 are sequence-specific transcriptional activators. In view of the high level expression of Ets1 in lymphoid cells, Ets1 could be part of the transcription complex which mediates the response to Tax1 and the control of HTLV-I replication.

PROJECT DESCRIPTION

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

John Brady	Chief, VTBS	LMV NCI
Scott Gitlin	Senior Staff Fellow	LMV NCI
Paul Lindholm	Research Associate (MSF)	LMV NCI
Susan Marriott	Biotechnology Fellow	LMV NCI
Michael Radonovich	Biologist	LMV NCI
Janet Duvall	BioLab Technician	LMV NCI

Objectives:

The main focus of this project is to understand and characterize viral and cellular transcription factors which regulate HTLV-I gene expression.

Methods Employed:

Recombinant DNA techniques, eukaryotic and bacterial expression vectors, eukaryotic tissue culture, transient gene expression assays, DNA and RNA and protein analyses, indirect immunofluoresence techniques, and protein purification techniques.

Major Findings:

1. Deletion of amino acids 2-59 of Tax1 results in a Tax1 protein which is unable to transactivate the HTLV-I long terminal repeat.

2. Deletion of amino acids 2-59 of Tax1 (Δ58 Tax1) results in a transdominant protein which inhibits the transactivation functions of wild type Tax1 on the HTLV-I long terminal repeat.

3. Tax1 mutant Δ58 does not localize to the nucleus of transiently transfected cells.

4. Tax1 mutant Δ58 blocks the nuclear localization of wild-type Tax1.

5. c-ets-1 and c-ets-2 gene products transactivate the HTLV-I LTR.

6. Ets1 binds specifically to the promoter/enhancer of the HTLV-I LTR. Ets1 binding correlates with transactivation activity.

Publications:

Bosselut R, Duvall JF, Gegonne A, Bailly M, Hemar A, Brady JN, Ghysdael J. The product of the c-ets-1 protooncogene and the related Ets2 protein act as transcriptional activators of the long terminal repeat of human T cell leukemia virus HTLV-I. EMBO 1990;9:3137-44.

Brady JN. Human retroviruses. JNCI (In Press).

Gitlin SD, Lindholm PF, Marriott SJ, Brady JN. Transdominant human T-cell lymphotropic virus type I Tax1 mutant that fails to localize to the nucleus. J Virol 1991;65:2612-21.

Marriott SJ, Lindholm PF, Brown KM, Gitlin SD, Duvall JF, Radonovich MF, Brady JN. A 36-kilodalton cellular transcription factor mediates an indirect interaction of human T-cell leukemia/lymphoma virus type I Tax1 with a responsive element in the viral long terminal repeat. Mol Cell Biol 1990;10:4192-201.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05605-03 LMV

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transformation by Human CMV

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

P.I.: J. Brady Chief, VTBS LMV NCI

COOPERATING UNITS *(if any)*
Department of Microbiology, Georgetown University, Washington, D.C.
(Dr. Leonard Rosenthal)

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.20	0.20	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 cytomegalovirus (CMV) infection has been associated with several neoplastic diseases, including prostatic carcinoma, adenocarcinoma of the colon, cervical carcinoma, and Kaposi's sarcoma. The morphological transforming region II (mtrII) of CMV Towne has been localized to a 980 base-pair fragment containing three putative open reading frames (ORFs) of 79, 83, and 34 amino acids (aa). Homologous regions exist in strains Towne, AD169 and Tanaka. However, Tanaka mtrII has reduced transforming activity when compared to Towne and AD169. A 79 aa (ORF) present in both Towne and AD169 is truncated in Tanaka, indicating that it may be crucial for efficient transformation.

The promoter activity of the 980 bp mtrII DNA fragment has been investigated. The experiments showed that the 980 bp colinear DNA acts as a promoter when inserted in the sense orientation with reference to the ORF but not in the opposite orientation. In further experiments, two distinct promoter regions were detected by separately testing the left-hand 440 bp and the right-hand 540 bp fragment. Promoter activity was shown to be similar in all three strains, implying that this is not a factor in transformation, thus emphasizing the importance of the 79 aa ORF.

PROJECT DESCRIPTION

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

John Brady | Chief, VTBS | LMV NCI

Objectives:

To genetically map transforming regions of CMV.

Methods Employed:

Tissue culture; DNA transfection; radiolabeling of DNA, RNA and protein; DNA cloning; polyacrylamide gel electrophoresis; Southern blotting; northern blotting and hybridization; S1 nuclease; transformation assays; and DNA sequencing.

Major Findings:

1. CMV morphological transforming region II (mtrII) has been localized to a 980 bp fragment containing three putative open reading frames of 79, 83 and 34 amino acids. DNA sequence analysis of wild-type CMV Towne and transformation-defective CMV Tanaka suggests an important role for the 79-amino acid protein in transformation.

2. The 980 bp mtrII transforming region contains an intrinsic promoter, but not enhancer activity.

3. Promoter activity of the mtrII is similar in human cytomegalovirus Towne, AdI69 and Tanaka, emphasizing the importance of the 79 aa in viral transformation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05643-02 LMV

PERIOD COVERED: October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Yeast as a Surrogate Organism to Study the Function of Viral Genes

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

P.I.:	R. Dhar	Research Chemist	LMV NCI
Others:	R. Toyama	Visiting Associate	LMV NCI
	J. Brown	IRTA Fellow	LMV NCI
	S. Bende	IRTA Fellow	LMV NCI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Laboratory of Molecular Virology

SECTION: Virus Tumor Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	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 fission yeast Schizosaccharomyces pombe has been utilized as a model system to study transcription factors. Yeast replicating vectors containing HIV-1 LTR-CAT (chloramphenicol acetyl transferase gene) and deletions of the HIV-LTR promoters were transfected in S. pombe cells. In a separate experiment, an SV40 promoter transcribing the TAT gene was used in a cotransfection assay. Our results show that HIV-LTR promoter is functional in yeast and RNA synthesis initiates from two sites. One of the 5′ ends of the RNA corresponds to the 5′ end of the HIV-mRNA observed in viral cells. Deletion analysis of the promoter suggests NF-kB binding sequences are required for promoter activity. However, the TAT gene product failed to transactivate transcription.

A number of yeast replicating vectors have been developed. Enhancers which are generally present on viral promoters, such as SP1, NF-kB, CRE, and AP1, have been shown to be functional in fission yeast. A number of viral promoters have been introduced into the yeast such as the human cytomegalovirus (CMV), human low density lipoprotein receptor (LDL), human chorionic gonadotrophin-α (HCG-α), α-crystallin and albumin. The level of activity of these promoters is dependent on the type of enhancer present upstream.

In our attempts to identify what causes the instability of HIV-gag mRNA, we observed that transcription of the HIV gag gene from the SV40 promoter in S. pombe results in a series of mRNAs that are less than full-length. These may result from poly-A signals within the coding region and are candidate crs sequences. Cis regulating sequences are responsible for dependence of HIV mRNAs on rev protein.

PROJECT DESCRIPTION

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

Ravi Dhar	Research Chemist	LMV NCI
Reiko Toyama	Visiting Associate	LMV NCI
Julie Brown	IRTA Fellow	LMV NCI
Steven Bende	IRTA Fellow	LMV NCI

Objectives:

Our major objective is to study the function of viral (e.g., HIV-1) and cellular gene products regulating cell growth and identify cellular factors with which they interact.

Methods Employed:

Yeast genetics, recombinant DNA technology, immunoprecipitation, Southern and northern blot analysis.

Major Findings:

1. A CMV promoter-based yeast replicating vector has been developed. This vector has been used to overproduce viral gene products in fission yeast.

2. CMV and SV40 promoter vectors that have been developed can be used as functional shuttle vectors between viral cells and fission yeast.

3. HIV-LTR promoter has been shown to be functional in fission yeast. The NF-kB enhancer is essential for the promoter activity. The HIV-1 TAT gene product failed to transactivate the viral promoter.

4. Potential crs sequences in the HIV gag gene, resulting in the instability of the HIV-gag mRNA, have been identified in fission yeast.

5. A number of viral promoters are functional in S. pombe: SV40 early, CMV, HIV-LTR, herpes thymidine kinase, human chorionic gonadotropin-α, somatostatin, α-crystallin and albumin.

6. A large number of viral enhancer sequences have been tested for functional activity in fission yeast; among the most active were SP1, NF-kB, CRE and AP1.

7. Yeast S. pombe vectors have been developed that can be used to isolate tissue-specific cDNA clones encoding transcriptional factors.

Publications:

Lisziewicz J, Brown J, Brevario D, Sreenath T, Ahmed N, Koller R, Dhar R. Transcriptional regulatory elements of the Ras2 bene of Saccharomyces cerevisiae. Nucleic Acids Res 1990;18:4167-74.

Lisziewicz J, Rappaport J, Dhar R. Tat-regulated production of multimerized TAR RNA inhibits HIV-1 gene expression. New Biol 1991;3:82-9.

Srikanth T, Dhar R, Bustin M. Expression of human chromosomal proteins HMG-14 and HMG-17 in Saccharomyces cerevisiae. Exp Cell Res 1990;191:71-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05691-01 LMV

PERIOD COVERED

October 1, 1990 through September 30, 1991

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

Soluble HTLV-I Tax1 Protein Stimulates Proliferation of Human Lymphocytes

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

P.I.:	J. Brady	Chief, VTBS	LMV NCI
Others:	S. Marriott	Biotechnology Fellow	LMV NCI
	P. Lindholm	Research Associate (MSF)	LMV NCI
	R. Reid	Medical Staff Fellow	MB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	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 T-cell lymphotropic virus type I (HTLV-I) is associated with two human diseases, adult T-cell leukemia (ATL) and tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM). Lymphocytes from both ATL and TSP/HAM patients display abnormal proliferation properties in culture. Purified, soluble Tax1 protein can be taken up by, and stimulate proliferation of, uninfected phytohemagglutinin-treated human peripheral blood lymphocytes (PBLs). Tax1 was 40-70% as active as IL-2 in stimulating proliferation of PBLs. Heat-inactivated or chloroform-extracted Tax1 failed to stimulate PBL proliferation. Tax1 could not stimulate proliferation of PBLs in the absence of PHA. Following an initial round of cell division, Tax1-treated PBLs exhibited prolonged sensitivity to interleukin-2-induced proliferation. These results indicate that Tax1 can stimulate lymphocyte proliferation in culture and imply that extracellular Tax1 may be involved in the spontaneous proliferation of TSP/HAM lymphocytes and the interleukin-2-dependent proliferation of ATL lymphocytes.

PROJECT DESCRIPTION

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

John Brady	Chief, VTBS	LMV NCI
Susan Marriott	Biotechnology Fellow	LMV NCI
Paul Lindholm	Research Associate (MSF)	LMV NCI
Robert Reid	Medical Staff Fellow	MB NCI

Objectives:

Analyze the role of extracellular Taxl in lymphocyte proliferation.

Methods Employed:

Recombinant DNA techniques, protein purification, gene expression using transient and permanent techniques, DNA replication assays, PCR, RNA and protein analysis.

Major Findings:

1. The Taxl protein can be recovered in the media of HTLV-I transformed cells.

2. Taxl can be taken up by primary human peripheral blood lymphocytes (PBLs).

3. Extracellular Taxl protein stimulates proliferation of PHA-treated PBLs. This effect can be blocked by chloroform extraction or anti-Taxl antibody clearing of the Taxl preparation.

4. Extracellular Taxl activates expression of a transiently transfected HTLV-I LTR CAT construct in PBLs.

5. Extracellular Taxl activates expression of the cellular IL-2R as well as a transiently transfected IL-2R promoter-CAT construct in PBLs.

Publications:

Marriott SJ, Lindholm PF, Reid RL, Brady JN. Soluble HTLV-I Taxl stimulates proliferation of human peripheral blood lymphocytes. New Biol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05692-01 LMV

PERIOD COVERED: October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Induction of NF-kB After Exposure of Lymphoid Cells to Soluble Tax1

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

P.I.:	J. Brady	Chief, VTBS	LMV NCI
Others:	P. Lindholm	Research Associate (MSF)	LMV NCI
	S. Marriott	Biotechnology Fellow	LMV NCI
	S. Gitlin	Senior Staff Fellow	LMV NCI
	C. Bohan	IRTA Fellow	LMV NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Molecular Virology

SECTION: Virus Tumor Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	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.)*

Purified HTLV-I Tax1 protein can be taken up by 70Z/3 lymphoid cells and localized in both the nuclear and cytoplasmic compartments. Introduction of the Tax1 protein into the growth medium of 70Z/3 cells resulted in the rapid and transient induction of NF-kB binding activity in the nuclear fraction. Tax1 activation of NF-kB was not sensitive to either staurosporin or prolonged stimulation with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, suggesting that Tax1-dependent NF-kB activation did not require the protein kinase C pathway. Purified Tax1 did not directly increase NF-kB binding activity in 70Z/3 cytoplasmic extracts, suggesting that NF-kB induction may require cellular factors. Western blot and competitive radioimmunoassays demonstrated that Tax1 protein was present in the tissue culture media of HTLV-I-transformed cell lines. These results show that extracellular Tax1 may regulate cellular gene expression in noninfected cells.

A purification protocol which involves sequential ammonium sulfate precipitation and zinc chelate chromatography to purify the HTLV-I Tax1 protein expressed in E. coli has been developed. The final Tax1 product is greater than 90% pure and the yield is approximately 1 mg per liter of liquid culture. The purified Tax protein is biologically active in indirect in vitro DNA binding assays, cellular NF-kB induction experiments and lymphocyte proliferation assays.

PROJECT DESCRIPTION

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

John Brady	Chief, VTBS	LMV NCI
Paul Lindholm	Research Associate (MSF)	LMV NCI
Susan Marriott	Biotechnology Fellow	LMV NCI
Scott Gitlin	Senior Staff Fellow	LMV NCI
Cindy Bohan	IRTA Fellow	LMV NCI

Objectives:

The objectives of this project are to understand the mechanisms by which purified soluble HTLV-I Taxl protein stimulates cellular growth and gene expression.

Methods Employed:

Recombinant DNA techniques, bacterial expression vectors, protein purification, gene expression using transient transfection assays, mobility shift assays, in vitro transcription assays, RNA and protein analysis, polymerase chain reaction, radioimmunoassay.

Major Findings:

1. HTLV-I Taxl is rapidly taken up by lymphoid cells. Approximately 80% of the internalized Taxl is localized in the nuclear fraction.

2. HTLV-I Taxl protein has been detected in the cell supernatant tissue culture medium of the HTLV-I transformed cells, MT4 and C81.

3. Introduction of HTLV-I Taxl protein into the growth medium of murine 70Z/3 pre-B cells resulted in rapid and transient induction of NF-kB DNA binding activity in the nuclear fraction.

4. The induction of nuclear NF-kB DNA binding activity by HTLV-I Taxl protein occurred in the absence of an intact protein kinase C pathway.

5. HTLV-I Taxl protein did not directly influence the stability of the cytoplasmic NF-kB IkB complex in vitro.

6. Stimulation of 70Z/3 cells by HTLV-I Taxl resulted in the amplification of immunoglobulin kappa light chain and lymphotoxin (TNF-β) genes, both of which contain NF-kB promoter/enhancer elements.

Publications:

Lindholm PF, Marriott SJ, Gitlin SD, Brady JN. Differential precipitation and zinc chelate chromatography purification of biologically active HTLV-I Taxl expressed in E. coli. J Biochem Biophys Methods 1991;22:233-41.

Lindholm PF, Marriott SJ, Gitlin SD, Brady JN. Induction of nuclear NF-kB DNA binding activity after exposure of lymphoid cells to soluble Tax1 protein. New Biol 1990;2:1034-43.

ANNUAL REPORT OF

THE LABORATORY OF TUMOR CELL BIOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 to September 30, 1991

The overall objective of the Laboratory of Tumor Cell Biology is to gain an understanding of the human blood cell growth and differentiation in health and disease. Of particular interest is the delineation of the changes and definition of mechanisms of blood or immune cell transformation (leukemias and lymphomas) or of other growth and functional abnormalities of immune cells such as the acquired immunodeficiency syndrome (AIDS). The role of human retroviruses in leukemia, lymphomas, human T lymphotropic virus (HTLV)-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and in AIDS continues to be a significant part of our investigative effort. Malignancies of other cell types are also under study, especially if they are associated with blood or immune cell abnormalities, for example, Kaposi's sarcoma (KS) associated with AIDS and B cell lymphomas. The ultimate goal is to bring the information and understanding gained in the laboratory to bear on the diagnosis, early detection, treatment or prevention of such disorders.

Progress made in the past year in various phases of this research effort is summarized below:

I. PATHOBIOLOGY OF AIDS-KS SPINDLE CELLS

AIDS-KS spindle cells have properties of primitive vascular cells, probably more closely related to smooth muscle cells, induce angiogenesis and cause the development of KS-like lesions in nude mice. Their growth is apparently regulated by corticosteroids and other growth factors and in turn produce factors which, deregulated, may contribute to the pathology of KS. Such factors include basic fibroblast growth factor (bFGF), interleukin-1 (IL-1), IL-6, and IL-8. AIDS-KS cells have receptors for IL-2, IL-6, platelet-derived growth factor (PDGF), bFGF, IL-1, tumor neurosis factor (TNF), hydrocortisone and human immunodeficiency virus type 1 (HIV-1) tat. Furthermore, cultured AIDS-KS cells induce vascular hyperpermeability response in nude mice and hairless guinea pigs. It is likely that AIDS-KS cells produce soluble factor(s) that may be involved in the regulation of edematous response in AIDS-KS lesion development.

Identification of the Cellular Origin of AIDS-KS Cells

The purpose was to define the spindle cells of KS and attempt to define their normal progenitor cells. Recently, in collaboration with Dr. Judah Folkman (Harvard Medical School, Boston, MA) we found that our cultured AIDS-KS cells reacted with a vascular smooth muscle actin (SMC-actin) specific antibody. These cells also expressed SMC-actin messenger ribonucleic acid (mRNA). Furthermore, these AIDS-KS cells appeared, by transmission electron microscopy, morphologically similar to a fetal-type of vascular smooth muscle cell. In addition, in collaborative studies with Dr. Gill (University of Southern California,

Los Angeles, CA), we found that spindle cells derived directly from KS lesions were also positive for SMC-actin. Thus, the AIDS-KS spindle-like cells have properties of primitive vascular cells, probably more closely related to smooth muscle cells.

Study of Angiogenesis Induced by AIDS-KS Cells In Vivo

As reported earlier (Science 1988;242:430-433), cultured or primary fresh AIDS-KS cells transplanted subcutaneously into the back of athymic nude mice, induce angiogenesis and cause the development of KS-like lesions on day 5 post-transplantation. Since then, we have been able to extend the model to another animal: when AIDS-KS cells were transplanted into hairless guinea pigs, they also induce a similar highly vascularized lesion.

Effect of Glucocorticoids on the Growth of AIDS-KS Cells

Utilizing our KS tissue culture system, we investigated the effects of corticosteroids and other hormones on the growth of AIDS-KS cells and their interaction with specific growth factors. The in vitro growth of these cells was significantly enhanced by several glucocorticoids, viz., hydrocortisone and dexamethasone, an effect that was inhibited by the addition of a steroid antagonist, U486. By comparison, the mineralocorticoid aldosterone had little or no effect. In contrast to their effect on AIDS-KS cells, these glucocorticoids did not augment the growth of normal vascular endothelial cells, fibroblasts, or aortic smooth muscle cells either alone or when used as a supplement to appropriate growth factors. The results suggest the existence of a specific interaction between certain corticosteroids and factors involved in the regulation of AIDS-KS cell growth and they provide a basis for more detailed studies of the mechanisms involved in corticosteroid-associated induction or promotion of KS lesion development in man. They also emphasize the need for judicious use of corticosteroids in patients at risk for developing KS.

Induction of Vascular Permeability by AIDS-KS Cells

Among other clinical symptoms, edema, pleural effusions, and continuous diarrhea are frequently observed in AIDS-associated KS patients. The mechanism(s) responsible for these changes is not clear, although it has been suggested that they are due to a simple mechanical obstruction of the lymphatic system capillaries. However, we have noted that long-term cultured AIDS-KS cells can induce a biphasic vascular hyperpermeability response in athymic nude mice. In vivo studies to evaluate the factor(s) produced by AIDS-KS cells which induces a vascular permeability response were performed in nude mice and hairless guinea pigs. To assay vascular permeability, dye was injected intravenously (IV) at various intervals after an initial subcutaneous or intradermal injection of AIDS-KS cells or serum-free AIDS-KS conditioned medium (CM). An AIDS-KS cell-related late phase response was observed 12 hours later. The activity in the late phase was non-dialyzable and stable for at least 3 weeks at 4°C. A similar vascular permeability response was also induced by the inoculation of freshly isolated primary AIDS-KS cells cultured for a short time without any additional growth factors. These findings indicate that AIDS-KS cells have been activated in the lesion and that such a phenotype has been maintained during growth in culture. These soluble mediators are, therefore, likely to be involved in the regulation of the edematous response in AIDS-KS lesion development. Preliminary molecular evidence suggests that vascular permeability factor is expressed by cultured

AIDS-KS cells, and that this factor may be the prime candidate for the induction of increased vascular permeability by AIDS-KS cells.

Characterization and Purification of Growth Factor(s) Produced by Cultured AIDS-KS Cells

Early studies have shown that the CM of cultured AIDS-KS cells has some autocrine effect on the growth of AIDS-KS cells. This CM contained a bFGF activity, IL-1 activity, and large amounts of IL-6. We also observed that exogenously added IL-1 stimulated the growth of AIDS-KS cells and that anti-IL-1 antibody, as well as anti-bFGF, partially inhibited the growth stimulatory activity found in AIDS-KS CM. Exogenously added IL-6 did not augment the growth of AIDS-KS cells. However, addition of polyclonal antibody to IL-6 or antisense oligonucleotides recognizing IL-6 sequences inhibit the growth of AIDS-KS cells. Also, IL-6 was found to be expressed at much higher levels in primary KS lesions than in adjacent skin tissues. Interestingly, glucocorticoids and the male sex hormone testosterone, markedly enhance IL-6 expression in these KS-derived cells, while estrogen downregulated their expression. These data suggest that IL-6 is another cytokine involved as an autocrine mechanism affecting the growth of AIDS-KS cells and possibly in the maintenance of the lesion in patients. These results provide a basis for future studies into the pathogenesis of KS and its predilection for the male sex hormone. The cultured AIDS-KS cells have also been found to express mRNA for IL-8 and to secrete that interleukin in their CM. The expression of IL-8, which is a chemotactic factor, may contribute to the pathogenesis of AIDS-KS.

Cytokine Receptors on the Membrane of AIDS-KS Cells

By immunofluorescence assay analysis, cytokine receptors were detected for IL-2, IL-6, PDGF, bFGF, IL-1, and TNF on the surface of AIDS-KS cells; cytoplasmic receptors were also found for hydrocortisone. Ligand binding assays revealed the presence of high affinity receptors for IL-6, IL-2, IL-1-α, TNF, bFGF, PDGF and hydrocortisone. We analyzed whether specific high-affinity cytokine receptor stimulation could induce the proliferation of AIDS-KS-derived cells. Our results indicate that the KS cells grow in the presence 100-1000 pM of recombinant IL-2 or 1 micromolar of hydrocortisone, but not in the presence of exogenously added recombinant IL-6. Our hypothesis is that IL-6 receptors on the AIDS-KS cells may already be saturated by their own endogenously produced IL-6. The IL-6 receptors on AIDS-KS cells were also found to be upregulated by treatment with hydrocortisone. Interestingly, a 69 kilodalton (kD) form of the IL-6 receptor was detected on AIDS-KS cells, which differs from the 80 kD form previously found on hepatoma cells. The IL-2 receptors on AIDS-KS cells and on endothelial cells are functional receptors, which could be involved in the immune responses generated by primary HIV-1 infection. The activation of IL-2 receptors in AIDS-KS-derived cells may be one factor responsible for growth and invasiveness of KS in vivo. Our findings raise the possibility that IL-2 is a growth factor for AIDS-KS-derived cells, and they support our model linking the development of AIDS-KS with immune stimulation.

Physiological Effect of Cytokines on AIDS-KS Cells

Our previous studies on AIDS-KS have elucidated some of the mechanisms in the formation of the KS lesion and demonstrated that the HIV-1 gene product, Tat, may play a role in KS pathogenesis. However, the reasons for the very high risk of KS development in HIV-1 infected homosexual or bisexual men are unclear. In the

early stages of HIV-1 infection, AIDS-KS patients often show signs of immunoactivation and are only marginally immunosuppressed suggesting that immune stimulation may play a pivotal role in the development of AIDS-KS. The HTLV cell line which is used as the source of CM for the long-term culture of the AIDS-KS cells expressed several cytokines normally produced during T cell activation, suggesting that T cell activation products may play a role in vivo in the pathogenesis of KS. To clarify the biological bases of these clinical-epidemiological and in vitro observations, and to evaluate the role of immunoactivation in AIDS-KS, we investigated whether CM from mitogen-activated primary immune cells could induce proliferation of cells derived from KS lesions of AIDS patients and of other cells of mesenchymal origin.

The CM reproducibly stimulated AIDS-KS and adult aortic smooth muscle cell growth, and low levels of H-UVE cell proliferation. Protein and mRNA analyses indicated that several cytokines were expressed by both types of activated T cells (HTLV-II CM and phytohemagglutinin (PHA)-T CM). IL-1α and -β, TNF-α and -β, PDGF and, to a lesser extent, IL-6 and granulocyte-macrophage colony stimulating factor promoted the growth of AIDS-KS cells at concentrations shown to be biologically active in other systems. A synthetic CM (made by combining the cytokines at the same concentration present in PHA-CM and HTLV-II CM, in vitro CM) stimulated a growth response very similar or identical to that obtained with CM from activated primary T cells. These results demonstrated that cytokines released from activated primary lymphocytes can induce growth of AIDS-KS cells and of normal mesenchymal cells present in the KS lesion, and that the combination of individually submitogenic levels of several cytokines had additive or synergistic growth effects on mesenchymal cells.

II. PURIFICATION AND CHARACTERIZATION OF THE 30 KD POTENT KS SPINDLE CELL GROWTH FACTOR

The goal of this study is to isolate and purify a factor that supports the long-term growth of AIDS-KS cells from human activated $CD4^+$ T cells (mitogen-stimulated normal peripheral blood mononuclear leukocytes as well as retrovirus-infected/transformed cells). This factor will then be thoroughly studied in order to determine its chemical and biological characteristics and will be used for the development of specific immunologic reagents.

We have used the culture media of these cells grown in serum-free conditions as a source of factor for purification. During purification, the biological activity was monitored using AIDS-KS cells in a biological assay. Factor has been purified through the stages of DEAE and CM-Sepharose ion exchange and reversed phase HPLC chromatography. The pooled active fractions consisted mainly of three silver staining bands on SDS-PAGE. Bioassays of material eluted from slices of the SDS-PAGE experiment indicated that the growth activity was associated with only one silver staining band which corresponded to a molecular weight of approximately 30,000 daltons. Incubation of AIDS-KS cells with ^{125}I-labeled material revealed that the labeled protein that bound to the cells had a molecular weight of approximately 30,000 daltons and was effectively competed by the addition of an excess of unlabeled factor to the binding reaction. This observation indicated that the AIDS-KS cells have specific receptors for the T cell-derived growth factor. The 30,000 dalton protein was effectively separated from remaining contaminants by metal ion chromatography (immobilized Ni^+) and the growth activity corresponded to this protein. The protein has now been purified to homogeneity

and the amino acid sequence is being determined. Purified protein will also be used in the future for molecular characterization, to study its mechanism of action, and to perform experiments designed to delineate its effect(s) on the development of KS.

III. MECHANISMS FOR MEDIATION OF AIDS-KS PATHOGENESIS

HIV-1 tat is capable of providing a growth stimulus to AIDS-KS cells in a complex dose-dependent manner. The HIV-1 tat molecule contains multiple independent segments which are capable of adhering to AIDS-KS cells, as well as other cell types, and have been used to identify the existence of a specific high-affinity receptor molecule on AIDS-KS cells. The identification of this and other possible receptor molecules is in progress, as is the possible contribution of AIDS-KS cell adhesion molecules to pathogenesis. Several drugs and compounds have been evaluated for effect on KS cell induced biological functions. The effect of SP-PG (a peptidoglycan natural product) on AIDS-KS cell growth and induction of vascular permeability or angiogenesis was promising. It was target specific with an effect on AIDS-KS and endothelial cells but not in fibroblasts.

Cell Growth Effects and Uptake of Recombinant HIV-1 Tat by Mesenchymal Cells

Recombinant purified Tat protein from different sources stimulated proliferation of target cells derived from KS lesions of AIDS patients at the picogram to nanogram concentrations. Interestingly, the curve of the dose-response with recombinant Tat shows two peaks of cell proliferation which are reproducibly observed with different protein preparations, one around 100 picograms/ml and a second one around 10 nanograms/ml. These data suggest that two different pathways for cell proliferation are activated by different concentrations of Tat, as has been shown for other peptides lacking a signal sequence for release. Similarly Tat stimulated a growth response on other mesenchymal-derived cells (normal human smooth muscle and endothelial cells), but only after preincubation and activation with T cell activation products. These results suggest that cell surface modifications are necessary to induce normal mesenchymal cell types to become responsive to Tat.

Recognition of a Cell Adhesion Domain at the C-terminus of HIV-1 Tat

Overlapping peptides representing HIV-1 HXB2 tat synthesized by automated PEPSCAN within microtiter plate wells were capable of specifically binding lymphocytes. The binding activity was determined to be within 24 amino acids from the carboxyl terminus. Contained within is an RGD sequence which has been identified as a member of the integrin binding family. The effect of these peptide sequences on normal and Kaposi cells will be studied. Recent studies have identified additional regions of the tat molecule which are capable of causing lymphocyte adherence. Experiments are in progress to determine the physiologic and biochemical outcome of lymphocyte adherence to HIV tat. Also in progress are experiments to determine the exact location of this new sequence, the degree and relevance of binding of vascular cells to this new sequence, and the physiologic outcome of this type adherence.

HIV-1 Tat Receptors on AIDS-KS Cells

Using cross-linking and Scatchard analysis, we have been able to demonstrate the presence of specific, high-affinity receptors for a peptide from the HIV-1

transactivator Tat protein. Those receptors could be saturated using an excess of unlabelled Tat peptide. We could also demonstrate that the Tat peptide, used in the Scatchard analysis, induced proliferation of the cultured AIDS-KS cells. These receptors are likely to mediate the proliferation of the AIDS-KS cells induced by the Tat protein.

Adhesion Molecules on AIDS-KS Cells

To date many of our studies of KS have concentrated on soluble mediators. However, cell-cell interactions and recognition of cells and factors will most likely also play an important role in the development of KS lesions. Factors stimulating endothelial cell growth and migration, relationships between endothelial and vascular smooth muscle cells (including AIDS-KS cells), processes which lead to edema, and interactions involving extravasated erythrocytes and the accumulation of inflammatory cells, such as macrophages and lymphocytes, are all related to the process of KS development. Adhesion molecules are known to have important regulatory effects on various cell functions and interactions, and cultured AIDS-KS cells as well as fresh cells associated with KS lesions were found to express the intercellular adhesion molecule-1. An analysis of the relationship between adhesion molecules and soluble mediators in KS lesions should lead to a better understanding of KS development.

Study of Endothelial Cell Chemotaxis and Chemoinvasion Inducing Activity Produced by AIDS-KS Cells

A coated basement membrane barrier assay performed in a Boyden chamber was used to study AIDS-KS cell migration. Also, CM from AIDS-KS cells were found to induce the invasiveness of normal endothelial cells. While classical bFGF can also induce such invasiveness, the major invasiveness-inducing activity in AIDS-KS CM was not inhibited by antibody to bFGF. Specific inhibitors of collagenase IV, however, were found to block the induction of endothelial cell invasiveness by AIDS-KS CM. It is possible that this in vitro phenomenon represents an early event of angiogenesis occurring in vivo. The secretion of a potent inducer(s) of endothelial cell invasiveness by AIDS-KS cells could, therefore, be related to the angiogenic process induced by AIDS-KS cells (in collaboration with NIDR).

Induction of Nerve Cell Degeneration by AIDS-KS Cells

Another activity found in serum-free AIDS-KS culture supernatant fluids induced degenerative morphological changes in the neuronal cell line PC12. Specifically, AIDS-KS CM induced the formation of hair-like cytoplasmic degenerative processes in a dose-dependent manner (in collaboration with NIDR).

Culture of a Factor-independent AIDS-KS Cell Isolate

Until now, all the different AIDS-KS-derived cell cultures were dependent on activated T cell CM for their long-term growth. However, recently a tetraploid mesenchymal cell population was cultured from the pleural effusion of an AIDS-KS patient. Those cells grow very well without the addition of any exogenous growth factor. Yet they show the same phenotype as the other AIDS-KS cell isolates: they are smooth muscle actin-positive and induce angiogenic lesions in nude mice. This cell population is being studied intensively for the following characteristics: long-term tumorigenesis in nude mice; reason for the apparent growth factor

independence; relevance for AIDS-KS in general (is it an isolated case or does it occur more frequently than realized until now?). The abnormal karyotype of the cells will also prompt us to assess whether KS is a polyclonal or a monoclonal proliferation.

Testing of Therapeutic Agents for AIDS-KS

Several drugs and compounds have been evaluated for their effect on KS cell induced biological function by our in vitro, in ovo and in vivo model systems. Compounds tested for effects on KS cell growth or other biological activities include: suramin, recombinant interferon-α (rIFN-α), pentosan polysulfate (SP-54), and SP-PG (a naturally occurring bacterial cell wall polysaccharide peptidoglycan product). Suramin and rIFN-α were found to inhibit the growth of KS cells; however, their effects on target cells did not appear to be entirely specific. Suramin at a concentration of 300 μg/ml was cytotoxic to cultured cells. Pentosan polysulfate (at concentrations less than 100 μg/ml) was target specific as it inhibited the growth of AIDS-KS cells and human endothelial cells. SP-PG was also target specific, and more effective on the growth of AIDS-KS cells and endothelial cells, while having no effect on fibroblasts. Its inhibitory effect occurred at concentrations of 3 μg/ml and 25 μg/ml for AIDS-KS cells and endothelial cells, respectively. Suramin (5 mg/mouse) or pentosan polysulfate (2 mg/mouse), injected into nude mice, did not inhibit the late phase vascular permeability response induced by AIDS-KS cells, unlike the in vitro effect seen with pentosan polysulfate. In contrast, a high dose of rIFN-α (10,000 U/mouse) inhibited the vascular permeability response to some degree. However, SP-PG (5 mg/mouse) completely inhibited the vascular permeability response induced by KS cells. This inhibitory effect was further stabilized in combination with tetrahydrocortisone. It was difficult to evaluate the effect of suramin on angiogenesis in mice since treatment with suramin was found to be very toxic, causing death in three of five mice inoculated. rIFN had only limited inhibitory effect on the angiogenesis in mouse models. SP-PG, however, was again found to be very promising as it exhibited a strong and complete inhibition of angiogenesis associated with inoculation of KS cells in nude mice. Following treatment with nontoxic levels of SP-PG, newly formed vasculature rapidly degenerated and spindle-shaped cells were not observed. In addition, SP-PG also inhibited the angiogenesis induced by AIDS-KS cells in the CAM assay. This effect in ovo was augmented by the combination of tetrahydrocortisone or hydrocortisone. SP-PG is, therefore, a potential candidate for clinical trials in the treatment of AIDS-KS. Further experiments are in progress to test inhibition in in ovo and in vivo systems. The drug testing program is now being expanded using the model systems described above. We will test many other agents, whose final effect will be an inhibition of angiogenesis.

Oncostatin M, originally identified, purified, and sequenced by Oncogen (Seattle, WA) was reported as a unique cytokine. It was shown to be produced by normal T cells, macrophages, and various T or macrophage cell lines. Like other cytokines, oncostatin M was found to inhibit the growth of various tumor cell lines, e.g., melanoma (hence its name), and stimulated the growth of fibroblasts to some degree. A comparison of this molecule with partially purified AIDS-KS growth factor from HTLV-II-transformed T cells also suggested similarities in its size (molecular weight of 30 kD) and in some biochemical/biological properties. Like AIDS-KS growth activity found in virally infected activated $CD4^+$ T cell cultures, the activity was more potent than that observed with other cytokines tested, e.g., IL-1 and TNF.

Secretion of Biologically Active Factors by HIV-1 Infected Macrophages

Primary human macrophages infected with a monocytotropic HIV-1 isolate were found to secrete one or more factors which had a significant effect on the rate of proliferation of cells derived from the synovial lining of uninfected donors. Quantitation of the cytokine present in the supernatants from infected macrophages showed that although several cytokines were expressed, none were present in amounts adequate to explain the proliferation of the test cells. These data suggest that not only may HIV-1 infected macrophages mediate disease expression in infected individuals, but that individually substimulatory concentrations of cytokines may operate addictively or synergistically.

IV. HIV GENE EXPRESSION

Defective HIV-1 Viruses, Recombination, Infectivity and Tropism

Defective proviruses are likely to be common in infected individuals. Genetic recombination among these proviruses and/or phenotypic mixing of the viruses may result in viruses with altered phenotype. This can be demonstrated in vitro by co-infection with more than one defective virus or co-transfection with defective proviral DNAs. Analysis of molecular clones of two defective viruses showed that one of these viruses (LW12.3) was defective in vpr and vif genes and showed preference for infecting macrophages. The other (MN-ST) had a defect in the vpu gene and in the gag p6 and infected both T-lymphocytes and macrophages. These partially defective viruses were able to trans complement each other, resulting in viruses with widened host range and increased cytopathic effects. Similarly, envelope defective HIV-1 proviruses could be complemented by providing competent envelope in trans.

Replication incompetent defective proviruses may also revert to replication competent proviruses. For example, when T-lymphocytes harboring Tat defective HIV-1 provirus were subjected to ultraviolet irradiation, replication competent virus was rescued. The progeny infectious virus was composed of a mixture containing the original mutant, new mutants and true revertants with the wild-type genotype. These results emphasize that the environmental factors can contribute to HIV-1 pathogenesis in more than one way.

Genetic Based HIV-1 Antiviral Approaches

Retroviral life cycle provides several targets for intervention. Exploitation of these targets with new approaches encompassing transdominant inhibition of viral functions, antisense RNA mediated arrest of viral protein synthesis and ribozyme mediated degradation of viral RNA may provide specific chemotherapeutic agents. The first target to be chosen was the transactivation (tat) function of HIV-1 which is essential for virus replication. The transactivation is mediated by interaction of Tat with the Tar element. An expression vector directing the synthesis of transcripts with multiple Tar elements clearly inhibited HIV-1 long terminal repeat directed gene expression in mammalian cells, suggesting transdominant inhibition of Tar.Tat function. To further evaluate the validity and usefulness of this approach permanent lymphocytic cell lines stably expressing multiple Tar elements have been obtained. These cell lines can now be challenged with HIV-1 to determine if this approach would successfully prevent HIV-1 infection. To further expand their usefulness, multiple Tar elements have been inserted into a retroviral vector as a gene therapy delivery vehicle and

lymphocytic cell lines infected with and expressing integrated multi-Tar elements are being obtained for a challenge test with HIV-1. Efforts are also underway to obtain retrovirus constructs which will combine transdominant inhibition by multi-Tar elements with ribozymes initially directed against HIV-1 Gag proteins. The antisense approach was used to target HIV-1 rev function. Rev is a second transactivator of viral expression ensuring balanced production of viral transcripts and its action is mediated by the rev response element (RRE). A phosphorothioate derivatized antisense oligonucleotide corresponding to the first 90 nucleotides of RRE strongly inhibited HIV-1 replication and syncytia induction in human lymphocytic cells without accompanying cellular toxicity. This oligonucleotide could also, to some extent, inhibit virus replication in chronically HIV-1 infected human lymphocytes.

Mature HIV-1 Virions Contain Viral DNA

The genetic information carried by the mature retrovirus particles is the RNA genome. Convincing evidence has now been obtained that virus particles of HIV-1 contain viral DNA in addition to the viral RNA. This viral DNA, an intermediate product of reverse transcription of viral RNA, is tightly associated with the reverse transcriptase (RT). Indeed, this particular intermediate DNA form co-purifies with RT through rather stringent purification procedures. This observation encourages certain interesting speculations. Is it possible that this viral DNA enclosed in the virion is the agent of latent infection, particularly of quiescent and nondividing cells (e.g., macrophages)? Productive infection with retroviruses requires integration of viral genome into the cellular chromosomes during cell division. This pathway may not be available in non- or minimally-dividing cells. Could it be that viral DNA:RT brought in by the infecting HIV-1 virions set up extrachromosomal replication of viral DNA in such cells? This could explain the unusually large quantities of unintergrated viral DNA observed in macrophages of infected brains. Thus, HIV-1 could adopt different strategies of replication, depending on the metabolic state of the cell or its state of differentiation.

HIV-2 Latency, Virulence and Pathogenicity

HIV-2 as a group is thought to be less virulent, more latent and less pathogenic than HIV-1. This, in part, may be related to the manner by which HIV-2 and HIV-1 expression is regulated. HIV-2 possesses two enhancers. It is conceivable that under certain conditions one of these enhancers functions maintains a low level of virus expression (e.g., latency). Under other conditions, both enhancers may function, resulting in a high level of virus expression and pathogenic effects. Furthermore, pathogenic potential of HIV-2 may, in part, be governed by its envelope structure. Studies with chimera between cytopathic and non-cytopathic HIV-2s have shown that fusiogenic and cytopathic epitopes are likely to be multiple and discontinuous (non-linear), the envelope of each virus may be unique in its ability to cause pathogenic effect, cytopathic potential may be coupled with the capacity to replicate, and in certain cells (e.g., monocytic cells) cytopathogenesis may involve single cell killing rather than massive induction of syncytia.

V. HIV MEDIATED IMMUNE RESPONSE AND VACCINE

HIV-1 Envelope Variability and Immune Response

An important aspect of the HIV-1 variability is its effect on immune recognition with implications for vaccine design. A neutralizing escape mutant provirus was previously described which contained a point mutation in gp41 away from the major neutralizing epitope (V3 loop). Analysis of this mutant showed that resistance was unlikely due to the direct effect on the antibody binding site but probably resulted from a global change in the tertiary or quaternary structure of the envelope. A new escape mutant has been obtained which also contains a point mutation upstream of the V3 loop in gp120. It will be interesting to determine if this mutation also induces global versus local changes in the envelope conformation.

To assess the detailed role of the V3 loop in immune recognition and response, chimeric proviruses containing the exchanges of V3 loops, and parts thereof, of HIV-1(IIIB) and HIV-1(MN) have been constructed. One such chimera which contains the V3 loop of HIV-1(MN) substituting the V3 loop of HIV-1(IIIB) is neutralized well by sera which fail to neutralize either parental virus, indicating that not only the loop content but its context is also important for neutralization. Analysis of these chimera should identify motifs within the V3 loop whose recognition results in neutralization.

Recent analysis of the DNA sequence of the envelope region of several HIV-1 isolates from a cohort of individuals from Zaire showed that the V3 loop of these isolates are much more closely related than expected--a divergence of 2-6 amino acid residues among the 32 residues of the loop compared with 10-20 residue differences for other African isolates. These results suggest that the V3 loop is not always as variable as the regions adjacent to it, and that varied changes within the V3 loop may be under functional constraints. This raises the hope that a broadly protective immune response may be attainable by eliciting reactivity toward a finite number of loop genotypes.

Neutralization of Monocyte/Macrophage Borne HIV

As monocytes/macrophages are an important reservoir for viral infection and latency, the role, if any, of neutralizing antibody in preventing infection of this cell type and the epitopes involved need to be addressed. We have established a system using fresh elutriated monocytes/macrophages, by which we can examine this question. Macrophage tropic isolates, or chimeric viruses rendered macrophage tropic by substitution of key components of the virus envelope, are assessed for neutralizability by natural human sera and specific hyperimmune and monoclonal antibodies. Our preliminary data suggest that neutralization of macrophage infection involves different epitopes than those participating in neutralization of T cell infection. The identification of these epitopes will be important for development of subunit vaccines.

Correlation of HIV-1 V3 Loop Antibody with Protection in Neonates

We have reexamined the role of antibodies to epitopes of the V3 PND in sera of children with AIDS, and have correlated reactivity with an amino terminal peptide of the V3 loop with lesser disease manifestation. Ongoing studies are aimed at further definition of such protective humoral responses.

Lack of Correlation between Neutralization and V3 Loop Homology in African HIV Isolates

The principal neutralizing determinant of HIV, the V3 loop, elicits highly type-specific neutralizing antibodies, complicating its use in vaccine preparations. To study the influence of viral heterogeneity on neutralization, we have analyzed neutralizing serotypes among Zairian isolates, using matched serum samples. Immunologic analysis of V3 loop sequences has been carried out using synthetic peptides and hyperimmune goat sera to the V3 loops in homologous and heterologous competition ELISA assays. As expected, highly related immunologic reactivity was associated with conservation of loop amino acid sequence. Nevertheless, in cross-neutralization analyses of Zairian isolates with matched sera, neutralizing serotypes did not correlate with homology in the V3 loop, suggesting the participation of alternate epitopes in broad neutralization. The role of envelope conformation in neutralization was also emphasized by studies on a chimeric virus composed of the V3 loop of the MN isolate substituted into the envelope of HXB2D. A panel of Zairian sera neutralized this chimeric virus with titers 20-fold higher compared to those against the MN isolate itself. Competition studies with V3 peptides showed this increase in titer could be attributed to better presentation and recognition of either the V3 loop itself or an alternate epitope. Thus, the context in which the V3 loop is presented is crucial for neutralization. The chimeric virus approach is being pursued both for enhancing immunogenicity and for further analysis of neutralizing epitopes. Additional studies aimed at elucidating the principal neutralizing determinant of HIV-2 have implicated a role for the homologous V3 region in this related virus.

HIV Recombinant Vaccine Vectors

A successful vaccine for AIDS will likely contain a cell mediated immune component in addition to neutralizing antibody. We are currently investigating epitopes which can elicit cell mediated immunity for inclusion in subunit vaccines. Initial studies on rhesus macaques infected with HIV-2 and HIV-2 accessory gene mutants have shown that while the animals are not immune compromised, they have a poor proliferative response to HIV-2 antigens, and lack circulating cytotoxic T-lymphocytes (CTLs) specific for HIV-2. Thus, the low viral load in these animals cannot be attributed to a strong immune response. These animals also lack neutralizing antibodies. We were able, however, to obtain HIV-2-specific CTL clones from several of the animals. These will be useful for mapping HIV-2 CTL epitopes for use in subsequent subunit vaccines.

In studies directly related to vaccine development, we have analyzed sera of dogs vaccinated with adenovirus constructs carrying the HIV-1 *env* gene. We observed a strong neutralizing antibody response in these animals following inoculation with two successive adenovirus constructs of different serotype. The antibody response declined over 12 weeks, but was readily boosted with purified recombinant envelope protein. These encouraging results form the basis for a vaccine trial in chimpanzees. If sufficiently high neutralizing antibody titers are achieved, the chimpanzees will be subjected to a live virus challenge. The cell mediated immune response in these chimpanzees will also be assessed. In additional studies the immune responses obtained following immunization of macaques with additional vectors carrying HIV or simian immunodeficiency virus (SIV) genes are being assessed.

Studies are being initiated to test the efficacy of bacterial (Salmonella and BCG) and viral (Vaccinia and Canarypox) vectors as delivery vehicles for HIV antigens/ immunogens to mice and rhesus macaques.

Characterization of Neutralization Reaction with Antibody Against HIV

Binding of glycoprotein gp120 to the T cell-surface receptor CD4 is a crucial step in CD4-dependent infection of a target cell by HIV. Earlier work done in our laboratory has revealed the entry process to a complex, prolonged event requiring some type of cooperative receptor-ligand interactions. It is this phase of viral entry which now can be targeted for vaccine design. Blocking some or all gp120 molecules on the viral surface should therefore inhibit infection; however, little is known about the molecular mechanisms. We have now quantitatively examined blocking by soluble CD4 in the hope of gaining insight into the complex process of viral blinding, adsorption and penetration. At low sCD4 concentrations, the inhibition in three HIV strains is proportional to the binding of gp120. For all three viral strains, the biological K(assoc) from infectivity assays is comparable to the chemical K(assoc). The inhibitory action of sCD4 at high concentrations, however, is not fully explained by simple proportionality with the binding to gp120. Positive synergy in blocking of infection occurs after approximately one-half the viral gp120 molecules are occupied, and is identical for all three viral strains, despite the large differences in K(assoc).

Furthermore, it was shown that a 20- to 50-fold difference in blocking activity could be observed for HIV-1 due to the content of the gp120/virion and the target-cell density. Thus, unappreciated variations in HIV stocks and assay conditions may hinder comparisons of blockers from laboratory to laboratory, and the age of HIV challenge stocks may influence studies of drug, vaccine efficacy, and antigenic variation studies. The results also suggest that blocking of viral particles in lymphoid compartments will require very high competitive blocker concentrations, which may explain the refractory outcomes from sCD4-based drug trials in humans.

Immunobiology of HIV-1: Antigenic Variation, Epitopes and Vaccine Development

Experimental studies done this past year have additionally demonstrated the V3 domain to be part of a contiguous conformational epitope as evidenced by the virus' ability to escape a V3-specific monoclonal antibody without changing any of the primary amino acids in the antibody binding site. Neutralizing antibody subsequently directed at the V3 region following infection is correlated in the selection of escape mutants which ultimately demonstrate V3-specific amino acid changes. The virus, therefore, has two mechanisms identified for evading the humoral immune system. Clonal expansion of a virus population is observed shortly in other acute or experimental infections in humans or chimpanzees, leading initially to a monotypic (type-specific) neutralizing response. This neutralizing response to the incoming virus appears to also clonally expand due to cross-reactivity in the invariant V3 domain of the emerging escape mutants. Similar results are observed with envelope immunization, suggesting an "original antigenic sin"-like phenomena may be operational during infection or vaccination. Fractionation studies using pooled human HIV-1 serum demonstrate that the majority of neutralizing activity is found associated with V3 and the CD4-binding site on gp120. The potency of broader CD4-mediated neutralization, however, is poorer than V3 and demonstrates strain-variability. Viruses passaged in vitro in primary lymphocyte cell lines select the most replication-competent virus subpopulations.

Escape mutants arise from less-replication-competent virus subpopulations. Mechanisms are operating in addition to a strong humoral response(s) in HIV-1 infected chimpanzees to restrict and possibly control the virus in this species. These include T8 suppressor activity, differences in their CD4 molecule and "relative" lack of infectability of their peripheral blood monocytes by HIV-1. This model appears to be very instructive for efficacy immunogenicity testing and challenge for examining the sterilizing immunity of a prototype HIV-1 vaccine. The use of the model for disease prevention is in serious doubt.

Refinement of System for Automated Peptide Design and Synthesis

New software allows synthesis of any peptide in any designated well by PC keyboard or file entry, facilitating design of peptide variants with specified deletions or substitutions. Conditions for preparation of organic solvent resistant microtiter plates with optimum spacing and density of functional amino groups has been achieved.

Analysis of Antibody Neutralization Structures of HIV

Complete loop structures (from C to C) have been prepared for 15 HIV variants--HXB2, MN, SC, SF2, NY5, CDC451, WMJ2, RF, BAL, Z3, Z6, ELI, MAL, JY1, ISY, SIV239. Goat anti-HXB2 loop peptide (subregion) preferentially recognizes HXB2 intact loop and goat anti-RF loop peptide (subregion) reacts more broadly among the other variants. Both goat antisera neutralize with type specificity. Analyses will proceed to the study of cross-reactivities which are non-neutralizing versus neutralizing.

Seroprevalence of Antibodies Reacting with HIV/SIV Variants

Natural HIV-positive serum reactivities against the intact loop peptides for two groups, HIV^+ and HIV^+/$HTLV\text{-}I^+$ drug abusers have been studied. Reactivities against the North American isolates were variable, with the HXB2 loop usually very weakly reacting. However, this is likely due to the structure. Some of the sera tested against loop subregions of HXB2 reacted much better with the subregions than the intact loop. There were no reactions with the Zairian isolates. This data will be used to begin modelling a functional topology of the HIV V3 loop.

PEPSCAN Analysis of HIV Antibody Epitopes

Given the possibility that the HIV V3 loop or analogous sequences for HIV-2 or SIV are necessary for a protective immune response, we have undertaken the complete epitopic analysis of a comprehensive series of HIV and SIV virus variants. The primary immunoreactive peptide sequence epitopes within the complete envelopes of HXB2, RF, several macaque and African green monkey variants, and ISY have been determined and catalogued. More variants will be done in the future.

Dynamics of Antigenic Drift and Immune Response In SIV-infected Macaques

Antibody recognition sites of the SIV envelope have been successfully characterized by automated PEPSCAN techniques for several SIV isolates using sera from wild-caught macaque and African green monkey species. Recognizable antibody response to SIV peptides in a group of recipients was found to consist of peptides recognized by all animals and peptides recognized occasionally by some of the animals. Future samples will be analyzed for progressive changes within animals.

VI. HTLV: ADULT T CELL LEUKEMIA (ATL) AND HAM/TSP AND THEIR ROLE IN AIDS

Characterization of HTLV-I and HTLV-II Prevalence in Normal Donor and Drug Abuser Populations

HTLV-I and HTLV-II envelopes were assayed for immune reactivity by peptide scanning and synthetic peptides capable of serologically discriminating between the viruses were prepared. We have run a small pilot test of 10 HTLV-I and HTLV-II peptides and have resolved a group of normal blood donor screen positives and a group of intravenous drug abuser (IVDA) screen positives. HTLV-II was ca 15% in blood donor screen positives and 55% in IVDA screen positives.

HTLV-I and T Cell Biology

The generation of mature lymphocytes can be studied in vitro by analyzing T cell colony formation in methylcellulose. This assay was applied to the peripheral blood mononuclear cells (PBMC) of patients infected with HTLV-I (healthy seropositive individuals, and patients with a disease associated with HTLV-I, namely HAM/TSP and ATL). We were prompted to investigate T cell colony formation in those patients, since a spontaneous lymphocyte proliferation was described in HAM/TSP patients and healthy HTLV-I carriers. We found that, in contrast to normal uninfected individuals, PBMC from HTLV-I-infected individuals contain T-lymphocyte colony-forming cells (T-CFC) with abnormal proliferative properties, since some of them grew spontaneously, in the absence of mitogen and IL-2 stimulation. The T-CFC from HTLV-I-infected individuals also show an abnormal differentiation in vitro, as evidenced by the $CD1^+$ T cells in the T-lymphocyte colonies generated in vitro. The T-CFC from infected individuals also seem to be infected, as shown by viral expression in the T cell colonies. The very rapid appearance of HTLV-I-expressing cells following cultivation in vitro is in contrast to previous studies, where liquid cultures of HTLV-I-infected cells only led to a gradual and slow selection of cells producing HTLV-I. Our interpretation is that the methylcellulose assay may select the target cell for infection, resulting in a rapid expression of HTLV-I. In this option, we believe that the T-CFC may be the target for infection by HTLV-I. Another interesting observation is that the HTLV-I transactivator Tax protein can stimulate the growth of the T cell colonies generated in methylcellulose from normal or HTLV-I-infected PBMC.

Control of Normal (Uninfected) T-lymphocyte Survival

A part of the lymphocytes are long-lived in vivo without undergoing proliferation for months or years. It is largely unknown how T cell survival is regulated. Practically all the current knowledge of T-lymphocyte biology revolves around activated and proliferating cells. When T cells are cultured in suspension without exogenous growth factors, they rapidly decline. Recently, the persistence for at least 2 months of mature B- and T-lymphocytes and plasma cells was demonstrated in the adherent layer of human long-term bone marrow cultures (HLTBMCs), in the absence of exogenously added growth factors. This long-term persistence is due to survival rather than to proliferation or generation from the hemopoietic stem cell. This long-term survival is stroma-associated, since it does not occur in unstimulated suspension cultures. It probably requires a close cell-cell and/or cell-extracellular matrix interaction. Recent experiments have shown that the surviving T cells retain their self-renewal capacity, as evidenced from the T-CFC assay. T-lymphocytes taken out of HLTBMCs are in a resting configuration, since they do not have activation markers and since they are

inactive in cytotoxicity assays. However, when they are stimulated, they regain their functional properties. The long-term survival of T-lymphocytes in HLTBMCs makes it possible, for the first time, to study this lymphocyte feature in vitro without tying it to T cell proliferation. Ultimately, our goal is to find out which factor(s) sustains the survival of the lymphoid cells in vitro and in vivo.

HTLV-I and ATL

The model of HTLV-I induced leukemogenesis holds that HTLV-I causes the initiation of transformation of T cells but is not required for the maintenance of the transformed phenotype. This is because the expression of HTLV-I genes, including the transactivator tax and rex genes, was not detectable in uncultured leukemic cells in vivo. At the same time, there was the persistent antibody response to viral proteins in ATL patients. This view now needs to be amended. Using more sensitive reverse polymerase chain reaction (PCR) technology, it was recently shown that ATL cells and other HTLV-I infected cells express HTLV-I genes and likely produce viral proteins, though at low levels, which could in part explain persistent antibody response. Interestingly, a diversity of transcripts corresponding to the regulatory genes, tax and rex, and arising from alternative splicing events, were observed--a situation reminiscent of HIV-1 transcript diversity, although it does not seem to be as extensive in HTLV-I as in HIV-1. In addition, other previously unidentified transcripts were detected. Some of these may correspond to the elusive p21 viral proteins related to Rex but of yet undetermined function. Further characterization of these transcripts, evaluation of their coding potential and abundance, and their role in leukemogenesis is being pursued.

HTLV-I and HAM/TSP

Though the etiology of HAM/TSP remains to be determined, there is high prevalence of HTLV-I antibodies in patients with HAM/TSP. Since HTLV-I is not known to infect cells of the central nervous system, it likely plays an indirect role in pathogenesis. HAM/TSP is accompanied by increase in spontaneous T cell proliferation, abnormal T cell differentiation, increase in CTLs specific for TSP, specially directed against HTLV-I antigens, unusually high anti-HTLV-I serum and cerebrospinal fluid antibody titers, and a high number of HTLV-I infected cells compared to healthy carriers. These abnormalities may be related to the active expression of HTLV-I genes in some T cells. As with ATL, using reverse-PCR technology, the expression of viral genes, particularly tax and rex, was demonstrated in PBMCs from individuals with HAM/TSP. Again, many different transcripts of tax and rex genes utilizing alternate splicing were detected, as were transcripts not previously identified. Interestingly, HTLV-I was being expressed in samples from individuals with varying duration of illness. This may imply that persistent expression of viral proteins may be important in pathogenesis. Further studies are in progress to characterize the protein product(s) of a novel transcript(s), to determine its in vivo expression and evaluate its role in pathogenesis.

Animal Model for HTLV-I and HAM/TSP

To obtain a better understanding of the role of HTLV-I in pathogenesis of HAM/TSP and later develop therapeutic modalities including vaccine, an animal model using rabbits is being developed. Rabbits have been previously shown to be sensitive to HTLV-I infection. Currently, using PBMCs from HAM/TSP patients, parameters such

as the route of inoculation and the dose of infected cells are being evaluated to achieve viral infection and pathogenic effects. From studies conducted thus far, it is apparent that rabbits can readily be infected with HTLV-I via HAM/TSP PBMCs inoculum. The animals seroconverted and virus could be readily isolated within a few weeks of inoculation. The inoculated animals developed behavioral changes and signs of neurological impairment reminiscent of encephalitis. Upon sacrifice, the presence of virus was detected in all the tissues examined except the brain stem and hypothalamus. Thus, the virus was widely distributed in tissues of the infected animals, presumably due to lymphocyte infiltrations. Because HAM/TSP at times develops in immunosuppressed individuals, it will be instructive to test immunosuppressed rabbits for infection-related pathological symptoms. Since in HAM/TSP patients, a high number of cytotoxic T cells specifically directed against HTLV-I Tax protein are found, evaluation of such cytotoxic T cells in infected rabbits will contribute towards the validity of the rabbit animal model. Such a model would be useful for the development of chemotherapeutic (e.g., antisense approach) measures and vaccine.

HTLV-I as a Tool of Molecular Anthropology

In contrast to HIV-1, HTLV-I provirus is genetically quite stable. Because of this property, analysis of proviruses from select populations separate in space and in time may not only increase our knowledge of the origin and evolution of HTLV-I but could possibly be useful in discerning ancient contacts between populations and their movements from one geographic region to the other. Recent molecular analysis of the proviruses from the same individual separated in time and different individuals from various parts of the world has shown that little or no genetic variation occurs in vivo regardless of the clinical status. Some regional variation is observed which could be diagnostic for the local geographic origin of the virus. In addition, a new quasi species of HTLV-I was identified in samples from Melanesia during these investigations. This HTLV-I isolate is only 92% identical to the protype HTLV-I, compared with 98-99% identity for isolates obtained from endemic and other regions.

HTLV-I Tax as a Growth Factor and HTLV-I as a Co-factor in AIDS

HTLV-I Tax can be instrumental in the induction of lymphokines important for T cell growth and function. Recently, it was shown that Tax itself can directly stimulate T cell proliferation. Furthermore, Tax is secreted by the HTLV-I infected cells. Combined with the observation of the expression of tax in vivo, this could imply that even if low levels of Tax are synthesized and released, it might promote the growth of leukemic cells and other T cells, perhaps increasing the probability of additional genetic changes culminating in the expansion of cells with transformed phenotype and thus leukemia. HTLV-I may also play a co-factor role in AIDS. Considerable epidemiological evidence now exists showing HTLV-I coinfection of HIV-1 infected individuals. Evidence also suggests that HTLV-I coinfection may hasten immune suppression. Extracellular Tax released by HTLV-I infected cells could promote T cell proliferation thus providing additional targets for HIV-1 infection. HTLV-I Tax may itself enhance HIV-1 expression. HTLV-I infection may also induce the production of cytokines that affect T cell growth and function on one hand and augment the expression of TNFα on the other. TNFα promotes the expression of HIV-1; which in turn may be important in progression from HIV-1 latency to active replication and progression to AIDS in dually infected individuals. It has been previously shown that HTLV-I and HIV-1 can infect the same cell, at least in vitro.

VII. HUMAN HERPESVIRUSES

Identification of the Target Cell for Infection by HHV-6 In Vitro

Mononuclear cell populations were obtained from normal human umbilical cord blood, adult peripheral blood, bone marrow, thymus, lymph node and tonsil, activated for 24 hours with PHA and exposed to HHV-6 at the approximate multiplicity of infection of 1. Infected cultures were monitored for the appearance of the typical morphologic changes induced by the virus and for the presence of intracellular HHV-6 antigens by indirect immunofluorescence (IF) analysis using specific monoclonal antibodies (mAb). Cord blood was more efficient than peripheral blood, perhaps as a consequence of the negligible number of specific anti-HHV-6 cytotoxic T-lymphocytes in the newborn. The vast majority of HHV-6-infected cells expressed phenotypic features of activated $CD4^+$ T-lymphocytes, exhibiting CD2, CD4, CD5, CD7, CD71 and, in a limited percentage, CD8, CD15 and class II MHC antigens (DR). The absence of surface CD3/TCR complex, together with the frequent co-expression of CD4 and CD8 could be related to cell immaturity or simply to phenotypic alterations induced by the infection. The expression of mRNA of the TCR indicated a mature origin for HHV-6-infected cells.

Identification of the Cellular Receptor for HHV-6

Despite the predominant tropism for $CD4^+$ T cells, HHV-6 does not use CD4 as a receptor to penetrate susceptible cells. We have performed competition experiments utilizing either mAb directed to human CD4 (OKT4, OKT4a, Leu3a) or the truncated, soluble form of the CD4 protein (sCD4) in an attempt to block viral infection. The mAb and sCD4 were maintained at the original concentration during the whole culture period. Virus expression was monitored by indirect IF, titration of released virus and antigen capture assays. No inhibition of HHV-6 expression was observed in any of the tests, while sCD4 and the mAb OKT4a and Leu3a completely abrogated HIV-1 infection. As further evidence for the use of different receptor structures by the two viruses, HHV-6 was shown to effectively superinfect human T cell lines chronically harboring HIV-1 (e.g., CEM-[IIIB]), whose membrane CD4 is completely downregulated or complexed with the gp120 envelope glycoprotein of HIV-1. We concluded that HHV-6 infection occurs via CD4-independent mechanism(s). The lack of interference between HHV-6 and HIV-1, at least in the initial phases of cellular infection, may be clinically relevant since the simultaneous presence of both viruses within the same target cell can permit HHV-6 to transactivate the HIV-1 regulatory sequences.

HHV-6 Modulation of CD4 Antigen Expression

A possible interaction between coinfecting viruses is at the level of receptor regulation. We have noticed that HHV-6-infected cells had consistently higher expression of CD4 surface antigen, i.e., the HIV-1 receptor. This observation suggested that the interaction between HIV-1 and HHV-6 might be more complex than coinfection of the same cell. To further dissect the possible mechanisms of HHV-6 interaction with HIV-1, we studied the effect of HHV-6 infection on the expression of the HIV-1 receptor, i.e., the CD4 glycoprotein.

The modulation of CD4 expression induced by HHV-6 infection was initially studied in the human neoplastic T cell line, Jurkat, a cell line with a low basal expression of CD4. Following HHV-6 infection, a dramatic upregulation of CD4 was observed by indirect IF analysis and fluorocytometry in parallel with the

expression of HHV-6-specific antigens. Treatment with CM from PHA-stimulated cord blood lymphocytes had no effect on CD4 expression by uninfected cells, whereas the combined action of PHA and phytohemagglutinin induced the downregulation of the antigen. No alteration of CD4 expression occurred during the course of infection with HIV-1 or human cytomegalovirus. Analogously, CD4 was not induced in the B lymphoblastoid cell line Raji after productive infection with Epstein-Barr virus. These data indicate that HHV-6 can favor the spread of HIV-1 infection by positively regulating its receptor on the cellular surface membrane. These observations were extended by blocking HHV-6 transcription at its later stages with the drug phosphonoformate (PFA). The lack of late gene product expression was documented using the monoclonal antibody 9A6D52, which recognizes a viral capsid component. Fewer than 1% of the infected cells were found to express late HHV-6 gene products. Jurkat cells infected with HHV-6 in the presence or in the absence of PFA were analyzed for CD4 expression at various times post-infection. A significant upregulation of CD4 was detected also in PFA-treated cells, despite the lack of expression of late virus products. This strongly indicates that immediate or early gene products are involved in the HHV-6-mediated CD4 upregulation.

To investigate whether HHV-6 could induce de novo CD4 expression, we subsequently analyzed the effects of HHV-6 infection on cells totally devoid of CD4, such as normal mature $CD8^+$ T cells. Although the CD8 genes are inducible by physiological stimuli, such as IL-4, no physiological agent capable of inducing CD4 has been previously identified. A productive infection by HHV-6 induced de novo expression of CD4 in these $CD8^+$ T cells. At any time point, the number of $CD4^+$ cells within the cultures correlated well with the number of cells expressing HHV-6 antigens. By two-color fluorocytometry, all the cells which acquired CD4 were simultaneously $CD8^+$. No alterations of either CD8 or CD2 antigen expression were detectable.

We subsequently studied T lymphocytic clones of $CD3^+CD4^-CD8^+$ phenotype. The clones were obtained by the limiting dilution technique from normal adult peripheral blood. In all the clones tested (n=5), productive HHV-6 infection induced de novo expression of CD4 antigens. By contrast, the expression of CD8 was unaltered. HHV-6 consistently failed to induce de novo CD8 expression in any of seven $CD3^+CD4^+CD8^-$ T cell clones tested as controls. In addition, HHV-6 induced CD4 expression in the human T cell line HSB-2, which displays an immature $CD2^-CD3^-CD4^-CD7^+CD8^-$ phenotype, as well as in the $HTLV\text{-}I^+$ $CD8^+$ mature T cell clone 67-I.

To determine whether HHV-6 was able to trigger de novo transcription of CD4 mRNA, Northern blot analysis was performed on RNA extracted from normal CD4-$CD8^+$ T cell populations before and after infection with HHV-6. A 3.0 kilobase signal specific for CD4 mRNA was dramatically induced in these $CD8^+$ T cells at day 8 post-infection. Simultaneous analysis of HHV-6 mRNA expression using a specific probe demonstrated that the induction of CD4 mRNA occurred in parallel with the transcription of viral proteins. These findings suggested that the mechanism of CD4 induction by HHV-6 was probably acting at the transcriptional level.

HHV-7

Part of the continuing efforts of this laboratory has been directed toward trying to isolate new viruses and to investigate the viral etiology of identifying the diseases of the hematopoietic cells, including lymphomas, leukemias and immunodeficiency. Out of one PBMC culture from a patient with chronic fatigue

syndrome a herpesvirus was isolated which seemed to be distantly related to, but significantly different from HHV-6. The difference between the new isolate and HHV-6 was apparent from immunological, molecular and biological studies. In a large survey of human sera, some did not react against the new isolate, while they disclosed a positivity against HHV-6. Using monoclonal antibodies to the HHV-6 protein, only 2 out of 9 reacted with cells infected with the new isolate. Molecular probes recognizing a number of human and simian herpesviruses were tested by Southern blot hybridization. Only HHV-6 reacted with no more than 31.5% similarity. Like HHV-6, infection with the new herpesvirus isolate is associated with the appearance of giant cells, which ultimately lyse. The infected cells are T-lymphocytes--$CD2^+$, $CD3^+$, $CD7^+$, $CD4^+$, and rarely $CD8^+$.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05534-05 LTCB

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Mechanisms of HIV-1 Pathogenesis, AIDS-associated Kaposi's Sarcoma

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

PI:	R. C. Gallo	Chief	LTCB NCI
Others:	F. Michaels	Staff Fellow	LTCB NCI
	P. Lusso	Visiting Associate	LTCB NCI
	B. Ensoli	Visiting Associate	LTCB NCI
	G. Barillari	Guest Researcher	LTCB NCI
	C. Balotta	Guest Researcher	LTCB NCI
	L. Bunonoguro	Guest Researcher	LTCB NCI
	H. K. Chang	Guest Researcher	LTCB NCI

COOPERATING UNITS (if any)
Department of Cell Biology, Advanced BioScience Labs., Inc., Kensington, MD (P. D. Markham, S. E. DeRocco); Laboratory of Immunogenetics, NIAID, NIH (M. Malnati); Laboratory of Immunoregulation, NIAID, NIH (A. DeMaria)

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Hematopoietic Cellular Control Mechanisms Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
11.0	7.0	4.0

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.)

Primary human macrophages infected with a monocytotropic human immunodeficiency virus type-1 (HIV-1) isolate were found to secrete one or more factors which had a significant effect on the rate of proliferation of cells derived from the synovial lining of uninfected donors. Quantitation of the cytokine present in the supernatants from infected macrophages showed that although several cytokines were expressed, none were present in amounts adequate to explain the proliferation of the test cells. These data suggest that not only may HIV-1 infected macrophages mediate disease expression in infected individuals, but that, individually, substimulatory concentrations of cytokines may operate addictively or synergistically.

The interaction of human herpesvirus-6 (HHV-6) and the pathogenic human retroviruses, human T lymphotropic virus type-I and HIV-1, was shown to result in both an expanded host cell range due to phenotypic mixing of the viruses as well as the modulation of CD antigens on infected cells. Although HHV-6 infection occurred only after cellular activation, the virus receptor appeared to be present continuously. Further studies showed that HHV-6 infection increased the surface density of the CD4 antigen as well as de novo expression on otherwise CD4- cells.

Project Description

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

R. C. Gallo	Chief	LTCB NCI
F. Michaels	Staff Fellow	LTCB NCI
P. Lusso	Visiting Associate	LTCB NCI
B. Ensoli	Visiting Associate	LTCB NCI
G. Barillari	Visiting Fellow	LTCB NCI
L. Bunonoguro	Guest Researcher	LTCB NCI
H. K. Chang	Guest Researcher	LTCB NCI
A. Garzino-Demo	Guest Researcher	LTCB NCI
F. Cocci	Guest Researcher	LTCB NCI
V. Fiorelli	Guest Researcher	LTCB NCI
R. Gendleman	Guest Researcher	LTCB NCI

Objectives:

Characterization of possible viral cofactors which can accelerate the dissemination of human immunodeficiency virus type-1 (HIV-1) and disease progression. Identification of the cellular receptors for human herpesvirus-6 (HHV-6). Elucidation of the role of immune stimulation and the interaction of HIV-1 proteins in the development and maintenance of acquired immunodeficiency syndrome-associated Kaposi's sarcoma (AIDS-KS). Identification of the factors released by HIV-1 infected human macrophages.

Methods Employed:

A variety of immunological, virological, molecular and cell biological techniques were employed. Some novel experimental tools, e.g., a human T lymphotropic virus (HTLV)-II-transformed virus-producer cell line (VevII), were developed in our laboratory.

Major Findings:

Identification of the Target Cell for Infection by HHV-6 In Vitro

Mononuclear cell populations were obtained from normal human umbilical cord blood, adult peripheral blood, bone marrow, thymus, lymph node and tonsil. The mononuclear populations were isolated by Ficoll-Hypaque gradient centrifugation, activated for 24 hours with 2 μg/ml purified phytohemagglutinin (PHA) and exposed to HHV-6 at the approximate multiplicity of infection (MOI) of 1. The cultures were kept at 37°C in complete medium in the absence of exogenous interleukin-2 (IL-2). In fact, ^{3}H-thymidine incorporation studies suggest that HHV-6-infected cells do not respond to exogenous IL-2. Infected cultures were monitored for the appearance of the typical morphologic changes induced by the virus and for the presence of intracellular HHV-6 antigens by indirect immunofluorescence (IF) analysis using specific monoclonal antibodies (mAb). The efficiency of infection, as measured by the ratio between the number of cells exposed to the virus and the

absolute number of cells expressing HHV-6 antigens at day 8 post-infection, varied among different mononuclear cell sources. Cord blood was more efficient than peripheral blood, perhaps as a consequence of the negligible number of specific anti-HHV-6 cytotoxic T lymphocytes in the newborn. The vast majority of HHV-6-infected cells expressed phenotypic features of activated $CD4^+$ T lymphocytes, exhibiting CD2, CD4, CD5, CD7, CD71 and, in a limited percentage, CD8, CD15 and class II MHC antigens (DR). The absence of surface CD3/TCR complex, together with the frequent coexpression of CD4 and CD8 could be related to cell immaturity or simply to phenotypic alterations induced by the infection. The expression of messenger RNA (mRNA) of the α and ß chains, but not the τ chain, of the TCR indicated a mature origin for HHV-6-infected cells.

Identification of the Cellular Receptor for HHV-6

Despite the predominant tropism for $CD4^+$ T cells, HHV-6 does not use CD4 as a receptor to penetrate susceptible cells. $CD4^+$ cells also constitute the major target of the human retroviruses discovered to date--namely, HTLV-I, HTLV-II, HIV-1 and HIV-2--and the CD4 molecule itself represents the membrane receptor for two of them (HIV-1 and HIV-2). We have performed competition experiments utilizing either mAb directed to human CD4 (OKT4, OKT4a, Leu3a) or the truncated, soluble form of the CD4 protein (sCD4) in an attempt to block viral infection. Normal, PHA-activated human cord blood mononuclear cells were pretreated for 1 hour at 4°C with the relevant mAb at 5 µg/ml, then exposed for 1 hour to either HHV-6 or to HIV-1, at an MOI of approximately 10^2 $TCID_{50}$ for either virus. The cells were subsequently washed several times and cultured for up to 20 days post-infection. To test the activity of sCD4, virus stocks were preincubated with the protein (at 3 µg/ml) for 1 hour at 4°C and subsequently used to infect PHA-activated cells. The mAb and sCD4 were maintained at the original concentration during the entire culture period. Virus expression was monitored by indirect IF, titration of released virus and antigen capture assays. No inhibition of HHV-6 expression was observed in any of the tests, while sCD4 and the mAb OKT4a and Leu3a completely abrogated HIV-1 infection. As further evidence for the use of different receptor structures by the two viruses, HHV-6 was shown to effectively superinfect human T-cell lines chronically harboring HIV-1 (e.g., CEM-[IIIB]), whose membrane CD4 is completely downregulated or complexed with the gp120 envelope glycoprotein of HIV-1. We concluded that HHV-6 infection occurs via the CD4-independent mechanism(s). The lack of interference between HHV-6 and HIV-1, at least in the initial phases of cellular infection, may be clinically relevant since the simultaneous presence of both viruses within the same target cell can permit HHV-6 to *trans*-activate the HIV-1 regulatory sequences.

HHV-modulation of CD4 Antigen Expression

A possible interaction between coinfecting viruses is at the level of receptor regulation. We have noticed that HHV-6-infected cells had consistently higher expression of CD4 surface antigen, i.e., the HIV-1 receptor. This observation suggested that the interaction between HIV-1 and HHV-6 might be more complex than coinfection of the same cell. To further dissect the possible mechanisms of HHV-6 interaction with HIV-1, we studied the effect of HHV-6 infection on the expression of the HIV-1 receptor, i.e., the CD4 glycoprotein. The modulation of CD4 expression induced by HHV-6 infection was initially studied in the human

neoplastic T-cell line, Jurkat, a cell line with a low basal expression of CD4. Following HHV-6 infection, a dramatic upregulation of CD4 was observed by indirect IF analysis and fluorocytometry in parallel with the expression of HHV-6-specific antigens. Treatment with conditioned medium (CM) from PHA-stimulated cord blood lymphocytes had no effect on CD4 expression by uninfected cells, whereas the combined action of PHA and phytohemagglutinin induced the downregulation of the antigen. To evaluate whether the ability to upregulate CD4 was common to other herpesviruses, Jurkat cells were infected with herpes simplex virus-1 (HSV-1) or human cytomegalovirus (HCMV). As previously documented, infection by HCMV in human T cells was nonproductive and virus expression was restricted to the immediate-early genes. No alteration of CD4 expression occurred during the course of infection with HSV-1 or HCMV. Analogously, CD4 was not induced in the B-lymphoblastoid cell line Raji after productive infection with Epstein-Barr virus. These data indicate that HHV-6 can favor the spread of HIV-1 infection by positively regulating its receptor on the cellular surface membrane. These observations were extended by blocking HHV-6 transcription at its later stages. When the drug phosphonoformate (PFA) was added at the concentration of 100 μg/ml, HHV-6 infection was blocked at the level of immediate early gene expression. The lack of late gene product expression was documented using the monoclonal antibody 9A6D52, which recognizes a viral capsid component. Fewer than 1% of the infected cells were found to express late HHV-6 gene products. Jurkat cells infected with HHV-6 in the presence or in the absence of PFA were analyzed for CD4 expression at various times post-infection. A significant upregulation of CD4 was detected also in PFA-treated cells, despite the lack of expression of late virus products. This strongly indicates that immediate or early gene products are involved in the HHV-6-mediated CD4 upregulation.

To investigate whether HHV-6 could induce de novo CD4 expression, we subsequently analyzed the effects of HHV-6 infection on cells totally devoid of CD4, such as normal mature $CD8^+$ T cells. It is well known that in the course of the intrathymic T-cell ontogenesis, functionally competent $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$ T lymphocytes develop from immature $CD4^-CD8^-$ thymocytes, following the transient acquisition of a double-positive, $CD4^+CD8^+$ phenotype. The partition between $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells is generally considered to be irreversible, although a small percentage of circulating $CD3^+$ T lymphocytes coexpressing CD4 and CD8 antigens has been identified. Although the CD8 genes are inducible by physiological stimuli, such as interleukin-4, no physiological agent capable of inducing CD4 has been previously identified.

Extensively purified (>99.9%) $CD3^+CD8^+$ T cells from normal adult peripheral blood mononuclear cell populations were prepared. A productive infection by HHV-6 induced de novo expression of CD4 in these $CD8^+$ T cells. At any time point, the number of $CD4^+$ cells within the cultures correlated well with the number of cells expressing HHV-6 antigens. For example, at day 8 post-infection, 51.0% of the cells expressed viral antigens by indirect IF, while CD4 was detected in 34.5%. By two-color fluorocytometry, all the cells which acquired CD4 were simultaneously $CD8^+$. No alterations of either CD8 or CD2 antigen expression were detectable.

To conclusively demonstrate that the observed CD4 induction was not due to the selective outgrowth and differentiation of a minor subset of immature double-negative T cells or, alternatively, to the presence of residual $CD4^+$ cells, we

subsequently studied T-lymphocytic clones of the $CD3^+CD4^-CD8^+$ phenotype. The clones were obtained by the limiting dilution technique from normal adult peripheral blood. In all the clones tested (n=5), productive HHV-6 infection induced de novo expression of CD4 antigens. In one clone, the percentage of HHV-6-expressing cells at day 8 post-infection was 58.0%, while CD4 increased from 0% to 40.9%. By contrast, the expression of CD8 was unaltered. HHV-6 consistently failed to induce de novo CD8 expression in any of seven $CD3^+CD4^+CD8^-$ T-cell clones tested as controls. In addition, HHV-6 induced CD4 expression in the human T-cell line HSB-2, which displays an immature $CD2^-CD3^-CD4^-CD7^+CD8^-$ phenotype, as well as in the HTLV-I^+ $CD8^+$ mature T-cell clone 67-I.

Studies were undertaken to identify the mechanisms by which HHV-6 acted to increase the cellular expression of CD4. To determine whether HHV-6 was able to trigger de novo transcription of CD4 mRNA, Northern blot analysis was performed on RNA extracted from normal $CD4^-CD8^+$ T cell populations before and after infection with HHV-6. As expected, no signal was detected in the uninfected cells. In contrast, a 3.0 kilobase signal specific for CD4 mRNA was dramatically induced in these $CD8^+$ T cells at day 8 post-infection. Simultaneous analysis of HHV-6 mRNA expression using a specific probe demonstrated that the induction of CD4 mRNA occurred in parallel with the transcription of viral proteins. These findings suggested that the mechanism of CD4 induction by HHV-6 was probably acting at the transcriptional level.

Secretion of Biologically Active Factors by HIV-1 Infected Macrophages

In order to elucidate the mechanisms responsible for the development of the HIV-associated arthritis, human synovial type A cells were isolated from the synovial lining of 13 patients undergoing total joint replacement for osteoarthritis. The cells were expanded in culture and phenotypically characterized. It was found that although the type A synovial cells exhibit several macrophage-like characteristics such as phagocytosis and the ability to act as accessory cells in an immune response, the cells could not be infected with monocytotropic isolates of HIV-1. This appears to be a significant difference between HIV-1 and caprine arthritis-encephalitis virus which has been shown to efficiently infect analogous type A cells isolated from goats. We were not able to show that human synovial type A cells express the T4 antigen at levels detectable with immunohistological techniques, suggesting that the T4 antigen may be obligatory for infection in a phagocytic cell. Further experiments showed that infectious virus could not be rescued from synovial type A cells by either primary human monocytes/macrophages or T lymphocytes, indicating that the cells did not become latently infected. These results indicated that the HIV-associated arthritis must be mediated by virus infected monocytes/macrophages or T lymphocytes, probably within the lesion. To further elucidate the mechanisms responsible for the HIV-associated arthritis, cultures of primary human monocytes/macrophages and T cells were infected with the T lymphotropic isolate HIV-1(IIIB) or the monocytotropic isolate HIV-1(BaL). The culture supernatants were collected at weekly intervals and virus removed by ultracentrifugation. The amount of HIV-1 expressed by the infected cells was quantified by both reverse transcriptase activity and p24 antigen capture assays. The expression of HIV-1(IIIB) was significantly less than that of HIV-1(BaL) by infected monocytes at all times after infection. The expression of HIV-1(IIIB) by T cells was significantly different in that the T tropic isolate was expressed

earlier and in larger quantities than the macrophage tropic BaL. However, the expression of both viruses declined rapidly, probably due to the death of the infected cells.

It has been shown by others that a strong correlation exists between the presence and progression of arthrogenic lesions, and the proliferative capacity of synovial lining cells (SLC) explanted from the lesions. Further, SLC are believed to contribute significantly to the progression of arthritis by the secretion of collagenase; an enzyme shown to be present in the synovium of patients with arthritis. Therefore, to test the arthrogenic potential of the supernatants from the infected T cells and macrophages, serial dilutions were added to cultures of SLC, and the proliferation of the synoviocytes quantified by the incorporation of ^{3}H-Tdr. Preliminary experiments had shown that SLC which had been exposed to 2000 rads of ionizing radiation remained viable but failed to incorporate significant amounts of the tracer, indicating that thymidine incorporation reflected mitotic activity. The supernatants from both HIV-1 infected and uninfected T cells had minimal to no stimulatory effect on the proliferation of SLC at any of the times tested. A statistically significant increase in SLC proliferation was observed in SLC cultures treated with CM from T cells collected at 21 days post-infection, but the difference between CM from infected and uninfected cultures probably represented the massive cell death noted in the infected cultures. Unlike the activity of T cell supernatants, however, supernatants collected from macrophage cultures had a significant mitogenic activity. This activity was present in CM from both infected and uninfected cells, indicating that the culture conditions used in these experiments stimulated the macrophages to secrete one or more factors which prompted the SLC to proliferate. Although no significant difference between HIV-1 infected and uninfected macrophage supernatants was evident at 7 days, statistically significant differences were observed at 14 and 21 days post-infection in supernatants from BaL infected cells; macrophages infected with HIV-1(IIIB) caused no more SLC proliferation than CM from uninfected cells. Although the increased SLC proliferation was modest when expressed as a percentage (22% to 29%), these numbers do not reflect the inherent mitogenic activity of supernatants collected from uninfected macrophages. This observation suggested that the relatively small magnitude of the SLC proliferation induced by CM from HIV-1(BaL) infected macrophages probably represents a significant pathogenic potential in vivo.

To further characterize the fundamental mediators responsible for the mitogenic response, two sets of experiments were undertaken. The first series of experiments examined the mitogenic potential of two conditioned supernatants previously shown to have significant activity of AIDS-KS cells, HTLV-II CM and CM from PHA treated T cells collected early after stimulation. In addition, several recombinant cytokines were examined for an ability to cause SLC to increase proliferation. Both HTLV-II CM and PHA CM demonstrated a significant mitogenic effect. Tumor necrosis factor-α (TNF-α), epidermal growth factor, and IL-1-α and -β all had the ability to mediate an increased rate of proliferation of SLC. TGF-β was found to significantly decrease the mitosis of the test cells at most concentrations; however, the suppression was reversed at the lowest concentrations tested. Cytokines also tested, but found to have no effect, included insulin-like growth factors 1 and 2, granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte-colony stimulating factor (M-CSF), IL-6 and IL-4.

The second series of experiments was designed to quantify the cytokines present in the CM from both macrophages and T cells infected with HIV-1 as well as the uninfected control cultures. These experiments are in progress, but preliminary data indicate that the concentrations of the two most mitogenically potent factors tested, TNF-α and IL-1-α, were not present in concentrations adequate to account for the proliferative effects observed. The low concentrations of IL-1 also indicate that the concentration of bacterial lipopolysaccharide was minimal in the cultures, demonstrating that the expression of the cytokines which caused the proliferation was the result of HIV-I infection. These data suggest that either an unrecognized factor is present, or that two or more recognized factors are working in an additive manner. In either case, the data presented indicate that macrophages infected with a monocytotropic isolate of HIV-1 secrete one or more agents that have a discernable biological effect of cells known to be central to a lesion clinically associated with HIV-1 infection.

Mechanism of Development of the KS Lesion: Role of Cytokines and Angiogenic Factors

To correlate the several biological activities expressed by cultured AIDS-KS cells with specific cytokines, we first analyzed these cells for mRNA expression of a number of candidate cytokines and growth factors. By comparison with normal endothelial cells and fibroblasts, AIDS-KS cells constitutively expressed very high levels of mRNA for basic fibroblast growth factor (bFGF) and IL-1-β, moderate levels of mRNA for GM-CSF, TGF-β, platelet-derived growth factor (PDGF)-β, and low levels of mRNA for a FGF and IL-1. Furthermore, mRNA for PDFG-A and IL-6 were also expressed by the AIDS-KS cells. There was no detectable expression of mRNA for other cytokines, such as TGF-α, angiogenin, M-CSF, TNF-α and -β, IL-2, and interferon (IFN). Since bFGF and IL-1 were expressed at much higher levels by the AIDS-KS cells compared to control cells, and in view of their activities on mesenchymal cells, on the vascular system and immune system, we further focused on these two cytokines, demonstrating that high levels of both proteins were synthesized and released by the AIDS-KS cells. Specific antisera to these molecules inhibited the basal growth of the AIDS-KS cells as well as the proliferation of human umbilical vein endothelial cells (H-UVE). Both forms of IL-1 (α and β), and PDGF induced significant proliferation of AIDS-KS cells. Low levels of cell growth are also stimulated by IL-6 and TGF-β. Smooth muscle cells proliferate in response to several of the molecules produced by AIDS-KS cell and inducing AIDS-KS cell growth (FGF, PDGF).

Role of Immunostimulation and Cytokine Release

Our previous studies on AIDS-KS have elucidated some of the mechanisms in the formation of the KS lesion, and demonstrated that the HIV-1 gene product, Tat, may play a role in KS pathogenesis. However, the reasons for the very high risk of KS development in HIV-1 infected homosexual or bisexual men are unclear. In the early stages of HIV-1 infection, AIDS-KS patients often show signs of immunoactivation and are only marginally immunosuppressed, suggesting that immune stimulation may play a pivotal role in the development of AIDS-KS. The HTLV cell line which is used as the source of CM for the long-term culture of the AIDS-KS cells expressed several cytokines normally produced during T cell activation, suggesting that T cell activation products may play a role in vivo in the

pathogenesis of KS. To clarify to biological bases of these clinical-epidemiological and in vitro observations, and to evaluate the role of immunoactivation in AIDS-KS, we investigated whether CM from mitogen activated primary immune cells could induce proliferation of cells derived from KS lesions of AIDS patients and of other cells of mesenchymal origin.

The CM reproducibly stimulated AIDS-KS and adult aortic smooth muscle cell (AA-SMC) growth, and low levels of H-UVE cell proliferation. Protein and mRNA analysis indicated that several cytokines were expressed by both types of activated T cells (HTLV-II CM and PHA-T CM). IL-α and -β, TNF-α and -β, PDGF and, to a lesser extent, IL-6 and GM-CSF promoted the growth of AIDS-KS cells at concentrations shown to be biologically active in other systems. A synthetic CM (made by combining the cytokines at the same concentration present in PHA-CM and HTLV-II CM, in vitro CM) stimulated a growth response very similar or identical to that obtained with CM from activated primary T cells. These results demonstrated that cytokines released from activated primary lymphocytes can induce growth of AIDS-KS cells and of normal mesenchymal cells present in the KS lesion, and that the combination of individually submitogenic levels of several cytokines had additive or synergistic growth effects on mesenchymal cells.

Cell Growth Effects and Uptake of Recombinant Tat by Mesenchymal Cells

Recombinant purified Tat protein from different sources stimulated proliferation of target cells derived from KS lesions of AIDS patients at the picogram to nanogram concentrations. Interestingly, the curve of the dose-response with recombinant Tat shows two peaks of cell proliferation which are reproducibly observed with different protein preparations, one around 100 picograms/ml and a second one around 10 nanograms/ml. These data suggest that two different pathways for cell proliferation are activated by different concentrations of Tat, as has been shown for other peptides lacking a signal sequence for release. Similarly Tat stimulated a growth response on other mesenchymal-derived cells (normal human smooth muscle and endothelial cells), but only after preincubation and activation with T cell activation products. These results suggest that cell surface modifications are necessary to induce normal mesenchymal cell types to become responsive to Tat.

Publications:

Ensoli B, Barillari G, Gallo RC. Pathogenesis of AIDS-associated Kaposi's sarcoma. Hematol Oncol Clin North Am 1991;5:281-95.

Franchini G, Markham PD, Gard E, Fargnoli K, Kueberuwa SS, Jagozinsky L, Robert-Gouroff M, Lusso P, Ford G, Wong-Staal F, Gallo RC. Persistent infection of rhesus macaques with a molecular clone of human immunodeficiency virus type 2: evidence of minimal genetic drift and pathogenetic effects. J Virol 1990;64:4462-7.

Gartenhaus R, Michaels F, Hall L, Gallo RC, Reitz MS Jr. Relative activities of HIV-1(IIIB) and HIV-1(BaL) tat in primary monocytes and lymphocytes. AIDS Res Hum Retroviruses (In Press).

Hattori N, Michaels FH, Fargnoli K, Marcon L, Gallo RC, Franchini G. The HIV-2 vpr gene is essential for productive infection of human macrophages. Proc Natl Acad Sci USA 1990;87:8080-4.

Lusso P. HHV-6 as a potential cofactor in AIDS. J Cell Biochem 1990;14:304.

Lusso P, DeMaria A, Mainati M, Lori F, DeRocco SE, Baseler M, Gallo RC. Induction of CD4 and susceptibility to HIV-1 infection in human $CD8^+$ T lymphocytes by human herpesvirus 6. Nature 1991;349:533-5.

Lusso P, DiMarzo Veronese F, Ensoli F, Ensoli B, Franchini G, Jemma C, DeRocco S, Kalyanaraman VS, Gallo RC. Pseudotypes in HIV-infected mice (technical comments). Science 1991;250:1152-4.

Lusso P, Lori F, Gallo RC. CD4-independent infection by human immunodeficiency virus type 1 after phenotypic mixing with human T-cell leukemia viruses. J Virol 1990;64:6341-4.

Marcon L, Michaels F, Hattori N, Fargnoli K, Gallo RC, Franchini G. Dispensable role of the human immunodeficiency virus type 2 vpx protein in viral replication. J Virol (In Press).

Michaels FH, Banks KL, Reitz MS Jr. Lessons from caprine and ovine retrovirus infections. Rheum Dis Clin North Am 1991;17:5-24.

Watkins BA, Dorn H, Kelly W, Armstrong R, Potts B, Michaels F, Kufta C, Dubois-Dalcq M. Specific tropism of HIV-1 for microglial cells in primary human brain cultures. Science 1990;249:549-53.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05535-05 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Retrovirus Infection and Treatment

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

PI:	P. S. Sarin	Research Chemist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI

COOPERATING UNITS *(if any)*
Worcester Foundation of Experimental Biology, Shrewsbury, MA (P. Zamecnik, J. Goodchild, S. Agrawal); Northwestern University, Chicago, IL (R. Letsinger)

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Hematopoietic Cellular Control Mechanisms Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	1.0	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.)*

Several approaches were utilized to identify drugs to block human immunodeficiency virus type 1 (HIV-1) replication in cell culture. Our studies show that palmitoil analogs of foscarnet can inhibit HIV reverse transcriptase and may be useful in the passage of the drug through the blood brain barrier. Chemically modified antisense oligonucleotides have been found to inhibit HIV replication in low concentration and without any toxic effect in cell culture.

The principal investigator, P. S. Sarin, departed LTCB in December 1990 and this project was terminated.

Project Description

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

P. S. Sarin	Research Chemist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI

Objectives:

Identify drugs that could interfere with human immunodeficiency virus (HIV) replication and to study their mechanism of action.

Methods Employed:

Standard virological, biochemical and cell biology techniques are utilized in the processing and culture of human peripheral blood. Retrovirus expression in the cell culture is measured by immunofluorescence with monoclonal antibodies against viral antigens, reverse transcriptase (RT) assay and syncytia formation.

Major Findings:

Inhibitors of RT has been a major target for blocking HIV replication. Foscarnet is currently undergoing clinical trials in several countries. We have prepared palmitoil analogs of foscarnet, which is also an effective HIV-1 inhibitor. The presence of the lipid group may be useful in the passage of the drug through the blood brain barrier.

Antisense oligodeoxynucleotides and analogs, such as methylphosphonates and phosphothioates were used as specific inhibitors of the acquired immunodeficiency virus. Several oligonucleotides (20-mers) complementary to HIV were tested. The most active oligomers were complementary to splice acceptor site (5358) and to splice donor site (5626). Methylphosphonate and phosphothioate analogs of the same target were about 10 times more effective than the unmodified oligomers, probably due to the increased stability.

Since HIV-1 has been shown to contain high cholesterol to phospholipid ratio compared to the normal lymphocyte membrane, we examined a number of oligomers bound to cholesterol. This modification may enhance the affinity of cholesterol oligomers for HIV infected cell membrane. We find that attachment of cholesterol residue to a oligomer (20-mer) complementary to the splice acceptor site (5358) changes the inhibitory doses about 10-50 times lower without affecting the toxicity, suggesting that these compounds will be nontoxic at therapeutic doses needed to block HIV replication.

The principal investigator, P. S. Sarin, departed LTCB in December 1990 and this project was terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05536-05 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Humoral and Cellular Immune Responses to HIV for Vaccine Development

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

PI:	M. Robert-Guroff	Research Biologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	A. Abimiku	Guest Researcher	LTCB NCI
	M. S. Reitz, Jr.	Senior Staff Scientist	LTCB NCI
	G. Franchini	Visiting Scientist	LTCB NCI
	W. A. Blattner	Chief, Viral Epidemiology Section	EEB NCI
	J. Goedert	Medical Officer	EEB NCI
	P. Pizzo	Chief, Pediatric Branch	DCT NCI

COOPERATING UNITS *(if any)*
Repligen Corp., Cambridge, MA (S. Putney); Advanced BioScience Lab., Kensington, MD (P. Markham); Univ. Pierre et Marie Currie, Paris (D. Zagury); Med-Immune, Inc., Gaithersburg, MD (T. Fuerst); Wyeth-Ayerst Research, Radnor, PA (P. Hung)

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Hematopoietic Cellular Control Mechanisms Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	2.0	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.)*

This project aims to delineate immunologic responses to the human immunodeficiency viruses (HIVs), and to identify viral subfragments which elicit protective humoral and cellular immunity to HIV-I, -2, and simian immunodeficiency virus (SIV). Neutralizing antibodies are being studied in monocyte/macrophage and T-cell systems. Protective immunity of nontransmitting HIV-seropositive mothers is also under investigation. To study the impact of viral heterogeneity on neutralization, serotypes were defined among Zairian isolates using matched sera. While immunologically related V3 loops (the principal HIV-1 neutralizing determinant) have conserved sequences, serotypes deduced by cross-neutralization were not based on V3 loop homology. Thus, alternate or conformational epitopes must play a role in broad neutralization. Enhanced neutralization by Zairian sera of an MN-V3 loop/HXB2D chimera was attributed to better presentation and recognition of either the V3 loop or an alternate epitope(s), depending on the serum studied. Thus, the context in which the V3 loop is presented affects neutralization. Study of neutralization resistant variants immune selected in vitro allows further elucidation of neutralizing epitopes. "Escape mutants" obtained to date exhibit changes outside the V3 loop, further implicating alternate or conformational epitopes. Cell mediated immune responses to the HIVs and SIVs are under investigation in several animal systems including rhesus macaques, mice, and dogs. The low viral load seen in HIV-2 infected macaques is not due to significant neutralizing or cell mediated immune responses. Nevertheless, cytotoxic T lymphocyte (CTL) clones obtained from these infected macaques will allow mapping of CTL epitopes. Humoral and cell mediated immune responses are under investigation in macaques immunized with vaccinia, avipox, or BCG vectors carrying HIV or SIV genes. For future vaccine programs, isolation and characterization of West African retroviruses have been initiated. These studies will elucidate viral heterogeneity in this region and possibly uncover novel retroviruses.

Project Description

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

M. Robert-Guroff	Research Biologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
A. Abimiku	Guest Researcher	LTCB NCI
M. S. Reitz, Jr.	Senior Staff Scientist	LTCB NCI
G. Franchini	Visiting Scientist	LTCB NCI
W. A. Blattner	Chief, Viral Epidemiology Section	EEB NCI
J. Goedert	Medical Officer	EEB NCI
P. Pizzo	Chief, Pediatric Branch	DCT NCI

Objectives:

The objectives of this project are to learn what natural immune surveillance mechanisms operate in human immunodeficiency virus type-1 (HIV-1) and HIV-2 infected individuals, whether such natural responses can be manipulated to enhance protection against virus infection or disease progression, and how to construct vaccine preparations to result in such protective immune responses. As retroviral neutralizing antibodies are known to result in protection against a viral challenge in animal systems, we initially focused on this humoral immune response. We subsequently pursued alternate humoral protective responses. More recently, we have undertaken investigation of the cellular immune response to the human immunodeficiency viruses. The specific goals of the present project are:

1. to determine to what extent HIV-1 and HIV-2 neutralizing antibodies are protective, focusing on monocytes/macrophages instead of T-cells;

2. to identify viral epitopes eliciting protective antibodies in seropositive mothers who fail to transmit HIV to their offspring;

3. to determine the influence of viral envelope heterogeneity on elicitation and function of neutralizing antibodies;

4. to identify regions of the virus envelope important for viral infectivity and neutralization;

5. to identify viral antigens which elicit cellular immune responses;

6. to determine the efficacy of viral antigen preparations, including individual gene products, recombinant constructions, viral subfragments, and synthetic peptides as vaccine materials; and

7. to characterize HIV isolates from potential vaccine target areas for use as immunogens and as analytical reagents, and to search for novel retroviruses in areas where human retroviruses are endemic.

Methods Employed:

Routine serologic assays for antibodies to HIV-1 and HIV-2 are carried out using the enzyme linked-immunosobent assay (ELISA) technique, Western blotting, radioimmunoprecipitation, and immune fluorescent assays. The method for assaying neutralization of monocyte/macrophage infection has been adapted from Robert-Guroff et al. (Nature 316:72, 1985). Fresh, elutriated monocytes/ macrophages are plated in microtiter plates. After allowing attachment for 2 days, the cells are infected with HIV previously incubated with test or control serum. Infection is monitored on tissue culture fluids by an antigen capture assay for p24 (Dupont, Boston, MA). Neutralization of T-cell infection is carried out as previously described (Robert-Guroff, in Aldovini, A. and Walker, B. (Eds.) Techniques in HIV Research, Stockton Press, New York, 1990, pp. 179-185).

The methodology for immune selection of HIV variants in vitro has also been described (Robert-Guroff et al., J. Immunol. 137:3306, 1986) as have procedures for their molecular and immunologic analysis (Reitz et al., Cell 54:57, 1988; Wilson et al., J. Virol. 64:3240, 1990). Immune selecting sera in the HIV-1 system include high titered neutralizing patient sera, and monoclonal antibodies to V3 loop peptides (supplied by S. Putney, Repligen Corporation, Cambridge, MA). Patient sera are also used to select escape mutants in the HIV-2 system. Parental viruses used in these experiments include the infectious molecular clones of HIV-1/HTLV-IIIB(HXB2D) and HTLV-III(MN); chimeric viruses consisting of HXB2D into which portions of other viral envelopes have been inserted; and the infectious molecular clones of HIV-2 (ISY and NIH-Z).

Stable stocks of viruses used are prepared and titered for use in cross-neutralization studies. Synthetic peptides corresponding to the V3 loops of various isolates are obtained from Multiple Peptide Systems, Inc., San Diego, CA, and are used to generate hyperimmune sera in goats, to screen sera by the ELISA technique, and to compete directly in neutralization assays. Homologous and heterologous competitive peptide ELISAs are carried out using synthetic V3 peptides to compete the binding of hyperimmune goat sera to V3 loop peptides. A peptide ELISA is similarly used to detect putative protective antibodies in sera of nontransmitting mothers.

The cellular immune response to antigenic stimulation is determined by thymidine incorporation assays to measure cell proliferation and by interleukin 2 (IL-2) production. The latter assay makes use of the IL-2-dependent mouse CTL cells. General stimuli include phytohemagglutinin and tetanus toxoid; specific stimuli include inactivated viral preparations, autologous cells infected with vaccinia constructs carrying viral genes and subsequently irradiated, purified viral proteins, and synthetic peptides representing portions of viral antigens. CTL are monitored using a 6 hour chromium release assay in which autologous B cell lines serve as targets following infection with vaccinia-HIV constructs or incubation with synthetic peptides. Systems under investigation include rhesus macaques infected with HIV-2 and HIV-2 accessory gene mutants; rhesus macaques immunized with vectors (including attenuated vaccinia, avipox, and BCG) carrying genes of HIV-1, HIV-2, and simian immunodeficiency virus (SIV); mice immunized with

vaccinia-HIV constructs; and dogs and chimpanzees immunized with adenovirus-HIV constructs. CTL clones are obtained by limiting dilution in the presence of monoclonal antibody to CD3 and IL-2.

Major Findings:

Previous studies from our laboratory (Robert-Guroff et al., Pediatr. Res. 21:547, 1987; Robert-Guroff et al., J. Immunol. 138:3731, 1987; Robert-Guroff et al., AIDS Res. Hum. Retroviruses 4:343, 1988) and other groups (Sawyer et al., AIDS Res. Hum. Retroviruses 6:341, 1990; Emini et al., J. Virol. 64:3674, 1990) which have shown that neutralizing antibodies are correlated with better disease outcome and suggest their role in effective immune surveillance, have been carried out on T-cell targets. As monocytes/macrophages are an important reservoir for viral infection and latency, the role, if any, of neutralizing antibody in preventing infection of this cell type and the epitopes involved need to be addressed. We have established a system using fresh elutriated monocytes/macrophages, by which we can examine this question. Macrophage tropic isolates, or chimeric viruses rendered macrophage tropic by substitution of key components of the virus envelope, are assessed for neutralizability by natural human sera and specific hyperimmune and monoclonal antibodies. Our preliminary data suggest that neutralization of macrophage infection involves different epitopes than those participating in neutralization of T-cell infection. The identification of these epitopes will be important for development of subunit vaccines.

HIV seropositive mothers who fail to transmit virus to their offspring possess high affinity antibody to the viral envelope protein, gp120, which is not correlated with neutralizing antibody (Goedert et al., Lancet ii:1351, 1989). Similar protective antibodies in nontransmitting mothers have been reported to be elicited by certain epitopes in the third variable domain (V3) of gp120 (Rossi et al., Proc. Natl. Acad. Sci. USA 86:8055, 1989; Devash et al., Proc. Natl. Acad. Sci. USA 87:3445, 1990). We have reexamined the role of these epitopes in sera of children with AIDS, and have correlated reactivity with an amino terminal peptide of the V3 loop with lesser disease manifestation. Ongoing studies are aimed at further definition of such protective humoral responses.

The principal neutralizing determinant of HIV, the V3 loop, elicits highly type-specific neutralizing antibodies, complicating its use in vaccine preparations. To study the influence of viral heterogeneity on neutralization, we have analyzed neutralizing serotypes among Zairian isolates, using matched serum samples. Immunologic analysis of V3 loop sequences has been carried out using synthetic peptides and hyperimmune goat sera to the V3 loops in homologous and heterologous competition ELISA assays. As expected, highly related immunologic reactivity was associated with conservation of loop amino acid sequence. Nevertheless, in cross-neutralization analyses of Zairian isolates with matched sera, neutralizing serotypes did not correlate with homology in the V3 loop, suggesting the participation of alternate epitopes in broad neutralization. The role of envelope conformation in neutralization was also emphasized by studies on a chimeric virus composed of the V3 loop of the MN isolate substituted into the envelope of HXB2D. A panel of Zairian sera neutralized this chimeric virus with titers 20-fold higher compared to those against the MN isolate itself. Competition studies with V3 peptides showed this increase in titer could be attributed to better presentation

and recognition of either the V3 loop itself or an alternate epitope. Thus, the context in which the V3 loop is presented is crucial for neutralization. The chimeric virus approach is being pursued both for enhancing immunogenicity and for further analysis of neutralizing epitopes. Additional studies aimed at elucidating the principal neutralizing determinant of HIV-2 have implicated a role for the homologous V3 region in this related virus.

Use of an in vitro system for immune selection of HIV-1 "escape mutants" (Robert-Guroff et al., J. Immunol. 137:3306, 1986) with subsequent analysis of the changes resulting in neutralization resistance provides a means for further elucidation of neutralization epitopes. A point mutation in the envelope gene of our original immune selected variant resulted in the substitution of a threonine for an alanine residue at position 582 of the transmembrane protein (Reitz et al., Cell 54:57, 1988). We further showed that the 582 region is not a neutralizing epitope itself, but rather that it influences a neutralizing epitope at another location (Wilson et al., J. Virol. 64:3240, 1990). Recently, the importance of this 582 region was emphasized by the emergence of a revertant virus following long-term culture of the variant in the absence of immune selecting serum. Molecular analysis of the revertant has shown the basis for the reversion to neutralization sensitivity was a simple back mutation at position 582. Conservation of sequence in this region suggests an important functional role. Analysis of a second immune selected variant also suggests that neutralization resistance is due to a change occurring outside the V3 loop. The fact that both these variants are neutralized equally well by a monoclonal antibody to the V3 loop further suggests the existence of an as yet unidentified neutralizing epitope outside the V3 region.

A successful vaccine for the acquired immunodeficiency syndrome will likely contain a cell mediated immune component in addition to neutralizing antibody. We are currently investigating epitopes which can elicit cell mediated immunity for inclusion in subunit vaccines. Initial studies on rhesus macaques infected with HIV-2 and HIV-2 accessory gene mutants have shown that while the animals are not immune compromised, they have a poor proliferative response to HIV-2 antigens, and lack circulating CTLs specific for HIV-2. Thus, the low viral load in these animals can not be attributed to a strong immune response. These animals also lack neutralizing antibodies. We were able, however, to obtain HIV-2-specific CTL clones from several of the animals. These will be useful for mapping HIV-2 CTL epitopes for use in subsequent subunit vaccines.

In studies directly related to vaccine development, we have analyzed sera of dogs vaccinated with adenovirus constructs carrying the HIV-1 env gene. We observed a strong neutralizing antibody response in these animals following inoculation with two successive adenovirus constructs of different serotype. The antibody response declined over 12 weeks, but was readily boosted with purified recombinant envelope protein. These encouraging results form the basis for a vaccine trial in chimpanzees. If sufficiently high neutralizing antibody titers are achieved, the chimpanzees will be subjected to a live virus challenge. The cell mediated immune response in these chimpanzees will also be assessed. In additional studies the immune responses obtained following immunization of macaques with additional vectors carrying HIV or SIV genes are being assessed.

A pilot study has shown that target populations in West Africa possess antibodies to HIV-1, HIV-2, and HTLV-I, indicative of the prevalence of all these viruses in that region. In addition, the number of indeterminate Western blot profiles obtained in the serologic analyses suggests the possible presence of novel human retroviruses in this region. These observations will form the basis for new studies aimed at isolation and characterization of HIV-1 and HIV-2 strains in West Africa. Information concerning the variability of isolates from this region is important for vaccine development. In addition, characterization of new retroviruses will be important not only with regard to retroviral evolution, but also for possible new disease associations.

Publications:

Franchini G, Markham P, Gard E, Fargnoli K, Keubaruwa S, Jagodzinski L, Robert-Guroff M, Lusso P, Ford G, Wong-Staal F, Gallo RC. Persistent infection of rhesus macaques with a molecular clone of human immunodeficiency virus type 2: evidence of minimal genetic drift and low pathogenetic effects. J Virol 1990;64:4462-7.

Robert-Guroff M. HIV neutralizing antibodies: epitope identification and significance for future vaccine. In: Kennedy RC, Koff WC, eds. Development of AIDS Vaccines. New York: Harwood Academic Publishers (In Press).

Robert-Guroff M. Host immune response: neutralizing antibodies. In: Aldovini A, Walker B, eds. Techniques in HIV research. New York: Stockton Press, 1990;179-85.

Robert-Guroff M, Gallo RC. The interaction of human T-cell leukemia and human immunodeficiency retroviruses. In: Srivastava P, ed. Current concepts in immunogenetics. Munich: Verlag Chemie International Publications (In Press).

Robert-Guroff M, Popovic M, Gartner S, Markham P, Gallo RC, Reitz MS. Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. J Virol 1990;64:3391-8.

Vujcic L, Katzenstein D, Martin M, Quinnan G, Collaborating Laboratories. International collaborative study to compare assays for antibodies that neutralize human immunodeficiency virus. AIDS Res Hum Retroviruses 1990;6:847-53.

Wilson C, Reitz MS Jr, Aldrich K, Klasse PJ, Blomberg J, Gallo RC, Robert-Guroff M. The site of an immune-selected point mutation in the transmembrane protein of human immunodeficiency virus type 1 does not constitute the neutralization epitope. J Virol 1990;64:3240-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05537-05 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunopathogenesis of Human RNA and DNA Viruses

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

PI:	W. C. Saxinger	Research Microbiologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	L. Channavajjala	IRTA Fellow	LTCB NCI
	Y. Lunardi-Iskandar	Visiting Scientist	LTCB NCI
	J. Smythe	Guest Researcher	LTCB NCI
	P. Browning	US Public Health Service Officer	LTCB NCI
	M. Klotman	US Public Health Service Officer	LTCB NCI
	Z. Berneman	Visiting Fellow	LTCB NCI

COOPERATING UNITS *(if any)*
Howard University Hospital, Washington, DC (W. Frederick); North Shore Hospital, Long Island, NY (S. Pahwa); New England Regional Primate Centar, Boston, MA (R. DesRosiers)

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Hematopoietic Cellular Control Mechanisms Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	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.)*

Molecular structure analysis of proteins related to human immunodeficiency virus/human T lymphotropic virus (HIV/HTLV) pathogenesis, related growth and regulatory factors, and prevention of disease: Aspects of cell-receptor binding, humoral and cellular immune response, and vaccine design are being studied by combined approaches of peptide synthesis, physiological function, and molecular modelling.

Viral pathogenesis: Work on the structure and function of viral proteins and peptides has led to the findings that sequences of HIV gp41 and nef can stimulate resting normal B-cells poly-clonally to differentiate and produce IgG. We have also found that the carboxyl terminus of tat can specifically bind to cells. Detailed characterization of the mechanism and function of these reactivities are in progress.

The HIV/simian immunodeficiency virus (SIV)/HTLV envelope binding sites of neutralizing, non-neutralizing, and cross-reactive antibodies have been identified and their structural and functional properties are being determined. Applications to vaccine design are being studied.

U.S. prevalence of HTLV-I and HTLV-II and relation to disease: A retrospective random sampling of the U.S. population (NHANES-II), a retrospective geographic drug abuser population, and a current drug abuser population have been tested previously for HTLV-I antibody. Analyses in progress will indicate frequency of infection and its rate of change in these populations. The range of clinical manifestations of HTLV-I and HTLV-II will be monitored in the current population of intravenous drug abusers.

Project Description

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

W. C. Saxinger	Research Microbiologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
L. Channavajjala	IRTA Fellow	LTCB NCI
Y. Lunardi-Iskandar	Visiting Scientist	LTCB NCI
J. Smythe	Guest Researcher	LTCB NCI
P. Browning	US Public Health Service Officer	LTCB NCI
M. Klotman	US Public Health Service Officer	LTCB NCI
Z. Berneman	Visiting Fellow	LTCB NCI
R. Gartenhaus	Senior Staff Fellow	LTCB NCI
B. Ensoli	Visiting Associate	LTCB NCI
M. Robert-Guroff	Research Biologist	LTCB NCI
M. Reitz	Senior Staff Scientist	LTCB NCI
G. Franchini	Visiting Scientist	LTCB NCI
D. Ablashi	Microbiologist	LCMB NCI
W. Blattner	Head, Virology Section	EEB NCI
P. Levine	Medical Officer	EEB NCI

Objectives:

Application of "PEPSCAN" procedure to studies of human immunodeficiency virus/human T lymphotropic virus (HIV/HTLV) and simian immunodeficiency virus/simian T cell leukemia virus (SIV/STLV) viral protein structure and function, recognition of viral peptides by the immune system and vaccine development.

Molecular Definition of Viral Protein Functions

To study functional aspects of viral protein interactions and specificity by the combined approaches of peptide synthesis, physiological function, and molecular modelling.

Modulation of T- and B-cell Function by Viral Proteins

Characterization of stimulatory and suppressive effects on human lymphocytes produced by HIV/HTLV proteins. Comparison of various HTLV variants with respect to these characteristics in support of studies of mechanisms important in the pathogenesis and spread of HIV/HTLV.

Identification of New Virus Clusters in Humans and Primates

Identification of the environmental distribution of HTLV or of variant viruses related to HTLV in support of studies of mechanisms important in the pathogenesis and spread of HIV/HTLV.

Relationship Between Antigenic Drift and Immune Response in SIV-infected Macaques

The antibody response to specific subregions of viral proteins, most notably viral envelope, will be measured at time intervals post-infection with a molecularly cloned pathogenic SIV and will be correlated with clinical and biological data collected at the New England Regional Primate Center, Boston, MA.

Immunological and Structural Characterization of Human B Cell Leukemia Virus (HBLV)

Development of a screening test for antibodies to HBLV in various human populations for studies of disease association. Identification of immunogenic viral proteins and characterization of human humoral response. Translate open reading frames of HBLV sequence into synthetic peptides to identify important viral proteins.

Characterization of Disease Associations with HBLV

Progression of viral antigen with a newly developed antigen capture test and IgG/IgM status will be monitored in candidate lymphoproliferative and immunosuppressive disease states, including acquired immunodeficiency syndrome.

Apply Principles of PEPSCAN Automation to Design a System Capable of Synthesis at the Milligram Level

To provide an automated procedure for determination of cellular immune response epitopes, provide a method for direct immunogen scanning and location of antibody neutralization sites or other functional epitopes, and to determine active sites of growth factors and cell surface receptors.

Methods Employed:

A variety of immunochemical, immunological, and microbiological techniques are used. Antigen-antibody reactions are measured by standard and in-house developed enzyme linked-immunosorbent assay, radioimmunoassay, Western blot, and a variety of other immunoprecipitation techniques. Purification of cellular and viral proteins is accomplished by a variety of chromatographic (gravity and high performance liquid chromatography), electrophoretic, and centrifugation techniques. Large-scale epidemiologic data analysis is performed using a lab-based personal computer coupled with an IBM mainframe system for demographic data entry and storage of immunological test data. Assays for cellular immunity and modulation are performed by standard in vitro tests for hematopoietic cell function by incorporation of radiolabelled, growth or specific plaque assays. New assays for cell cytolysis and growth which allow the substitution fluorescent reporter molecules for the typical radioisotopic substrates have been installed. Peptide syntheses are performed by standard (9-fluorenylmethyoxycarbonyl amino group protection) chemical procedures integrated with automation devices developed within the LTCB.

PEPSCAN peptides representing an entire protein sequence or arbitrarily designed peptides to test the effect of amino acid deletion or variation are synthesized,

attached to the wells of 96 well microtiter plates and used directly to detect the binding of antibodies, of cells with specific receptors for the peptides, or of any other type of ligand which might bind to the peptides. Synthesis procedures are automated using a Biomek robotic workstation. The process for converting specific peptide sequences into instructions for the Biomek is accomplished automatically by programs written for an IBM PC.

Major Findings:

Refinement of System for Automated Peptide Design and Synthesis

New software allows synthesis of any peptide in any designated well by PC keyboard or file entry, facilitating design of peptide variants with specified deletions or substitutions. Conditions for preparation of organic solvent resistant microtiter plates with optimum spacing and density of functional amino groups has been achieved.

Analysis of Antibody Neutralization Structures of HIV

Complete loop structures (from C to C) have been prepared for 15 variants--HXB2, MN, SC, SF2, NY5, CDC451, WMJ2, RF, BaL, Z3, Z6, ELI, MAL, JY1, ISY, SIV239. Goat anti-HXB2 loop peptide (subregion) preferentially recognizes HXB2 intact loop and goat anti-RF loop peptide (subregion) reacts more broadly among the other variants. According to Dr. Robert-Guroff both goat antisera neutralize appropriately. Analyses will proceed to the study of cross-reactivities which are non-neutralizing versus neutralizing soon.

Seroprevalence of Antibodies Reacting with HIV/SIV Variants

Natural HIV-positive serum reactivities against the intact loop peptides for two groups, HIV^+ and HIV^+/$HTLV\text{-}I^+$ drug abusers have been studied. Reactivities against the North American isolates were variable, with the HXB2 loop usually very weakly reacting. However, this is likely due to the structure. Some of the sera tested against loop subregions of HXB2 reacted much better with the subregions than the intact loop. There were no reactions with the Zairian isolates. This data will be used to begin modelling a functional topology of the HIV V3 loop.

Recognition of a Cell Adhesion Domain at the C-terminus of HIV-1 Tat

Overlapping peptides representing HIV-1 HXB2 tat synthesized by automated PEPSCAN within microtiter plate wells were capable of specifically binding lymphocytes. The binding activity was determined to be within 24 amino acids from the carboxyl terminus. Contained within is an RGD sequence which has been identified as a member of the integrin binding family. The effect of these peptide sequences on normal cell and Kaposi cells will be studied. Recent studies have identified additional regions of the tat molecule which are capable of causing lymphocyte adherence. Experiments are in progress to determine the physiologic and biochemical outcome of lymphocyte adherence to HIV tat. Also in progress are experiments to determine the exact location of this new sequence, the degree and relevance of binding of vascular cells to this new sequence, and the physiologic outcome of this type adherence.

Characterization of HTLV-I and HTLV-II Prevalence in Normal Donor and Drug Abuser Populations

We have run a small pilot test of 10 HTLV-I and HTLV-II peptides and have resolved a group of normal blood donor antibody screen positives and a group of intravenous drug abuser (IVDA) screen positives. HTLV-II was ca 15% in blood donor screen positives and 55% in IVDA screen positives.

Dynamics of Antigenic Drift and Immune Response In SIV-infected Macaques

Antibody recognition sites of the SIV envelope have been successfully characterized by automated PEPSCAN techniques for several SIV isolates using sera from wild-caught macaque and African green monkey species. Recognizable antibody response to SIV peptides in a group of recipients was found to consist of peptides recognized by all animals and peptides recognized occasionally by some of the animals. Future samples will be analyzed for progressive changes within animals.

Publications

Ablashi DV, Salahuddin SZ, Balachandran N, Luka J, Henry B, Komaroff A, Josephs SF, Buchbinder A, Saxinger C, Kaplan M, Manak M, Pearson GR, Krueger GRF, Horwitz CA, Gallo RC. Immunobiology of human herpesvirus-6 (HHV-6). AIDS Res Hum Retroviruses 1990;6:143.

Blattner WA, Saxinger C, Riedel D, Hull B, Taylor G, Cleghorn F, Gallo R, Blumberg B, Bartholomew C. A study of HTLV-I and its associated risk factors in Trinidad and Tobago. J AIDS 1990;3:1102-8.

Buchwald D, Freedman AS, Ablashi DV, Sullivan JL, Caligiuri M, Weinberg DS, Hall CG, Ashley RL, Saxinger C, Balachandran N, Ritz J, Nadler LM, Komaroff AL. A chronic "postinfectious fatigue syndrome associated with benign lymphoproliferation, B-cell proliferation, and active replication of human herpesvirus-6. J Clin Immunol 1990;10:335-44.

Castro O, Saxinger C, Barnes S, Alexander S, Flagg R, Frederick W. Prevalence of antibodies to human immunodeficiency virus and to human T cell leukemia virus type I in transfused sickle cell disease patients. J Infect Dis 1990;162:743-5.

Levine PH, Saxinger WC, Ablashi DV, Salahuddin SZ, Connelly RR. Recent studies on the seroepidemiology of human herpesvirus-6 (human B-lymphotropic virus). AIDS Res Hum Retroviruses 1990;6:145.

Reeves WC, Levine PH, Cuevas M, Quiroz E, Maloney E, Saxinger WC. Seroepidemiology of human T cell lymphotropic virus in the Republic of Panama. Am J Trop Med Hyg 1990;42:374-9.

Saxinger C. Development of a system for automated peptide design and synthesis. AIDS Res Hum Retroviruses 1990;6:138.

Saxinger C, Ayub J, Frederick W. Seroprevalence of human antibodies reacting with HIV/SIV variant loop structures. AIDS Res Hum Retroviruses 1991;7:146.

Saxinger C, Frederick W, Bianco C. Representation of HTLV-I and HTLV-II seropositivity in normal blood donors and drug abusers based on synthetic peptide assays. AIDS Res Hum Retroviruses 1991;7:250.

Saxinger C, Lee S, Gallo R, Wong-Staal F. Characterization of a cell surface interaction with HIV-1 Tat. AIDS Res Hum Retroviruses 1991;7:196.

Saxinger C, McQuillan G, Haddon W, Murphy R, Bodner A, Gallo RC. HTLV-I antibody prevalence in a U.S. population survey: NHANES-II. AIDS Res Hum Retroviruses 1990;6:137.

Srivastava BIS, Gonzales C, Loftus R, Fitzpatrick JE, Saxinger, CW. Examination of HTLV-I ELISA-positive leukemia/lymphoma patients by Western blotting gave mostly negative or indeterminate reaction. AIDS Res Hum Retroviruses 1990;6:617-27.

Steeper TA, Horwitz CA, Ablashi DV, Salahuddin SZ, Saxinger C, Saltzman R, Schwartz B. The spectrum of clinical and laboratory findings resulting from human herpesvirus-6 (HHV-6) in patients with mononucleosis-like illnesses not resulting from Epstein-Barr virus or cytomegalovirus. Am J Clin Pathol 1990;93:776-83.

Patents:

Saxinger, WC. US Patent Pending: An Automated Peptide Design and Synthesis.

Saxinger, WC, Gallo, RC. US and European Patents Pending: Detection of Human T-cell Leukemia Virus Type III.

Saxinger, WC, Gallo, RC, Ablashi, DV, Salahuddin, SZ. US and European Patents Pending: Diagnostic Test for HBLV.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05538-05 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
HIV-1 Envelope Gene Variability

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

PI:	M. Reitz	Senior Staff Scientist	LTCB NCI
Others:	B. Watkins	Guest Researcher	LTCB NCI
	M. Robert-Guroff	Research Biologist	LTCB NCI
	F. Lori	Guest Researcher	LTCB NCI
	H.-G. Guo	Visiting Scientist	LTCB NCI
	R. C. Gallo	Chief	LTCB NCI
	W. Blattner	Chief, Viral Epidemiology Section	EEB NCI

COOPERATING UNITS *(if any)*
Rocky Mountain National Laboratory, Hamilton, MT (S. Pincus); Program Resources Inc., Frederick, MD (D. Waters)

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Molecular Genetics of Hematopoietic Cells Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	5.0	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.)*

One of the most important aspects of human immunodeficiency virus type 1 (HIV-1) env variability concerns its effect on immune recognition, which has profound implications for vaccine design. We are characterizing env determinants which are important for an effective immune response and studying how variability affects such a response. One approach involves transmitting and culturing molecularly cloned HIV-1 strains in the presence of natural antisera containing high titers of group- or class-specific neutralizing antibodies. Such viruses are resistant to neutralization by the selecting antiserum. We have characterized one such variant as well as the nature of the altered site, and are now characterizing others. We have developed a system for introducing the principal neutralization determinant (PND) loop from any virus into an infectious molecular clone of HIV-1. This will allow us to study the contribution of this loop to neutralization by natural sera. We have constructed a number of loop chimerae and have obtained an escape variant of one. We continue studies which involve viral isolates obtained from defined risk groups in Zaire, such as virus isolation, determination of env sequences, and analyses of appropriate sera for reactivity to peptides representing different parts of the envelope and ability to neutralize different wild-type and envelope chimeric viruses. We have established 16 Zairian isolates and sequenced portions of the gp120 of multiple clones from each isolate, including the PND neutralization loop. We have analyzed genetic variability which occurs in time within single individuals, and are beginning to correlate this with changes in neutralization specificity. We have obtained several different infectious molecular clones which differ in their biologic properties, such as cell tropism and cytopathic effect. We have initiated a project to molecularly clone and express human antibodies to HIV-1 in E. coli, and hope to be able to obtain pure natural neutralizing antibodies. We have begun studies designed to elucidate the mechanisms of neutralization by different antibodies. It is hoped this will lead to a better understanding of what constitutes a protective neutralizing response.

Project Description

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

M. Reitz	Senior Staff Scientist	LTCB NCI
B. Watkins	Guest Researcher	LTCB NCI
M. Robert-Guroff	Research Biologist	LTCB NCI
F. Lori	Guest Researcher	LTCB NCI
H.-G. Guo	Visiting Scientist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
W. Blattner	Chief, Viral Epidemiology Section	EEB NCI

Objectives:

This work is an effort to understand how the genetic variability of human immunodeficiency virus type 1 (HIV-1), most notably of the env gene, affects various attributes of viral function, especially as it relates to the ability of the virus to be recognized by an effective immune response, but also how it relates to other properties, such as cell tropism. The work also involves an effort to understand how divergent genomes interact to give different biological properties from those of either variant alone. We hope to understand the molecular basis for virus neutralization.

Methods Employed:

Standard techniques of molecular biology and virology are being used to pursue these investigations. These include gene cloning, transfection, transfer and expression, DNA sequence analyses, site-directed mutagenesis, virus culture and neutralization, radioimmunoprecipitation and immunofluorescence assays, restriction enzyme mapping, Northern and Southern blotting, and molecular hybridization.

Major Findings:

In Vitro Immunoselection of HIV-1 Variants

One approach to define the determinants important for recognition by neutralizing antibodies and the effect of variability on recognition involves the transmission and cultivation of virus from infectious molecular clones of HIV-1 in the continuous presence of human antisera with high titers of neutralizing activity. Occasionally, after 4-6 weeks, virus is expressed which is resistant to the selecting antiserum but remains sensitive to the majority of other neutralizing antisera. All variants obtained to date derive from HIV-1(IIIB), so it is likely that with most human sera tested, a broadly neutralizing antibody response is what is being measured; indeed, most of these sera neutralize HIV-1(MN) and (RF) in addition to (IIIB). We have previously described one such variant in which an ala to thr substitution in the gp41 at amino acid 582 results in neutralization resistance. Mutations which change the local primary or predicted secondary structure do not confer resistance. The epitope in question is thus likely to be

conformational and dependent on tertiary or quaternary structure. The area of the mutation is believed to be involved in association of gp41 into oligomers, and threonine is bulkier than alanine. It is thus possible that the mutation affects the distance between gp120 subunits as well. We are introducing other bulkier residues such as valine in order to test this possibility.

We have now obtained a second HIV-1(IIIB)-derived immune escape mutant, selected with another human antiserum with neutralizing antibodies. Several clones of the env gene from this variant have been obtained and sequenced. One amino acid change is common to all the clones; namely, an ala to val at position 281, immediately upstream of the V3 loop, which has been shown to be a target for neutralization. These envelope genes have been inserted separately into the wild-type plasmid and are being tested to see whether they both confer neutralization resistance. If so, we will use site-directed mutagenesis to try to understand the structural basis for recognition by the neutralizing antibody. Similar studies are now under way using biologically active molecular clones of HIV-1(MN), which is a completely different strain from IIIB, and of LW, which is highly related to IIIB, to broaden the range of epitopes which can be observed. Moreover, since the HIV-1(MN) clone infects macrophages, neutralization of infection of macrophages can also be studied.

Variability and Immune Recognition of Zairian HIV-1 Isolates

We have obtained blood and sera from Dr. Daniel Zagury (Universite Pierre et Marie Currie, Paris, France) from a cohort of individuals from a relatively restricted area in Zaire. This group manifests an HIV-1 seroconversion rate of approximately 10% a year. Virus isolates have been obtained from 14 of these individuals, and multiple DNA clones have been obtained from each isolate by polymerase chain reaction (PCR) amplification and sequenced. These clones constitute a 600-1000 base pair (bp) of env, including the principal neutralization determinant (PND) loop implicated in type-specific neutralization. In general, the degree and kind of overall variability seen among these isolates is similar to what has been reported for other African isolates. In contrast, the PND loops are much more closely related than expected. Within the 32 amino acids of the loop, all the above isolates differ among themselves by between two and six residues, compared with 10-20 residues for other African isolates. These data, obtained from within a fairly broad cohort, indicate that the PND loop is not always as variable as the regions adjacent to it, and that rapid change within it may be under serious functional constraints. They further suggest that it may be possible to obtain a broadly protective immune response by eliciting reactivity toward a finite number of loop genotypes.

Construction of the PND Loop Cassette

We have found that many of the Zairian sera fail to neutralize either the MN or human T lymphotropic virus (HTLV)-IIIB strains of HIV-1 well, even though they generally recognize a peptide based on the MN loop. In order to be able to assess the contribution of the PND loop to neutralization by natural antibodies, it was desirable to be able to easily substitute any given loop into an infectious clone of known sequence and neutralization properties. For this purpose, we used PCR amplification of the regions immediately upstream and downstream of the PND loop

to introduce unique Mlu I and Hpa I restriction sites at the borders of the PND loop, while maintaining the original amino acid sequence of HXB2 outside the region. These fragments were colligated into a plasmid by means of a common Pst I site, which in turn was inserted into the Sal I-BamHI env-containing fragments of pHXB2gpt. The coding region for any PND loop can then be synthesized as a series of oligonucleotides (typically four) and ligated into the loop cassette after cleavage with Mlu I and Hpa I. This has been used to construct viruses which have the PND loops on the MN, BaL, RF and L321 (Zairian) isolates within the HXB2 envelope framework. Surprisingly, the MN loop-containing virus is well neutralized by a variety of sera which do not neutralize either HXB2 or the original MN strains well. This indicates that both content and context of the PND loop may be of importance in neutralization by natural antisera and that these kinds of viral constructs should be extremely useful in delineating natural neutralizing targets. We are in the process of constructing HXB2 viruses with hybrid loops derived in part from MN and in part from HXB2 to define the recognition site on the loop for these natural sera. We are also substituting portions of the non-PND loop portions of the MN envelope in order to determine the region which abrogates recognition of the MN loop in a neutralization reaction.

Variability Over Time Within Infected Individuals

Virus was obtained from peripheral blood cells drawn from one patient (FO) in 1981 and 7 years later, in 1988. The patient remained well during this time period. DNA fragments of the env gene were amplified by PCR and compared. Different clones from the 1981 sample were 98.5-100% identical over 875 bases. The extent of variation of this region within the recovered virus population in the 1988 sample was no greater. The nef genes within both samples showed no greater variability than did the env genes.

In contrast, a comparison of any given env gene clone from the 1981 sample with any clone from the 1988 sample showed a greater than 12% difference in nucleotide sequence identity over 600 nucleotides. This degree of divergence is equivalent to that obtained comparing isolates from different individuals in the United States or Europe. This strongly supports the idea that drift in the env gene which occurs over an extended time within a single individual is sufficient to account for much of the variability in this region among viruses from different individuals. The major part of evolution of this region may thus occur by selection within the individual.

Inspection of which parts of this fragment tend to remain constant and which tend to change most extensively shows that the same regions which vary the most among isolates from different individuals vary the most over time within a single individual. For example, 5 of 32 amino acids within the PND loop are different. The effect that this and the other changes have on neutralization is currently under investigation.

In contrast to the large drift seen with the env gene, the nef gene is relatively more conserved (93-94%) over this 7 year period. This finding was unanticipated for two reasons. First, it implies that nef has an important function, but nef has been shown to be disposable for productive infection, and its function is not at all clear. On the other hand, results from this laboratory, presented in

Annual Report Z01CP07419-08 LTCB, show that nef is highly expressed under a variety of conditions, which is also consistent with it having an important function. Second, nef varies among HIV-1 isolates to nearly the same extent as env does. It is clear, however, that within this patient the nef gene has not been free to drift. Similar results have recently been reported by others on isolates obtained from a single individual within a 4 year time period. The rate of divergence in the env gene from our data is between 1-2% per year at the amino acid level.

We have analyzed isolates obtained from a second individual at several different time points. This person was a laboratory worker (LW) who became infected with HIV-1(HTLV-IIIB) sometime in 1985. An envelope clone obtained shortly after infection in 1985 and sequenced was as similar to various clones of HIV-1(HTLV-IIIB) as these clones were to each other. A complete infectious provirus was obtained from a 1987 blood sample from LW and completely sequenced. This clone, LW12.3, although closely related to the various members of the HTLV-IIIB group of HIV-1, none of which grows well in macrophages, appears to grow well in macrophages. A comparison with the putative infecting virus shows that a small amount of change has already occurred relative to the 1985 sample, and is greatest in the env gene. A complete env gene was also obtained and sequenced from a 1990 blood sample. A comparison of the three envelope protein sequences show that by 1990, divergence in the envelope amino acid sequence has reached approximately 5% from that of the putative input virus, suggesting a rate of change of about 1% per year. This is slower than the rate of divergence seen in patient MA; the somewhat lower rate could reflect initial infection with a more homogenous inoculum. Alternatively, the rate of change may not be constant, but rather, may increase later in the course of infection, when virus replication is more extensive and the viral gene pool is more diverse. Other blood samples were used to obtain either λ clones of proviral DNA or PCR fragments of the env gene from LW. The sequences of these DNAs is consistent with the conclusions drawn from the full envelope sequences.

The third set of samples are more problematic, but further establish the rate of drift of the env gene at 1% to 2% a year, and that of the nef gene as less. The individual studied was the patient BRU, from whom LAV-1/BRU was purportedly isolated. Over the past year, due to our interest in the extent in single individuals of genetic change in HIV-1 which occurs over time, we have characterized samples received by the LTCB in 1983, including some from the research group involved in acquired immunodeficiency syndrome (AIDS) research at the Pasteur Institute.

Extensive searches through freezers were carried out to locate the 1983 samples for analysis. Dr. Hahn, upon leaving the NCI, retained the July JBB/LAV DNA and we obtained a portion of it directly from her. The C306 freeze of JBB/LAV was located and used for attempted transmission to CEM cells and PBLs by Dr. Waters (Program Resources, Inc.) in May 1990. Virus could not be transmitted to the CEM cells, but the PBLs became productively infected, as judged by media reverse transcriptase (RT) assays and the appearance of cytopathic changes. DNA was prepared from the infected PBLs. Part of the September 22nd JBB/LAV had been retained by Dr. Popovic who left the LTCB later in 1989, and was used by him in February 1989 in transmission attempts to H9 cells and PBLs. Transmission to H9

was not successful, but the PBLs became infected by the criteria of RT, antigen expression, and cytopathic changes. We obtained these infected cells for DNA purification in July 1989. Nothing remained of the M2T/B supernatant, and we have not been able to locate any derivatives from it in our freezers, nor have we been able to obtain any additional samples of this culture from the original sources.

DNA from the JBB/LAV(C306) and the JBB/LAV grown by Dr. Popovic in 1989 were analyzed by Southern blotting. There was insufficient JBB/LAV DNA from Dr. Hahn for Southern blotting. All three samples were also used to obtain multiple clones of 900 bp generated from the envelope gene by the PCR, except for C306, from which 670 bp clones were obtained. In addition, multiple 600 bp PCR-generated clones of another portion of the envelope gene were obtained from JBB/LAV (Popovic), and multiple 900 bp clones were generated by PCR of the nef gene of JBB/LAV(C306). The 1987 BRU sample was used to generate multiple clones of 900 bp from the env and nef genes from the same regions as the JBB/BRU clones. The DNA sequence of these clones were analyzed by standard Sanger sequencing protocols.

A comparison of env clones from all of the 1983 JBB/LAV shows 98.5-100% amino acid sequence identity. In contrast, all of these clones differ from the same regions of HTLV-III and LAV-1/BRU by 9% by sequence identity. This suggests that none of the JBB/LAV samples were the origin of HTLV-IIIB or LAV-1/BRU, with the possible exception of the M2T/B sample. It should be emphasized, however, that all four samples were represented by the sources from which they were obtained as deriving from the patient BRU. Since three independent samples gave highly related envelope sequences, it is likely that they, in fact, represent the majority virus from BRU. It should also be noted that the data do not exclude that HTLV-IIIB/LAV-1(BRU) could have also derived from patient BRU and been present as a very small proportion of the analyzed samples.

The sequences from multiple clones of the 1987 BRU sample were determined and, in view of the uncertainty noted above, were compared with those of both the 1983 JBB/BRU samples and clones of HTLV-III and LAV-1/BRU. Amino acid sequence divergence of the env clones from the JBB/BRU samples were about 6%, for a divergence rate of about 1.5% per year. The divergence from HTLV-III and LAV-1/BRU was 9%, which would represent a divergence rate of slightly more than 2% per year. Both numbers are consistent with the divergence rate seen in the other two individuals analyzed, although the latter number is slightly higher than either. Sequence comparisons were also made with clones from the nef gene. The divergence of the 1987 BRU nef sequences from the JBB/BRU nef sequences was quite small (2.5-3%). The divergence from the same nef sequences of HTLV-III and LAV/BRU was much greater (6.5-7%). Both magnitudes of difference were lower than those obtained from similar comparisons of the env genes. The closer similarity of JBB/BRU to the 1987 BRU sample strongly suggests, however, that this comparison is the valid one.

Although it is not possible to be certain that the comparison of the 1987 BRU sample with the 1983 JBB/BRU rather than HTLV-III and LAV-1 is the correct one, either comparison leads to approximately the same conclusions as those obtained from the comparisons of MA and LW. These are that the env gene diverges from 1-1.5% per year in infected individuals, and that the nef gene does not diverge as rapidly.

Presumably, the rate of change in the env gene is driven by the host immune response to a greater extent than in the nef change. The relative conservation of nef also implies that it is functionally important to the virus.

Genetic Determinants for Macrophage Tropism

We are interested in which genetic determinants enable HIV-1 to grow in macrophages. We have looked at the possible contributions of tat and the long terminal repeat (LTR), and find that these regions do not seem to play a part in the selective ability to grow in macrophages, as outlined in greater detail in Annual Report Z01CP07149-08 LTCB. We are further investigating this problem by developing sets of infectious molecular clones from single individuals which differ in their ability to grow in macrophages.

One HIV-1 strain for which an infectious clone exists is (IIIB). Virus derived from the pHXB2 plasmid grows in T cell lines but not in macrophages. We have now made an infectious clone (LW12.3) from an HIV-1 isolate obtained from the laboratory worker accidentally infected with HIV-1(IIIB). This isolate grows well on macrophages. The infectious molecular clone grows in the Sup T1 human T cell line but not in peripheral blood monocytes or lymphocytes. We have obtained the complete DNA sequence of this clone, and find that it differs very little from HIV-1(IIIB), except for a deletion of 130 bp which results in the premature truncation of vif and the elimination of the amino terminus and initiation codon of vpr. Our hypothesis is that these genes are required for growth on peripheral blood cells, and that their lack in this clone may be a cloning or tissue culture artifact. We are therefore replacing the deleted region and will test the resultant virus for its ability to grow on macrophages. If successful, the low degree of difference between pLW1 and pHXB2 and the nearly complete conservation between the two of restriction endonuclease cleavage sites will make it relatively easy to construct viral chimerae which can be used to map the determinants important for growth in macrophages.

We have also isolated an infectious clone from HIV-1(MN-ST). This clone grows well on peripheral blood macrophages and T cells as well as on T cell lines, but has a phenotype which manifests itself by the presence of abundant budding particles and few mature virions for a lag phase of 3 weeks, at which point production dramatically increases. The clone has been completely sequenced, and contains a premature stop codon in vpu and a frame shift mutation in gag p6, both of which have been implicated in virion morphogenesis. We are currently repairing these defects to ascertain their contribution to the observed production lag phase. We are now concentrating on a obtaining molecular clone of MN which will be unable to grow on macrophages. Besides the utility of this clone for vaccine and related studies, it should also be extremely useful, similar to the LW12.3-pHXB2 pair, for dissecting out determinants for growth on macrophages.

MN-ST and LW12.3 have also been used in complementation studies to see how genetic variability in virus populations may give the population properties not predicted from either virus alone. Our preliminary experiments show that each virus can trans-complement the other to give a virus population of increased cellular host range, growth rate and cytopathogenicity.

Development of an Improved HIV-1 Infectivity Assay

Much of the research with HIV-1, including antibody neutralization experiments, involves the use of infectivity assays. These are rather labor intensive, and an easier and quicker assay would be extremely valuable. We have therefore attempted to develop a simple calorimetric assay based on the presence in infected cells of the bacterial lacZ gene products, which can be assayed calorimetrically. If this gene is put under the control of the HIV-1 LTR, its expression should only be induced in the presence of tat. Therefore, only if a cell containing this type of construct is infected with HIV-1 and expresses tat will a sufficient level of the lacZ gene product be induced for detection by color development. Infected cells will appear blue, while uninfected cells will remain colorless.

The vector for lacZ expression was constructed by taking the pHXB2gpt and removing the coding region for pol and most of gag and env by digestion with Cla I and BamHI. The Cla I site was made blunt and the resultant fragment was ligated to a BamHI-HindIII fragment containing the lacZ gene in which the HindIII site was made blunt, resulting in a fusion of the lacZ reading frame with the first few amino acids, including the initiation codon, of the gag gene of pHXB2gpt. The resultant plasmid, pHXB2-lacZ, thus expresses the bacterial gene β-galactosidase as an HIV-gag fusion protein.

pHXB2-lacZ was transfected into $CD4^+$ HeLa cells (a gift from Dr. Paul Maddon, Columbia University, New York) with the gpt selectable marker. Mycophenolic acid and xanthine resistant cells were selected by single cell cloning. In preliminary experiments, expression of the fusion protein was detected by transactivation of the HIV LTR-lacZ gene when cells selected for the pHXB2lacZ were infected with either HIV-1, HIV-2, or simian immunodeficiency virus. These cells can either be histologically detected (microscopically) as blue cells using the chromogenic 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside substrate or with a fluorometer capable of reading 96 well plates using 4-methyl-umbelliferyl-β-D-galactoside as substrate. This fluorometric assay is very sensitive and can detect as few as 5 molecules of enzyme per cell in a 20,000 cell sample. In addition, cells can be viably sorted using a fluorescent activated cell sorter using the fluoro-di-β-D-galactoside (FDG) substrate to identify β-galactosidase producing transactivated cells. The FDG substrate is intracellularly cleaved by β-galactosidase and the fluorescein product detected following laser excitation.

This assay system exhibits a sensitivity similar to PBL's and will speed and simplify a variety of studies requiring the assay of live virus, agents or molecular constructs capable of activating the HIV-1 LTR. This adherent cell, nonradioactive, 72 hour assay system can be performed in 96 well plates and will be used for a variety of studies with HIV-1 and HIV-2, including neutralization assays.

Analysis of Anti-HIV-1 Immunoglobulins on Infected Individuals

A major problem in studying neutralizing antibodies in infected humans is that sera contain mixtures of antibodies to the envelope protein, some of which neutralize virus, some of which do not, and some of which may facilitate virus infection. For this reason, we have begun to clone PCR amplified cDNA for

immunoglobulins from B cells of infected people using the recombinatorial library technique reported by others. In this way we hope to obtain pure populations of neutralizing antibodies which can be studied with respect to their target determinants. In addition, they can be used to elucidate the mechanisms by which neutralization occurs.

In order to analyze the immune responses of AIDS patients to HIV-1, we intend to utilize a bacterial vector that has been used to express mouse immunoglobulins (Ig) in bacteria (a gift from Dr. Richard Lerner, Scripps Clinic, La Jolla, CA). The basis of the technique is to use PCR to amplify the variable domain and first constant domain from light and heavy chains (approximately the entire light chain and the amino terminal half of the heavy chain). Expressed in a vector designed to give the correct folding, this technique essentially allows active recombinant Fab fragments to be produced. This technique has tremendous possibilities, and has already been used to clone neutralizing antibodies to influenza hemagglutinin from immunized mice.

Ribonucleic acid (RNA) will be isolated from lymphocyte cultures, reverse transcribed into cDNA and then subjected to PCR using primers for the variable (VL, VH) and first constant (CL, CH1) domains of either light (VL and CL) or heavy (VH and CH1) Ig chains. These amplified fragments will be cloned into expression vectors, and then combined into a vector which produces recombinant proteins that mimic the specificity of the original human antibodies. In doing this, we will be extending the application of the technique from mouse to human Igs. For this purpose, we have designed a series of oligonucleotide primers for PCR amplification of human Igs by comparing the available sequences in regions of human Ig messenger RNAs (mRNAs) which are homologous to those used in the design of the murine system. Primers have been developed for both κ (which represents about 60% of human active light chains) and λ (representing about 40% of human expressed light chains) mRNA. The sense primers are homologous to the mRNA coding regions for the amino end of the respective variable regions. The λ and κ chain antisense primers are complementary to the carboxyl terminus of the respective constant regions, and the γ chain antisense primer is derived from the γ chain hinge region. In the case of the γ chain sense primers, it was not possible to obtain a single consensus sequence. The four primers are designed to be used in four separate reactions to obtain an amplification of a consensus region. Restriction sites are built in Xho I and Spe I for the γ chain sense and antisense primers, respectively, and Sac I and Xba I in the case of the light chain primers.

Recombinant proteins produced in this system will be screened initially for binding to HIV-1 gp160. Positive clones will then be analyzed for neutralizing activity as described in Annual Report Z01CP05536-05 LTCB. It is likely that to achieve neutralization, the remainder of the heavy chain gene will need to be added to the recombinant Ig construct. These clones will then be analyzed to determine the recognition site of neutralizing antibodies on gp160. It will also be possible to analyze the immune response in individual patients to different variants of the gp120, for example, between monocyte/macrophage tropic (e.g., BaL) and T cell tropic (e.g., HTLV-IIIB) isolates of HIV-1. Analysis of isolate-specific antibodies during the progression of AIDS may yield valuable information on the natural progression of the disease, in terms of viral mutation and

selection. The same analysis will be used in examining the response of animals immunized against HIV in animal vaccine trials.

Recombinant proteins with high neutralizing activity may be evaluated as possible therapeutic agents, either as a form of immunoglobulin therapy, or for raising anti-idiotype antibodies in mice, which could then be cloned using the same procedure, for use as immunogens. Anti-idiotypes would hopefully mimic the neutralizing epitope of gp160.

Cloning anti-envelope antibodies may also be the basis for a strategy for determining whether systematic differences in response to a pathogen can lead to differences in the course of the disease. Using this technique to examine differences in the immune response to the HTLV-I envelope protein between HTLV-I-associated myelopathy/tropical spastic paraparesis and adult T cell leukemia patients may help to understand the differences in the pathogenesis of the two diseases.

We also are beginning to study neutralization by antibodies at the molecular level. Any molecularly cloned neutralizing antibody obtained as described above can be used in these studies. At first, however, it will be necessary to use available neutralizing monoclonal antibodies, hyperimmune polyclonal neutralizing antisera, and natural neutralizing antisera. The first are more amenable to study since they contain only a single antigenic reactivity. The latter two will be useful primarily to see if there are fundamental differences in the way immunized animals and infected humans neutralize HIV-1.

Receptor binding is readily assayed using recombinant CD4 on immunobeads or by performing reverse PCR on cells exposed to virus and removed by centrifugation. Cell fusion is morphologically determined. Penetration can be assayed using a combination of limited proteolysis and reverse PCR; release of viral RNA becomes refractory to trypsin after internalization of the virus core. Uncoating and RT can be assayed by a variety of PCR assays, including amplification of the LTR-LTR junction of circular proviral DNA. An anchor PCR assay is being developed to detect integrated viral DNA. Expression of early virus gene products (specifically tat) will be detected using the cell lines containing the HIV-1-LTR driving a lacZ reporter gene, as described above. These methods should allow us to pinpoint the precise mechanisms of neutralization by a variety of antibodies and to determine whether alternate mechanisms exist.

Publications:

Gallo RC, Reitz MS. Retroviruses and human disease. A historical perspective. In: Gallo RC, Wong-Staal F, eds. Retrovirus biology and human disease. New York: Marcel Dekker, 1990;1-8.

Guo H-G, Chermann J-C, Waters D, Hall L, Louie A, Gallo RC, Streicher H, Reitz MS, Popovic M, Blattner W. Nucleotide sequence analysis of the original isolate of HIV-1. Nature 1991;349:745-6.

Pal R, Reitz MS Jr, Tschachler E, Gallo RC, Sarngadharan MG, Veronese FD. Myristoylation of Gag proteins of HIV-1 plays an important role in virus assembly. AIDS Res Hum Retroviruses 1990;6:721-30.

Pincus SH, Wehrly K, Tschachler E, Hayes SF, Buller RS, Reitz M. Variants selected by treatment of human immunodeficiency virus-infected cells with an immunotoxin. J Exp Med 1990;172:745-57.

Wilson C, Reitz MS Jr, Aldrich K, Klasse PJ, Blomberg J, Gallo RC, Robert-Guroff M. The site of an immune-selected point mutation in the transmembrane protein of human immunodeficiency virus type 1 does not constitute the neutralization epitope. J Virol 1990;64:3240-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05539-05 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Determinants of Latency and Pathogenicity of Human Retroviruses in AIDS

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

PI:	S. K. Arya	Research Biologist	LTCB NCI
Others:	R. Sadaie	Senior Staff Fellow	LTCB NCI
	M. Klotman	Guest Researcher	LTCB NCI
	R. C. Gallo	Chief	LTCB NCI

COOPERATING UNITS *(if any)*
University of Alabama, Birmingham, AL (B. Hahn); University of Pennsylvania, Philadelphia, PA (J. Hoxie); Advanced BioScience Laboratories, Inc., Kensington, MD (V. S. Kalyanaraman)

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Molecular Genetics of Hematopoietic Cells Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	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.)*

Human immunodeficiency viruses (HIVs) may comprise a spectrum of human retroviruses with varying potential for latency, virulence and pathogenicity. These capacities may be governed, in part, by their genetic structure. This project aims to gain an understanding of pathogenicity by analyzing the structure and function of the genomes of the highly pathogenic (HIV-2[ROD]) and weakly pathogenic (HIV-2[ST]) HIVs. Since much of the virus regulation is centered in its long terminal repeat (LTR), relevant to latency and virulence, and the envelope gene plays a major role in pathogenicity, the focus of this study is the LTR-based regulatory elements and regulatory genes (tat), and the envelope gene. Also relevant are the properties of these viruses to be activated by cofactors, e.g., T cell activation. We recently presented the novel observation of two enhancer elements in HIV-2 that regulate virus expression. Apparently, the activation of HIV-2 with the second enhancer as the target does not involve the NFκB pathway followed for HIV-1 activation. The presence of two enhancers in HIV-2 raises the possibility that these enhancers may function differentially depending on the cell phenotype and environmental factors and this may underline virus latency. In addition to the previously known regulatory elements, our studies show that HIV-2 contains sequence elements downstream of the transcriptional initiation site that have repressive effect on gene expression. Studies mapping the functional domains of HIV-2 TAT suggest that the amino terminus region of the TAT protein may be important for its function and specificity. In an attempt to map determinants of fusogenicity and pathogenicity of HIV, analysis of the chimeric proviruses between noncytopathic HIV-2(ST) and cytopathic HIV-2(ROD) revealed that the env gene is a major determinant of cytopathicity and presumably pathogenicity. Apparently, fusogenicity and cytopathogenicity determinants encompass multiple and discontinuous epitopes, and their ability to cause cytopathic effects may be coupled with the capacity to replicate and this may be cell-type dependent.

Project Description

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

S. K. Arya	Research Biologist	LTCB NCI
R. Sadaie	Senior Staff Fellow	LTCB NCI
M. Klotman	Guest Researcher	LTCB NCI
R. C. Gallo	Chief	LTCB NCI

Objectives:

The major objective of this project is to evaluate the determinants of the latency, virulence, and pathogenicity of human immunodeficiency virus (HIV) and to utilize this information for developing therapeutic modalitites. An additional and related objective is to dissect the mechanisms of the regulation of HIV gene expression that may be relevant to its pathogenicity.

Methods Employed:

Molecular cloning in expression vectors, DNA sequencing, DNA-mediated transfection analysis, transcriptional and translational analyses, DNA:protein gel shift assays, reverse transcriptase assays, CD4 binding and modulation, syncytia formation and other standard methodologies of gene cloning and recombinant DNA technology.

Major Findings:

HIV-I is etiologically associated with acquired immunodeficiency syndrome (AIDS). More recently, new human retroviruses (termed HIV-2) have been isolated from sick and healthy individuals resident in West Africa. Some of these HIV-2 isolates may be less pathogenic in the natural host. Thus, HIVs may comprise a spectrum of viruses with varying potential for latency and pathogenicity. Our studies explore the premise that the latency and pathogenic potential of these viruses is governed, at least in part, by their genetic structure (e.g., env gene) and that viral gene expression (e.g., LTR, tat) underlies pathogenesis.

Regulatory Elements

Detailed mutational analysis of the long terminal repeat (LTR)-based regulatory elements of HIV-2 has revealed the existence of two enhancer elements which may be the targets of T cell activation signals. The activation of HIV-1 gene expression which contains only one enhancer involves the NFκB pathway where p50 protein of NFκB complex interacts with the NFκB binding site contained in the enhancer element. Although the enhancers of HIV-2 have some sequence similarity with the HIV-1 enhancer, they do not seem to interact with nuclear protein of 50 kilodalton (kD) size in vitro. Instead, they apparently bind with proteins of apparent mobility of 27kD and 85kD proteins. Furthermore, the HIV-2 enhancer more closely related to the HIV-1-enhancer does not function effectively in HIV-2. It appears to be constrained by the sequence context. It is important to note that the mere

existence of a consensus regulatory elements does not always mean that it is functionally important. Thus, there may be subtle but important differences in the mechanisms of HIV-1 and HIV-2 gene regulation and this may be relevant to the longer latency and lesser virulence and pathogenicity of HIV-2.

The activity of the genes is controlled by several regulatory elements, including promoters. The promoters interact with a complex of cellular transcriptional factors that modulate gene expression. The promoters of HIV also require cellular factors for their operation. It was therefore reasonable to think that promoters of other genes (cellular or viral) when supplied in trans will inhibit HIV LTR-directed gene expression by competition for the available transcriptional factors. This indeed is the case in vitro. We are now exploiting this observation to delineate transcriptional factors that are shared by HIV and other viral genes such as those by DNA viruses.

TAT Gene

One mechanism by which Tat protein transactivates HIV gene expression is to overcome abortive transcription or premature transcriptional pausing. This implies that sequence elements downstream of the transcriptional initiation site function operationally as repressive elements. We have now obtained evidence that deletion of some downstream elements enhances LTR-directed gene expression in the absence of TAT supplied in trans. Thus, the downstream elements corresponding to the U5 region of the LTR may constitute repressive elements. It is conceivable that viral genomes can arise during the course of infection where these elements have been deleted and such genomes may replicate more actively contributing to virulence of HIV infection.

We have previously reported that whereas HIV-1 TAT can transactivate both HIV-1 and HIV-2 LTR, HIV-2 tat transactivates its own LTR more effectively than HIV-1 LTR. Apparently, HIV-2 has more stringent requirements for its function and it may be less promiscuous than HIV-1 Tat. Recent functional domain mapping studies suggest that amino acid residues located towards the amino acid terminus of HIV-2 TAT may be important for its function and specificity.

Envelope Gene

HIV-2 (strain ST), isolated from a healthy individual at risk, is nonfusogenic and noncytopathic. HIV-2 (strain ROD), from an AIDS patient, is fusogenic and cytopathic. These two isolates, respectively, serve as a prototype for noncytopathic and cytopathic HIVs and provide the tools for investigating the determinants of cytopathicity and pathogenicity. The premise here is that dissectible domains exist that are largely responsible for the pathogenicity of HIV. Our approach entails the engineering of biologically active, replication competent chimeric genomes between HIV-2(ST) and HIV-2(ROD) and testing their ability to cause fusion, cytopathic effects and binding to the CD4 molecule in the context of $CD4^+$ human T cells. Several chimeric proviruses have been created where the env gene and parts thereof have been exchanged. Analysis of these chimeric proviruses performed thus far suggest that (i) fusogenic and cytopathic epitopes are likely to be multiple and discontinuous in the envelope; (ii) the envelope of each virus may be unique in its ability to induce syncytia and

pathogenic effects, probably related to the particular conformation; (iii) the putative "fusogenic domain" by itself is not the sole determinant of fusogenicity; (iv) premature termination or truncation of gp41 is not a major factor in causing cytopathic effect; (v) truncation of the nef gene or 3'-LTR may play some role in fusogenicity and cytopathicity; (vi) fusogenic and cytopathic potential may be coupled with the capacity to replicate to high levels and may be cell type dependent; and (vii) some isolates of HIV-2 (i.e., ROD) may cause a cytopathic effect without extensive syncytia (single cell killing) in some cell types (i.e., monocytic cells).

More recently, fusogenic and cytopathic variants of HIV-2(ST) have been obtained by continuous passage of the virus in culture. We have used a replication competent molecular clone of one of these variants to construct envelope-based chimeric proviruses of noncytopathic and cytopathic variants where much of the genetic composition is the same. Investigation of phenotypic properties of the chimeric proviruses of the variants of HIV-2(ST), combined with studies of HIV-2(ST) and HIV-2(ROD) chimera, may allow a more precise definition of the determinants of cytopathicity. Thus, well-defined chimeric genomes will be available for further delineation of the determinants of pathogenicity in animal model systems. These studies may provide tools for more targeted development of chemo- and immunotherapeutic modalities, including antisense approaches and vaccine.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05614-03 LTCB

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Immunobiology of HIV-1: Antigenic Variation, Epitopes and Vaccine Development

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

PI:	P. L. Nara	Expert	LTCB NCI
Others:	R. R. Garrity	Molecular Biologist	LTCB NCI
	N. M. Dunlop	Microbiologist	LTCB NCI
	M. J. Merges	Microbiologist	LTCB NCI
	R. C. Gallo	Chief	LTCB NCI

COOPERATING UNITS *(if any)*: Program Resources, Inc., Frederick, MD (S. Conley); Academic Medical Center, Amsterdam (J. Goudsmit, L. Smit, W. Krone)

LAB/BRANCH: Laboratory of Tumor Cell Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.50	0.25	1.25

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.)*

Experimental studies done this past year have demonstrated the V3 domain to be part of a contiguous conformational epitope as evidenced by the virus' ability to escape a V3-specific monoclonal antibody without changing any of the primary amino acids (a.a.) in the antibody binding site. Neutralizing antibody subsequently directed at the V3 region following infection is correlated in the selection of escape mutants which ultimately demonstrate V3-specific a.a. changes. The virus therefore has two mechanisms identified for evading the humoral immune system. Clonal expansion of a virus population is rapidly observed in other acute or experimental infections in humans or chimpanzees, leading initially to a monotypic (type-specific) neutralizing response. This neutralizing response to the incoming virus appears to also clonally expand due to cross-reactivity in the invariant V3 domain of the emerging escape mutants. Similar results are observed with envelope immunization, suggesting an "original antigenic sin" - like phenomena may be operational during infection or vaccination. Fractionation studies using pooled human HIV-1 serum demonstrate that the majority of neutralizing activity is found associated with V3 and the CD4-binding site on gp120. The potency of broader CD4-mediated neutralization, however, is poorer than V3 and demonstrates strain-variability. Viruses passaged in vitro in primary lymphocyte cell lines select the most replication-competent virus subpopulations. Escape mutants arise from less replication-competent virus subpopulations. Mechanisms are operating in addition to a strong humoral response(s) in human immunodeficiency virus (HIV)-1 infected chimpanzees to restrict and possibly control the virus in this species. These include T8 suppressor activity, differences in their CD4 molecule and "relative" lack of infectability of their peripheral blood monocytes by HIV-1. This model appears to be very instructive for efficacy immunogenicity testing and challenge for examining the sterilizing immunity of a prototype HIV-1 vaccine. The use of the model for disease prevention is in serious doubt.

Project Description

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

P. L. Nara	Expert	LTCB NCI
R. R. Garrity	Molecular Biologist	LTCB NCI
N. M. Dunlop	Microbiologist	LTCB NCI
M. J. Merges	Microbiologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI

Objectives:

The viral envelope is considered a prime target for a subunit vaccine approach to human immunodeficiency virus (HIV)-1. The humoral immune response of the host to the envelope as well as those evolved/selected features which allow its survival in the host are the factors which have to be understood. The objectives therefore were to:

1. Continue studying the distant-site charges responsible for escape from V3-specific neutralizing antibody.

2. Fractionate polyclonal human sera from HIV-1 positive patients to determine the spectrum and epitopic diversity of neutralizing antibody.

3. Study the relationships between peptide-based affinity and neutralization potency of antibody to the V3 loop.

4. Evaluate various envelope subunit immunization strategies such as native, nonreduced CHO-derived recombinant gp120, gp160/V3 peptides, etc., by studying the neutralizing antibody profile following experimental vaccination.

5. Study the role of an immunodominant cross-reactive epitope on the humoral immune system.

6. Study the role of the peripheral blood monocyte/macrophage in chimpanzees, following in vitro infection with HIV-1.

Methods Employed:

The method for assaying the various human and chimpanzee sera for HIV-1 neutralizing antibodies has been previously described by Nara et al. (AIDS Res Hum Retroviruses 3:283, 1987; Nature 331:469, 1988). Detailed kinetic studies were also done as described by Nara (Vaccines, Cold Spring Harbor, 1989, p. 137). E. coli-expressing recombinant proteins were produced containing V3 amino acid (a.a.) sequences of three HIV-1 variants p168-1, p168-10 and IIIB (Goudsmit et al., Vaccines, Cold Spring Harbor, 1990, p. 291).

Neutralization resistant variants (NRV) of HIV-1(IIIB) were reisolated in serial fashion from experimentally inoculated chimpanzees every 2 weeks by standard

phytohemagglutinin/interleukin-2 stimulation/cocultivation procedures. V3 sequences from select NRVs were sequenced directly and cloned prior to polymerase chain reaction analysis as described previously (Nara et al., J Virol 63:2118, 1990).

Routine serologic analysis was carried out on all serum samples using radioimmunoprecipitation (RIPA), PEPSCAN, enzyme linked-immunosorbent assay (ELISA), competitive radioimmunoassay, Western blotting and immunofluorescence. (Nara et al., J Virol 63:2118, 1990).

Specific monoclonal typing sera (0.5B) used for neutralization analysis were obtained from Dr. H. Mitsuya (Clinical Oncology Program, NCI).

Major Findings:

To characterize the initial and protracted neutralizing antibody responses following HIV-1 infection, chimpanzees were intravenously given large volume inoculums of cell-free and cell-associated laboratory strains of HIV-1. Serum samples taken weekly, monthly and yearly were evaluated for their ability to neutralize the inoculum virus and other less related isolates. A high titered type-restricted neutralization response develops first followed by a lower titered response able to neutralize other laboratory strains. The neutralization titer to both inoculated and heterologous virus strains maintain themselves at a ceiling titer (1.512-2048) for years. More refined-infectious dose titration studies in subsequent animal studies demonstrated that the ability to neutralize other laboratory strains was a titer dependent phenomena, suggesting either the development of a subpopulation of cross-neutralizing high affinity antibody and/or a neutralizing response of other conserved functional epitope(s). In collaboration with the Academic Medical Center (Amsterdam), this early type-specific neutralization response mapped to the V3 domain (a.a. 296-331) as indicated by antibody-reactive peptide scanning (PEPSCAN) and solution V3-peptide competition assays. The region was also independently mapped in collaboration with Scott Putney (Repligen, Cambridge, MA) using antisera to E. coli-derived recombinant proteins. Addition of homologous V3-specific peptides to polyclonal derived anti-V3 serum reduced the neutralization titers in a concentration dependent fashion. These V3 peptides were also effective in reducing a lower titered neutralizing response to a heterologous virus strain. This latter finding suggested that the broader neutralization seen during HIV-1 infection was in part due to cross-neutralizing antibody seroreactivity to different V3 peptide sequences. This was confirmed by individual HIV-1 infected chimpanzees. The same results were seen with a large number of human sera; however, as we observed earlier a predominantly MN/SC/168.1 V3-specific epitope (IHIGPGRAFY) dominated in Europe and the United States.

As described earlier in this report, early in experimental infection of chimpanzees with HIV-1 strains LAV-1, HTLV-III(B) and HTLV-III(RF), antibodies are elicited that bind to the V3 region of gp120 of the inoculum strain, resulting in a blocking of virus replication and cell fusion. Subsequently, additional HIV-1 strains are included in the specificity of the neutralizing antibody response. This broadening apparently occurs sometimes in the absence of detectable binding to peptides corresponding to the V3 domain of the viruses included in the

neutralization response. The binding to the peptide corresponding to the a.a. sequence of the inoculum strain, however, persists.

To compare the serologic responses of HIV-1 infection to immunization with the viral envelope glycoprotein, we purified the native viral envelope glycoprotein from HIV-1(IIIB) and RF in 1984-85. These were purified by standard immunoaffinity columns using high titered HIV-1 positive human serum. These viral envelopes were formulated into complete Freund's adjuvant and immunized into goats and horses. Some animals were hyperimmunized with just one viral envelope and other received an additional viral envelope following their first booster series. The results clearly demonstrated a highly type-specific/restricted neutralizing response following hyperimmunization. Under no conditions in these experiments and those carried out in chimpanzees, did the neutralizing response broaden. Also observed in these studies was the relatively short half-life of the neutralizing response (10-25 days). The maintenance of the humoral response appeared to be as big a problem as the restricted nature of the humoral response. An interesting paradoxical anamnestic recall phenomena was observed during sequential immunizations with divergent viral envelopes indicating a unique cross-reactivity. To improve on the humoral responses observed in these earlier studies the gp120 was formulated into ISCOMS and injected into rhesus monkeys. The encouraging finding in this study was both the persistence of the precipitating and neutralizing titers over time period of approximately 22-25 weeks and a lower titered broadening of the neutralization response. Similar results have been obtained from our collaborative studies using CHO-derived gp120 from HIV-1 SF2 in baboons. However, in these studies slightly higher titers have been observed and the degree of cross-reactivity included one additional divergent isolate, HIV-1 CC. Preliminary studies in collaboration with Jay Berzofsky (NCI) suggest, in addition to novel adjuvants and more native glycoprotein structure, that priming with various conserved T cell epitopes can enhance the antibody response to the viral envelope.

Following infection with HIV-1, neutralization escape mutants with invariant V3-domains evolve rapidly de novo. To further correlate the neutralization resistant phenotype of isolate V32 with conformational structure, the expression of various envelope epitopes was analyzed using well-defined monoclonal antibodies (mAb) by RIPA in comparison to the inoculum. Five HX10 monoclonal antibodies against 5 different epitopes on the envelope were tested. One has neutralizing activity against HX10 (mAb 178.1). Three monoclonals were overt on lysate HX10 and covert on Variant V32 (mAbs 2.1, 136.1 and 213.1). A topographical rearrangement of these epitopes had apparently occurred in the envelope of variant V32. The two other mAbs (mAbs 187.1 and 178.1), of which one had neutralizing activity against HX10, were overt on both, in spite of the lack of neutralizing capacity of the variant. Epitope mapping of 3 mAbs which differ in epitope recognition comparing HX10 and V32, showed that 2 of the 3 mAbs (2.1 and 213.1) could not be mapped to a clear epitope in the PEPSCAN. The third mAb (136.1) was directed against a conserved epitope (between HX10 and V32) localized at a.a. position 376-381. From these data we conclude that the region containing the binding site for monoclonal C (136.1) plays a role in determining the conformation of the HIV-1 envelope. Conformational changes might have occurred due to the observed substitutions resulting in neutralization resistance to both broadly neutralizing human sera and V3-specific antibodies. This occurs without loss of antibody binding. Additional

studies of these neutralization resistant variants demonstrated the host had a limited ability to mount an equivalent neutralization response.

It appears that the V3 domain is a sequential epitope with inherent contiguous conformational structure and is part of an assembled conformational epitope requiring additional molecular structures for its overall antigenic nature. It was therefore of interest to characterize the dominant type-specific neutralization response of human serum through fractionation and neutralization studies. Serum randomly selected from an HIV-1 infected asymptomatic male homosexual exhibited a predominant HIV-1 MN-like serological neutralization and ELISA profile as reported previously. The serum was extensively fractionated by immobilized peptide affinity column chromatography using full-length and truncated V3 peptides. The purified IgG from whole serum exhibited a starting 90% neutralization titer of 1:490, 1:128, and 1:75 for laboratory strains HIV-1(MN), RF, and IIIB, respectively. Fractionation of the serum separately over MN, IIIB, and RF V3 peptide columns resulted in the specific loss of 95%, 90% and 50% of the MN, IIIB, and RF neutralization titers. The bound antibody was eluted and tested again for neutralization. A significant amount (1:180-220) of MN neutralizing activity was recovered from the MN V3 peptide columns. Interestingly, MN neutralizing activity was recovered from the IIIB and RF columns of titers with 1:77 and 1:22, respectively. Antibodies eluted from the IIIB and RF peptide columns were unable to neutralize their respective virus strains. It thus appears that V3 cross-reactive antibodies present in the serum of an HIV-1-infected individual are able to contribute to the cross-neutralization profile of the serum. These cross-reactive MN neutralizing antibodies were unable to recognize their homologous V3 sequences by ELISA, suggesting a different presentation of the peptides under these conditions. Further fractionation using MN V3 peptides on the amino-terminal (INCTRPNNNTRKSI) and carboxyl terminal (IGDIRQAHCNIS) sides of the conserved tetrapeptide (GPGR) and the 11-a.a. central core demonstrated that the central V3 core plays a significant role in the adsorbed neutralization activity.

To understand the contribution of other anti-gp120 humoral neutralizing responses in host defense against HIV infection, we have fractionated and characterized, by neutralization and binding assays, polyclonal anti-gp120 antibodies present in HIV-infected individuals. Total anti-gp120 antibodies (TAGA) were purified from a pool of 4 sera by affinity chromatography on a gp120 SF2-Sepharose column and tested for their neutralizing activities. The results indicated that TAGA exhibited both type- and group-specific neutralizing activities. CD4 attachment site-specific antibodies (CAGA) were isolated from TAGA using a CD4 blocked gp120SF2-Sepharose column and were tested for their neutralizing activities. Collectively, this study indicated that group-specific neutralizing anti-gp120 antibodies exist in lower potency in the sera of HIV-infected individuals and that these antibodies are specific for the CD4 attachment site as well as possibly other unknown epitopes on gp120, which independently or in concert act to inhibit HIV-1 infection of $CD4^+$ cells.

Publications:

Back NKT, Wolfs TFW, Sun W, Ramavtarsing C, Smit L, Goudsmit J, Nara PL, Bruck C. Conformational rearrangement of the HIV-1 envelope renders viruses resistant to type-specific and broadly neutralizing antibodies. In: Lerner RA, Ginsberg H, Channock RM, Brown F, eds. Vaccines 91. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1991;179-82.

Emini E., Nara PL, Schleif WA, Lewis JA, Davide JP, Lee DR, Kessler J, Conley S, Matsushita S, Putney SD, Gerety RJ, Eichberg JW. Antibody-mediated in vitro neutralization of human immunodeficiency virus type 1 abolishes infectivity for chimpanzees. J Virol 1990;64:3674-8.

Gendelman HE, Ehrlich G, Baca LM, Conley S, Ribas J, Kalter DC, Meltzer MS, Poiez B, Nara P. The inability of human immunodeficiency virus to infect chimpanzee monocytes can be overcome by serial viral passage in vivo. J Virol 1991;65:3853-63.

Girard M, Kieny MP, Pinter A, Barre-Sinoussi F, Nara P, Kolbe H, Kusumi K, Chaput A, Reinhart T, Muchmore E, Ronco J, Kaczorek M, Gomard E, Gluckman JC, Fultz PN, Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus. Proc Natl Acad Sci USA 1991;88:542-6.

Goudsmit J, Back N, Zwart G, Smit L, Dunlop N, Nara PL. Characteristics of the HIV-1 neutralizing antibody response during infection and immunization. In: Girard M, Valette L, eds. 5th Coll. DesCent Gardes. Paris: Pasteur Merieux, 1991;73-9.

Haigwood N, Nara PL, VanNest G, Scandella CJ, Eichberg JW, Steimer KS. Vaccination of primates with native recombinant HIV-1 gp120 generated broad spectrum neutralizing antibodies. In: Lerner RA, Ginsberg H, Channock RM, Brown F, eds. Vaccines 91. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1991;51-8.

Hosmalin A, Nara PL, Zweig M, Lerche N, Cease K, Gard E, Markham P, Putney S, Daniel M, Desrosiers R, Berzofsky J. Priming with T helper cell epitope peptides enhances the antibody response to the envelope glycoprotein of HIV-1 in primates. J Immunol 1991;146:1667-73.

Johnson VA, Nara PL. Quantitative assay for virus infectivity: quantitative infectivity syncytium-forming assay, In: Aldovini A, Walker B, eds. Techniques in HIV research. New York: Stockton Press, 1990;77-86.

Kang CY, Nara P, Chamat S, Caralli V, Ryskamp T, Haigwood N, Newman R, Kohler H. Evidence for non-V3 specific neutralizing antibodies in HIV-1 infected humans which interfere with gp120/CD4 binding. Proc Natl Acad Sci USA (In Press).

Nara PL, Goudsmit J. Clonal dominance of the neutralizing response to the HIV-1 V3 epitope: evidence for "original antigenic sin" during vaccination and infection in animals, including humans. In: Lerner RA, Ginsberg H, Channock RM, Brown F, eds. Vaccines 91. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1991;37-50.

Nara PL, Smit L, Dunlop N, Hatch W, Merges M, Waters D, Kelliher J, Gallo RC, Fischinger PJ, Goudsmit J. Emergence of viruses resistant to neutralization by V3-specific antibodies in experimental human immunodeficiency virus type 1 IIIB infection of chimpanzees. J Virol 1990;64:3779-91.

Nara PL, Smit L, Dunlop N, Hatch W, Merges M, Water D, Kelliher J, Krone W, Goudsmit J. Evidence for rapid selection and deletion of HIV-1 subpopulations in vivo by V3-specific neutralizing antibody: A model of humoral-associated selection. Dev Biol 1990;72:315-41.

Profy AT, Salinas PA, Eckler LI, Dunlop NM, Nara PL, Putney SD. Epitopes recognized by the neutralizing antibodies of an HIV-1 infected individual. J Immunol 1990;144:4642-7.

Vujcic L, Katzenstein D, Martin M, Quinnan G, Collaborating Laboratories. International collaborative study to compare assays for antibodies that neutralize human immunodeficiency virus. AIDS Res Hum Retroviruses 1990;6:847-53.

Zwart G, VanDerHoek L, Langedijk H, Back N, Ramautarsing C, Wolfs T, DeJong J, DeRonde T, Nara P, Goudsmit J. Antigenicity and antigenic variation of the V3 neutralization domain on the envelope glycoprotein gp120 of HIV-1. In: Lerner RA, Ginsberg H, Channock RM, Brown F, eds. Vaccines 91. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1991;45-50.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05616-03 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Anti-HIV Factors in Animal Sera and CD4 Anti-receptor Therapy for HIV-1

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

PI:	P. L. Nara	Expert	LTCB NCI
Others:	N. M. Dunlop	Microbiologist	LTCB NCI
	W. P. Tsai	Microbiologist	LBP NCI

COOPERATING UNITS *(if any)*
Laboratory of Cell Biology, NIMH, NIH (L. Eiden, D. Rausch); Genelabs Inc., Redwood City, CA (J. Lifson, K. Hwang); FDA, Bethesda, MD (B. Fraser); USUHS, Bethesda, MD (T. Borsos); George Washington University, Washington, D.C. (V. Hy)

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	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.)*

The synthetic derivatized CD4 peptides discussed in the last annual report are currently being used as molecular tools to dissect the complex stages of viral entry. Much of this work is now being done by collaborators through technology transfer from our laboratory. No further details are provided. A current summary of the work is referenced in Rausch et al. (Ann NY Acad Sci 616:125-148, 1991).

This project has been terminated.

Project Description

Names, Titles, Laboratory and institute Affiliations of Professional Personnel Engaged on the Project:

P. L. Nara	Expert	LTCB NCI
N. M. Dunlop	Microbiologist	LTCB NCI
W. P. Tsai	Microbiologist	LBP NCI

The synthetic derivatized CD4 peptides discussed in the last annual report are currently being used as molecular tools to dissect the complex stages of viral entry. Much of this work is now being done by collaborators through technology transfer from our laboratory. No further details are provided. A current summary of the work is referenced in Rausch et al. (Ann NY Acad Sci 616:125-148, 1991).

This project has been terminated.

Publications:

Rausch DM, Hwant KM, Padget M, Voltz A-H, Rivas A, Engleman E, Gaston I, McGrath M, Fraser B, Kalyanaraman VS, Nara PL, Dunlop N, Martin L, Murphey-Corb M, Lifson JD, Eiden L. Peptides derived from the CDR3-homologous domain of the CD4 molecule are specific inhibitors of HIV-1 and SIV infection, virus-induced cell fusion, and post-infection viral transmission in vitro. Ann NY Acad Sci 1991;616:125-48.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05645-02 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Epidemiology and Biological Determinants of HTLV-I

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

PI:	G. Franchini	Visiting Scientist	LTCB NCI
Others:	A. Gessain	Visiting Fellow	LTCB NCI
	I. Koralnik	Guest Researcher	LTCB NCI
	E. Boeri	Guest Researcher	LTCB NCI
	R. C. Gallo	Chief	LTCB NCI

COOPERATING UNITS *(if any)*
Advanced BioScience Laboratories, Inc., Kensington, MD (P. Markham)

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Molecular Genetics of Hematopoietic Cells Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.75	2.75	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 aim of this project is to study the human T lymphotropic virus type I (HTLV-I) origin and evolution, and its pathogenetic role in HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). To study the origin, evolution and HTLV-I dissemination in the world, we analyzed viral sequence from ex vivo samples of infected individuals, whose origin, clinical diagnosis and general background was known. Using this approach, we were able to identify a novel HTLV-I variant in the southwestern Pacific Islands and formally prove that HTLV-I was disseminated in the New World through slave trade events. We plan to extend our genetic studies to the close HTLV-I relatives (simian T cell leukemia virus) which are found in naturally infected monkeys. We are investigating the pathogenetic role of HTLV-I in HAM/TSP from three different angles: 1) by studying which viral genes are expressed in the cells of infected patients. For example, in this study we identify novel HTLV-I spliced mRNAs; 2) by developing an animal model (rabbits first and perhaps monkeys later) for HAM/TSP using field viral isolates from patients rather than culture adapted HTLV-I; and 3) testing the effect of special antiviral therapy (antisense oligonucleotides or retroviral vector carrying molecular decoys) in vitro and in vivo (in rabbits) targeting specific HTLV-I genes.

Project Description

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

G. Franchini	Visiting Scientist	LTCB NCI
A. Gessain	Visiting Fellow	LTCB NCI
I. Koralnik	Guest Researcher	LTCB NCI
E. Boeri	Guest Researcher	LTCB NCI
R. C. Gallo	Chief	LTCB NCI

Objectives:

We will study the role of retroviral genes in infectivity, disease induction and maintenance, and the studies on the prevention of retroviral infection and diseases.

Methods Employed:

Standard molecular biology, immunological and virological techniques, basic knowledge in animal handling and procedures.

Major Findings:

The Low Degree of Human T Cell Leukemia Virus Type-I (HTLV-I) Genetic Drift In Vivo as a Means to Follow Viral Transmission and Movement of Ancient Populations

We have studied the genetic variation of HTLV-I in the same individuals in time, as well as in HTLV-I isolates from various parts of the world. The nucleotide sequence from 2-13 molecular clones containing an HTLV-I DNA amplified fragment of 522 base pair (bp) was obtained from each isolate. The viral DNA fragment encompassed the carboxy terminus of the gp46 and almost the entire gp21 protein. The samples, obtained from native inhabitants of five African countries, two South American countries, China, the French West Indies and Haiti, included 14 HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) cases, 10 adult T-cell leukemia (ATL) cases, one non-Hodgkin's lymphoma case and three healthy HTLV-I seropositive individuals. The analyses of a total of 65 kilobases of HTLV-I sequence demonstrated that: 1) little or no genetic variation occurred in vivo in the same individual or in different hosts carrying the same virus regardless of their clinical status; 2) some nucleotide position changes were diagnostic for the geographic origin of the viruses; 3) HTLV-Is from West African countries (Mauritania, Guinea Bissau) and some from the Ivory Coast and the Central African Republic were virtually identical to those from the French West Indies, Haiti, French Guyana and Peru. These data demonstrated that at least some HTLV-I strains were introduced in the New World through infected individuals during the slave trade; and 4) the Zairian HTLV-I isolates, in which we also observed intrastrain variability, are more divergent to the other HTLV-Is and may represent a separate HTLV-I cluster. Because of the low genetic variability of HTLV-I in vivo, the study of the proviral DNA sequence in selected populations of

infected individuals will increase our knowledge on the origin and evolution of HTLV-I and could possibly be useful in anthropological studies.

A New HTLV-I Quasi Specie in Melanesia

Some of the most remote, inaccessible population groups on earth can be found in Melanesia, the ethnographic region which includes New Guinea, the Solomon Islands, Vanuatu and New Caledonia. It is therefore not surprising that the reportedly high seroprevalence of antibodies against HTLV in isolated Melanesian population groups was initially viewed with skepticism by some investigators. The high frequency of indeterminate Western immunoblots in Melanesia, was interpreted by other investigators as indicative of the existence of HTLV-I variants. The identification of a case of HTLV-I myeloneuropathy in a life-long resident of the Solomon Islands and the isolation of HTLV-I-like viruses from the Hagahai, a recently contacted group in the fringe highlands of Papua New Guinea, and from unrelated Melanesians from widely separated provinces in the Solomon Islands validate this original conjecture. To determine the relationship of these variant viruses to prototype HTLV-I, we cloned and sequenced a polymerase chain reaction (PCR)-amplified 522 bp env gene segment encoding the carboxy terminus of the major envelope glycoprotein gp46 and nearly the entire transmembrane protein gp21, using DNA extracted from fresh or cultured peripheral blood mononuclear cells (PBMC) or virus-infected T cell lines derived from HTLV-I-seropositive natives of Papua New Guinea and the Solomon Islands.

Viral DNA sequences were analyzed from eight individuals, all of whom were seropositive by Western immunoblot, possessing antibodies against HTLV-I Gag-encoded proteins p19 and p24 and env gene products gp46 and/or gp21. Except for one individual with HTLV-I myeloneuropathy, all of the other samples were obtained from clinically healthy individuals. The Hagahai individual (RB100) from whom the HTLV-I variant designated PNG-1 was obtained, and his seropositive mother (RB105) are inhabitants of the Madang Province in Papua New Guinea. Samples were obtained from two individuals with residence in Guadalcanal, one of which is a 38 year old male with HAM/TSP. Another patient, 30, is a 60 year old Melanesian woman living in Marau, who was married to an HTLV-I seropositive man originally from Rendovo. In this case, we do not know if patient 30 was already infected before marrying the man from Rendovo or whether her husband transmitted the virus to her. Lastly, two patients from a Polynesian outlier, Bellona Island, were studied.

The results indicated the existence of an novel quasi specie of retroviruses, phylogenetically related to the HTLV-I prototype, but distinct in their nucleotide sequence, in Melanesia. The various members of this new HTLV family are 92% related to, but distinct from Japanese and West Indies isolates which, in turn, are 98-99% related among themselves. The HTLV-I variant from the remote Hagahai population of Papua New Guinea differs by 4% from the HTLV-I variants from Melanesians, including a patient with HTLV-I myeloneuropathy from three widely separated provinces in the Solomon Islands. However, HTLV-I strains from inhabitants of the Polynesian outlier, Bellona Island, were more closely related (97%) to the HTLV-I prototype. These data uncover the existence of HTLV-I as a quasi specie in nature and pose new questions on the origin and evolution of HTLV-I. The Melanesian HTLVs and the HTLV-I(ATK) prototype are equally genetically distant to the simian T cell leukemia virus (STLV)-I(mac) virus, which

was isolated from Japanese macaques and is very closely related to HTLV-I. In contrast, a difference in the genetic relationship of the Melanesian isolates and the HTLV-I prototype to HTLV-II is evident (27% versus 30% variability on the average) and interestingly, the HTLV-I isolates from the Polynesian outlier also cluster with the HTLV-I(ATK) prototype when compared to HTLV-II.

The vast majority (more than 85%) of nucleotide changes among the Melanesian HTLV-Is and HTLV-I prototype occur in the third codon position and do not induce a change in the encoded amino acid. Among the amino acid changes, one-half are conservative changes and the remaining one-half are nonconservative. These data further indicate that the HTLV-I envelope structure is probably subjected to a high genetic constraint and that few amino acid changes are compatible with the survival of the virus in a given infected population. Some of the amino acid changes observed are shared among all the Melanesian viruses as well as STLV-I and/or HTLV-II.

The identification of these HTLV-I quasi specie in the Southwestern Pacific poses new questions about the origin and evolution of HTLV-I. The detection of three main strains of HTLV-I, one in the Hagahai of Papua New Guinea, another in the Solomon Islands and a third, more closely related to the HTLV-I prototype in the Bellona Islands, suggests that HTLV-I has been present in these isolated populations for thousands of years.

HTLV-I Expression in Fresh PBMCs from Patients with HAM/TSP

HAM/TSP is a chronic neurological illness epidemiologically associated with HTLV-I infection. In HAM/TSP viral replication appears to be more directly related with disease development than it does in ATL because: 1) rapid onset of disease in a few well-documented cases of "transfusion associated" HAM/TSP; 2) presence of IgG and IgM oligoclonal bands in the serum and the cerebrospinal fluid (CSF), some of which are directed against HTLV-I antigens; 3) higher titers of serum and CSF antiviral antibodies; and 4) a higher number of circulating HTLV-I infected cells when compared to healthy HTLV-I carriers. To gain some insight into the pathogenesis of HAM/TSP, we investigated qualitative and quantitative viral expression in fresh uncultured PBMCs from HAM/TSP patients. Genomic DNA from the PBMCs of all the patients studied carried HTLV-I provirus, but viral expression was not detected by Northern blot analysis of total cellular PBMCs' ribonucleic acid (RNA). When the reverse transcriptase (RT)-PCR technique was employed using primers specific for the tax/rex messenger RNA (mRNA), all the samples were positive, regardless of the duration of the illness (range 2-13 years). The same splice junction for the tax/rex mRNA described in cases of HTLV-I induced ATL (position 5183 of the envelope and 7302 of the px region) was present in all three HAM/TSP cases studied. To ascertain whether viral expression occurred at a low level in many cells or at a high level in a few permissive cells, we performed in situ hybridization on fresh PBMCs from two patients (2 and 7 years from clinical diagnosis) for HTLV-I RNA. Our finding indicated that in vivo HTLV-I expression occurred at a high level in a few cells (one of every 5000 PBMCs) in both cases studied. The fact that all six HAM/TSP patients cells were positive for viral expression, regardless of the time lag from diagnosis, suggests that persistent expression of viral product(s) may be pivotal in the pathogenesis of HAM/TSP.

Novel Protein Isoforms Encoded by HTLV-I Alternatively Spliced mRNAs

Viruses have developed fine post-transcriptional mechanisms, which allow the maximal utilization of their limited genomes. Using the RT-PCR techniques, we studied the HTLV-I spliced mRNAs in HTLV-I primary cell culture and fresh cells from patients with HAM/TSP, to uncover the degree of the HTLV-I genome complexity.

Here we report the identification of a novel acceptor splice site which is used in the generation of a single and a double spliced mRNA species. By the alternative splicing mechanism, two protein isoforms of 99 and 152 amino acids could be encoded by the open reading frame I (orf I) in the pX region. Translation of the single spliced mRNA could initiate at the internal AUG codon of the orf I, whereas the latter double spliced mRNA acquires the AUG codon from the Rex open reading frame. Interestingly, the juxtaposition of the first 20 amino acids of the Rex protein to the orf I would confer to this protein a nucleolar targeting signal sequence.

A single species of double spliced polysixtronic mRNA (pXmRNA), encoding for the Tax, Rex and the p21(Rex) protein, has been previously identified. Our study uncovered the presence of a separate single spliced monocistronic mRNA which could encode for the p21(Rex) protein. Lastly, a novel acceptor splice site was found to be used in the generation of alternative spliced mRNAs for both the envelope and the pX region. Since the use of this acceptor splice site does not alter the coding potential of these mRNAs, the functional meaning of these alternatively spliced mRNAs is unknown. Thus, likely in human immunodeficiency virus (HIV), HTLV-I genomic complexity is greatly enhanced by post-transcriptional events. These novel RNA species were found not only in the cytoplasm of an HTLV-I infected cell line, but also in the RNA obtained from fresh PBMCs of HAM/TSP patients.

HTLV-I Infection of Rabbits as a Possible Model for HAM/TSP

HAM/TSP is a progressive myelopathy associated with neurological impairment. The association between HTLV-I infection and the development of HAM/TSP invokes HTLV-I as the causative agent. Little information is available regarding the HTLV-I tissue localization in infected individuals because those patients usually die late in life, and the disease is rare. To understand the biology of HTLV-I infection, and to study the viral-host interaction, we attempted to develop an animal system where some of these questions could be addressed.

We have chosen the rabbit for infectivity studies in vivo because evidence exists for rabbit sensitivity to HTLV-I infection and rabbits are easily manipulated and readily available. In contrast to other investigators, we have used early passages (2 months) of primary human cell lines (from HAM/TSP patients from the West Indies) to inoculate rabbits. At the time of inoculation, about 30% of the cells from both patients (HTLV-I[BOU], HTLV-I[cor]) expressed the viral Gag antigen p19. In the first experiment, two rabbits (oryctolagus cuniculus) were inoculated intravenously with $5x10^4$ cells from each HTLV-I infected cell culture. A third animal received $5x10^4$ cells of HTLV-I(BOU) in the CSF. The animals were tested for seroconversion against viral antigens by Western blot analysis of serum samples obtained every month and various attempts were performed to isolate

HTLV-I from the PBMCs of the infected animals. All animals seroconverted within a month from inoculation. Virus recovery was easily obtained from the three animals infected with the HTLV-I(BOU) at 2, 4, 8 weeks and thereafter, whereas virus isolation was positive in animals C2071 and C2059, infected with HTLV-I(cor) only after 8 weeks. In three of the infected animals we observed peculiar clinical signs. Approximately 8 weeks from inoculation animal C2068 displayed behavioral changes. The animal became very aggressive toward the animal handlers and had to be sacrificed at 10 weeks post-inoculation. The presence of HTLV-I in the tissues of the sacrificed animal was monitored by virus isolation from the minced tissue as well as PCR analysis of tissue DNA. From most cultured tissues, with the exception of the heart, cerebellum, bladder, uterus and kidney, virus in cell culture was detected by either RT or antigen capture assays. The results of viral detection in vitro in the cultured tissues did not agree totally with the PCR findings. The PCR analysis showed viral sequence in almost all the samples with the exception of the brain stem and the hypothalamus, both of which were positive for viral isolation. Thus, the animal had detectable HTLV-I in most tissues at the time of death.

Of the remaining rabbits, by 24 weeks post-inoculation, rabbit C2059 inoculated with HTLV-I(cor) and rabbit C2044 inoculated with HTLV-I(BOU) developed similar clinical pictures. In addition, C2044 showed signs of neurological impairment (pupil dilatation, aggressiveness, and hyperventilation at rest). Both rabbits are still under observation. To investigate whether the peculiar clinical signs, reminiscent of encephalitis, present in animals C2068 and C2059 could be transmitted, the blood of the animal at the time of death was used to inoculate two naive rabbits. To rule out the possibility of transmission of other infectious agents with the blood of animal C2068, the rabbit cell line carrying the HTLV-I(BOU) obtained from the same animal (C2069) was also used to inoculate one animal. Given that in humans, HAM/TSP at times develops in iatrogenically immunodepressed patients, to test whether we could accelerate the clinical manifestations observed, we immunodepressed the infected animals with cortisone at the time of inoculation. The inoculation protocol involved three animals. Animals C2072 and C2073 received 5 and 15 ml of whole blood from animal C2068. Animal C2074 received 10^4 cells from the HTLV-I(BOU) positive primary cell line developed from the C2068 rabbit. Reproduction of the clinical symptoms observed in animal C2068 in all these animals would more conclusively indicate a causative role of HTLV-I(BOU).

Publications:

Gessain A, Louie A, Gout O, Gallo RC, Franchini G. HTLV-I expression in fresh peripheral blood mononuclear cells from patients with HTLV-I associated myelopathy/tropical spastic paraparesis. J Virol 1991;665:1628-33.

Gessain A, Yanagihara R, Franchini G, Garruto RM, Jenkins CL, Ajdukiewicz AB, Gallo RC, Gajdusek DC. Highly divergent molecular variants of human T-lymphotropic virus type I from isolated populations in Papua New Guinea and Solomon Islands. Proc Natl Acad Sci USA (In Press).

Lusso P, diMarzo-Veronese F, Ensoli B, Franchini G, Jemma C, DeRocco SE, Kalyanaraman VS, Gallo RC. Expanded HIV-I cellular tropism by phenotypic mixing with murine endogenous retroviruses. Science 1990;247:848-52.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05688-01 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular Approaches for Development of an HIV Vaccine: Rhesus Macaques as a Model

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

PI:	G. Franchini	Visiting Scientist	LTCB NCI
Others:	E. Boeri	Guest Researcher	LTCB NCI
	M. Robert-Guroff	Research Biologist	LTCB NCI
	R. C. Gallo	Chief	LTCB NCI

COOPERATING UNITS (if any)
Advanced BioScience Laboratories, Inc., Kensington, MD (P. Markham); Program Resources, Inc., Frederick, MD (A. Aggarwal); Virogenetic, Albany, NY (E. Paoletti); MedImmune Vaccine, Inc., Gaithersburg, MD (T. Fuerst).

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Molecular Genetics of Hematopoietic Cells Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	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 aim of this project is to test in rhesus macaques the efficacy of various live vectors carrying human immunodeficiency virus type-1 (HIV-1), HIV-2 and simian immunodeficiency virus (SIV) antigens in eliciting a protective immunoresponse. We are testing three different delivery systems: 1) attenuated vaccinia and avipox carrying HIV and SIV antigens; 2) attenuated Salmonella typhimurium strain and the Bacille of Calmette and Guerin (BCG), as a bacterial vaccine, carrying HIV/SIV antigens. We are also planning to further stimulate the immunoresponse in the immunized animals using purified viral antigens (either native or recombinant viral subunits). We will use rhesus macaques which are susceptible to the infection of both SIV(mac251) and HIV-2(SBL/ISY). The end point of our studies will be the protection from infection by either SIV or HIV-2. Vectors containing HIV-1 antigens will also be evaluated for their immunogenicity in rhesus macaques. The infectivity of HIV-2 mutants performed in vitro and in vivo provided a better choice for the HIV-2 virus to be used in homologous challenge experiments.

Project Description

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

G. Franchini	Visiting Scientist	LTCB NCI
E. Boeri	Guest Researcher	LTCB NCI
M. Robert-Guroff	Research Biologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI

Objective:

Investigate various approaches for the development of an HIV vaccine.

Methods Employed:

Basic molecular and immunological techniques. Knowledge on animal care.

Major Findings:

Dispensable Role of the Human Immunodeficiency Virus Type-2 (HIV-2) Vpx Protein in Viral Replication

The HIV-2 is similar in its genetic organization to HIV-1, but contains a unique gene (vpx) encoding a 16 kilodalton protein. A replication competent molecular clone of HIV-2 (HIV[SBL/ISY]), which infects human primary cells in vitro as well as rhesus macaques, was used to generate three mutants in the vpx gene. In the first mutant, the vpx open reading frame was truncated at amino acid 20, the second mutant was tailored to eliminate the proline rich carboxyl terminus of the protein, and the third mutant was obtained by the addition of 4 amino acids (KDEL) to the carboxyl terminus of the protein to provide a retention signal in the endoplasmic reticulum. The kinetics of viral infection with the three mutant viruses and the isogeneic HIV-2(SBL/ISY) on the Sup T1 cell line were similar. When primary human peripheral blood mononuclear cells (PBMCs) were used as a target, a slight impairment was observed with the vpx mutant viruses in the early phase of viral replication. All the vpx mutant viruses were able to productively infect macrophages,indicating that vpx expression is dispensable for HIV-2 replication in human macrophages.

The Viral Infectivity Gene (vif) of HIV-2

The vif gene is conserved in the genome of HIV-1 and HIV-2 as well as in the simian immunodeficiency viruses (SIV) identified in many monkey species (SIV[mac], SIV[agm], SIV[sm] and SIV[man]). The vif gene appears to play a key role in the mechanism of viral production, although its mechanisms of action are poorly understood. Previous studies performed in human neoplastic cell lines hypothesized that the vif gene of HIV-1 might be required either in early or late phases of viral infection. Using a biologically active HIV-2 molecular clone (HIV-2[SBL/ISY]) which is infectious in primary human T-cells and macrophages in vitro as well as in vivo in rhesus macaques, we generated mutated genotypes in the

vif gene. The HIV-2 vif minus virions were generated by transfecting the mutated proviral DNA in HeLa/tat(+) cells, and the resulting virus was successfully propagated in the Sup T1 T-cell line. The cell free virus from the Sup T1 cell line was infectious as the wild-type HIV-2(SBL/ISY) in Sup T1 cells, but not the other human neoplastic T-cell lines that we used (MOLT 3, CEM, HUT 78, MT-2), which are very sensitive to infection by HIV-2(SBL/ISY) wild-type virus. Since all the cell lines tested expressed the receptor for HIV-2 (the CD4 molecule), our data suggested that Sup T1 cells may contain a cellular factor that can substitute for vif and enable viral replication. Supernatant from the Sup T1 cells was used to infect human fresh PBMC as well as a purified population of primary macrophages. Very high levels of viral replication and production were observed in macrophages as early as 3 days after infection, as shown by reverse transcriptase (RT) in the supernatant and labelled viral protein. In the fresh PBMCs, a peak of RT activity and labelled viral protein was also seen day 3 after infection, but no long-term productive viral infection was established. These data might indicate that the viral replication observed in PBMCs might be due to the presence of remaining macrophage in the Ficoll-purified PBMC. In summary, our data indicates that the effects of the vif gene in viral replication are dependent on the target cell type. In T-cells, the presence of the vif gene enabled productive viral expression, while in macrophages vif appeared to repress viral expression initially because its removal appeared to boost viral expansion within a few days after infection of human macrophages. This peculiar behavior of the HIV-2 vif minus virus in primary human cells correlated with our results of HIV-2 vif minus infectivity of rhesus macaques. Early seroconversion occurred in the infected animal but the immune response faded after 4 weeks from inoculation, suggesting that viral replication had probably ceased.

Infection of Rhesus Macaques with an HIV-2 Molecular Clone

In an attempt to generate a suitable animal model to study the infectivity and possible pathogenicity of HIV, we intravenously inoculated juvenile rhesus macaques and African green monkeys with a molecularly cloned virus, HIV-2(SBL/ISY), as well as with the uncloned HIV-2(NIH-Z) virus. Infection was monitored by virus recovery from the peripheral blood cells and by seroconversion against HIV-2 antigens measured by Western blot, radioimmunoprecipitation and enzyme linked-immunosorbent assay (ELISA). We successfully infected two out of two macaques with the molecularly cloned virus, and one macaque out of two with the HIV-2(NIH-Z). No evidence of infection was seen in the African green monkeys with either virus. We followed the infected animals for 2 years. The animals remained healthy, although we observed intermittent lymphadenopathy and a transient decrease in the absolute number of circulating $CD4^+$ T lymphocytes in both animals infected with the molecularly cloned virus. Virus isolation from the peripheral blood cells of the infected animals was successful only within the first few months after inoculation. Evidence of persistent infection was provided by the detection of proviral DNA by polymerase chain reaction (PCR) analysis of the blood cells of the inoculated animals and by the stability of antiviral Ab titers. To evaluate the genetic drift of the proviral DNA, we molecularly cloned viruses which were reisolated 1 and 5 months post-inoculation from one of these animals. Comparison of the DNA sequence of the envelope genes of both these isolates indicated that a low degree of variation (0.2%) in the envelope protein had occurred in vivo during the 5 month period. These data suggest that the use

of HIV-2(SBL/ISY) in rhesus macaques may represent a good animal model system to study prevention of viral infection. In particular, molecularly cloned virus can be manipulated for functional studies of viral genes in the pathogenesis of AIDS, and provides a reproducible source of virus for vaccine studies.

Biological Features of HIV-2(SBL/ISY) Rhesus Macaque Infection

As we have previously shown, the animals infected with HIV-2 displayed minimal signs of disease development within 2 years from the experimental inoculation. Because of the difficulty of virus recovery from the infected animals and the constant level of immune response, we believe that HIV-2(SBL/ISY) causes a persistent, but latent infection in rhesus macaques. Three animals (#172, #177, and #180) which have been infected with HIV-2 for 21, 24, and 26 months, respectively, were sacrificed. The DNA of the tissues collected at necroscopy was analyzed by PCR for HIV-2 sequences. In animal #180, virus isolation was also performed from several of the collected tissues. Viral sequences were detected from most tissues of the sacrificed animals with the exception of the heart, liver, testis, and salivary glands. Viral sequences in various sections of the brain were tested in all the animals. Virus recovery was positive from several tissues from animal #180. Genetic analysis (by Southern blot) of the virus recovered in culture from animal #180 indicated that it was indistinguishable from the inoculum virus. Thus, we conclude that HIV-2 persistently infects rhesus macaques. The virus appears to be sequestered in the tissues very shortly after infection as indicated by the difficulty of virus recovery from the seropositive monkeys' PBMC. The immunological mechanisms that could mediate this phenomenon is not clear since the animals do not develop substantial titers of neutralizing antibodies and they do not appear to have circulating cytotoxic T lymphocytes (CTL) cells. It is conceivable that the virus is sequestered in the animals' macrophages and that the tissue CTLs siege the infected macrophages. We will explore the hypothesis by searching for CTL in the spleen of the infected animals. The hypothesis that macrophage versus T-cell infection by HIV-2(SBL/ISY) might be prevalent in these animals is also supported by the fact that the HIV-2 vpr mutant does not infect rhesus macaques, and we have shown that the vpr gene is crucial for viral expression in primary macrophages.

Infectivity of HIV-2 Mutants in Rhesus Macaques

The HIV-2 mutants in the vif, vpr, nef, vpx, as well as the viruses containing a combination of mutations in their genome (vpx-vpr; vpr-nef; and vpr, vpx, nef) were used to infect rhesus macaques. Viral stocks were prepared in the human neoplastic cell line HUT 78, and in Sup T1 cell line for the HIV-2 vif mutants. The viral stocks were titered in vitro and two rhesus macaques were intravenously inoculated with 2.5×10^4 $TCID_{50}$ of each virus. The serum of each animal was tested every month for the presence of antibodies against HIV-2 antigens by plate ELISA and Western blot. The results for the first 4 months are summarized. The animals inoculated with the wild-type HIV-2(SBL/ISY) and the HIV-2 nef virus seroconverted between the second and third month after inoculation. The animals inoculated with the vpx, vpx-vpr and vpx-vpr-nef seroconverted within the first and second months from inoculation. In contrast, no seroconversion was evident in animals inoculated with the HIV-2 vpr virus. The HIV-2 vif virus instead induced a temporary immune response that faded 4 months after inoculation. Virus isolation

from the PBMC of the inoculated animals was performed using various tamet cells (monkey CD8-depleted PBMC plus CD8-depleted human PBMC or purified monkey macrophages). PCR analysis of viral sequences was performed on the monkey PBMC DNAs at various times from inoculation. Several months following inoculation virus was recovered from the animals inoculated with the vpx, nef-vpx and vpx-nef-vpr viruses. The same animals (#194, #195, #196, #197, #199 and #207) also had a more stable immunoresponse. Of those, only animals #207 and #197 repetitively scored positive for the presence of viral sequences in their PBMCs by PCR analysis. Virus isolation and PCR analysis on PBMC cells from the animals inoculated with the HIV-2 vif and HIV-2 vpr virus were consistently negative. These data, in addition to the lack of seroconversion in animals #189-#193 and the lack of a permanent immunoresponse in animals #200 and #202 indicate that those animals were probably not infected by the inoculated viruses. The results obtained with the HIV-2 nef, nef-vpr and vpx-vpr mutants are somewhat less interpretable at present. To rule out that animals #189, #193, #200 and #202 did not seroconvert because of some problem with the viral inocula, we reinoculated all the animals in the study 8 months after the first inoculation, with $1x10^5$ $TCID_{50}$ of the same viruses used at the start of the experiments. Interestingly, all the animals in the study had an anamnestic immune response, with the exception of the two control animals and the animals which received the HIV-2 vif and the HIV-2 vpr viruses. These data are indicative of a real difference in the biological activity of the HIV-2 mutant viruses.

Giardia Lamblia and Entamoeba Histolytica: Human Parasites--Potential Carriers of HIV-1

Giardia lamblia and Entamoeba histolytica infections are frequent in male homosexuals, most likely because of sexual practices which lead to increased ingestion of cysts in contaminated feces. Infections with these two intestinal parasites are often asymptomatic in AIDS patients. These parasites pass from person-to-person directly by fecal-oral routes or through food and water. If other infectious agents were present, these protozoa may serve as vectors of transmission. We investigated whether these parasites could play a role in transmission of HIV-1 by testing the capability of trophozoites to support viral replication. The results showed that trophozoites could internalize the virus and support its replication and that HIV-1 could be transmitted by cocultivation of infected trophozoites with human $CD5^+$ T-cells. These findings raise the possibility that these parasites could play an active role in spreading HIV-1 infection, especially in developing countries where these parasites are endemic. However, if such an event would occur, it would probably be very rare since epidemiological data argue against transmission of HIV-1 through routes other than sexual, mother-to-child and blood.

Publications:

Chakrabarti S, Mizukami T, Franchini G, Moss B. Synthesis, oligomerization, and biological activity of the human immunodeficiency virus type 2 envelope glycoprotein expressed by a recombinant vaccinia virus. Virology 1990;178:134-42.

Franchini G, Markham P, Gard E, Fargnoli K, Keubaruwa S, Jagodinski L, Robert-Guroff M, Lusso P, Ford G, Wong-Staal F, Gallo RC. Persistent infection of rhesus macaques with a molecular clone of human immunodeficiency virus type 2: evidence of minimal genetic drift and low pathogenic effects. J Virol 1990;64:4462-7.

Franchini G, Wong-Staal F. Conserved and divergent features of human and simian immunodeficiency virus. In: Putney S, Bolognesi D, eds. AIDS vaccine research and clinical trials. New York: Marcel Dekker, 1990;107-19.

Hattori N, Michaels F, Fargnoli K, Marcon L, Gallo RC, Franchini G. The human immunodeficiency virus type 2 vpr gene is essential for productive infection of human macrophages. Proc Natl Acad Sci USA 1990;87:8080-4.

Marcon L, Hattori N, Franchini G. A comparison of the genetic and biological features of human and non-human immunodeficiency lentivirus. In: Giraldo G, ed. Advanced course on AIDS and associated tumors. Basel: Karger (In Press).

Marcon L, Michaels F, Hattori N, Fargnoli K, Franchini G. Dispensable role of the human immunodeficiency virus type-1 Vpx protein in viral replication. J Virol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05689-01 LTCB

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): HTLV-I and Adult T Cell Leukemia: Pathophysiology of HTLV-I Infection

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

PI:	M. E. Klotman	US Public Health Service Officer	LTCB NCI
Others:	Z. Berneman	Visiting Scientist	LTCB NCI
	R. Gartenhaus	Staff Fellow	LTCB NCI
	M. Reitz	Senior Staff Scientist	LTCB NCI
	Y. Lunardi-Iskandar	Visiting Scientist	LTCB NCI
	W. Blattner	Chief, Viral Epidemiology Section	EEB NCI
	A. Manns	Senior Clinical Investigator	EEB NCI

COOPERATING UNITS (if any): University of Vienna Medical School, Vienna, Austria (E. Tschachler); University of the West Indies, Kingston, Jamaica (B. Harchand, B. Cranston)

LAB/BRANCH: Laboratory of Tumor Cell Biology

SECTION: Molecular Genetics of Hematopoietic Cells Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	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.)

Seroepidemiologic data as well as molecular analysis firmly establishes the association between the human retrovirus human T cell leukemia virus type-I (HTLV-I) and the diseases adult T cell leukemia/lymphoma (ATL) and HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP). In contrast to human immunodeficiency virus type-1 (HIV-1) infection where the majority of those infected develop disease, only a small percentage of those infected with HTLV-I will develop ATL. Because of the inability to detect virus expression by standard ribonucleic acid (RNA) and protein analyses in patients, the exact role the virus plays in disease pathogenesis remains unclear. Newer methodologies have allowed the reexamination of the role of the virus in pathogenesis. Reverse transcriptase-polymerase chain reaction amplification of specific HTLV-I cDNAs resulted in the mapping of at least three new cDNAs, one of which is a candidate for the monocistronic expression of an HTLV-I protein, p21 rex, of unknown function and two contain potential open reading frames for new proteins. When RNA from peripheral blood lymphocytes of HTLV-I infected patients were examined by similar methods, virus expression was detected in the majority of samples analyzed. To further support the role of continued virus expression in disease pathogenesis, recombinant Tax protein could be taken up by noninfected lymphocytes and results in increased proliferation of cells of lymphoid and glial origin. A number of cofactors may play a role in HTLV-I-associated disease pathogenesis. Specific viral proteins from the herpesvirus, cytomegalovirus, significantly down regulates the expression from the HTLV-I promoter. Viral expression and, specifically, expression of Tax protein enhances the expression of tumor necrosis factor β in vitro and this cytokine may result in some of the pathologic effects such as severe hypercalcemia associated with ATL. Understanding the control of viral expression in vivo during diseases will give insight into novel approaches for therapy.

Projection Description

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

M. E. Klotman	US Public Health Service Officer	LTCB NCI
Z. Berneman	Visiting Scientist	LTCB NCI
R. Gartenhaus	Staff Fellow	LTCB NCI
M. Reitz	Senior Staff Scientist	LTCB NCI
Y. Lunardi-Iskandar	Visiting Scientist	LTCB NCI
W. Blattner	Chief, Viral Epidemiology Section	EEB NCI
A. Manns	Senior Clinical Investigator	EEB NCI

Objectives:

Although the associations between human T cell leukemia virus type-I (HTLV-I) and the diseases adult T cell leukemia/lymphoma (ATL) and HTLV-associated myelopathy/ tropical spastic paraparesis (HAM/TSP) have been clearly established, the exact role the virus plays in disease pathogenesis remains unclear. In contrast to human immunodeficiency virus type-1 (HIV-1) infection where the majority of those infected ultimately develop disease, only a small percentage of those infected with HTLV-I will develop ATL. The objective of these studies was to gain a better understanding of factors that might modulate viral expression in the host and to examine the role that HTLV-I virus expression plays in determining the outcome of infection. The following studies focus on further elucidation of the role of specific viral gene expression in disease, especially ATL, the effects of soluble viral proteins, specifically the major transcriptional transactivator tax, on infected and uninfected target cells, the potential role of viral cofactors on HTLV-I expression, the role virally-induced cytokines play in the hypercalcemia associated with ATL, and the mechanism of spontaneous proliferation of T cells from HTLV-I infected patients.

Methods Employed:

HTLV-I virus expression was measured using a new sensitive assay for specific viral messages called reverse transcriptase-polymerase chain reaction (RT-PCR). Ribonucleic acid (RNA) from HTLV-I-infected cell lines and samples from HTLV-I-infected patients is converted to cDNA using RT with specific antisense primers recognizing sequences in the pX region of HTLV-I. The cDNA is amplified by PCR, using a sense primer recognizing a sequence in the 5′ long terminal repeat (LTR). The PCR products are analyzed by polyacrylamide gel electrophoresis and detected by specific oligonucleotide probes. The reverse PCR products are cloned into Bluescript SK plasmid for further analysis by DNA sequencing and expression in eukaryotic cells.

Standard proliferative assays using ^{3}H-thymidine incorporation are used for analysis of the proliferative effects of the HTLV-I Tax protein on cells of lymphoid and neural origin. The Tax protein is analyzed for immunoreactivity by Western blot, for intracellular localization using indirect immunofluorescence, for activity by standard chloramphenicol acetyl-transferase (CAT) detection and

for extracellular release by radioimmunoprecipitation. Spontaneous proliferation of lymphocytes from HTLV-I infected patients is measured by a methyl-cellulose assay.

The interrelationship of HTLV-I expression and the expression of specific cytokines is studied using Northern hybridization, radioimmunoprecipitation and CAT detection when the promoter for the gene of interest is placed upstream from the CAT gene.

Major Findings:

Utilizing RT-PCR, we detected four types of spliced messages coded for by the pX region of HTLV-I in the HTLV-I infected cell line C10/MJ. Three of these spliced messages have not been previously described and one such message is the prime candidate for a monocistronic message for $p21^{rex}$, an HTLV-I protein of unknown function. Another message contains a small open reading frame not previously described, potentially coding for a polypeptide of 14 amino acids. All four different pX messages were found in different HTLV-I-infected cell lines, both interleukin-2 (IL-2)-dependent (N1186, G11/MJ, ECL55) and IL-2-independent (HUT 102, MT-2, C10/MJ, NS1).

RNA was analyzed from uncultured, unstimulated peripheral blood mononuclear cells from 10 patients with ATL using RT-PCR. Nine out of 10 samples were positive for the classical pX message (message 1) and/or the other 3 alternatively spliced pX messenger RNAs (mRNA). The classical and alternative pX splice junctions are the same in the ATL samples as in the HTLV-I-producing cell lines. Thus, not only does this demonstrate expression of HTLV-I in vivo, an observation not appreciated by previously employed less sensitive techniques, but there is alternative splicing in vitro and in vivo.

A preparation of Tax protein was prepared from E. coli containing a Tax-expressing plasmid and was found to be >90% pure by protein gel analysis and of normal immunoreactivity by Western blot. Activity and uptake of the protein was assayed by introducing the protein to the media of cells in a transient transfection assay. Target cells contained the CAT gene under the control of the HTLV-I LTR containing the promoter. Introduction of Tax protein to the media resulted in a significant stimulation of the HTLV-I promoter as indicated by increased CAT activity indicating that the protein was taken up by the cells and had the expected biologic activity. Primary peripheral blood lymphocytes (PBL) exposed to Tax protein showed uptake of the protein with specific fluorescence localized primarily in the nucleus.

The soluble Tax protein is mitogenic to primary PBL as well as SVG cells (an immortalized astroglial cell line). The addition of Tax protein to the media resulted in a 2-fold increase in ^{3}H-thymidine incorporation when compared to medium alone or control E. coli extract. The proliferative response to Tax protein required prior activation of the PBL's with phytohemagglutinin. Cotreatment of the cells with Tax protein and specific monoclonal antibody (prepared directly from large scale culture of the hybridoma cell line) to Tax protein resulted in a significant reduction in ^{3}H-thymidine incorporation. Anti-Tac antibody

significantly blocked the proliferative response to IL-2 but had no effect on the response to Tax protein, suggesting that the mechanisms of stimulation of proliferation differ.

We assessed the effect of the products of the human cytomegalovirus (HCMV) IE-2 region on HTLV-I promoter function in primary PBL as well as cell lines and showed that the expression of these CMV proteins resulted in a 3-fold reduction in HTLV-I promoter activity. In contrast these same CMV proteins enhanced the expression from the HIV-1 promoter. Therefore, the HCMV IE-2 region specifically repressed the HTLV-I promoter in both HTLV-I producing cell lines where tax, the major transactivating protein of HTLV-I, is expressed as well as in PBL. RNA analysis was consistent with a specific repression at the level of transcription or a decrease in the stability of the message. Both HTLV-I and HCMV are known to infect these cells in vivo. The observed unique repressive effect of one viral gene product on the promoter of another introduces another potential role of viral cofactors in possibly altering disease expression in the host.

Since hypercalcemia is a major factor contributing to the morbidity of HTLV-I associated ATL, we investigated the potential role of cytokines that might be involved in this abnormal response. Tumor necrosis factor β (TNFβ), which is known to activate osteoclast activity is secreted by HTLV-I infected cells in vitro. Furthermore, media from HTLV-I infected cell lines assayed for TNF activity by their ability to kill mouse L929 cells show killing with serial dilutions of culture fluid and RNA from cell lines established from normal cord blood T cells by infection with HTLV-I are positive for the expression of TNFβ mRNA. This TNFβ mRNA expression is not associated with gene rearrangement and is activated by the HTLV-I tax gene specifically.

Selection of T cell colonies from circulating peripheral blood cells of normal donors requires stimulation, whereas there is spontaneous growth of such colonies from HTLV-I infected patients who are asymptomatic or have HAM/TSP. These colonies express HTLV-I proteins. Furthermore, the phenotype of these cells are distinct from the characteristics of the induced colonies of normal donors. These findings indicate that there is abnormal growth and differentiation of certain HTLV-I infected T cells in infected patients.

Publications:

Berneman ZN, Gartenhaus RB, Reitz MS, Gallo RC, Klotman ME. Expression and alternative splicing of human T-lymphotropic virus, HTLV-I, type I in adult T-cell leukemia/lymphoma and ATL. Blood 1991;76:484a.

Gartenhaus RB, Berneman ZN, Reitz MS, Gallo RC, Klotman ME. Soluble Tax can stimulate proliferation of primary lymphocytes. Blood 1990;76:468a.

Tschachler E, Gallo RC, Reitz MS Jr. Constitutive expression of lymphotoxin (tumor necrosis factor β) in HTLV-I-infected cell lines. In: Blattner WA, ed. Human retrovirology: HTLV. New York: Raven Press, 1990;105-13.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05690-01 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of the Neutralization Reaction with Antibody Against HIV-1

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

PI:	P. L. Nara	Expert	LTCB NCI
Others:	M. J. Merges	Microbiologist	LTCB NCI
	N. M. Dunlop	Microbiologist	LTCB NCI
	R. C. Gallo	Chief	LTCB NCI

COOPERATING UNITS *(if any)*
Los Alamos National Laboratories, Los Alamos, NM (S. P. Layne, M. Nembo); Dyna Corp, Frederick, MD (S. Conley); National Center for Biotechnology Information, Bethesda, MD (J. L. Spouge); Ortho Diagnostics, Inc., Raritan, NJ (Y. Devash)

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	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.)*

Binding of glycoprotein gp120 to the T cell-surface receptor CD4 is a crucial step in CD4-dependent infection of a target cell by the human immunodeficiency virus (HIV). Earlier work done in our laboratory has revealed the entry process to a complex, prolonged event requiring some type of cooperative receptor-ligand interactions. It is this phase of viral entry which now can be targeted for vaccine design. Blocking some or all gp120 molecules on the viral surface should therefore inhibit infection; however, little is known about the molecular mechanisms. We have now quantitatively examined blocking by soluble CD4 in the hope of gaining insight into the complex process of viral blinding, adsorption and penetration. At low sCD4 concentrations, the inhibition in three HIV strains is proportional to the binding of gp120. For all three viral strains, the biological K(assoc) from infectivity assays is comparable to the chemical K(assoc). The inhibitory action of sCD4 at high concentrations, however, is not fully explained by simple proportionality with the binding to gp120. Positive synergy in blocking of infection occurs after approximately one-half the viral gp120 molecules are occupied, and is identical for all three viral strains, despite the large differences in K(assoc).

Furthermore, it was shown that a 20- to 50-fold difference in blocking activity could be observed for HIV-1 due to the content of the gp120/virion and the target-cell density. Thus, unappreciated variations in HIV stocks and assay conditions may hinder comparisons of blockers from laboratory to laboratory, and the age of HIV challenge stocks may influence studies of drug, vaccine efficacy, and antigenic variation studies. The results also suggest that blocking of viral particles in lymphoid compartments will require very high competitive blocker concentrations, which may explain the refractory outcomes from sCD4-based drug trials in humans.

Project Description

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

P. L. Nara	Expert	LTCB NCI
M. J. Merges	Microbiologist	LTCB NCI
N. M. Dunlop	Microbiologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI

Objectives:

The objectives of this project are:

1. to develop a quantitative infectivity/neutralization assay for cell-free and cell-associated human immunodeficiency virus (HIV)-1 and -2 to identify and map neutralization and antifusion antibody to biologically relevant epitopes;

2. to determine the basic molecular, physical-chemical and cellular requirements for viral infectivity, vaccine and antiviral interventive strategies; and

3. to study the bimolecular kinetic rates, valency and molecular mechanistic details of the neutralization reaction in vitro.

Methods Employed:

The methods employed for all these analyses have been previously described and published by Nara et al. (AIDS Res Hum Retroviruses 3:283, 1987; Nature 331:469, 1988) and Layne et al. (Nature 346:277, 1990; J Virol, In Press). Solid phase antibody K(assoc) and K(disassoc) constants are being measured by plasmon resonance spectroscopy.

Major Findings:

We successfully developed and rigorously tested a quantitative microtiter infectivity assay for use with multiple laboratory strains of HIV-1 and -2 in 1987. This assay was adopted by the World Health Organization in 1989 as one of only two quantitative assays available to the field. Since its publication, the Virus Biology Unit (VBU) has served as a national and international standardizing laboratory and more than 100 investigators have requested and received the cells and protocols. As well, the VBU has trained over 35 groups from the United States and around the world in the assay's application to the acquired immunodeficiency syndrome research.

Using this assay two important and fundamental observations were made which served to renew the field's interest in neutralizing antibody. First, neutralization with HIV-1 infected human and chimpanzee sera was shown for the first time to be biologically relevant in vitro as determined by its kinetic analysis. Second, it was discovered that neutralization of HIV-1 could be mediated in a post-CD4 binding manner. The ability of naturally acquired HIV-1 neutralizing antibody to

prevent cellular infection in this fashion becomes more important when it is understood that the affinity binding constant (K[assoc]) of the viral glycoprotein to the cellular CD4 receptor may be on the order of 10 to 100 times higher than most neutralizing or other binding antibodies. Using polyclonal serum and monoclonal antibodies, this post-CD4 binding neutralization was subsequently mapped to the third variable region (V3) of the envelope viral glycoprotein. The mapping of the neutralizing activity of the post-CD4 binding to the V3 domain suggested three important criteria: 1) that biologically relevant neutralizing antibody is made during HIV-1 infection as opposed to the neutralizing antibody described earlier for visna-maedie virus of sheep; 2) that a "potentially" linear peptide-epitope or domain could be identified; and 3) that the post-CD4 binding neutralization mediated by the V3 domain may imply some biologic or functional role for viral infectivity.

These results indicated that a thorough understanding of the molecular interaction of viral binding and adsorption with the CD4 receptor would be necessary to further characterize bimolecular interactions of antibody and viral envelope glycoprotein. Furthermore, the ability to measure a "relative affinity" of a molecule (such as antibody) directly from an infectivity assay would provide an important means for "classifying" these antibodies as high, moderate or low affinity. These could then be subsequently compared to the results obtained from experimental viral challenges of immunized chimpanzees.

During the past 2 years, numerous quantitative infectivity assays were conducted in our laboratory with the goal of understanding how individual variables influence HIV neutralization assay results. In broad terms, these variables characterize both cellular assay conditions in vitro and the physical state of HIV stocks. Assay analysis was facilitated by a kinetic (mathematical) model of HIV infection designed to analyze the effects of individual variables in the infectivity assays. For all studies, sCD4 was used as the targeted antiviral since it is a well-characterized (competitive) monovalent blocker that interferes with initial viral envelope and cell receptor interactions. This form of soluble cellular receptor permits detailed analysis of the viral-associated gp12-'s relationship to binding, adsorption, and fusion to a target cell. At low sCD4 concentrations, the inhibition in three HIV strains is proportional to the binding of gp120. The biological association constant (gp120-sCD4 K[assoc]) for HIV-2(NIH-Z) is $8.5 \pm 0.5 \times 10^{7}\ M^{-1}$, whereas K(assoc) for HIV-1(HXB3) $1.4 \pm 0.20 \times 10^{9}\ M^{-1}$ and HIV-1(MN) $1.7 \pm 0.1 \times 10^{9}\ M^{-1}$ are 15- to 20-fold larger. For all three viral strains, the biological K(assoc) from infectivity assays is comparable to the chemical K(assoc). The inhibitory action of sCD4 at high concentrations, however, is not fully explained by simple proportionality with the binding to gp120. Positive synergy in blocking infection occurs after approximately one-half of the viral gp120 molecules are occupied and is identical for all three viral strains despite the large differences in K(assoc). Our method of measuring the viral-cell receptor K(assoc) directly from infectivity assays is applicable to immunoglobulins, to other viruses and to assays using primary or transformed cell lines.

So far this novel approach has shown that HIV particles infect at a rate proportional to the number of gp120 molecules (or oligomeric spikes) on their surface. Thus, the results of viral infectivity assays depend on both the

concentration of target cells in the assay and the density of cellular CD4 receptors. Also, adding sCD4 to infectivity assays uncovers new relationships between infection and viral-associated gp120 molecules. When a small fraction of gp120 molecules are blocked by sCD4, infection is proportional to the number of unblocked gp120 molecules. When a large fraction of gp120 molecules are blocked by sCD4, infection is disproportionate to the number of unblocked gp120 molecules. Hence, it appears some small number of gp120 molecules cooperate in infection. This number is small, however, compared to the total number of gp120 molecules that are present on HIV's surface. It appears that the HIV envelope is covered by a highly redundant number of gp120 molecules which act by interacting (metastable) capsid polypeptide subunits and interacting glycoprotein subunits, respectively, which present relatively few critical neutralization sites. When a fraction of these sites are blocked by neutralizing antibodies, a nonlocal transition in subunit orientation is induced by binding of the antibody which inactivates the virus. This property may contribute to the humoral efficacy of vaccines against polio and influenza. Neutralizing HIV seems to be fundamentally different.

The combination of cooperating and redundant gp120 molecules on HIV's surface suggests that the physical state of a virion as well as the target cell density will significantly influence its blocking or inactivation characteristics. To understand the role of these fundamental variables in therapy and immunity, we used the quantitative infectivity assay to study how the blocking activity of a model antiviral agent, such as sCD4 is influenced by increasing $CD4^+$ target cell density and viral stock age. During incubation with 20 nanomolar(nM) sCD4, HIV-1(HXB3) stocks underwent irreversible inactivation. By contrast, inactivation with 2nMsCD4 was almost entirely reversible. At lower sCD4 concentrations ($\leq$2nM) and target cell densities of 6.25×10^4 ml^{-1}, sCD4 blocking activity for HIV-1(HXB3) gave a gp120-sCD4 association constant (K[assoc]) of 1.7×10^9 M^{-1}, which agrees with previous biological and chemical measurements. At the higher density of 1.6×10^7 cells ml^{-1}, the blocking activity was 20-fold smaller. During incubation (37°C) of HIV-1(HXB3) stock (optimized for infectivity by acute harvest), sCD4 blocking activity increased 20-fold during a 3 hour window. Our results show competitive blocking activity depends strongly on target cell density and viral age. The effects of viral age may be due to spontaneous shedding of the viral envelope. Further, the blocking of viral particles in lymphoid compartments will require very high competitive blocker concentrations. This explains, in part, the refractory outcomes from sCD4-based drug trials in humans. Unappreciated variations in HIV stocks and assay conditions will hinder comparisons of blockers from lab to lab, and the age of HIV challenge stocks will influence estimates of vaccine efficacy. Primary field isolates of HIV and other viruses may behave similarly.

Studies using synthetically-derived 25 mer peptides mimicking the CD4 molecule have been used to further dissect the complex regions of the gp120 molecule and CD4 receptor. It appears that more than one CDR combining site is involved in cooperative binding adsorption and fusion. The role of the CDR3 domain identified by our studies implies multiple complex interactions which might serve as immunogenic targets for vaccine design.

Publications:

Layne SP, Merges MJ, Dembo M, Spouge JL, Nara PL. HIV requires multiple gp120 molecules for CD4-mediated infection. Nature 1990;346:277-9.

Layne SP, Merges MJ, Dembo M, Spouge JL, Nara PL. Blocking of human immunodeficiency virus infection depends on cell density and viral stock age. J Virol 1991;65:3293-3300.

Rausch DM, Hwant KM, Padgett M, Volta AH, Engleman E, Gaston I, McGrath M, Fraser B, Kalyanaraman VS, Nara PL, Dunlop N, Martin L, Murphey-Corb M, Lifson JD, Eiden L. Peptides derived from the CDR3-homologous domain of the CD4 molecule are specific inhibitors of HIV-1 and SIV infection, virus-induced cell fusion, and post-infection viral transmission in vitro. Ann NY Acad Sci 1991;616:125-48.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP07148-08 LTCB

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies on T Cell Malignancies, Lymphomas and AIDS

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

PI:	R. C. Gallo	Chief	LTCB NCI
Others:	Z. Berneman	Visiting Fellow	LTCB NCI
	P. Browning	US Public Health Service Officer	LTCB NCI
	Y. Lunardi-Iskandar	Visiting Scientist	LTCB NCI
	S. Nakamura	Visiting Scientist	LTCB NCI
	S. Sakurada	Visiting Scientist	LTCB NCI
	D. Ablashi	Senior Investigator	LCMB NCI

COOPERATING UNITS (if any) NIDR, NIH (P. Klotman, H. Kleinman, B. Weeks, A. Albinini, E. Thompson); FDA-CBER (J. G. Judde); USC, Los Angeles, CA (P. Gill); Harvard Univ. Med. School, Boston, MA (A. Komaroff); Mt. Sinai School of Med., NY, NY (I. Brus); Univ. of Kansas, Kansas City, KS (N. Balachandran)

LAB/BRANCH: Laboratory of Tumor Cell Biology

SECTION: Hematopoietic Cellular Control Mechanisms Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.3	3.8	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.)

With the in vitro, in ovo and in vivo models for acquired immunodeficiency syndrome-Kaposi's sarcoma (AIDS-KS), developed in our laboratory, we have been able to demonstrate that: 1) the growth of AIDS-KS-derived cells is enhanced by corticosteroids; 2) AIDS-KS cells secrete factors, which not only induce angiogenesis, but also increase vascular permeability; 3) AIDS-KS cells produce interleukin (IL)-6 and IL-8; 4) AIDS-KS cells have high affinity receptors for IL-1, IL-2, IL-6, platelet-derived growth factor, basic fibroblast growth factor, tumor necrosis factor α, hydrocortisone and the human immunodeficiency virus type 1 Tat protein. We have purified and begun the sequencing of the activated T-cell-derived 30 kilodalton protein, which is a very potent stimulator of KS cell growth. Different drugs have been tested for their effect on KS cell growth in vitro and induction of KS-like lesions in nude mice. So far, one compound (SP-PG, a bacterial cell wall peptidoglycan), has shown the highest specificity and the lowest toxicity, while inhibiting KS growth and development. This drug is therefore a potential candidate for clinical trials in the treatment of AIDS-KS.

The study of T-cell colony formation in methylcellulose of peripheral blood mononuclear cells from HTLV-I-infected individuals, strongly suggests that the T-lymphocyte colony-forming cells are infected by HTLV-I and may be an important reservoir for that retrovirus.

We have isolated a herpesvirus, which is similar to the recently described human herpesvirus 7. Our findings confirm the existence of a herpesvirus, distantly related to, but significantly different from human herpesvirus 6.

Project Description

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

R. C. Gallo	Chief	LTCB NCI
Z. Berneman	Visiting Fellow	LTCB NCI
P. Browning	US Public Health Service Officer	LTCB NCI
Y. Lunardi-Iskandar	Visiting Scientist	LTCB NCI
S. Nakamura	Visiting Scientist	LTCB NCI
S. Sakurada	Visiting Scientist	LTCB NCI
D. Ablashi	Senior Investigator	LCMB NCI

Objectives:

1. One of the major goals of this laboratory is to study the cell biology of tumors in general, and of tumors associated with viruses in particular. We have mainly focused on two areas related to retroviral infection. The first deals with Kaposi's sarcoma (KS), a highly vascularized tumor occurring with a high frequency in acquired immunodeficiency syndrome (AIDS) patients. Our aim is to understand the pathogenesis of KS in AIDS patients. This could lead to the development of specific, nontoxic therapeutic approaches for the treatment of KS. Information accumulated on KS should provide valuable insights into angiogenesis in general which may also be helpful in understanding the role of angiogenesis in other diseases, like metastatic cancer and diabetic retinopathy.

As outlined in our previous annual reports, we have developed a culture system to grow what we believe are the relevant cells in KS, namely the KS spindle cells. Those cells are not infected by human immunodeficiency virus (HIV), but they can induce biological actions which can explain the features of KS in vivo. Our studies emphasize the importance of growth factor-mediated mechanisms, with autocrine and paracrine effects, in the development of AIDS-KS lesions. These observations suggest that AIDS-KS could be a reactive and possibly controllable lesion. The information gathered about the cytokine dysregulation in KS are also likely to shed some light on the many clinical disorders which result from abnormal cytokine regulation.

The other retroviral model which received attention is human T lymphotropic virus type I (HTLV-I), which is the etiological agent of adult T-cell leukemia/lymphoma (ATL) and a demyelinating neurological disorder, HTLV-I-associated myelopathy/ tropical spastic paraparesis (HAM/TSP). Our efforts currently are directed to understand how HTLV-I infection affects the T-lymphocytes with a self-renewal capacity, the T-lymphocyte colony-forming cells (T-CFC).

2. Another goal of this laboratory is to look for new viruses, and to investigate the viral etiology of disease. These efforts recently led to the isolation of a new herpesvirus isolate, which turned out to be similar to human herpesvirus 7 (HHV-7), very recently reported by another laboratory.

Methods Employed:

Cell cultures were carried out in suspension and in methylcellulose. Viral expression was monitored by diverse assays such as immunofluorescence (IF), electron microscopy, and reverse transcriptase (RT) activity. Molecular studies were performed using Southern and Northern blot hybridization, and RT-polymerase chain reaction amplifications. Receptor studies were carried out by ligand binding and internalization assays, cross-linking, Scatchard plot analysis and proliferation studies. Growth factors and cytokines were also assayed by enzyme linked-immunosorbent assay techniques. Growth factor purification was carried out by chromatographic methods. In vivo studies were performed in nude mice and hairless guinea pigs; in ovo studies were performed in the chicken chorionic allantois membrane (CAM) assay.

Major Findings:

1. KS in AIDS

Identification of the Cellular Origin of AIDS-KS Cells

The purpose was to define the spindle cells of KS and attempt to define their normal progenitor cells. Recently, in collaboration with Dr. Judah Folkman (Harvard Medical School, Boston, MA) we found that our cultured AIDS-KS cells reacted with a vascular smooth muscle α actin (SMC-actin) specific antibody. These cells also expressed SMC-actin messenger ribonucleic acid (mRNA). Furthermore, these AIDS-KS cells appeared, by transmission electron microscopy, morphologically similar to a fetal-type of vascular smooth muscle cell. In addition, in collaborative studies with Dr. Gill (University of Southern California, Los Angeles, CA), we found that spindle cells derived directly from KS lesions were also positive for SMC-actin. Thus, the AIDS-KS spindle-like cells have properties of primitive vascular cells, probably more closely related to smooth muscle cells.

Study of Angiogenesis Induced by AIDS-KS Cells In Vivo

As reported earlier (Science 1988;242:430-433), cultured or primary fresh AIDS-KS cells transplanted subcutaneously into the back of athymic nude mice, induce angiogenesis and cause the development of KS-like lesions on day 5 post-transplantation. Since then, we have been able to extend the model to another animal: when AIDS-KS cells were transplanted into hairless guinea pigs, they also induce a similar highly vascularized lesion.

Effect of Glucocorticoids on the Growth of AIDS-KS Cells

Utilizing our KS tissue culture system, we investigated the effects of corticosteroids and other hormones on the growth of AIDS-KS cells and their interaction with specific growth factors. The in vitro growth of these cells was significantly enhanced by several glucocorticoids, viz., hydrocortisone and dexamethasone, an effect that was inhibited by the addition of a steroid antagonist, U486. By comparison, the mineralocorticoid aldosterone had little or no effect. In contrast to their effect on AIDS-KS cells, these glucocorticoids

did not augment the growth of normal vascular endothelial cells, fibroblasts, or aortic smooth muscle cells either alone or when used as a supplement to appropriate growth factors. The results suggest the existence of a specific interaction between certain corticosteroids and factors involved in the regulation of AIDS-KS cell growth and they provide a basis for more detailed studies of the mechanisms involved in corticosteroid-associated induction or promotion of KS lesion development in man. They also emphasize the need for judicious use of corticostreoids in patients at risk for developing KS.

Induction of Vascular Permeability by AIDS-KS Cells

Among other clinical symptoms, edema, pleural effusions, and continuous diarrhea are frequently observed in AIDS-associated KS patients. The mechanism(s) responsible for these changes is not clear, although it has been suggested that these are due to a simple mechanical obstruction of the lymphatic system capillaries. However, we have noted that long-term cultured AIDS-KS cells can induce a biphasic vascular hyperpermeability response in athymic nude mice. In vivo studies to evaluate the factor(s) produced by AIDS-KS cells which induces a vascular permeability response were performed in nude mice and hairless guinea pigs. To assay vascular permeability, the dye (Evans blue, 100 μl of a 5 mg/ml solution) was injected intravenously (IV) at various intervals after an initial subcutaneous or intradermal injection of AIDS-KS cells or serum-free AIDS-KS conditioned medium (CM). Fifteen minutes after injecting the dye (IV for mice, or intracardial for hairless guinea pigs), dye was found to be exuded into sites of AIDS-KS cells or AIDS-KS CM. The nonspecific early phase of this response reached its maximum at 30 minutes post-inoculation of KS cells and an AIDS-KS cell-related late phase response was observed 12 hours later. The early phase was completely inhibited by the histamine antagonist triprolidine. In contrast, the late phase was resistant to triprolidine and also to treatment with indomethacin. It was, however, inhibited by treatment with a high dose of dexamethasone (10 mg/kg). Cell-free CM-derived from AIDS-KS cell cultures also induced a vascular permeability response in a biphasic but accelerated manner, i.e., the histamine-dependent, nonspecific response peaked 15 minutes post-inoculation and the histamine-independent response, 1 hour post-inoculation. The response induced by AIDS-KS cell CM was not reduced by prophylactic treatment. The activity in the late phase was nondializable and stable for at least 3 weeks at 4°C. A similar vascular permeability response was also induced by the inoculation of freshly isolated primary AIDS-KS cells cultured for a short time without any additional growth factors. These findings indicate that AIDS-KS cells have been activated in the lesion and that such a phenotype has been maintained during growth in culture. These soluble mediators are, therefore, likely to be involved in the regulation of the edematous response in AIDS-KS lesion development. Preliminary molecular evidence suggests that vascular permeability factor (VPF) is expressed by cultured AIDS-KS cells, and that this factor may be the prime candidate for the induction of increased vascular permeability by AIDS-KS cells.

Characterization and Purification of Growth Factor(s) Produced by Cultured AIDS-KS Cells

Early studies have shown that the CM of cultured AIDS-KS cells has some autocrine effect on the growth of AIDS-KS cells. This CM contained a basic fibroblast

growth factor (bFGF) activity, interleukin (IL)-1 activity, and large amounts of IL-6. We also observed that exogenously added IL-1 stimulated the growth of AIDS-KS cells and that anti-IL-1 antibody as well as anti-bFGF partially inhibited the growth stimulatory activity found in AIDS-KS CM. While exogenously added IL-6 did not augment the growth of AIDS-KS cells, addition of polyclonal antibody to IL-6 or antisense oligonucleotides reorganizing IL-6 sequences to AIDS-KS cell culture inhibits the growth of AIDS-KS cells. Also, IL-6 was found to be expressed at much higher levels in primary KS lesions than in adjacent skin tissues. Interestingly, glucocorticoids and the male sex hormone testosterone markedly enhanced IL-6 mRNA expression and IL-6 protein synthesis in these KS-derived cells, while estrogen downregulated their expression. These data suggest that IL-6 is another cytokine involved as an autocrine mechanism affecting the growth of AIDS-KS cells and possibly in the maintenance of the lesion in patients. These results provide a basis for future studies into the pathogenesis of KS and its predilection for the male sex hormone. The cultured AIDS-KS cells have also been found to express mRNA for IL-8 and to secrete that interleukin in their CM. The expression of IL-8, which is a chemotactic factor, may contribute to the pathogenesis of AIDS-KS.

Cytokine Receptors on the Membrane of AIDS-KS Cells

By IF, cytokine receptors were detected for IL-2, IL-6, platelet-derived growth factor (PDGF), bFGF, IL-1, and tumor necrosis factor (TNF)-α on the surface of AIDS-KS cells; cytoplasmic receptors were also found for hydrocortisone. Ligand binding assays revealed the presence of high affinity receptors for IL-6, IL-2, IL-1-α, TNF-α, bFGF, PDGF and hydrocortisone. We analyzed whether specific high-affinity cytokine receptor stimulation could induce the proliferation of AIDS-KS-derived cells. Our results indicate that the KS cells grow in the presence 100-1000 pM of recombinant IL-2 or 1 micromolar of hydrocortisone, but not in the presence of exogenously added recombinant IL-6. Our hypothesis is that IL-6 receptors on the AIDS-KS cells may already be saturated by their own endogenously produced IL-6. The IL-6 receptors on AIDS-KS cells were also found to be upregulated by treatment with hydrocortisone. Interestingly, a 69 kilodalton (kD) form of the IL-6 receptor was detected on AIDS-KS cells, which differs from the 80 kD form previously found on hepatoma cells. The IL-2 receptors on AIDS-KS cells and on endothelial cells are functional receptors, which could be involved in the immune responses generated by primary HIV-1 infection. The activation of IL-2 receptors in AIDS-KS-derived cells may be one factor responsible for growth and invasiveness of KS in vivo. Our findings raise the possibility that IL-2 is a growth factor for AIDS-KS-derived cells, and they support our model linking the development of AIDS-KS with immune stimulation.

Tat Receptors on AIDS-KS Cells

Using cross-linking and Scatchard analysis, we have been able to demonstrate the presence of specific, high-affinity receptors for a peptide from the HIV-1 transactivator Tat protein. Those receptors could be saturated using an excess of unlabelled Tat peptide. We also could demonstrate that the Tat peptide, used in the Scatchard analysis, induced proliferation of the cultured AIDS-KS cells. These receptors are likely to mediate the proliferation of the AIDS-KS cells induced by the Tat protein.

Effect of Oncostatin M on the Growth of AIDS-KS Cells

Oncostatin M, originally identified, purified, and sequenced by Oncogen (Seattle, WA) was reported as a unique cytokine. It was shown to be produced by normal T cells, macrophages, and various T or macrophage cell lines. Like other cytokines, oncostatin M was found to inhibit the growth of various tumor cell lines, e.g., melanoma (hence its name), and stimulated the growth of fibroblasts to some degree. A comparison of this molecule with partially purified AIDS-KS growth factor from HTLV-II-transformed T cells also suggested similarities in its size (molecular weight of 30 kD) and in some biochemical/biological properties. Like AIDS-KS growth activity found in virally infected activated $CD4^+$ T cell cultures, the activity was more potent than that observed with other cytokines tested, e.g., IL-1 and TNF-α. Further collaborative studies are ongoing.

Purification and Characterization of the 30 kD Potent KS Spindle Cell Growth Factor Present in CM of Activated $CD4^+$ T Cells and in CM from HTLV-immortalized T Cells

The goal of this study is to isolate and purify a factor that supports the long-term growth of AIDS-KS cells from human activated $CD4^+$ T cells (mitogen-stimulated normal peripheral blood mononuclear leukocytes as well as retrovirus-infected/transformed cells). This factor will then be thoroughly studied in order to determine its chemical and biological characteristics and will be used for the development of specific immunologic reagents. Purification was carried out with our contract collaborators from Advanced BioScience Laboratories (Kensington, MD).

We have used the culture media of these cells grown in serum-free conditions as a source of factor for purification. During purification, the biological activity was monitored using AIDS-KS cells in a biological assay. Large volumes of CM were concentrated by ultrafiltration. The concentrated material was then subjected to ion exchange chromatography on DEAE columns. This step effectively removed albumin, a major constituent of medium proteins. Subsequent chromatography on CM-Sepharose substantially increased the specific activity with a recovery of up to 85% of the initial activity. The material obtained after CM-chromatography was further purified using acid/organic solvent conditions previously used for purification of IL-2. After acidification, the factor retained approximately 55% of its activity. The active material was eluted with a linear gradient of acetonitrile. The pooled active fractions consisted mainly of three silver staining bands on SDS-PAGE. Bioassays of material eluted from slices of the SDS-PAGE experiment indicated that the growth activity was associated with only one silver staining band which corresponded to a molecular weight of approximately 30,000 daltons. Incubation of AIDS-KS cells with ^{125}I-labeled material revealed that the labeled protein that bound to the cells had a molecular weight of approximately 30,000 daltons and was effectively competed by the addition of an excess of unlabeled factor to the binding reaction. This observation indicated that the AIDS-KS cells have specific receptors for the T-cell-derived growth factor. A series of experiments were performed to determine the ideal conditions for the purification of the factor to homogeneity. Immobilized metal ion affinity chromatography (IMAC), a specific adsorption method for the purification of a variety of proteins was used to purify the growth factor. The ^{125}I-labeled material (pooled active fractions after rechromatography

on HPLC) was loaded onto a column of immobilized $Ni2^{+}$ on Sepharose and the factor was eluted from the column with a decreasing pH gradient. The fractions were analyzed by SDS-PAGE. The unlabeled factor was chromatographed separately under the same conditions in order to determine the fractions with growth activity. The 30,000 dalton protein was effectively separated from the contaminants by this method and the growth activity corresponded to this protein.

The protein has now been purified to homogeneity and the amino acid sequence is being determined. Purified protein will also be used in the future for molecular characterization, to study its mechanism of action, and to perform experiments designed to delineate its effect(s) on the development of KS. If the protein is novel, oligonucleotides based on its sequence will be used to clone its gene and its mRNA. This will allow the large scale production of the 30 kD factor by recombinant techniques. Experiments to prepare specific antibodies to the 30 kD factor are also in progress and once available these will be used to facilitate factor purification and detection and quantification of the levels of growth factor in sera from various disease states, possibly yielding insights into disease association and involvement in pathogenesis. The distribution of growth factor in tissues will also be determined by immunohistochemical localization.

Adhesion Molecules on AIDS-KS Cells

To date many of our studies of KS have concentrated on soluble mediators. However, cell-cell interactions and recognition of cells and factors will most likely also play an important role in the development of KS lesions. Factors stimulating endothelial cell growth and migration, relationships between endothelial and vascular smooth muscle cells (including AIDS-KS cells), processes which lead to edema, and interactions involving extravasated erythrocytes and the accumulation of inflammatory cells, such as macrophages and lymphocytes, are all related to the process of KS development. Adhesion molecules are known to have important regulatory effects on various cell functions and interactions, and cultured AIDS-KS cells as well as fresh cells associated with KS lesions were found to express the intercellular adhesion molecule-1. An analysis of the relationship between adhesion molecules and soluble mediators in KS lesions should lead to a better understanding of KS development. Studies of these processes are being carried out in collaboration with Dr. Weeks and Dr. Klotman (National Institute of Dental Research).

Study of Endothelial Cell Chemotaxis and Chemoinvasion Inducing Activity Produced by AIDS-KS Cells

A coated basement membrane barrier assay performed in a Boyden chamber was used to study AIDS-KS cell migration. Also, CM from AIDS-KS cells was found to induce the invasiveness of normal endothelial cells. While classical bFGF can also induce such invasiveness, the major invasiveness-inducing activity in AIDS-KS CM was not inhibited by antibody to bFGF. Specific inhibitors of collagenase IV, however, were found to block the induction of endothelial cell invasiveness by AIDS-KS CM. It is possible that this in vitro phenomenon represents an early event of angiogenesis occurring in vivo. The secretion of a potent inducer(s) of endothelial cell invasiveness by AIDS-KS cells could, therefore, be related to the

angiogenic process induced by AIDS-KS cells. This project was carried out in a collaboration with Dr. Albini and Dr. Thompson (National Institute of Dental Research).

Induction of Nerve Cell Degeneration by AIDS-KS Cells

A study of biological activities expressed by cultured AIDS-KS cells is part of a continuing collaborative project with Drs. B. Weeks and H. Kleinman (National Institute of Dental Research). One activity found in serum-free AIDS-KS culture supernatant fluids induced degenerative morphological changes in the neuronal cell line PC12. Specifically, AIDS-KS-CM induced the formation of hair-like cytoplasmic degenerative processes in a dose-dependent manner. Further analysis of this phenomenon is ongoing.

Culture of a Factor-independent AIDS-KS Cell Isolate

Until now, all the different AIDS-KS-derived cell cultures were dependent on activated T-cell CM for their long-term growth. However, recently a tetraploid mesenchymal cell population was cultured from the pleural effusion of an AIDS-KS patient. Those cells grow very well without the addition of any exogenous growth factor. Yet they show the same phenotype as the other AIDS-KS cell isolates: they are smooth muscle actin-positive and induce angiogenic lesions in nude mice. This cell population is being studied intensively for the following characteristics: long-term tumorigenesis in nude mice; reason for the apparent growth factor independence; relevance for AIDS-KS in general (is it an isolated case or does it occur more frequently than realized until now?). The abnormal karyotype of the cells will also prompt us to assess whether KS is a polyclonal or a monoclonal proliferation.

Testing of Therapeutic Agents for AIDS-KS

Several drugs and compounds have been evaluated for their effect on KS-cell induced biological function by our in vitro, in ovo and in vivo model systems. Compounds tested for effects on KS cell growth or other biological activities include: suramin, recombinant interferon-α (rIFN-α), pentosan polysulfate (SP-54), and SP-PG (a naturally occurring bacterial cell wall polysaccharide peptidoglycan product). Suramin and rIFN-α were found to inhibit the growth of KS cells; however, their effects on target cells did not appear to be entirely specific. Suramin at a concentration of 300 μg/ml was cytotoxic to cultured cells. Pentosan polysulfate (at concentrations less than 100 μg/ml) was target specific as it inhibited the growth of AIDS-KS cells and human endothelial cells. SP-PG was also target specific, more effective on the growth of AIDS-KS cells and endothelial cells, while having no effect on fibroblasts. Its inhibitory effect occurred at concentrations of 3 μg/ml and 25 μg/ml for AIDS-KS cells and endothelial cells, respectively. Suramin (5 mg/mouse) or pentosan polysulfate (2 mg/mouse), injected into nude mice, did not inhibit the late phase vascular permeability response induced by AIDS-KS cells, unlike the in vitro effect seen with pentosan polysulfate. In contrast, a high dose of rIFN-α (10,000 U/mouse) inhibited the vascular permeability response to some degree. However, SP-PG (5 mg/mouse) completely inhibited the vascular permeability response induced by KS cells. This inhibitory effect was further stabilized in combination with

tetrahydrocortisone, which Dr. Folkman and his colleagues have shown as augmenting the potency of several angiogenesis inhibitors.

It was difficult to evaluate the effect of suramin on angiogenesis in mice since treatment with suramin was found to be very toxic, causing death in three of five mice inoculated. rIFN-α had only limited inhibitory effect on the angiogenesis in mouse models. SP-PG, however, was again found to be very promising as it exhibited a strong and complete inhibition of angiogenesis associated with inoculation of KS cells in nude mice. Following treatment with nontoxic levels of SP-PG, newly formed vasculature rapidly degenerated and spindle-shaped cells were not observed. In addition, SP-PG also inhibited the angiogenesis induced by AIDS-KS cells in the CAM assay. This effect in ovo was augmented by the combination of tetrahydrocortisone or hydrocortisone. SP-PG is, therefore, a potential candidate for clinical trials in the treatment of AIDS-KS. Experiments with SP-PG have been performed under a confidentiality agreement with Daiichi Pharmaceutical Co., Ltd., Japan. Saporin, a ribosome inhibitory protein which has a potent inhibitory effect on protein synthesis, and bFGF, tested independently or as a saforin-bFGF conjugate (also called mitotoxin), were also tested for an effect on the in vitro growth of AIDS-KS cells. While bFGF, and saporin alone, had only limited effect on the growth of AIDS-KS cells, the conjugate, mitotoxin, at a concentration of 10 nM completely inhibited AIDS-KS cell growth. In contrast to the conjugated form, a simple mixture of the two compounds required 100 nM for inhibition. While promising results were obtained, the effective dose range for inhibition was very narrow, 1 nM through 10 nM. Also, the use of mitotoxin in vivo may be complicated by the fact that many types of cells have receptors for bFGF. Further experiments are in progress to test inhibition in in ovo and in vivo systems. The drug testing program is now being expanded using the model systems described above. We will test many other agents, whose final effect will be an inhibition of angiogenesis.

2. Human T-lymphotropic Virus Type-I (HTLV-I) and T-cell Biology T-CFC and HTLV-I

The generation of mature lymphocytes can be studied in vitro by analyzing T-cell colony formation in methylcellulose. This assay was applied to the peripheral blood mononuclear cells (PBMCs) of patients infected with HTLV-I (healthy seropositive individuals, and patients with a disease associated with HTLV-I; namely, HAM/TSP and ATL). We were prompted to investigate T-cell colony formation in those patients, since a spontaneous lymphocyte proliferation was described in HAM/TSP patients and healthy HTLV-I carriers. We found, that in contrast to normal uninfected individuals, PBMCs from HTLV-I-infected individuals contain T-CFC with abnormal proliferative properties, since some of them grew spontaneously, in the absence of mitogen and IL-2 stimulation. The T-CFC from HTLV-I-infected individuals also show an abnormal differentiation in vitro, as evidenced by the $CD1^+$ T-cells in the T-lymphocyte colonies generated in vitro. The T-CFC from infected individuals also seem to be infected, as shown by viral expression in the T-cell colonies. The very rapid appearance of HTLV-I-expressing cells following cultivation in vitro is in contrast to previous studies, where liquid cultures of HTLV-I-infected cells only led to a gradual and slow selection of cells producing HTLV-I. Our interpretation is that the methylcellulose assay may select the target cell for infection, resulting in a rapid expression of

HTLV-I. In this option, we believe that the T-CFC may be the target for infection by HTLV-I. Another interesting observation is that the HTLV-I transactivator Tax protein can stimulate the growth of the T-cell colonies generated in methylcellulose from normal or HTLV-I-infected PBMCs.

Control of Normal (Uninfected) T-lymphocyte Survival

A part of the lymphocytes are long-lived in vivo without undergoing proliferation for months or years. It is largely unknown how T-cell survival is regulated. Practically, the whole current knowledge of T-lymphocyte biology revolves around activated and proliferating cells. When T-cells are cultured in suspension without exogenous growth factors, they rapidly decline. Recently, the persistence for at least 2 months of mature B- and T-lymphocytes and plasma cells was demonstrated in the adherent layer of human long-term bone marrow cultures (HLTBMCs), in the absence of exogenously added growth factors. This long-term persistence is due to survival rather than to proliferation or generation from the hemopoietic stem cell. This long-term survival is stroma-associated, since it does not occur in unstimulated suspension cultures. It probably requires a close cell-cell and/or cell-extracellular matrix interaction. Recent experiments have shown that the surviving T-cells retain their self-renewal capacity, as evidenced from the T-CFC assay. T-lymphocytes taken out of HLTBMCs are in a resting configuration, since they do not have activation markers and since they are inactive in cytotoxicity assays. However, when they are stimulated, they regain their functional properties. The long-term survival of T-lymphocytes in HLTBMCs makes it possible for the first time to study this lymphocyte feature in vitro, without tying it to T-cell proliferation. Ultimately, our goal is to find out which factor(s) sustains the survival of the lymphoid cells in vitro and in vivo.

3. HHV-7

Part of the continuing efforts of this laboratory has been directed toward trying to isolate new viruses and to investigate the viral etiology of identifying the diseases of the hematopoietic cells, including lymphomas, leukemias, and immunodeficiency. Out of one PBMC culture from a patient with chronic fatigue syndrome (CFS) (sent to us by Dr. Brus, Mt. Sinai School of Medicine, New York, NY), a herpesvirus was isolated which seemed to be distantly related to, but significantly different from HHV-6. The difference between the new isolate and HHV-6 was apparent from immunological, molecular and biological studies. In a large survey of human sera, some did not react against the new isolate, while they disclosed a positivity against HHV-6. Using monoclonal antibodies to HHV-6 protein, only two out of nine reacted with cells infected with the new isolate. Molecular probes recognizing a number of human and simian herpesviruses were tested by Southern blot hybridization. While those probes identified their respective positive controls, they did not hybridize with the DNA of the new isolate. However, a probe recognizing a repetitive sequence of both Marek's disease virus (a herpesvirus infecting chickens and inducing lymphoma) and HHV-6 recognized some sequences in the new isolate. In order to evaluate the relationship to HHV-6, 13 probes recognizing HHV-6 sequences were tested on DNA from the new isolate. Together, these plasmid probes represent approximately 87.7 kilobases, i.e., 51.6% of the HHV-6 genome. Only two of these HHV-6-specific probes hybridized to the DNA of the new isolate. This represents a homology of,

at most, 31.5% with HHV-6. Like HHV-6, infection with the new herpesvirus isolate is associated with the appearance of giant cells, which ultimately lyse. Double IF, utilizing a combination of specific cell membrane markers and cytoplasmic/nuclear staining with patient's serum, demonstrated that the infected cells are T-lymphocytes--they are $CD2^+$, $CD3^+$, $CD7^+$, $CD4^+$, and rarely $CD8^+$. The appearance of large cells occurs later when the cells are infected cell-free with the new isolate as compared to the infection by HHV-6 (generally 9-12 days for the new isolate, 5-7 days for HHV-6). A molecular comparison between the new isolate and the recently described HHV-7 (Frenkel et al. PNAS 1990;87:748-752) showed that our virus is similar to HHV-7. Our isolate is thus the first HHV-7 isolate obtained independently of the laboratory from which it was first reported. We are currently carrying out a large seroepidemiological study to determine the distribution of the virus in the general population. An initial study, performed in collaboration with Dr. Komaroff (Harvard University, Boston, MA) comparing the IgG antibody titers to HHV-7 between normal controls and patients with CFS (47 sera in all), did not reveal a statistically significant difference between the two populations, when a two-tailed t-test was used. We are also currently studying the tropism of HHV-7 in vitro.

4. Mycosis Fungoides

Further Characterization of a Monocytoid Cell Line from a Patient with Mycosis Fungoides

Previously Dr. Philip Browning described a monocytoid cell line isolated from a patient with mycosis fungoides named PB338. This cell line had evidence of RT activity in the culture supernatant. We have not been able to isolate a new retrovirus from this cell line. The cell line does respond to the recently purified 30kd protein which is important for the KS spindle cell growth. We feel that the use of this factor to isolate similar cell lines from such patients will be useful. We are looking to identify a viral etiology for this disease.

Publications:

Berneman ZN, Ablashi DV, Salahuddin SZ. Isolation of a potentially new herpesvirus from a patient with chronic fatigue syndrome. AIDS Res Hum Retroviruses 1991;7:236.

Browning PJ, Trepel J, Fletcher M, Sechler J, Salahuddin SZ, Gallo RC. Establishment of continuously growing myelomonocytic cells from a patient with mycosis fungoides with reverse transcriptase activity. AIDS Res Hum Retroviruses 1991;7:224.

Browning PJ, Trepel JB, Sechler JM, Eger-Fletcher M, Salahuddin SZ, Gallo RC. Establishment of continuously growing monocytoid cells from a patient with mycosis fungoides. Blood 1990;76:344a.

Gartenhaus RB, Berneman Z, Gallo RC, Klotman ME. Soluble tax can stimulate proliferation of primary lymphocytes. Blood 1990;76:486a.

Lunardi-Iskandar Y, Nakamura S, Gill P, Sakurada S, Gallo RC, Salahuddin SZ. Cytokine and cytokine receptor expression in AIDS Kaposi's sarcoma-derived cells. AIDS Res Hum Retroviruses 1991;7:219.

Lunardi-Iskandar Y, Nakamura S, Gill P, Sakurada S, Salahuddin SZ, Gallo RC. Structure and function of interleukin 2-receptors (IL-2R) on spindle cells of AIDS-associated Kaposi's sarcoma (AIDS-KS). J Cell Biochem 1991;15F:133.

Nakamura S, Sakurada S, Gill P, Lunardi-Iskandar Y, Gallo RC, Salahuddin SZ. Glucocorticoid enhanced in vitro growth of AIDS-KS associated Kaposi's sarcoma-derived cells. AIDS Res Hum Retroviruses 1991;7:216.

Sakurada S, Nakamura S, Gallo RC, Salahuddin SZ. Cultured Kaposi's sarcoma cells express activity inducing vascular permeability. AIDS Res Hum Retroviruses 1991;7:217.

Sakurada S, Nakamura S, Salahuddin SZ, Gallo RC. Long-term cultured and freshly isolated Kaposi's sarcoma cells express activity inducing vascular permeability. J Cell Biochem 1991;15:254.

Sechler JM, Browning PJ, Gallo RC, Nakamura S. Spindle-like cells derived from the blood of patients with AIDS related Kaposi's sarcoma. Blood 1990;76:372a.

Thompson EW, Nakamura S, Shima TB, Melchiori A, Martin GR, Salahuddin SZ, Gallo RC, Albini A. Supernatants of acquired immunodeficiency syndrome-related Kaposi's sarcoma cell induce endothelial cell chemotaxis and invasiveness. Cancer Res (In Press).

Weich HA, Salahuddin SZ, Nakamura S, Gallo RC, Folkman J. AIDS-Kaposi's sarcoma-derived cells in long-term culture express and synthesize smooth muscle a-actin. Am J Pathol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP07149-08 LTCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Biological Studies on Human Pathogenic Viruses

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

PI:	M. S. Reitz	Senior Staff Scientist	LTCB NCI
Others:	M. Klotman	US Public Health Service Officer	LTCB NCI
	J. Lisziewicz	Visiting Associate	LTCB NCI
	M. Thomson	Guest Researcher	LTCB NCI
	J. Smythe	Postdoctoral Fellow	LTCB NCI
	F. Lori	Guest Researcher	LTCB NCI
	A. Cara	Visiting Fellow	LTCB NCI
	R. Sadaie	Senior Staff Fellow	LTCB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Molecular Genetics of Hematopoietic Cells Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
11.0	8.0	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 major efforts of our group at present are directed at studies on the molecular level into the life cycle of the human immunodeficiency viruses (HIV), especially type 1. The following areas are addressed: (1) analyses of the viral regulatory genes, especially tat and rev, their interactions with cellular factors which influence their activity, and their effects on expression of cellular genes; (2) studies on the regulation of viral RNA polymerase promoters in different cell types; (3) studies on the regulation of viral gene expression at the level of RNA splicing in different cell types, in different diseases and their stages of progression, and under the influence of extrinsic factors such as cytokines; (4) correlation of proviral structures and integration status with viral gene expression; and (5) development of antiviral treatments based on the above studies.

Project Description

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

M. S. Reitz	Senior Staff Scientist	LTCB NCI
M. Klotman	US Public Health Service Officer	LTCB NCI
J. Lisziewicz	Visiting Associate	LTCB NCI
M. Thomsen	Guest Researcher	LTCB NCI
J. Smythe	Postdoctoral Fellow	LTCB NCI
F. Lori	Guest Researcher	LTCB NCI
A. Cara	Visiting Fellow	LTCB NCI
R. Sadaie	Senior Staff Fellow	LTCB NCI
Z. Berneman	Visiting Fellow	LTCB NCI
P. Browning	US Public Health Service Officer	LTCB NCI
R. Gartenhaus	Senior Staff Fellow	LTCB NCI
M. Robert-Guroff	Research Biologist	LTCB NCI
S. Colombini	Biotechnology Fellow	LTCB NCI
F. Michaels	Staff Fellow	LTCB NCI
R. Gallo	Chief	LTCB NCI
C. Gurgo	Biotechnology Training Program Fellow	LTCB NCI

Objectives:

The major objectives of this project are directed towards studies on the life cycle of human immunodeficiency virus type 1 (HIV-1). This includes studies of the following: (a) viral regulatory genes, including tat and rev, and factors which may modulate their activity; (b) activity of viral transcriptional promoters in different cell types and how their level of activity is regulated; (c) regulation of gene expression as manifested by ribonucleic acid (RNA) splicing patterns, both in vitro and in vivo; (d) the relationship of proviral structure and integration status and biological activity; and (e) possible antiviral treatments directed against any of the above.

Methods Employed:

Standard methods of molecular and cell biology, virology, and immunology are used in these studies. These include Southern and Northern blotting, restriction endonuclease mapping, polymerase chain reaction (PCR) amplification of DNA and RNA, gene cloning and DNA sequencing, site directed mutagenesis, oligonucleotide gel shift assays, transfection, radioimmunoprecipitation assays, and tissue culture.

Major Findings:

Gene Therapy in HIV-1

One goal is to develop recombinant DNA constructs for gene therapy (intracellular immunization) to protect human cells from HIV-1 infraction in individuals who are already infected. Work in this and other laboratories has shown that activation

of the viral promoter by the tat gene product is essential for virus replication, and a stretch of 44 nucleotides immediately downstream from the RNA initiation site, called the tat activation response (TAR) element, provides the essential regulatory target for tat-mediated activity.

We have concentrated our efforts on using overproduction of this region by cloning multimerized TAR elements under the control of a modified HIV-1 long terminal repeat (LTR) lacking the negative regulatory element of the wild-type LTR. This construct has the advantage of being expressed only in the presence of Tat protein; thus, uninfected cells should not express it.

The effect of multimerized TAR elements on HIV-LTR directed gene expression was measured by cotransfection with a reporter gene linked to the HIV-1 LTR and various tat expression vectors. We showed that under these conditions, multiple TAR elements clearly inhibited the HIV-1 LTR. The degree of inhibition was roughly proportional to the number of tandem TAR elements in the construct up to a number of 25, which decreased expression by 94%. Addition of further TAR elements did not have a further inhibitory effect on expression, possibly because inhibition was already 100% in cells successfully transfected with all three plasmids.

HIV-1 is largely an infection of blood cells, which are derived from hematopoietic stem cells. It could be possible to introduce the protective gene using high efficiency retrovirus-mediated gene transfer either into circulating T cells and monocytes/macrophages or directly into stem cells. We have inserted an LTR-50TAR into the 3′ LTR of a double copy (DC) retroviral vector (a generous gift from Dr. Eli Gilboa, Memorial Sloan Kettering Cancer Center, New York, NY) and the resultant plasmid was called DC-LTR-50TAR. DC-LTR-50TAR was converted into corresponding virus by transfection into the F2 murine ecotropic retroviral packaging line, followed by infection of the PA317 cell line by the resultant ecotropic retrovirus. PA317 is a murine amphotropic retroviral packaging line. G418 resistance colonies were isolated, expanded to cell lines and tested for virus production. CEM cells were infected with the DC-LTR-50TAR-containing murine amphotropic virus and with murine amphotropic virus made from the DC-vector alone. After G418 selection, resistance clones are being pooled and tested for resistance to infection with HIV-1.

This construct is also being used as a basis for further constructs. Other antiviral activities will be added to the inhibition of gene expression. These activities can interfere with other steps of the viral replication, such as translation of viral RNA or assembly of virions. This sort of "combination therapy" would give a greater probability of effective inhibition of virus replication and make it less likely that resistant HIV-variants would be generated.

Expression of Alternately Spliced mRNA in HIV-1 Infection: Role of Rev

The rev gene also has been shown by this laboratory and others to be indispensable for HIV-1 replication. It facilitates the transport of unspliced and singly spliced viral mRNA from the cell nucleus. Since these transcripts code for all the virion proteins, rev expression is critical for virion production and virus

spread. The multiply spliced transcripts code for the viral regulatory genes, including tat, nef, and rev itself. Thus, rev is at the center of a web of regulatory activities which control virus expression, replication, and likely pathogenesis.

We have therefore undertaken to understand the role played by rev in viral latency, replication and pathogenesis. We have analyzed the patterns of expression of transcripts for the viral regulatory proteins and characterized their structure. We have developed a sensitive reverse transcriptase-polymerase chain reaction (reverse PCR) assay to discriminate among the different multiply spliced transcripts and cloned and sequenced them. In H9 cells chronically infected with HIV-1(HTLV-IIIB), we find a complex pattern of splicing among these transcripts. Rev, tat, and nef all contain their unique splice acceptors within the first tat-rev coding exon; in addition, a minority of the nef transcripts lack the middle exon completely. Nef is the most abundant transcript, representing about 80% of the total multiply spliced transcripts, suggesting that the Nef protein plays an important role in the viral life cycle. Rev (18%) and tat (2%) are minority transcripts. The same general pattern is seen with peripheral blood lymphocytes infected with the IIIB strain and with macrophages infected with HIV-1(BA-L), except that in the latter case the nef transcript lacking the middle exon is relatively more abundant. Nef transcripts are also the most abundant transcript shortly after infection (4-12 hours). High levels of nef transcripts thus seem to be a robust feature of HIV-1 gene expression. These techniques can now be used to evaluate gene expression in clinical samples, such as blood cells from infected individuals in various stages of disease progression or being treated with different antiviral therapies.

Mechanism of Trans-activation by Rev

The possibility that the activity of rev was mediated through direct binding to the region of RNA containing the rev responsive element (RRE) was tested using recombinant Rev from E. coli and an in vitro synthesized 249 nucleotide (nt) transcript encompassing the RRE in a gel retardation assay. A Rev-RRE complex could be detected with as little as 50 ng of protein. By looking at transcripts with progressive deletions, the binding site was shown to be contained within a 90 nt region corresponding to nt 7755-7845, within the env gene of the HIV-1 genome. Interestingly, this region shares some homology (90% over 20 nt) with human U2 small nuclear RNA, which is thought to be involved in the splicing of cellular mRNA. Studies on the mechanism of action of rev may thus have a more general applicability to control of splicing and transport of cellular mRNA in addition to holding promise in the development of antiviral strategies.

Possible Mechanisms in Tissue Targeting of HIV-1 Infected Cells

It is known that T lymphocytes posses cell surface receptors for extracellular matrix components, known as integrins, which recognize fibronectin, fibrinogen, vitronectin, laminin and collagen IV. Lymphocyte-specific integrins are responsible for the tissue specific localization of lymphocytes. Recently it has been shown that the very late activation antigen expressed during T cell activation is also a member of the integrin super gene family and the cell-to-cell contact between cytotoxic T lymphocytes and infected lymphocytes is mediated

through an integrin. Thus, it appears that this family of receptors plays an important role in tissue targeting, activation and cellular recognition by lymphocytes.

We examined the role that integrin expression on HIV-1 infected lymphocytes might play in specific tissue targeting of the lymphocytes and ultimately in determining disease manifestations. HIV-1 infected CD4+ lymphocytes (H9/HTLV-IIIB) exhibit enhanced binding to extracellular matrix components. Chronically infected H9 cells appear to attach to the basement membrane components laminin, collagen IV and fibronectin to a greater extent than uninfected H9 lymphocytes. The enhanced attachment was most striking to fibronectin and least striking to laminin. No enhanced binding was observed to interstitial collagen I. This attachment was associated with striking morphological changes characterized by marked spreading and by the production of numerous projections in the presence of fibronectin. Attachment occurred within 1 hour of plating the infected cells onto the substrate and reached a peak by 3 to 5 hours. The binding was not blocked in the presence of cycloheximide indicating that exposure to fibronectin did not induce synthesis of a receptor.

The specificity of this binding to HIV-1 infected lymphocytes was tested using a number of cell lines and virus strains. H9 lymphocytes chronically infected with the HIV-1 strains HTLV-IIIB and MN showed enhanced binding over uninfected controls while H9 cells chronically infected with HIV-1 strain RF and HIV-2 (strain NIH-Z) failed to show enhanced attachment.

Role of the LTR and Tat in Cell Tropism

In order to evaluate the role of the viral promoter and its transactivation by tat in the ability of HIV-1 to grow in different cell types, we cotransfected the LTRs from a T cell tropic (IIIB) and a macrophage tropic (BA-L) strain of HIV-1 with plasmids expressing tat from either virus into different cell types. The IIIB LTR was consistently the more active, regardless of which cell type or which tat was used. The IIIB tat was at least as effective in transactivation of either LTR in both cell types. We conclude, then, that lack of ability to grow in macrophages is not determined by failure of effective transactivation of the viral promoter by tat.

HIV-1 in Terminally Differentiated Cells: Latency and Integrative States

We are interested in mechanisms of latency for HIV-1 in infected nondividing cells, particularly as it relates to the state of integration of viral DNA. Cell division is thought to be critical for integration. Although integration is thought to be a prerequisite for retroviral replication, HIV-1 is able to infect cells, such as macrophages, which do not normally divide. We have tried to understand the way in which HIV-1 deals with this problem. The presence of high levels of unintegrated DNA in HIV-1 infected cells suggests that integration may not be required in some cell types, such as macrophages, for productive infection.

We have shown by site directed mutagenesis that the viral integrase gene is necessary for productive infection of T cells by HIV-1(IIIB). The IIIB clone (HXB2) used in this study, however, does not grow in macrophages, so it cannot be used to address the question of whether integrase is required for infection of macrophages. To help answer this question, we have isolated several molecular clones which are infectious for macrophages. We are introducing stop codons into the integrase gene of these clones to ascertain whether or not integration is required for infection of macrophages.

We have also studied the temporal relationship of different steps in the synthesis of viral DNA to events in the infection process. We have found that purified reverse transcriptase has a DNA polymerase activity in the absence of added template (endogenous activity). Surprisingly, this endogenous template is viral DNA, rather than RNA, and represents most or all of the minus strand of DNA plus a partially complete plus strand of DNA. Characterization of the endogenous product shows that much of the endogenous synthesis initiates from the polypurine tract in the middle of the viral genome, which serves as one of the two primers for initiation of synthesis of plus strand viral DNA. This complex appears to exist within extracellular virions, and may even be synthesized before release of particles from infected cells.

If HIV-1 is able to partially convert genomic RNA to viral DNA prior to entry of the target cell, it may provide a means for the viral genetic material to persist in the target cell for extended periods of time, remaining latent until the onset of favorable conditions, such as activation of T cells, for continuing the viral life cycle. It is further possible that this mechanism allows HIV-1 to replicate in some cell types without integration at all. This may thus be a way in which HIV-1 deals with the problem of infecting cells which do not normally replicate. In addition to the use of integrase negative infectious clones, other experiments are underway to further investigate these possibilities.

Publications:

Berneman ZN, Gartenhaus RB, Reitz MS, Gallo RC, Klotman ME. Expression and alternate splicing of human T-lymphotropic virus, HTLV-I, type I in adult T-cell leukemia/lymphoma and ATL. Blood 1991;76:484.

Daefler S, Klotman ME, Wong-Staal F. The trans-activating rev protein of the human immunodeficiency virus 1 interacts directly and specifically with its target RNA. Proc Natl Acad Sci USA 1990;87:4571-5.

Gartenhaus RB, Berneman ZN, Reitz MS, Gallo RC, Klotman ME. Soluble Tax can stimulate proliferation of primary lymphocytes. Blood 1990;76:486a.

Guo HG, Chermann JC, Waters D, Hall L, Louie A, Gallo RC, Streicher H, Reitz MS, Popovic M, Blattner W. Nucleotide sequence analysis of the original isolate of HIV-1. Nature 1991;349:745-6.

Klotman ME, Henry SC, Greene RC, Klotman PE, Hamilton JD. Detection of murine cytomegalovirus nucleic acid in latently infected mice by in vitro enzymatic amplification and oligonucleotide hybridization. J Infect Dis 1990;161:220-5.

Klotman ME, Kim S, Buchbinder A, DeRossi A, Baltimore D, Wong-Staal F. Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infection of lymphocytes and monocytes. Proc Natl Acad Sci USA (In Press).

Klotman ME, Wong-Staal F. HIV-1 gene structure and genetic variability. In: Gallo RC, Jay G, eds. The human retroviruses. Orlando: Academic Press (In Press).

Lisby G, Frederiksen J, Olesen J, Reitz MS. Absence of HTLV-I DNA in blood from patients with multiple sclerosis. AIDS Res Hum Retroviruses 1991;6:1219.

Lisziewicz J, Rappaport J, Dhar R. Tat regulated production of multimerized TAR RNA inhibits HIV-1 gene expression. New Biol 1991;3:1-8.

Looney DJ, Hayashi S, Nicklas M, Redfield RR, Broder S, Wong-Staal F, Mitsuya H. Differences in the interaction of HIV-1 and HIV-2 with CD4. J AIDS 1990;3:649-57.

Morgan RA, Looney DJ, Muenchau DD, Wong-Staal F, Gallo RC, Anderson WF. Retroviral vectors expressing soluble CD4: a potential gene therapy for AIDS. AIDS Res Hum Retroviruses 1990;6:183-91.

Okamoto T, Benter T, Josephs SF, Sadaie MR, Wong-Staal F. Transcriptional activation from the long-terminal repeat of human immunodeficiency virus in vitro. Virology 1990;177:606-14.

Pal R, Reitz MS Jr, Tschachler E, Gallo RC, Sarngadharan MG, Veronese FD. Myristoylation of gag proteins of HIV-1 plays an important role in virus assembly. AIDS Res Hum Retroviruses 1990;6:721-30.

Robert-Guroff M, Popovic M, Gartner S, Markham P, Gallo RC, Reitz MS. Structure and expression of tat-, rev-, and nef-specific transcripts of HIV-1 in infected lymphocytes and macrophages. J Virol 1990;64:3391-8.

Sadaie MR, Mukhopadhyaya R, Benaissa ZN, Wong-Staal F. Conservative mutations in the putative metal-binding region of human immunodeficiency virus tat disrupt virus replication. AIDS Res Hum Retroviruses 1990;6:1257-63.

Sadaie MR, Tschachler E, Valerie E, Rosenberg M, Felber BK, Pavlakis GN, Klotman ME, Wong-Staal F. Activation of tat-defective human immunodeficiency virus by ultraviolet light. New Biol 1990;2:479-86.

Shaunak S, Albright RE, Klotman ME, Henry SC, Bartlett JA, Hamilton JD. Amplification of HIV-1 provirus from cerebrospinal fluid and its correlation with neurological disease. J Infect Dis (In Press).

Tada H, Rappaport J, Lashgari M, Amini S, Wong-Staal F, Khalili K. Trans-activation of the JC virus late promoter by the tat protein of type 1 human immunodeficiency virus in glial cells. Proc Natl Acad Sci USA 1991;87:3479-83.

Tschachler E, Gallo RC, Reitz MS. Constitutive expression of lymphotoxin (tumor necrosis factor beta) in HTLV-I-infected cell lines. In: Blattner WA, ed. Human retrovirology: HTLV. New York: Raven Press, 1990;105-13.

Weeks BS, Klotman ME, Dhawan S, Kibbey M, Rappaport J, Kleinman HK, Yamada KM, Klotman PE. HIV-1 infection of human T-lymphocytes results in enhanced $\alpha(5)\beta(1)$ integrin expression. J Cell Biol (In Press).

CONTRACT IN SUPPORT OF PROJECTS Z01CP05534-05 LTCB, Z01CP05536-05 LTCB, Z01CP05645-02 LTCB, Z01CP05688-01 LTCB, Z01CP07148-08 LTCB

ADVANCED BIOSCIENCE LABORATORIES, INC. (NO1-CP8-7213)

Title: Provision of Animal Facilities and Performance of Routine Experiments and Tests

Current Annual Level: $662,913

Person Years: 2.73

Objectives: This contract provides a well-equipped and maintained animal facility with biohazard containment for small laboratory animals and up to 30 nonhuman primates. In addition, the contract provides routine veterinary care, routine inoculations of virus and antigens, bleeding, surgical procedures, post-mortem examinations, routine blood chemistry tests, histological examination of tissue and -70°C storage of tissue are provided. The contract also provides for the use of mice, guinea pigs, rabbits and goats to produce antisera to purified viral and cellular proteins and for the use of mice for hybridoma antibody production.

Major Contributions: This contract provides essential support for research conducted by LTCB investigators, especially in the provision of animals for AIDS vaccine research; a more detailed discussion of the research supported by this contract can be found in the discussions of the LTCB projects listed above.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05536-05 LTCB, Z01CP05688-01 LTCB

SEMA (NO1-CP1-5644)

Title: Provision of Subhuman Primate Facilities to Test Immune Response to Viral Antigens

Current Annual Level: $161,366

Person Years: 1.55

Objectives: This contract provides an animal facility, with appropriate biohazard containment, for the maintenance of 15 nonhuman primates.

Major Contributions: This contract provides support for AIDS vaccine research; a more detailed description of the research can be found in the discussions of the individual LTCB projects listed above.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05534-05 LTCB, Z01CP05536-05 LTCB, Z01CP05537-05 LTCB, Z01CP05645-02 LTCB, Z01CP05688-01 LTCB, Z01CP07148-08 LTCB, Z01CP07149-08 LTCB,

ADVANCED BIOSCIENCE LABORATORIES, INC. (NO1-CP7-3723)

Title: Provision of Hematopoietic Cell Cultures, Growth Factors and Retroviral Proteins

Current Annual Level: $444,728

Person Years: 4.45

Objectives: This contract has three major purposes: (1) to provide purified and partially purified growth factors from conditioned media produced by culture of a variety of cell types, (2) to provide cultured cells in support of LTCB programs, and (3) to purify retroviral proteins and cellular proteins to homogeneity and produce monoclonal antibodies to them.

Major Contributions: This contract provides important services for research conducted by LTCB investigators, particularly in support of AIDS vaccine and Kaposi's sarcoma projects; a more detailed description of the research can be found in the discussions of the individual LTCB projects listed above.

CONTRACT IN SUPPORT OF PROJECTS ZO1CP05534-05 LTCB, ZO1CP05536-05 LTCB, ZO1CP05645-02 LTCB, ZO1CP05688-01 LTCB, ZO1CP07148-08 LTCB, ZO1CP07149-08 LTCB

ADVANCED BIOSCIENCE LABORATORIES, INC. (NO1-CP7-3722)

Title: Conduct of Routine Tests in Support of Tumor Cell Biology

Current Annual Level: $352,272

Person Years: 5.75

Objectives: This contract provides a variety of services, tests and assays to detect and/or characterize viruses, antibodies, cells, growth factors, cytokines and other regulatory factors, and viral proteins.

Assays, tests and services provided include:

ELISA	Syncytium inhibition assays
Western blot	Reverse transcriptase assays
Radioimmunoprecipitation (RIP)	Antigen capture assays
Radioimmunoassays (RIA)	Growth factor assays
Immunofluorescent assays (IFA)	Cytokine activity assays
Cell phenotyping	Assays of cell growth
Virus neutralization assays	Production of viruses

Major Contributions: This contract provides important services for research conducted by LTCB investigators; a more detailed description of the research can be found in the discussions of the individual LTCB projects listed above.

CONTRACT IN SUPPORT OF PROJECTS ZO1CP05536-05, ZO1CP05537-05, ZO1CP05688-01 LTCB

ADVANCED BIOSCIENCE LABORATORIES, INC. (NO1-CP1-5643)

Title: Purify and Characterize HIV Viral Proteins

Current Annual Level: $248,923

Person Years: 4.75

Objectives: This contract provides for the purification and characterization of native envelope proteins of HIV-1, HIV-2 and SIV from the supernatant of cultures of human T cell lines chronically infected with defective viruses and secreting envelope glycoproteins.

Major Contributions: This contract provides purified native envelope protein for AIDS vaccine research.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05534-05 LTCB, Z01CP05536-05 LTCB, Z01CP05688-01 LTCB, Z01CP07148-08 LTCB

ADVANCED BIOSCIENCE LABORATORIES, INC. (NO1-CP7-3711)

Title: Procurement of Fresh Cells from Monocytes, Macrophages and T and B Cell Lines

Current Annual Level: None

Person Years: 1.77

Objectives: This contract provides for the culture of lymphocytes and monocyte-macrophages from peripheral blood, bone marrow, etc., and for the culture of lymphoid and/or myeloid cell lines, both uninfected and viral-infected. The contract supplies the culture supernatants from these cultures, as needed, for growth factor studies. The contract also supplies radiolabeled viral nucleic acids and proteins from viral-infected cells for analysis.

Major Contributions: This contract provides important services for research conducted by LTCB investigators; a more detailed description of the research can be found in the discussions of the individual LTCB projects listed above.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05534-05 LTCB, Z01CP05536-05 LTCB, Z01CP05537-05 LTCB, Z01CP05688-01 LTCB, Z01CP07148-08 LTCB, Z01CP07149-08 LTCB

ADVANCED BIOSCIENCE LABORATORIES, INC. (NO1-CP7-3725)

Title: Preparation and Purification of Viral Components

Current Annual Level: $149,874

Person Years: 1.20

Objectives: This is a virus production contract which provides biocontainment (BL3) facilities for the growth and density gradient purification of large quantities (up to 30 liters/week) of virus. The contract provides the capability to produce virus under GLP conditions and to simultaneously produce more than one virus.

Major Contributions: This contract provides purified virus for several studies conducted by LTCB investigators; a more detailed description of the research supported by this contract can be found in the discussions of the LTCB projects listed above.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05534-05 LTCB, Z01CP05538-05 LTCB, Z01CP05539-05 LTCB, Z01CP07148-08 LTCB

CAMBRIDGE-BIOTECH RESEARCH LABORATORIES, INC. (N01-CP7-3724)

Title: Preparation and Supply of Fresh and Cultured Mammalian Cells

Current Annual Level: $47,000

Person Years: 0.75

Objectives: This contract provides the capability to grow and supply cultures of normal cells, neoplastic cells and mammalian tissue culture cell lines, both uninfected and infected with retroviruses. The contract also provides for the supply of human peripheral blood and bone marrow samples, either fresh or after short term culture and for the performance of reverse transcriptase assays on cultures. The contract also provides for the storage and maintenance of tissue culture cell line stocks and hybridoma stocks, and for the storage and distribution of molecular clones.

Major Contributions: This contract provides significant services for research conducted by LTCB investigators; a more detailed description of the research can be found in the discussions of the individual LTCB projects listed above.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05534-05 LTCB, Z01CP05536-05 LTCB, Z01CP05689-01 LTCB, Z01CP07148-08 LTCB, Z01CP07149-08 LTCB

CAMBRIDGE-BIOTECH RESEARCH LABORATORIES, INC. (N01-CP0-5686)

Title: Support Services in Virology, Tissue Culture and Immunology

Current Annual Level: $232,000

Person Years: 3.25

Objectives: This contract provides the capability to perform karyotype analysis on cell cultures, and to perform tests for mycoplasma contamination of cell cultures. The contract also provides nude mice for tumorigenicity studies. The contract provides the capability to perform immunological tests (ELISA and Western blot assays) to detect the presence of retrovirus in infected cells or the presence of human or primate retroviral antibodies in serum, provides for the production and purification of antibodies, and also produces and supplies small quantities of human retroviruses, predominately for use as antigens for ELISA and Western blot tests.

Major contributions: This contract provides significant services for research conducted by LTCB investigators; a more detailed description of the research can be found in the discussions of the individual LTCB projects listed above.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05689-01 LTCB, Z01CP07149-08 LTCB

UNITED BIOTECHNOLOGIES, INC. (N01-CP9-5626)

Title: Production of Purified Recombinant Human Viral Proteins in E. coli

Current Annual Level: $353,000

Person Years: 1.4

Objectives: The contractor expresses recombinant proteins in an E. coli expression system and purifies and supplies the proteins. The contractor also makes monoclonal antibodies to the purified proteins.

Major Contributions: This contract has provided purified, biological active HTLV-I regulatory proteins to LTCB investigators; a more detailed description of the research supported by this contract can be found in the discussions of the LTCB projects listed above.

ANNUAL REPORT OF

THE LABORATORY OF TUMOR VIRUS BIOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

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.

The Biochemical Oncology Section (1) plans and executes research on the biochemical and molecular mechanisms of oncogene and proto-oncogene mediated cellular transformation; (2) studies the interaction of viral oncogenes with cellular regulatory proteins; (3) studies the interactions of cellular proto-oncogenes with other cellular proteins; (4) studies the regulation of expression and the activity of oncogenes and proto-oncogenes; and (5) studies the functions of cellular proto-oncogenes in growth and development.

Among the primary objectives of the Laboratory of Tumor Virus Biology is the evaluation of the role of the human papillomaviruses in carcinogenesis. A major focus of the Laboratory is on the molecular biology of the papillomaviruses. It maintains a strong interest in the molecular biology of the bovine papillomavirus type 1 (BPV-1), one of a subgroup of papillomaviruses which readily transforms a variety of rodent cells in tissue culture. This virus serves as the prototype for the systematic study of the molecular biology and genetics of the papillomaviruses. The papillomaviruses are transcriptionally complex and encode a series of transcriptional regulatory factors. The bovine papillomavirus genome remains as a stable multicopy plasmid in transformed cells and is faithfully partitioned to the daughter cells at cell division. BPV-1 has biologic characteristics which make it an excellent model for study of the cellular transformation and latent viral infections which are properties of the papillomaviruses.

Bovine Papillomavirus

Our studies with bovine papillomaviruses type 1 (BPV-1) have focused on the mechanisms of transcriptional regulation, transformation, and DNA replication. With regard to transformation, the virus encodes two transforming genes, E5 and E6. Both of these genes are required for efficient and full transformation of mouse C127 cells. Our research effort the past year has focused primarily on the transcriptional regulation of this virus.

Transcriptional transactivation and repression by the viral E2 proteins are important regulatory mechanisms for the virus. The E2 proteins play a direct role in the transcriptional regulation as well as viral DNA replication for the virus. We have now demonstrated that the BPV-1 P_{2443} promoter is transactivated by E2-responsive elements in the long control region (LCR). We have characterized the cis elements involved in P_{2443} regulation and have shown that a single E2 binding site directly upstream of P_{2443} is not required for the E2 transactivation or for E2 repression of the basal or transactivated activity of this promoter. Further definition of the cis regulatory elements of this promoter indicated that a binding site for the transcriptional factor, SP1, exists directly upstream of the P_{2443} TATA box and is critical for the basal and transactivated levels from this promoter. Disruption of this SP1 site in the background of the viral genome demonstrated the importance of this element and its promoter in the expression of the viral functions. Furthermore, the data suggests the possibility that the E2 transactivator and the cellular SP1 factors may directly interact. Another transcriptional regulatory sequence, which we refer to as a constitutive enhancer, has been mapped in the viral LCR. This constitutive enhancer is most active in primary bovine fibroblasts and has little activity in other cell types. A deletion mapping has localized this activity to a 113 base pair fragment within the viral LCR in a region of the viral genome that also contains the P_{7185} promoter and an E2 binding site at nucleotide 7203. The enhancer activity of this element could be positively modulated by the full-length transactivator or negatively modulated by the E2 repressor. Site directed mutagenesis has defined two cis elements within the constitutive enhancer and recent evidence has demonstrated that the cellular factor SP1 binds to one of these factors. Furthermore, competitive binding between the E2 repressor and the cellular factor at one of the cis elements has been demonstrated.

The E2 gene products of the papillomavirus encode factors important for viral replication and transcriptional regulation. The E2 open reading frame of BPV-1 encodes at least three different proteins. The full-length gene product is a transcriptional activator and is required for plasmid DNA replication. The 3′ half of the open reading frame encodes two smaller proteins that repress E2 mediated transactivation. All three proteins share a common C-terminal DNA binding domain that also contains sequences which mediate E2 dimer formation. An amino terminal domain unique to the transactivator encodes the transactivation domain. Little is known about the regions of the E2 protein important for DNA replication. We have shown that the BPV-1 E2 polypeptides are phosphorylated primarily on two serine residues. Mutations have been engineered resulting in amino acid substitution at these serine residues. Mutants with an amino acid substitution at serine residue 301 in the E2 protein replicate to a high copy number, much greater than that of the wild type genome. This provides evidence for a direct role for E2 in replication. We have also demonstrated that DNA binding can be regulated in vitro by

oxidation-reduction of a single highly conserved cysteine residue in each subunit of the DNA binding domain.

Transcription of the 8 kb circular DNA genome of bovine papillomavirus type 1 (BPV-1) is unidirectional. During productive infection, the structural (late) genes, which lie immediately downstream of the early poly(A) site, are expressed from a late promoter which lies upstream of the early region near nt 7250. In nonproductively infected (transformed) mouse C127 cells, steady state levels of late region mRNAs, are approximately four orders of magnitude lower than those of early region mRNAs. Analysis of BPV-1 transcription rates in BPV-1 transformed cells showed a 10- to 20-fold exponential decrease in the rate of transcription between the early and late poly(A) sites. A series of deletion mutations of various sizes have been made between the early and late poly(A) sites to determine if there are specific sequences which are required for transcription termination. Nuclear run-on analysis of cell lines harboring these BPV-1 mutants are in progress. However, analysis of steady state levels of late cytoplasmic mRNAs from these cell lines indicates that the amount of late mRNA shows an exponential relationship with deletion size. This data is consistent with transcription termination between the early and late poly(A) sites having a direct effect on late poly(A) site usage and suggests that there are no specific sequences between the two poly(A) sites which are required for transcription termination. An alternative interpretation is that deletions within the late region decrease the time that the early poly(A) site is the only poly(A) site available on the nascent transcript and that transcription termination between the two poly(A) sites is the consequence of cleavage and polyadenylation at the early poly(A) site. Distinguishing between these two models is complicated by the fact that early poly(A) site mutants [E. Andrews and D. DiMaio, personal communication; Burnett et al., Plasmid 20:61-74, 1988] show a strong preference for use of cryptic poly(A) sites within the vicinity of the early poly(A) site rather than for use of the late poly(A) site. In contrast, the late poly(A) site is both efficiently and accurately utilized in nonproductively infected cells when it is positioned within 500 bp downstream of the early poly(A) site, and, in addition, is used almost exclusively when placed 472 bp upstream of the early poly(A) site. In these tandem poly(A) site constructions, use of the distal late poly(A) site is increased as the level of transcription increases, suggesting that the early to late shift in expression during the viral life cycle may be partially due to limiting polyadenylation factors required for utilization of the early poly(A) site.

Our data also suggests that there may be interactions between splicing and polyadenylation. In productively infected keratinocytes of the wart, mRNAs transcribed from the late promoter can be spliced from the late leader into 3′ splice sites at either nt 3225 or 3605 and can be polyadenylated at either the early poly(A) site (nt 4203) or the late poly(A) site (nt 7175). However, the majority of transcripts which are spliced into nt 3225 are polyadenylated at the early poly(A) site, whereas most transcripts spliced into nt 3605 are polyadenylated at the late poly(A) site, suggesting that splice site selection may determine poly(A) site choice. In addition, only transcripts which splice into nt 3605 can be further spliced from nt 3764 to nt 5609, removing the early poly(A) site. The 5′ splice site at nt 3764 (AAGGCAAGA) deviates from the consensus. To study the interactions of splicing and polyadenylation, two SV40 cDNA expression vectors were constructed based on the L2-L and L2-S mRNA structures, except that BPV-1 genomic sequences were substituted at the late

poly(A) site. These vectors contain intact early and late polyadenylation regions as well as the entire late region and differ only by the inclusion of extra exon sequences between nt 3225 and 3605 in L2-L. Both vectors express abundant early mRNAs when transfected into CV-1 or COS-1 cells. The L2-L vector consistently produces extremely low levels of late mRNA and these late mRNAs are predominantly unspliced. In contrast, the L2-S vector produces significantly higher levels of late mRNA and these mRNAs are both spliced and unspliced. Mutation of the nt 3764 5′ splice site to a consensus splice site in the L2-L vector stimulates the production of both spliced and unspliced late mRNAs, although early mRNAs still greatly predominate over late mRNAs. The same mutation in the L2-S vector almost completely blocks polyadenylation at the early poly(A) site, favoring synthesis of late mRNAs. The effect of this splice site mutation on polyadenylation occurs even in the absence of the 3′ splice site and effectively suppresses polyadenylation at poly(A) sites at least as far downstream as 900 nt. These data suggest that binding of spliceosomes or other factors at the nt 3764 5′ splice site can have a direct influence on polyadenylation at the early poly(A) site and that the efficiency of this splice site is modulated by exon sequences between nt 3225 and 3605.

Human Papillomaviruses

The "high risk" papillomaviruses include HPV-16 and HPV-18. These viruses can be differentiated from "low risk" papillomavirus such as HPV-6 and HPV-11 by their ability to efficiently immortalize primary human keratinocytes. Efficient immortalization of these cells requires the combination of both the E6 and E7 gene products which are now considered oncoproteins. The E7 oncoprotein shares properties with the E1A transforming protein of adenovirus E1A and the large T antigen encoded by the polyomaviruses in that it can complex with a group of cellular proteins which include the product of the retinoblastoma tumor suppressor gene (RB). The biochemical and biological properties of the high risk and low risk HPVs were studied and compared. Chimeric proteins were constructed between these high risk and low risk E7 proteins and their biochemical and biological properties studied. Biological properties important to cellular transformation were mapped to the amino terminus of the HPV-16 E7 protein. This amino terminus is required for efficient immortalization, high affinity binding to the retinoblastoma gene product (RB), and the abrogation of the TGF-beta mediated transcriptional repression of c-myc expression. In contrast, both the HPV-6 and HPV-16 E7 proteins can efficiently transactivate the adenovirus E2 promoter. We have carried out additional experiments characterizing the cellular proteins associated with HPV-16 E7. In addition to pRB binding HPV-16 E7, another protein with the same electrophoretic ability as the adenovirus E1A associated protein, p107, was noted. A series of additional cellular proteins have been observed and their identities are under investigation.

In collaboration with Drs. Harold Moses and Jennifer Pietenpol (Vanderbilt Medical School), we have previously shown that the HPV-16 E7 can abrogate the TGF beta induced transcriptional repression of c-myc expression. Further studies have shown that this property is dependent upon the intact pRB binding site on E7. This has implicated pRB, or another cellular protein capable of binding E7 through this same domain, as an essential component in the pathway of transcriptional modulation of the c-myc gene by TGF- beta. Transient expression of pRB also leads to the repression of c-myc expression. Using a series of deletions of the c-myc promoter, a 22 base pair element has been

defined and shown to be necessary to mediate the repression of the c-myc promoter by TGF-beta and pRB. Gel shift analysis with this sequence element has led to the detection of several specific DNA protein complexes. Formation of one of these complexes can be regulated by TGF-beta. This regulation is not observed in the presence of SV40 large T antigen or in cells containing a defective RB gene. This implicates RB directly in this regulation. Studies are in progress to further characterize the proteins involved in mediating the TGF-beta transcriptional modulation of c-myc expression. We have investigated whether one of the targets of TGF-beta may be the cell cycle dependent protein kinase that regulates the biologic activity of pRB at the G1/S boundary of the cell cycle. Inactivation of this protein kinase by TGF-beta would be the accumulation of unphosphorylated pRB leading consequently to G1 growth arrest.

We have continued our studies exploring the functional consequences of the interaction of the E6 oncoproteins encoded by HPV-16 and HPV-18 with the cellular tumor suppressor gene product, p53. Insight into the mechanism by which E6 functions in oncogenesis is provided by the observation that the E6 protein encoded by HPV-16 and -18 can complex with the wild type p53 protein in vitro. The wild type p53 gene has tumor suppressor properties and is a target for several of the oncoproteins encoded by the DNA tumor viruses. We have demonstrated that the E6 proteins of the oncogenic type that bind p53 stimulate the degradation of p53 in vitro. The E6 promoted degradation of p53 is ATP dependent and involves the ubiquitin-dependent protease system. Selective degradation of cellular proteins such as p53 with the negative regulatory function provides a novel mechanism of action for dominant acting oncoproteins. We are in the process of investigating whether the p53 binding by E6 is a direct protein-protein interaction or involves additional cellular proteins.

In order to investigate the relevance of p53 and RB as targets of the E6 and E7 oncoproteins, we have investigated the state of these genes in human cervical carcinoma cell lines. We have examined a series of cell lines that are either positive or negative for HPV sequences. Approximately 85% of human cervical carcinomas can be demonstrated to contain HPV DNA sequences. We have investigated five HPV positive cell lines and have shown that the RB and p53 genes are wild type in these cell lines. This result is consistent with the hypothesis that the normal functions of the tumor suppressor gene products, pRB and p53, are abrogated as a consequence of the complex formation with the HPV E6 and E7 oncoproteins. In contrast, mutations were identified in the p53 and RB genes expressed in the two HPV negative cervical carcinoma cell lines examined. In RB, the mutations mapped to the domain involved in complex formation with the oncoproteins of the DNA tumor virus. Mutations in the RB genes that affect this portion of the protein have been noted in a variety of different human cancers. Mutations in the p53 gene were likewise in regions commonly mutated in human cancers. These results support the hypothesis that the inactivation of the normal function of the tumor suppressors, pRB and p53, are important steps in human cervical carcinogenesis, either by mutation or as a consequence of complex formation with the virally encoded oncoproteins.

An important step in progression to cervical carcinoma from a preneoplastic intraepithelial dysplasia appears to be the integration of the HPV genome into the host chromosome. Integration occurs in a manner to disrupt the expression of the E2 gene product. Transcription of the transforming genes E6 and E7 of

the HPVs can be repressed by the E2 gene product. We have studied the cis elements in the HPV-16 and HPV-18 promoters that are responsive to this E2 mediated repression. In HPV-16 and in HPV-18, it is the P_{97} and P_{105}, respectively, that are responsible for expression of the E6 and E7 oncoproteins. These promoters can be specifically repressed by the full-length E2 protein. Mutational analysis of the E2 binding sites in the upstream regulatory region of each promoter revealed that the E2 repression was mediated through the E2 binding sites immediately proximal to each promoter. In the context of a mutated E2 binding site at the proximal promoter, each of these promoters can be transactivated by E2. This transcriptional repression occurs at a transcriptional initiation step and it is likely that the interaction of the E2 with the promoter proximal E2 binding sites is important for the controlled expression of the viral oncoproteins regulated by these promoters in virally infected cells.

HPV-16 and HPV-18 immortalize primary human keratinocytes with different efficiencies. HPV-18 is more efficient in this immortalization process and its function has been mapped at the LCR/E6/E7 region of the genome. We have carried out a study to examine the basis for this difference in transformation efficiency between HPV-16 and HPV-18 and have shown that it maps to the transcriptional control region upstream of the E6 and E7 genes.

Tyrosine Protein Kinases

Signal transduction collectively encompasses the diverse biochemical reactions involved in the modulated programmed cellular responses to both external and internal stimuli. Among the cellular enzymes involved in this complex process, the tyrosine protein kinases appear to play key roles in mediating signaling events. We have shown that several members of the src-family of non-receptor tyrosine protein kinase proto-oncogenes function as signaling components of diverse receptors in cells of hematopoietic origin.

Addition of interleukin-2 (IL-2) to IL-2 dependent T-cells results in tyrosine protein kinase signal transduction events even though the IL-2 receptor alpha and beta chains lack intrinsic enzymatic activity. We have found that addition of IL-2 to IL-2 dependent human T-cells transiently stimulates the specific activity of p56lck, a member of the src family of non-receptor tyrosine protein kinases expressed at high levels in T lymphocytes. The ability of IL-2 to induce p56lck activation was found to be independent of the capacity of p56lck to associate with either CD4 or CD8. Following IL-2 treatment, p56lck was found to undergo serine/threonine phosphorylation modifications which resulted in altered mobility of the lck gene product on polyacrylamide gels. These observations raise the possibility that p56lck participates in IL-2-mediated signal transduction events in T-cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP00543-13 LTVB

PERIOD COVERED: October 1, 1990 to September 30, 1991

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
Others:	J. Byrne	Biologist	LTVB	NCI
	S. Van de Pol	Biotechnology Fellow	LTVB	NCI
	J. Benson	Guest Researcher	LTVB	NCI

COOPERATING UNITS *(if any)*: University of California, San Francisco, CA (D. Hanahan); Pasteur Institute, Paris, France (M. Yaniv)

LAB/BRANCH: Laboratory of Tumor Virus Biology

SECTION: Viral Oncology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	2.4	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 papillomaviruses are a group of small DNA viruses associated with benign proliferative lesions in a variety of higher vertebrates. A subgroup of viruses are associated with lesions which can progress to malignancy. There are currently 65 different human papillomaviruses and six different bovine papillomaviruses that have been described. The life cycle of these viruses is closely linked to the differentiation program of squamous epithelial cells which are the natural host cell for these viruses. To date, no culture system has yet been developed for the propagation of any papillomaviruses. The bovine papillomavirus type 1 (BPV-1) has served as the prototype of the papillomaviruses for genetic and molecular studies. BPV-1 virus readily transforms a variety of rodent cells in tissue culture, and a unique feature of the transformed cells is that the viral DNA often remains as the stable extrachromosomal plasmid within the cells. Our studies have been designed to focus on the molecular biology of the BPV-1 for understanding the normal virus-host cell interaction with the idea of gaining insight into the viral and cellular factors involved in viral gene expression and carcinogenic progression. Within the viral system, the E2 gene products have served as critical factors in regulating viral gene expression. The E2 open reading frame of BPV-1 encodes three distinct proteins with DNA binding properties. These proteins have both negative and positive effects on the regulation of viral gene expression. Our studies have been designed to understand the viral cis-elements and encoded factors involved in transcriptional regulation and replication of the virus.

PROJECT DESCRIPTION

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

P.M. Howley	Chief	LTVB	NCI
J. Byrne	Biologist	LTVB	NCI
S. Van de Pol	Biotechnology Fellow	LTVB	NCI
J. Benson	Guest Researcher	LTVB	NCI

Objectives:

1. To analyze the molecular biology of the papillomaviruses using the bovine papillomavirus as a model system.
2. To localize the transcriptional elements in the viral genome involved in the control of viral gene expression.
3. To determine the factors, both viral and cellular, involved in the control of virus-specific gene expression for the papillomaviruses.
4. To analyze the "early" papillomavirus protein products expressed in transformed cells.
5. To determine the cis and trans functions required for autonomous extrachromosomal plasmid replication.
6. To determine the nature of the molecular events involved in the progression of a benign papillomavirus lesion into a malignant lesion.

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, primary extensions.
4. DNA sequencing.
5. Immunoprecipitation, immunoblotting and immunofluorescence of viral proteins.
6. Transfer of DNA into mammalian cells using standard calcium precipitation, DEAE dextran or electrophoration technology.
7. Transgenic mice.
8. Genetic analysis of papillomavirus functions in saccharomyces cerevisiae.
9. Standard biochemical techniques for characterizing the viral gene products.

Major Findings:

1. BPV-1 long control region (LCR) contains DNA sequence elements involved in the regulation of viral transcription and replication. Differences in the levels of transcription have previously been noted between BPV-1 infected rodent cell lines and bovine cells. To investigate these differences in levels of expression, fragments of the LCR were cloned into an enhancer-deleted chloramphenicol acetyl transferase expression vector and assayed for enhancer activity. A strong constitutive enhancer was found in the upstream

portion of the LCR that was most active in primary bovine fibroblasts and had little activity in other cell types. Deletion mapping localized most of the activity to a 113 base pair fragment within the viral LCR in a region of the genome sequence that also contains the p_{7185} promoter and an E2-binding site at nucleotide 7203. The enhancer activity of this element could be positively modulated by the full-length E2 transactivator or negatively modulated by the E2 repressor. Site directed mutagenesis defined two cis elements, referred to as CE1 and CE2, were necessary for this enhancer activity. These elements overlap the E2 binding site at nucleotide 7203. In vitro DNA binding studies revealed a special gel retardation complex associated with cellular factor binding at CE1 and a correlation between enhancer activity and the binding activities of factors to the CE1 element. Competitive binding between the E2 repressor and the cellular factor at this CE1 element, which we have shown is bound by the transcription factor SP1, was demonstrated. A manuscript describing these studies was published in The Journal of Virology.

2. The products encoded by the E2 open reading frame (ORF) of the papillomaviruses are DNA binding transcription factors involved in the positive or negative regulation of multiple viral promoters. To further understand the mechanisms by which the same transcription factor may act differentially, the full-length BPV-1 E2 protein was expressed and purified from these saccharomyces cerevisiae. The purified E2 protein was assayed in vitro for its capacity to modulate transcription. This protein stimulated transcription of the HSV thymidine kinase promoter when E2 binding sites were positioned in an enhancer configuration about 100 base pairs upstream of the promoter start site. In contrast, the same full-length E2 protein repressed transcription of the HPV-18 p105 promoter. This repression was mediated through binding to the E2 binding site immediately upstream of the p_{105} promoter TATA box and could be abrogated by pre-incubation of the HPV-18 p_{105} promoter template with nuclear extract allowing formation of the pre-initiation complex. The in vitro DNA binding experiments with purified E2 and TFIID showed the binding of E2 to its DNA target placed at different positions with respect to the TATA box differentially affects binding of TFIID to its cognate site. In these respects, E2 is similar to bacteriophage lambda, a repressor which can act either as a repressor or as an activator of transcription depending on the position of its binding sites relative to the promoter sequences. These studies have been carried out in collaboration with Dr. Moshe Yaniv at the Pasteur Institute and a manuscript has been submitted for publication. Dr. John Benson in the laboratory has designed a selection scheme in saccharomyces cerevisiae in which E2 expression can be regulated and lethal in saccharomyces cerevisiae. Using this selective scheme, he used identifying cellular factors in yeast which interact with the papillomavirus E2 gene product.

Publications:

Hanahan D, Wetzel E, Skowronski J, Sippola-Thiele M, Lindgren V, Howley P. Tumorigenic latency and separable stages during fibrosarcoma development in transgenic mice carrying papillomavirus genomes. In: Knudson AG, Stanbridge EJ, Sugimura T, Terada M, Watanabe S, eds. Proceedings of the 20th international Princess Takamatsu cancer research fund. Tokyo: Japan Scientific Societies Press, 1990;289-96.

Van de Pol SB, Howley PM. A bovine papillomavirus constitutive enhancer is negatively regulated by the E2 repressor through competitive binding. J Virol 1990;64:5420-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP00565-09 LTVB

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Structure Function Studies on the Human Papillomavirus E7 Oncoprotein

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

P.I.:	Karl Münger	Visiting Associate	LTVB	NCI
Others:	Peter Howley	Chief, LTVB	LTVB	NCI
	Donald Heck	Howard Hughes Institute Fellow	LTVB	NCI
	Carole Yee	Biologist	LTVB	NCI

COOPERATING UNITS *(if any)*: Vanderbilt Medical School, Nashville, TN (J. Pietenpol and H. Moses); Massachusetts General Hospital Cancer Center, Charlestown, MA (N. Dyson and E. Harlow)

LAB/BRANCH: Laboratory of Tumor Virus Biology

SECTION: Viral Oncology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	2.2	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.)*

Of the more than 65 different human papillomavirus types (HPVs), about 20 are associated with lesions of the anogenital tract. The anogenital HPVs can be further subdivided into two groups: the "low risk" HPVs (e.g., HPV-6 and HPV-11) which are associated with benign lesions such as condyloma acuminata and the "high risk" HPVs (e.g., HPV-16 and HPV-18) which are associated with cervical carcinomas. The E7 gene of the "high risk" HPVs is consistently expressed in CINs as well as in cervical carcinomas and the derived cell lines. It encodes a 98 amino acid nuclear phosphoprotein that shares amino acid similarity with the adenovirus (Ad) E1A transforming proteins as well as the large tumor antigens (TAg) of the polyomaviruses. The biological properties that have been described for the HPV-16 E7 protein include transformation of established rodent fibroblast cell lines, cooperation with a ras oncogene to transform primary baby rat kidney cells, cooperation with the E6 oncoprotein to transform primary human epithelial cells, abrogation of the TGF beta mediated repression of c-myc expression and transactivation of the Ad E2 promoter. Biochemical studies have revealed that the HPV-16 E7 protein can form a specific complex with the retinoblastoma tumor suppressor gene product pRB which is phosphorylated at serine residues by casein kinase II and exhibits anomalous migration properties on SDS polyacrylamide gels. The biological properties have been mapped to specific regions of the E7 protein and studies with chimeric HPV-6/HPV-16 E7 proteins have revealed differences in the biological and biochemical properties of E7 proteins derived from "high risk" HPVs and "low risk" HPVs.

PROJECT DESCRIPTION

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

Karl Münger	Visiting Associate	LTVB	NCI
Peter Howley	Chief, LTVB	LTVB	NCI
Donald Heck	Howard Hughes Institute Fellow	LTVB	NCI
Carole Yee	Biologist	LTVB	NCI

Objectives:

1. To study the biological and biochemical properties of the E7 proteins derived from "high risk" and "low risk" HPVs.
2. To map the biological properties of HPV E7 proteins to specific sequences of E7.
3. To study the biological consequences of HPV E7 expression in epithelial cells.
4. To study E7 associated cellular proteins.
5. To study the mechanisms by which the E7 associated proteins are inactivated.

Methods Employed:

1. Standard recombinant DNA technologies.
2. Standard protein chemical methods.
3. Expression of proteins in eukaryotic and prokaryotic cells.
4. Immunoblotting of cellular proteins.
5. Screening of cDNA expression libraries.
6. Analysis of protein/DNA interactions.
7. Transient and stable DNA transfection of primary and established cells.

Major Findings:

1. The biochemical and biological properties of a "high risk" HPV (HPV-16) and a "low risk" HPV (HPV-6) derived E7 protein were studied. Two chimeric E7 proteins were also constructed: the HPV-16/6 E7 protein contains the amino terminal half of HPV-16 E7 and the carboxyl-terminal half of HPV-6; the HPV-6/16 E7 protein contains the amino terminal half of HPV-6 E7 and the carboxyl-terminal half of HPV-16 E7. The corresponding E7 genes were inserted in an isogenic background and tested for a variety of biological and biochemical parameters. In contrast to HPV-16 E7, the HPV-6 E7 is transformation impaired, cannot abrogate the TGFb mediated transcriptional repression of c-myc expression and exhibits normal electrophoretic properties on SDS poly-acrylamide gels. In contrast, however, both HPV-6 and HPV-16 E7 efficiently transactivate the AdE2 promoter. The analysis of the chimeric E7 proteins showed that the observed differences in the biological and biochemical properties of the "low risk" and "high risk" HPV derived E7 proteins is determined by amino acid sequences in the amino terminal portion of the E7 proteins. A manuscript describing these data is in press in The Journal of Virology. A further dissection of the amino terminal portion of the E7 proteins was performed by construction of additional chimeric E7 proteins.

Analysis of these recombinant E7 proteins revealed that the anomalous electrophoretic migration properties of HPV-16 E7 are determined by sequences located in the first 12 amino acid residues of HPV-16 E7. Furthermore, the difference in the transforming potential of HPV-6 and HPV-16 E7 is mostly determined by the relative binding potential of E7 to the retinoblastoma tumor suppressor protein pRB. A manuscript describing these findings is in preparation. In addition, a mutagenic analysis of the HPV-16 E7 protein was performed. A number of specific point mutations, small deletions and premature termination signals were introduced throughout the HPV-16 E7 sequence. Studies with these mutant E7 proteins revealed that the ability of E7 to cooperate with ras to transform BRK cells and to transactivate the AdE2 promoter are overlapping activities and primarily depend on amino terminal sequences of E7 including the pRB binding site. Transformation and pRB binding correlate in that every mutation that is impaired for pRB binding is also impaired for cellular transformation. In contrast, however, some mutations located at the extreme amino terminus of E7 are transformation deficient, although they bind to pRB with high affinity. This implies that pRB binding may be necessary but not sufficient for cellular transformation. A manuscript describing these findings is in preparation.

2. To further study HPV-16 E7 associated proteins, mixing experiments with labeled cell extracts and purified recombinant HPV-16 E7 protein synthesized in bacteria were performed. These experiments showed that in addition to pRB, other cellular proteins are also found in complex with HPV-16 E7. One of these proteins exhibits the same electrophoretic mobility as the AdE1A associated protein, p107. A protein of the same molecular size was also co-precipitated with anti-E7 antibodies from HPV-16 transformed keratinocyte cell lines. The p107 protein interacts with AdE1A and polyomavirus TAg and this interaction requires similar sequences on these oncoproteins as those required for complex formation with pRB. In collaboration with Nick Dyson (Massachusetts General Hospital Cancer Center), synthetic peptides corresponding to the previously mapped pRB binding site of HPV-16 E7 were synthesized. These peptides were able to compete for binding of p107 to AdE1A. In addition, an interaction between p107 and the E7 derived peptides was also directly demonstrated using immobilized peptides. An additional protein that binds to AdE1A using very similar sequences as pRB is cyclin A. The interaction of cyclin A with HPV-16 E7 was studied by mixing experiments using ^{35}S methionine labeled cyclin A synthesized by in vitro transcription/in vitro translation and various HPV-16 E7 containing cell extracts. A manuscript describing these findings is in preparation. Further studies are in progress to identify additional cellular proteins that interact with E7 through sequences other than the pRB binding site.

3. In collaboration with Jennifer Pietenpol and Harold Moses (Vanderbilt University), we have previously shown that HPV-16 E7 can abrogate the TGF-beta induced transcriptional repression of c-myc expression. Additional studies with mutant E7 proteins have now revealed that this property is dependent on an intact pRB binding site on E7. This has implicated pRB, or another cellular protein which is targeted by E7 through the pRB binding site, as an essential component in the pathway of the transcriptional modulation of the c-myc gene by TGF-beta. Transient expression of pRB also leads to the repression of c-myc expression. Using a series of deletions of the c-myc

promoter, a 22 bp element was defined that is necessary to mediate the repression of the c-myc promoter by both TGF-beta and pRB. Gel shift analyses with this sequence element led to the detection of several specific DNA/protein complexes. Formation of one of these complexes is regulated in response to TGF-beta treatment. This regulation, however, is not observed in SV40 TAg expressing cells or in cells with a defective RB gene. A manuscript describing these findings has been submitted for publication. Studies are in progress to further characterize the proteins involved in mediating the TGF beta mediated transcriptional modulation of c-myc expression. We have investigated whether one of the targets of TGF-beta may be the cell cycle dependent protein kinase that regulates the biological activity of pRB at the G1-S boundary of the cell cycle. Inactivation of this protein kinase by TGF-beta would lead to the accumulation of unphosphorylated pRB and consequently G1 growth arrest. This growth arrest could be overcome in HPV-16 E7 containing cells by formation of a biologically inactive pRB-E7 complex resulting in a TGF-beta resistant phenotype. In agreement with our earlier study, we found the primary human epithelial cells were G1 growth arrested by TGF-beta treatment but HPV-16 E7 transformed keratinocytes were not. Immunoblotting studies revealed that the phosphorylation status of pRB was strictly correlated with cell growth, i.e., accumulation of unphosphorylated pRB was only detected in TGF-beta sensitive cells but not in TGF-beta resistant cells. These experiments argue that either the protein kinase regulating pRB phosphorylation at the G1-S boundary is not a direct target of TGF-beta or that the activity of this protein kinase is regulated by TGF-beta in a similar manner as c-myc transcription and that HPV-16 E7 is also able to annul this regulation.

Publications:

Howley PM, Münger K, Werness BA, Phelps WC, Schlegel R. Molecular mechanisms of transformation by the human papillomaviruses. In: Knudson AG, Stanbridge EJ, Sugimura T, Terada M, Watanabe S, eds. Proceedings of the 20th international Princess Takamatsu cancer fund. Tokyo: Japan Scientific Societies Press; 1990;199-206.

Münger K, Scheffner M, Huibregtse J, Howley PM. Papillomaviruses and their interactions with tumor suppressor gene products. Cancer Surv (In Press).

Münger K, Werness BA, Cowsert LM, Phelps WC. Papillomaviruses and neoplastic transformation. In: Dulbecco R, ed. Encyclopedia of human biology. New York: Academic Press (In Press).

Münger K, Yee CL, Phelps WC, Pietenpol JA, Moses HL, Howley PM. Biochemical and biological differences between E7 oncoproteins of the high and low risk HPVs are determined by amino terminal sequences. J Virol (In Press).

Scheffner M, Münger K, Byrne JC, Howley PM. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc Natl Acad Sci USA (In Press).

Werness BA, Münger K, Howley PM. The role of the human papillomavirus oncoproteins in transformation and carcinogenic progression. In: DeVita VT, Hellman S, Rosenberg SA, eds. Philadelphia: JB Lippincott (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP00898-08 LTVB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
Others:	J. Lichy	Biotechnology Fellow	LTVB	NCI
	H. Romanczuk	Guest Researcher	LTVB	NCI
	C. Yee	Biologist	LTVB	NCI
	J. Huibregtse	IRTA Fellow	LTVB	NCI
	M. Scheffner	Guest Researcher	LTVB	NCI
	J. Brokaw	IRTA Fellow	LTVB	NCI

COOPERATING UNITS *(if any)*

Princeton University, Princeton, NY (A. Levine); Pasteur Institute, Paris, France (Francoise Thierry); Georgetown Medical School, Washington, D.C. (R. Schlegel); Ludwig Institute, San Paolo, Brazil (Luisa Villa)

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.9	6.4	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 papillomaviruses are associated with naturally occurring cancers in humans. Over 65 different papillomaviruses (HPVs) have been identified. Approximately 25 of these have now been associated with genital tract lesions. The HPVs that affect the anogenital area can be separated on the basis of their clinical associations into two distinct groups. One group, including HPV-6 and HPV-11, is generally associated with benign anogenital warts that infrequently progress to cancer and is referred to as "low risk" viruses. The "high risk" group, including HPV-16 and HPV-18, is associated with intraepithelial neoplastic lesions that are at high risk for malignant progression. We have been studying the role that HPV may play in the progression of a benign lesion to a malignant lesion. In HPV-16 and HPV-18 associated human cancers, the viral genome is generally found to be integrated. Integration often occurs in the E1 or E2 open reading frame, such that it disrupts the expression of the E2 open reading frame. The E2 open reading frame of the papillomaviruses encodes a DNA binding protein which is involved in the regulation of viral promoters. Integration into the E2 ORF is believed to lead to the deregulation of the viral promoter upstream of the E6 and E7 open reading frames. The E6 and E7 ORFs of HPV-16 and HPV-18 have been shown to encode transforming proteins. Expression of both the E6 and E7 gene products are required for efficient immortalization of human keratinocytes. The E7 proteins of the genital tract HPVs have been shown to associate with the product of the retinoblastoma tumor susceptibility gene. The E6 protein of the "high risk" genital HPVs has been shown to complex with the p53 cellular protein, which is also believed to be a tumor suppressor gene. The biochemistry of these interactions has been a major focus of this project.

PROJECT DESCRIPTION

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

P. M. Howley	Chief	LTVB	NCI
J. Lichy	Biotechnology Fellow	LTVB	NCI
H. Romanczuk	Guest Researcher	LTVB	NCI
C. Yee	Biologist	LTVB	NCI
J. Huibregtse	IRTA Fellow	LTVB	NCI
M. Scheffner	Guest Researcher	LTVB	NCI
J. Brokaw	IRTA Fellow	LTVB	NCI
F. Del Vecchio	IRTA Fellow	LTVB	NCI

Objectives:

1. To analyze human squamous cell carcinomas from a variety of sites for the presence of HPV DNAs and the expression of HPV-specific mRNAs.
2. To characterize the HPV RNAs expressed in HPV-associated carcinomas.
3. To characterize the HPV-16 and HPV-18 genes which can transactivate transcriptional regulatory sequences within the viral genome and within the host cell.
4. To identify and characterize the HPV-16 and HPV-18 gene products involved in transformation and immortalization of a variety of cell types.
5. To determine the viral promoters active in benign HPV-16 and HPV-18 associated lesions and in cervical carcinomas.
6. To determine the cellular factors involved in regulating viral gene expression.
7. To identify the cellular factors which interact with the viral transcriptional, transregulatory, and transforming gene products.
8. To identify human genes that are inactivated in progression of benign papillomavirus lesions to malignancy.

Methods Employed:

1. Standard recombinant DNA technologies.
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.
7. Transient and stable DNA transfection techniques.
8. Cellular transformation using primary and established cell lines.
9. PCR analysis.

Major Findings:

1. We have continued our studies exploring the functional consequences of the interaction of the E6 oncoproteins encoded by the "high risk" human papillomaviruses with the cellular protein p53. The E6 encoded by the oncogenic HPV types 16 and 18 is one of two viral products expressed in HPV

cancers. E6 is an oncoprotein which cooperates with E7 to immortalize primary human keratinocytes. Insight into the mechanism by which E6 functions in oncogenesis is provided by the observation that the E6 protein encoded by HPV-16 and HPV-18 can complex with the wild type p53 protein in vitro. The wild type p53 gene has tumor suppressor properties, and is a target for several of the oncoproteins encoded by the DNA tumor viruses. We have demonstrated that the E6 proteins of the oncogenic HPVs that bind p53 stimulate the degradation of p53 in vitro. The E6 promoted degradation of p53 is ATP dependent and involves the ubiquitin-dependent protease system. Selective degradation of cellular protein such as p53 with negative regulatory functions provides a novel mechanism of action for dominant acting oncoproteins. A manuscript describing these studies was published in Cell during this past year.

2. In order to investigate whether p53 and the retinoblastoma gene products are the relevant targets of the E6 and E7 oncoproteins encoded by the "high risk" viruses, we have investigated the state of the p53 and RB genes in human cervical carcinoma cell lines. The cell lines which were either positive or negative for HPV DNA sequences were analyzed for evidence for mutation of the p53 and RB genes. Approximately 85% of human cervical carcinomas can be demonstrated to contain HPV. We have investigated a total of seven cervical carcinoma cell lines, five of which are positive for HPV and two of which are negative for HPV sequences. Each of the five HPV positive cell lines expressed normal pRB and low levels of wild type p53. These functions are presumed to be altered as a consequence of their association with the HPV E7 and E6 oncoproteins, respectively. In contrast, mutations were identified in the p53 and RB genes expressed in the two HPV negative cervical carcinoma cell lines (C-33A and HT-3). Mutations in the p53 genes were mapped to codon 273 and codon 245 in the two cell lines, respectively, which are located in the highly conserved regions of p53 where mutations have been found in a variety of human cancers. Mutations in RB occurred at splice junctions resulting in in-frame deletions affecting exons 13 and 20 in these two cell lines, respectively. These mutations resulted in aberrant sized proteins that were not phosphorylated and were not able to complex with the adenovirus E1A oncoprotein. These results support the hypothesis that inactivation of the normal functions of the tumor suppressors pRB and p53 are important steps in human cervical carcinogenesis, either by mutation or as a consequence of complex formation with HPV E6 and E7 oncoproteins. A manuscript describing these studies is in press in Proceedings of the National Academy of Sciences.

3. Transcription of the transforming genes E6 and E7 of the "high risk" human papillomaviruses can be repressed by the product of the E2 open reading frame. We have studied the cis elements that is in the HPV-16 and HPV-18 promoters that are responsive to this E2 mediated repression. In HPV-16 and HPV-18, it is the P_{97} and P_{105} promoters that are responsible for the E6 and E7 expression and it is these two promoters which can be repressed by the full-length E2 protein. In transfected primary human keratinocytes, each promoter had a basal activity that can be repressed by this product. Mutational analysis of the E2 binding sites in the LCR upstream of each promoter revealed that the E2 repression was mediated through the E2 binding sites proximal to each promoter. In the context of mutated E2 binding sites at the proximal promoter position, each of these promoters can be transactivated by E2. This

repression is primarily a transcriptional affect and it is likely that the interaction of E2 with promoter-proximal E2 binding sites will be important for the controlled expression of viral genes transcribed from these promoters in infected human genital epithelial cells. Manuscripts describing these data were published in The Journal of Virology and The New Biologist.

4. The human papillomavirus type 16 and 18 can immortalize primary human keratinocytes. The region of the viral genome responsible for this function mapped to the E6 and E7 genes and their respective upstream transcriptional regulatory sequences which are referred to as the long control regions (LCR). HPV-18 is more efficient in this immortalization function than HPV-16, and this function can be mapped to the LCR/E6/E7 regions of the genome. We have carried out a study designed to examine the basis for the difference in HPV-16 and HPV-18 immortalization efficiencies. The E6 and E7 genes of either HPV-16 or HPV-18, when expressed from the heterologous promoter, can immortalize primary human keratinocytes with approximately the same efficiency, suggesting that the difference in immortalization activities was not due to intrinsic properties of the E6 or E7 gene products themselves but rather to the transcriptional regulatory regions upstream of these transforming gene sequences. The analysis of the series of chimeric genes confirm this observation and further map the viral element responsible for the major difference in immortalization efficiency to the transcriptional regulatory region upstream of the E6 and E7 genes. A manuscript describing these data has been published in The Journal of Virology.

5. The tumorigenicity of the cervical carcinoma cell line (HeLa) can be suppressed by the addition of a normal human chromosome 11 in somatic cell hybrids as originally shown in Eric Stanbridge's laboratory at the University of California, Irvine. We have attempted to reproduce this phenomena by transfecting a cDNA expression library into a tumorigenic HeLa-fibroblast hybrid. A cell line was isolated which displayed morphologic features of the non-tumorigenic hybrids, demonstrated reduced tumorigenicity in nude mice, and showed an 85% reduction in alkaline phosphatase, a consistent marker of the tumorigenic phenotype in these cells. F2 contained a single exogenous cDNA which was recovered by PCR and designated HTS4 "hypothetical tumor suppressor." The HTS gene was shown to be expressed from human chromosome 11 by analysis of appropriate human hamster hybrid RNAs. A polymorphism was identified by sequencing the 3' terminal portion of the cDNA isolated from several lines which permitted the demonstration that one allele of the HTS gene had been deleted in two independent tumorigenic cell lines. Analysis of the parental cell lines of these hybrids suggested that the deleted segment derived from the tumorigenic parent. The results suggest that loss of information from the HeLa derived chromosome 11 may be a common feature of these tumorigenic segregants, and suggest that the HTS gene may play a role in expression of tumorigenicity in this system. A manuscript describing these data has been submitted for publication.

Publications:

Howley PM. The role of the human papillomaviruses in human cancer. Cancer Res 1991 (In Press).

Howley PM, Münger K, Werness BA, Phelps WC, Schlegel R. Molecular mechanisms of transformation by the human papillomaviruses. In: Knudson AG, Stanbridge EJ,Sugimura T, Terada M, Watanabe S, eds. Proceedings of the 20th international Princess Takamatsu cancer research symposium. Tokyo: Japan Scientific Society Press, 1990;199-206.

Romanczuk H, Thierry F, Howley PM. Mutational analysis of cis-elements involved in E2 modulation of human papillomavirus type 16 P_{97} and type 18 P_{105} promoters. J Virol 1990;64:2849-59.

Romanczuk H, Villa LL, Schlegel R, Howley PM. The viral transcriptional regulatory regions upstream of the E6 and E7 genes is a major determinant of the differential immortalization activities of human papillomavirus types 16 and 18. J Virol 1991;65:2739-44.

Scheffner M, Münger K, Byrne JC, Howley PM. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc Natl Acad Sci USA (In Press).

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus 16 and 18 promotes the degradation of p53. Cell 1990;63:1129-36.

Schiffman MH, Bauer HM, Lorincz AT, Manos MM, Byrne JC, Glass AG, Cadell DM, Howley PM. Comparison of southern blot hybridization and polymerase chain reaction methods for detection of human papillomavirus DNA. J Clin Microbiol 1991;29:573-7.

Thierry F, Howley PM. Functional analysis of E2 mediated repression of the HPV-18 P_{105} promoter. New Biol 1991;3:90-100.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05481-06 LTVB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biochemical Regulation of Tyrosine Protein Kinases

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

P.I.:	J.B. Bolen	Chief, Biochemical Oncology Section	LTVB	NCI
Others:	I.D. Horak	Medical Staff Fellow	MB	NCI
	P.A. Thompson	Medical Staff Fellow	LTVB	NCI
	J. Pyper	Senior Staff Fellow	LTVB	NCI
	E. Eiseman	IRTA Fellow	LTVB	NCI
	E. Horak	Microbiologist	LTVB	NCI
	A.L. Burkhardt	Microbiologist	MB	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Tumor Virus Biology

SECTION
Biochemical Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.8	3.0	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors

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

Signal transduction collectively encompasses the diverse biochemical reactions involved in modulating programmed cellular responses to external and internal stimuli. Among the cellular enzymes involved in this complex process, the tyrosine protein kinases appear to play key roles in mediating signaling events that aid in the regulation of cell growth and differentiation. We have shown that several members of the src family of non-receptor tyrosine protein kinase proto-oncogenes function as signaling components of diverse receptors in cells of hematopoietic origin.

PROJECT DESCRIPTION

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

J. B. Bolen	Chief, Biochemical Oncology Section	LTVB	NCI
I. D. Horak	Medical Staff Fellow	MB	NCI
P. A. Thompson	Medical Staff Fellow	LTVB	NCI
J. Pyper	Senior Staff Fellow	LTVB	NCI
E. Eiseman	IRTA Fellow	LTVB	NCI
E. M. Horak	Microbiologist	LTVB	NCI
A. L. Burkhardt	Microbiologist	MB	NCI
Z-H. Li	Visiting Fellow	MB	NCI

Objectives:

1. Mechanism of regulation of the src family of tyrosine protein kinase members in normal and transformed cells.

Methods Employed:

1. Standard recombinant DNA technology.
2. Cell culture.
3. Transcriptional analysis of RNA.
4. Construction and analysis of cDNAs.
5. Polymerase chain reaction.
6. Flow cytometry.
7. Generation of peptide-specific polyclonal antibodies.
8. Protein kinase analysis.
9. Immunoblot analysis.
10. Phosphoamino acid analysis.
11. Two-dimensional peptide analysis.

Major Findings:

Addition of interleukin-2 (IL-2) to IL-2 dependent T-cells results in tyrosine protein kinase signal transduction events even though the IL-2 receptor alpha and beta chains lack intrinsic enzymatic activity. We have found that addition of IL-2 to IL-2 dependent human T-cells transiently stimulates the specific activity of p56lck, a member of the src family of non-receptor tyrosine protein kinases expressed at high levels in T lymphocytes. The ability of IL-2 to induce p56lck activation was found to be independent of the capacity of p56lck to associate with either CD4 or CD8. Following IL-2 treatment, p56lck was found to undergo serine/threonine phosphorylation modifications which resulted in altered mobility of the lck gene product on polyacrylamide gels. These observations raise the possibility that p56lck participates in IL-2-mediated signal transduction events in T-cells.

Publications:

Bolen JB. Signal transduction by the src family of tyrosine protein kinases in hematopoietic cells. Cell Growth Dev (In Press).

Bolen JB, Thompson PA, Eiseman E, Horak ID. Expression and interactions of the src family of tyrosine protein kinases in T lymphocytes. Adv Cancer Res (In Press).

Burke TR Jr, Li Z-H, Bolen JB, Chapekar M, Gang Y, Glazer RI, Rice KC, Marquez VE. Examination of the possible mediation of antineoplastic effects of opiates through the inhibition of tyrosine-specific protein kinases. Biochem Pharmacol (In Press).

Burke TR Jr, Li Z-H, Bolen JB, Marquez VE. Structural influences of styryl-based inhibitors on epidermal growth factor receptor and p56lck tyrosine-specific protein kinases. Bioorg Med Chem Lett (In Press).

Burke TR Jr, Li Z-H, Bolen JB, Marquez VE. Phosphonate containing inhibitors of tyrosine protein kinases. J Med Chem (In Press).

Burkhardt AL, Bolen JB. Signal transduction by the src family of tyrosine protein kinases in lymphocytes. In: Brugge J, Curran T, Harlow E, McCormick F, eds. Symposium on the origins of human cancer. Cold Spring Harbor, Cold Spring Harbor Press (In Press).

Burkhardt A, Brunswick M, Bolen JB, Mond JJ. Anti-immunoglobulin stimulation of B lymphocytes activates src-related tyrosine protein kinases. Proc Natl Acad Sci USA (In Press).

Eiseman E, Bolen JB. src-Related tyrosine protein kinases as signaling components in hematopoietic cells. Cancer Cells 1990;2:303-10.

Horak ID, Gress RE, Lucas PJ, Horak EM, Waldmann TA, Bolen JB. T lymphocyte IL-2 dependent tyrosine protein kinase signal transduction involves the activation of p56lck. Proc Natl Acad Sci USA 1991;88:1996-2000.

Horak ID, Popovic M, Horak EM, Lucas PJ, Gress RE, June CH, Bolen JB. No T-cell tyrosine protein kinase signaling and calcium mobilization following CD4 association with HIV-1 and HIV-1 gp120. Nature 1990;348:557-60.

Huang MM, Bolen JB, Barnwell J, Shattil SJ, Brugge JS. Membrane glycoprotein IV (CD36) is tightly associated with the fyn, lyn, and yes protein tyrosine kinases in human platelets. Proc Natl Acad Sci USA (In Press).

Kassis S, Juszczak RJ, Turchin H, Truneh A, Bolen JB, Hanna N. Activation of protein kinase C results in the dissociation of p56lck from CD4 in human T lymphocytes. EMBO J (In Press).

Li Z-H, Burke TR Jr, Bolen JB. Analysis of styryl-based inhibitors of the lymphocyte tyrosine protein kinase p56lck. J Biol Chem (In Press).

Thompson PA, Biddison WE, Ledbetter JA, Bolen JB. The T-cell protein tyrosine kinase p56lck is activated during antigen presentation. J Immunol (In Press).

Veillette A, Sleckman BP, Ratnofsky S, Bolen JB, Burakoff SJ. The cytoplasmic domain of CD4 is required for stable association with the lymphoid-specific tyrosine protein kinase p56lck. Eur J Immunol 1990;20:1351-5.

Yi T, Bolen JB, Ihle JN. Hematopoietic cells express two forms of lyn kinase differing by 21 amino acids in the amino terminus. Mol Cell Biol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05482-06 LTVB

PERIOD COVERED
October 1, 1990 to September 30, 1991

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	Acting Chief, CRT Section	LTVB	NCI
Others:	P. A. Furth	Senior Staff Fellow	LTVB	NCI
	S. Barksdale	Medical Staff Fellow	LP	NCI

COOPERATING UNITS *(if any)*
National Institute of Diabetes and Digestive and Kidney Diseases, NIH (L. Hennighausen); Oak Ridge National Laboratories, Oak Ridge, TN (R. Woychik)

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:
2.0	2.0	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 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. We have used bovine papillomavirus type 1 (BPV-1) as a model system for the study of papillomavirus late gene expression and its control. Transcriptional mapping data indicates that the late mRNAs which encode the major and minor capsid proteins are expressed from a strong viral transcriptional promoter (called the late promoter) which is active only in productively infected epithelium. Late mRNAs are almost undetectable in nonproductively infected (transformed) cells. Analysis of BPV-1 mutants with deletion of most of the late region demonstrated that the BPV-1 late polyadenylation site can be efficiently utilized even in transformed cells. Eukaryotic expression vectors have also been used to show that the late poly(A) site is a more efficient poly(A) site than the early poly(A) site. The inefficient use of the late poly(A) site when it is positioned in its normal context 3 kb downstream of the early poly(A) site may be due partially to transcription termination or pausing which has been shown to occur between the early and late poly(A) sites in transformed cells and partially to a short inhibitory element in the late 3'UTR. This late 3'UTR element has been shown not to function by destabilizing late mRNAs as was previously thought and may function at an RNA processing or transport level. We also have evidence that BPV-1 poly(A) site choice could be regulated by splicing factors. BPV-1 late promoter transcripts can be spliced into acceptors at nt 3225 or 3605 and this splice site choice is differentially regulated in nonproductively and productively infected cells. We have shown, using transfection studies, that exon sequences between these two splice sites modulate the use of the splice donor at nt 3764. In addition, we have shown that efficient use of the nt 3764 splice site in the absence of these exon sequences can suppress polyadenylation at the early poly(A) site.

PROJECT DESCRIPTION

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

C. C. Baker	Acting Chief, CRT Section	LTVB	NCI
P. A. Furth	Senior Staff Fellow	LTVB	NCI
S. Barksdale	Medical Staff Fellow	LP	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 determine the viral and/or cellular factors involved in the trans-activation of the major late viral transcriptional promoter.
3. To identify the cis-acting sequence elements involved in the control of the major late promoter.
4. To construct eukaryotic expression vectors suitable for the identification and mapping of cis-acting regulatory elements which lie within and 3′ to transcription units.
5. To identify the cis-acting sequence elements in the late region of BPV-1 which may control late gene expression through transcription termination, polyadenylation, nuclear transport, and/or mRNA stability.
6. To identify the viral and/or cellular trans-acting factors which interact with late region sequence elements to control late gene expression.
7. To identify cis-acting elements which control BPV-1 alternative splicing.
8. To identify the trans-acting cellular and/or viral factors which regulate BPV-1 alternative splicing during keratinocyte differentiation.
9. To determine the mechanism by which poly(A) site choice is modulated by splice site regulation.

Methods Employed:

1. Tissue culture for the preparation of viral mRNA from non-productively 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 and polymerase chain reaction analysis, Northern blotting, primer extension, nuclease S1 protection and nuclear run-off analysis.
5. Construction of eukaryotic expression vectors using standard recombinant DNA technology.
6. Control element mapping by the generation of successive deletions in viral control regions subcloned into eukaryotic expression vectors using the exonuclease III deletion method.
7. 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.

8. Selection of stable cell lines containing recombinant expression vectors for the analysis of transcription termination by nuclear run-off analysis and for the analysis of mRNA stability by actinomycin-D chase analysis.
9. Preparation of crude nuclear and cytoplasmic extracts from HeLa cells, bovine fibropapillomas and BPV-1 transformed cells to assay for trans-acting factors in in vitro transcription and RNA processing systems and to identify DNA and RNA binding proteins.
10. Analysis of the effects of keratinocyte differentiation on papillomavirus late gene expression in transgenic mice.
11. Control element mapping by the generation of deletions in the BPV-1 genome, establishment of BPV-1 transformed cell lines, and analysis of BPV-1 transcription.
12. Construction of minigene expression plasmids for the analysis of BPV-1 RNA processing signals in vivo and in vitro.

Major Findings:

1. Bovine papillomavirus type 1 (BPV-1) virions contain multiple minor capsid (L2) proteins. We have identified two mRNAs from BPV-1 infected fibropapillomas which could encode the BPV-1 L2 protein. The polymerase chain reaction was used to demonstrate that these two mRNAs are most likely transcribed from the late promoter with splicing from the common late leader 5′ splice site at nt 7385 to 3′ splice sites at either nt 3225 or nt 3605. These mRNAs are capable of encoding only a single L2 gene product, however. Four monoclonal antibodies (MoAbs) which react exclusively with BPV-1 L2 protein epitopes were used in Western blots to compare the mass of BPV-1 minor capsid protein(s) purified from denatured virions by immunoaffinity chromatography with the BPV-1 L2 protein synthesized by in vitro translation. L2 proteins purified from denatured BPV-1 particles had apparent molecular weights of 76, 68, and 55-kDa. The in vitro synthesized gene product of the BPV-1 L2 open reading frame (ORF) had an apparent molecular weight of 76-kDa and was immunoprecipitated by all four anti-BPV-1 L2 MoAbs. On the basis of these studies, we concluded that the 76-kDa peptide represents the primary gene product of the BPV-1 L2 ORF. This work was carried out in collaboration with the laboratory of Dr. Bennett Jenson of the Department of Pathology, Georgetown University School of Medicine. A manuscript describing these results has been submitted to Virology.

2. Transcription of the 8 kb circular DNA genome of bovine papillomavirus type 1 (BPV-1) is unidirectional. During productive infection, the structural (late) genes, which lie immediately downstream of the early poly(A) site, are expressed from a late promoter which lies upstream of the early region near nt 7250. In nonproductively infected (transformed) mouse C127 cells, steady state levels of late region mRNAs are approximately four orders of magnitude lower than those of early region mRNAs. Previous studies from this laboratory showed that there is a 10- to 20-fold exponential decrease in the rate of BPV-1 transcription between the early and late poly(A) sites in BPV-1 transformed cells. A series of deletion mutations of various sizes have been made between the early and late poly(A) sites to determine if there are specific sequences which are required for transcription termination. Nuclear run-on analysis of cell lines harboring these BPV-1 mutants are in progress,

However, analysis of steady state levels of late cytoplasmic mRNAs from these cell lines indicates that the amount of late mRNA shows an exponential relationship with deletion size. This data is consistent with transcription termination between the early and late poly(A) sites having a direct effect on late poly(A) site usage and suggests that there are no specific sequences between the two poly(A) sites which are required for transcription termination. An alternative interpretation is that deletions within the late region decrease the time that the early poly(A) site is the only poly(A) site available on the nascent transcript and that transcription termination between the two poly(A) sites is the consequence of cleavage and polyadenylation at the early poly(A) site. Distinguishing between these two models is complicated by the fact that early poly(A) site mutants [E. Andrews and D. DiMaio, personal communication; Burnett et al., Plasmid 20:61-74, 1988] show a strong preference for use of cryptic poly(A) sites within the vicinity of the early poly(A) site rather than for use of the late poly(A) site. In contrast, the late poly(A) site is both efficiently and accurately utilized in nonproductively infected cells when it is positioned within 500 bp downstream of the early poly(A) site, and, in addition, is used almost exclusively when placed 472 bp upstream of the early poly(A) site. In these tandem poly(A) site constructions, use of the distal late poly(A) site is increased as the level of transcription increases, suggesting that the early to late shift in expression during the viral life cycle may be partially due to limiting polyadenylation factors required for utilization of the weaker early poly(A) site. A manuscript describing these results is in preparation.

3. Data from this lab suggests that there may be interactions between splicing and polyadenylation. In productively infected keratinocytes of the wart, mRNAs transcribed from the late promoter can be spliced from the late leader into 3′ splice sites at either nt 3225 or 3605 and can be polyadenylated at either the early poly(A) site (nt 4203) or the late poly(A) site (nt 7175). However, the majority of transcripts which are spliced into nt 3225 are polyadenylated at the early poly(A) site, whereas most transcripts spliced into nt 3605 are polyadenylated at the late poly(A) site, suggesting that splice site selection may determine poly(A) site choice. In addition, only transcripts which splice into nt 3605 can be further spliced from nt 3764 to nt 5609, removing the early poly(A) site. The 5′ splice site at nt 3764 (AAGGCAAGA) deviates from the consensus. To study the interactions of splicing and polyadenylation, two SV40 cDNA expression vectors were constructed based on the L2-L and L2-S mRNA structures, except that BPV-1 genomic sequences were substituted at the late poly(A) site. These vectors contain intact early and late polyadenylation regions as well as the entire late region and differ only by the inclusion of extra exon sequences between nt 3225 and 3605 in L2-L. Both vectors express abundant early mRNAs when transfected into CV-1 or COS-1 cells. The L2-L vector consistently produces extremely low levels of late mRNA and these late mRNAs are predominantly unspliced. In contrast, the L2-S vector produces significantly higher levels of late mRNA and these mRNAs are both spliced and unspliced. Mutation of the nt 3764 5′ splice site to a consensus splice site in the L2-L vector stimulates the production of both spliced and unspliced late mRNAs, although early mRNAs still greatly predominate over late mRNAs. The same mutation in

the L2-S vector almost completely blocks polyadenylation at the early poly(A) site, favoring synthesis of late mRNAs. The effect of this splice site mutation on polyadenylation occurs even in the absence of the 3′ splice site and effectively suppresses polyadenylation at poly(A) sites at least as far downstream as 900 nt. These data suggest that binding of spliceosomes or other factors at the nt 3764 5′ splice site can have a direct influence on polyadenylation at the early poly(A) site and that the efficiency of this splice site is modulated by exon sequences between nt 3225 and 3605. A manuscript describing these results is in preparation.

4. We have continued to explore the function of a 53 basepair fragment from the BPV-1 late 3′untranslated region (3′UTR) which was found to inhibit expression of CAT mRNA and CAT protein when cloned in the sense orientation in the 3′UTR of a pOBCAT expression plasmid. We investigated the function of this element on BPV-1 L1 expression by cloning a wild-type L1 expression vector (BPVL1) and a vector in which the 53 bp element was deleted (BPVL1Δ3′UTR). These expression vectors utilize the cytomegalovirus immediate early (CMVIE1) gene promoter and contain an L1 gene consisting of a fully spliced cDNA linked to 3′ genomic sequences which include the BPV-1 late polyadenylation signal and approximately 200 bp of downstream sequences. We found that deletion of this fragment from the late 3′UTR in a BPV-1 L1 expression vector resulted in a sixfold increase in levels of L1 mRNA. This difference was not due to an effect on cytoplasmic RNA stability as measured by actinomycin D chase experiments. We are currently investigating the possibilities that this element acts in the nucleus on stability and/or polyadenylation or on nuclear transport. A manuscript describing these results has been submitted to the Journal of Virology.

5. We continue to work on developing a permissive system to study BPV-1 late gene regulation. In order to introduce the BPV-1 late genes into fully differentiated skin, we have generated several lines of transgenic mice containing the BPV-1 genome in collaboration with Robert Wall, Ph.D. at the United States Department of Agriculture. We will now investigate BPV-1 expression in these transgenic mice.

In an effort to identify a strong promoter which we could utilize to study gene expression in terminally differentiated keratinocytes, we have investigated the tissue specific activity of the CMVIE1 promoter in transgenic mice. We have analyzed expression of a CMVIE1-CAT transgene in three lines of transgenic mice. These studies were done in collaboration with Lothar Hennighausen at NIH and Rick Woychik at the Oak Ridge National Laboratories.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05518-05 LTVB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transformation and Gene Regulation of the Hamster Papovavirus

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

PI:	J.B. Bolen	Chief, Biochemical Oncology Section	LTVB	NCI
Others:	P.M. Howley	Chief, LTVB	LTVB	NCI
	R. Levis	Guest Researcher	LTVB	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Tumor Virus Biology

SECTION
Biochemical Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.20	1.2	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.)*

Hamster polyomavirus (HaPV) is a small, circular DNA virus which belongs to the papovavirus family. HaPV was originally isolated from skin epitheliomas originating from hair follicle epithelial cells in Syrian golden hamsters. High levels of virus DNA replication and progeny virion production has been detected in epitheliomas of infected hamsters. In some infected animals, HaPV can also cause lymphomas. The viral genome is defective in lymphoid tumors and although there is a high level of DNA replication, no progeny virion are produced. The genome of HaPV has three distinct regions: the early protein coding region which codes for three proteins expressed directly after infection; the late protein coding region which codes for the three viral structural proteins; the noncoding region which contains all of the sequences which control transcription of both the early and late region messenger RNAs and sequences which affect viral DNA replication. Studies have been continued to elucidate the important biological roles for each of these regions in virus replication and cellular transformation.

PROJECT DESCRIPTION

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

J. B. Bolen	Chief, Biochemical Oncology Section	LTVB	NCI
P. M. Howley	Chief	LTVB	NCI
R. Levis	Guest Researcher	LTVB	NCI

Objectives:

1. To identify and characterize the HaPV encoded proteins required for oncogenic transformation and tumor formation in hamster and other rodent cells.
2. To identify and characterize the HaPV cis-acting elements responsible for the control of early and late gene expression in different types of rodent cells and to determine what trans-acting viral encoded proteins are required for HaPV gene expression.

Methods Employed:

1. Standard recombinant DNA technology.
2. Cell culture.
3. Transfer of DNA into rodent cells.
4. Transcriptional analysis of RNA.
5. Polymerase chain reaction.
6. Generation of viral fusion proteins and antibody production in rabbits.
7. Immunoprecipitation analysis and immunoblot analysis.

Major Findings:

Analysis of regulatory sequences in the noncoding region. The early and late coding regions of HaPV are divergent in the viral genome and are separated by sequences which act as the cis-acting regulatory sequences for controlling either early or late gene expression. Clones have been constructed in which the bacterial chloramphenicol acetyltransferase (CAT) gene has been inserted downstream from either the early or the late promoter such that the ATG from the CAT gene is in the same position as the ATG for the early or the late proteins. These clones were transfected into a variety of cell types and analyzed for basal levels of CAT activity. They were also transfected in the presence of the viral large T protein which is important for regulating early versus late protein expression and DNA replication. Although some differences were seen in the basal levels of CAT expression in different cell types, the results show that the level of expression from the early promoter is generally much lower than from the late promoter. The addition of the large T ORF to these transfection experiments inhibits CAT activity from the early promoter, presumably by affecting CAT message transcription. However, the presence of large T stimulates CAT activity from the late promoter, presumably due to the large T protein stimulating DNA replication. These results are consistent with promoter activity in the presence or absence of large T for other polyomaviruses. Further analyses are being done to determine the trans-affect that the large T protein is having on the early and late promoters and whether

or not the protein is regulating transcription or replication. Deletions and mutations are also being constructed in the noncoding region to identify cis-acting sequences which are important for both early and late gene expression.

Analysis of virus replication in lymphoid cells. HaPV causes lymphomas in infected animals. It has not yet been determined whether these tumors are of B- or T-cell origin. To address this question, the HaPV genome has been transfected into two factor dependent T-cell lines, HT2 cells and CTLL2 cells. Analysis of protein expression in the T-cell lines show that early after transfection (two days) both early and late proteins are being expressed. However, at 21 days after transfection late proteins are no longer being expressed and the level of early genes being expressed is much higher. This suggests that, as is seen in the animal, the viral genome has become defective and can no longer express the late genes. Further analysis of these cells will include whether the cells become transformed, what the state of the viral genome is in these cells, and whether cellular proteins are associated with viral proteins or the viral genome. Similar experiments are being carried out in a factor independent, murine B-cell line, M12 cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05662-01 LTVB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Characterization of the Papillomavirus Regulatory Proteins

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

P.I.:	A. A. McBride	Visiting Associate	LTVB	NCI
Others:	T. R. Sarafi	Biologist	LTVB	NCI
	P. L. Winokur	Biotechnology Fellow	LTVB	NCI
	P. M. Howley	Chief	LTVB	NCI

COOPERATING UNITS *(if any)*

National Institute of Child Health and Human Development, NIH (R. Klausner)

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	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 E2 genes of the papillomaviruses encode polypeptides important for viral DNA replication and transcriptional regulation. The E2 open reading frame of the bovine papillomavirus type 1 encodes at least three polypeptides. The full-length gene product functions as a transcriptional transactivator and is required for episomal DNA replication. The 3′ half of the open reading frame encodes two smaller polypeptides that repress E2-mediated transactivation. All three E2 proteins share a common carboxyl-terminal domain that encodes a specific DNA binding function and which contains sequences which promote E2 dimer formation. An amino terminal domain, which is unique to the full-length transactivator polypeptide, encodes the transactivation domain. Less is known about the regions of the E2 protein important for DNA replication. We have previously demonstrated that the BPV-1 E2 polypeptides are phosphorylated primarily on two serine residues at a site adjacent to the DNA binding domain. We have mutated these residues and have shown that viruses containing substitutions at serine residue 301 in the E2 polypeptide replicate to a copy number that is much greater than that of the wild-type genome. It has recently been demonstrated that both the full-length E2 polypeptide and the E1 polypeptide are both necessary and sufficient for BPV-1 replication and that these polypeptides interact to form a complex. An attractive model is that phosphorylation could regulate the formation of this complex and we are currently investigating this hypothesis and analyzing which regions of the E2 polypeptide are important for replication and complex formation. In addition, we are examining which protein kinase is responsible for the phosphorylation of the serine residue at position 301. We have demonstrated that DNA binding can be regulated in vitro by oxidation reduction (redox) of a single highly conserved cysteine residue in each subunit of the DNA binding domain. E2 polypeptides containing mutations in this highly conserved residue are still able to bind DNA but are defective in transactivation. We are currently investigating the defect in these mutated polypeptides.

PROJECT DESCRIPTION

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

A.A. McBride	Visiting Associate	LTVB	NCI
T.R. Sarafi	Biologist	LTVB	NCI
P.L. Winokur	Biotechnology Fellow	LTVB	NCI
P.M. Howley	Chief	LTVB	NCI
R.D. Klausner	Chief	CBMB	NICHHD

Objectives:

1. To characterize the viral gene products involved in the transcriptional regulation and replication of the bovine papillomavirus.
2. To determine the role of phosphorylation of the viral E2 gene products.
3. To determine how E2 phosphorylation is regulated and identify the kinases and phosphatases involved.
4. To analyze the interaction between the BPV E1 and E2 polypeptides and to determine which regions of these proteins are important for formation of this complex and for viral DNA replication.
5. To identify cellular and/or viral proteins which interact with the BPV E2 polypeptides.
6. To identify amino acid residues in the E2 C-terminal domain important for sequence specific DNA binding and/or dimerization.
7. To analyze redox regulation of the E2 polypeptides.
8. To identify residues in the E2 polypeptides important for transactivation and replication functions.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of papillomavirus mutant and hybrid plasmids.
2. Tissue culture techniques including several gene transfer techniques and focus formation assays.
3. Expression of papillomavirus proteins in vitro and from recombinant viruses such as baculovirus and SV40.
4. Expression of papillomavirus proteins in bacteria for use both in biochemical studies and for the production of antisera.
4. Analysis of viral proteins by immunoprecipitation, immunoblotting, immunofluorescence and 2D gel electrophoresis.
5. Analysis of viral transcription by Northern blot analysis.
6. Analysis of viral DNA replication by transient replication assays and Southern blot analysis.
7. Analysis of transcriptional transactivation and repression using transient transfection assays and CAT assays.
8. Analysis of DNA binding using mobility shift assays and DNA-protein immunoprecipitation assays.
9. Standard biochemical techniques for characterizing proteins.

Major findings:

1. We have previously demonstrated that the major phosphorylation sites of the BPV-1 E2 polypeptides are two serine residues located in a region adjacent to the carboxyl-terminal DNA binding domain, which is present in all three E2 proteins. BPV-1 genomes mutated in these residues have been analyzed for focus formation, viral gene expression and extrachromosomal viral DNA replication. Viruses containing amino acid substitutions at serine residue 301 in the E2 polypeptide replicate to a copy number that is much greater than that of the wild-type genome. Several approaches are being used to determine whether this phenotype results from a direct role of E2 phosphorylation in the regulation of DNA replication or is due to an indirect effect on viral gene expression. It has recently been demonstrated that both the full-length E2 polypeptide and the E1 polypeptide are both necessary and sufficient for BPV-1 replication and that these polypeptides interact to form a complex. An attractive model is that phosphorylation could regulate the formation of this complex and we are currently investigating this hypothesis. In addition we are examining which protein kinase is responsible for the phosphorylation of the serine residue at position 301.

2. Little is known about the regions of the E2 polypeptide important for DNA replication and interaction with the E1 replication protein. Preliminary studies show that E2 polypeptides that are unable to bind DNA are incapable of supporting DNA replication. Using a number of other existing E2 mutations, these studies are being extended to determine which regions of the E2 gene product are important for the interaction with the E1 polypeptide and for episomal DNA replication.

3. The C-terminal domain of the E2 polypeptides does not contain any of the common DNA binding or dimerization motifs such as zinc fingers, helix-loop-helix structures or leucine zippers. We have demonstrated that DNA binding can be regulated *in vitro* by oxidation-reduction (redox) of a single highly conserved cysteine residue in each subunit of the DNA binding domain. E2 polypeptides containing mutations in this highly conserved residue are still able to bind DNA but are unable to activate transcription from certain enhancer promoter configurations *in vivo*. We are currently investigating the defect in these mutated polypeptides.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05663-01 LTVB

PERIOD COVERED
October 1, 1990 to September 30, 1991

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

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

PI:	B. Spalholz	Senior Staff Fellow	LTVB	NCI
Others:	J. Quintero	Biologist	LTVB	NCI
	S. Van de Pol	Biotechnology Fellow	LTVB	NCI

COOPERATING UNITS *(if any)*

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:
2.2	1.2	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 papillomaviruses are widespread in nature and are generally associated with benign epithelial and fibroepithelial lesions in their natural hosts, including humans. Although there is no system to study papillomavirus infection in tissue culture, bovine papillomavirus type 1 (BPV-1) has provided an excellent model system for a systematic study of the molecular biology of the papillomaviruses. BPV-1 readily transforms a variety of rodent cells in tissue culture. In these transformed cells, viral genes are expressed which are responsible for the transformed state as well as for other functions such as viral DNA replication and episomal maintenance of the viral genome. Our studies have been designed to focus on the molecular biology of the BPV-1/host cell interaction. Critical to the investigation of this interaction is the identification of the promoters responsible for the expression of the various viral gene products and a characterization of the elements which regulate expression from those promoters. We have previously described the important role the viral E2 gene products play in viral gene expression and have now shown that many of the BPV-1 promoters are regulated by these products, including the promoters responsible for expression of the E2 gene products themselves. Similarly, we have begun to identify cellular factors which also play a role in regulating viral gene expression and have found that the ubiquitous transcription factor SP1 plays in important role. Equally important as transcriptional control in understanding BPV/host cell interactions are the mechanisms controlling viral replication, particularly as preliminary localization of replicating cis elements map very near major transcriptional elements. Characterization of the elements involved in viral replication is therefore also underway.

PROJECT DESCRIPTION

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

B.A. Spalholz	Senior Staff Fellow	LTVB	NCI
S. Van de Pol	Biotechnology Fellow	LTVB	NCI
J. Quintero	Biologist	LTVB	NCI

Objectives:

1. To localize and characterize the BPV-1 transcriptional elements in the viral genome.
2. To identify the specific transcriptional promoters responsible for expression of particular viral genes.
3. To identify viral and cellular factors which interact with the transcriptional elements to determine patterns of viral gene expression.
4. To identify *cis* elements required for the replication of the viral genome and to identify the factors with which they interact.
5. To investigate the interdependence of viral replication and transcriptional mechanisms.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of papillomavirus containing plasmids.
2. Tissue culture techniques involving established cell lines, primary cultures, and morphological assays for cellular transformation.
3. Standard calcium phosphate coprecipitation and electroporation transfection technology.
4. DNA analysis including Southern blotting and DNA sequencing.
5. Northern blot, primer extension and S1 endonuclease protection analyses.
6. Analysis of transient viral replication by DpnI resistance assays.
7. Immunoprecipitation and western blotting for the detection of proteins.
8. DNA-protein interaction analysis including, mobility shift experiments, McKay assays, and DNase footprinting.

Major Findings:

Transcriptional transactivation and repression by the viral E2 proteins are important regulatory mechanisms for the papillomaviruses. We have now demonstrated that the BPV-1 P2443 promoter is transactivated by E2-responsive elements in the long control region (LCR). Our characterization of the *cis* elements involved in P2443 regulation indicated that the single E2-binding site directly upstream of P2443 was not required for either the E2 transactivation or for any E2 repression of the basal or transactivated activity of this promoter. Therefore, we feel that cooperative interactions bewteen E2 bound 2000 nucleotides upstream in the LCR and E2 bound near P2443 do not have any role in the regulation of this promoter. Further definition of the *cis* regulatory elements of this promoter indicated that a binding site for

the transcriptional factor SP1 exists directly upstream of the P2443 TATA box and is critical for the basal and transactivated levels of transcription from this promoter. These data were published in The Journal of Virology. Disruption of this SP1 site in the background of the viral genome confirmed the importance of this promoter in the expression of viral functions. This promoter was suspected to be the major promoter for the E2 transactivator and the E5 transforming genes. Mutation of this site led to a loss in transforming ability and in all full-length E2 dependent functions, including replication functions and transcriptional transactivation properties. These results confirmed that the P2443 promoter is the major promoter responsible for the expression of the E2 and E5 genes.

Publications:

Spalholz BA, Vande Pol S, Howley PM. Characterization of the cis elements involved in basal and E2-transactivated expression of the bovine papillomavirus P2443 promoter. J Virol 1991;65:743-53.

ANNUAL REPORT OF

THE LABORATORY OF VIRAL CARCINOGENESIS
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

FUNCTIONAL STATEMENT: The Laboratory of Viral Carcinogenesis (LVC) has as its charge 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. 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. In addition, the combined maturation of human genetics and molecular biology of infectious disease have permitted the implementation of experiments designed to study interaction of viruses and genetic structure of human populations. 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 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. (2) The normal functional role of several of these oncogenes during tissue development has recently been elucidated in several systems, as well as a role in signal transduction, phosphorylation and in stimulation of cellular differentiation or proliferation. (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 6,000 different loci comprising the human genetic map. In addition to the proto-oncogene loci, recessive tumor suppressors and additional described loci are thought to participate in neoplastic transformation in man (e.g., growth factors, cell surface antigens, retroviral receptors, 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) In the aftermath of a long history of unsuccessful attempts to isolate human tumorigenic viruses, we are now in a position to recognize viral etiologies for several very important human neoplasias: namely, HIV-associated acquired immune deficiency syndrome; papillomavirus-associated cervical carcinoma; HTLV-I-associated adult T-cell leukemia-tropical spastic paralysis; and hepatitis B virus, the primary etiologic agent for development of heptocellular carcinoma. (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 immunodeficiency syndrome [SAIDS]) and in FIV-plus feline leukemia virus-infected cats (feline acquired immunodeficiency 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. Development of a universal genetic method for tracking the progress of physical, genetic and sequence mapping of the Human Genome Initiative. Much of the data collected by the Human Genome Project (HGP) is designed to augment the current status of the human gene map. However, there are many types of maps of human genetic information, and there is no universal coordinate system for comparison or quantification of the various mapping efforts. Furthermore, the goals set forth by the various agencies coordinating the HGP have not been translated into targets that can be quantified. We have developed a means of quantifying progress toward a complete, integrated map of the human genome. Current estimates of the total number of loci, the number of base pairs (bp), and the relative lengths of chromosomes and their bands are very crude. The algorithms employed in our quantitative analysis were designed to be flexible for anticipated improvements in each of these parameters. Analyses of the current status of the HGP were summarized graphically and specifically and will be updated annually by invitation from Science.

2. Application of high resolution restriction fragment length polymorphism (RFLP) methods to detecting heritable mutants in human inborn errors and in candidate genes for pathogen resistance. The field of human genetics has been revolutionized by the development of a large collection of RFLPs. Even though there are now 2300 RFLPs characterized, there are only 500 polymorphisms in coding genes. Thus, there is a need to develop new methods to allow polymorphisms to be characterized in specific genes. We have characterized a number of polymorphisms in biologically important human genes using both standard approaches, as well as new methods that employ the polymerase chain reaction (PCR). Polymorphisms have been described in a gene that alters the morphology of papillomavirus transformed cells (p1596); and the immunoglobulin Fc receptor, which can also serve as a receptor for HIV. The PCR has been employed to amplify specific regions of genes, such as the introns or the 3' untranslated region. The resulting DNA products are then assayed for variation by one of four approaches: (1) digestion with frequently cutting (4 bp recognition site) enzymes; (2) analysis of simple sequence (microsatellite)

loci on high resolution gels; (3) single-stranded conformation polymorphism (SSCP) analysis, a method for detecting single bp changes in DNA; or (4) heteroduplex analysis, a method developed by this project for detecting sequence variation. Using these approaches we have been able to identify and characterize polymorphisms in several different genes including the KIT oncogene, the insulin-like growth factor receptor 1, the dopamine D2 receptor, the gamma aminobutyric acid receptor and the cystic fibrosis, CFTR, gene. All of these polymorphisms have been typed in the 40 large human pedigrees provided by the Centre D'Etude du Polymorphisme Humain for the construction of human genetic linkage maps. These methods should allow virtually any human gene to be developed as a genetic marker, and should greatly increase the possibility for understanding complex human disorders with at least a partial genetic basis.

3. Development of high resolution DNA typing of HLA class I and class II loci. HLA typing at the DNA level is capable of delineating more extensive allelic polymorphisms than those detected by serology. Using PCR technology, consensus primers, and ASO probes, the distribution of 8 alleles at the DQα locus and 13 alleles of DQβ were determined in the HIV-1-infected homosexual male populations. Several new techniques and strategies for HLA DNA typing have been developed. A method for delineating the various alleles (and potential new alleles) was developed using single-stranded conformational polymorphism. Heteroduplex formation of amplified alleles was found to be capable of discriminating non-major histocompatibility complex (MHC) identity not detected by serology. A technique for DNA HLA typing was developed using serum and plasma as source material.

4. HLA haplotype is associated with progression to AIDS-related Kaposi's sarcoma. The HLA-B35, Cw4, DR1, and DQ1 alleles, as determined by serologic methods, were elevated in frequency in 77 individuals with the diagnosis of Kaposi's sarcoma, relative to the frequency of these alleles in controls consisting of HIV-1 seropositive individuals without disease (462 homosexual men). In analysis of disease progression defined by years from seroconversion to decline of CD4+ cells below 20%, individuals with HLA-DR1 had more rapid disease progression relative to individuals without this phenotype, while CD4+ cell loss was less rapid in individuals with the HLA-DR7 antigens.

5. Discovery of the pivotal selective role that infectious disease plays in generation and maintenance of antigenic diversity in mammalian species. The MHC has been studied using molecular techniques as an approach to comparative genome organization of this important gene cluster. The MHC in most mammals consists of two classes of genes, classes I and II, which play special roles in presenting antigens to T-cell receptors. Comparative sequence analysis of MHC revealed strong conservation of invariant and variant amino acid residues in antigen-binding and T-cell recognition sites among human, mouse, and domestic cat. The pattern and mode of genetic variation of sequence domains revealed the participation of four major factors in the evolution of the feline MHC class I genes. These include: (1) a gradual accumulation of spontaneous mutational substitution, (2) negative selection for functional constraints of class I genes, (3) positive selection in favor of persistence of polymorphism, and (4) periodic intragenic and intergenic DNA recombination. Sequence analysis of MHC class I molecules from two other feline species (cheetah and ocelot) showed that feline MHC class I molecules have highly mosaic structures in their entire coding regions. We identify at least 15 mosaic regions where each has at least two polymorphic sequence motifs. Some of these sequence motifs are conserved not only in Felidae MHC class I sequences but also in some of human, orangutan, and bovine MHC class I sequences. These data revealed that modern polymorphic sequence motifs found in mammalian MHC class I molecules are extremely ancient and shuffling of

these motifs by DNA recombination plays a pivotal role in producing novel polymorphic MHC class I molecules in mammals.

6. The raf proto-oncogene is frequently altered in mouse and human lung carcinoma and as such can be considered as a potential diagnostic target. We have focused on Raf-family proto-oncogenes because of their original isolation in this lab and because their behavior in the mouse suggested to us a potential involvement in human lung cancer, the most frequent cancer in man. We have discovered that the c-raf-1 gene is selectively and consistently mutated in a mouse model for lung cancer and is also frequently mutated in human lung carcinoma in which they are also overexpressed. Overexpression can contribute to malignant transformation, as we have found that wild-type forms of raf and ras synergize in transformation. Our results demonstrated that Raf-1 protein kinase is an essential component of growth signaling pathways used by many growth factors and from this position controls the mitogenic and oncogenic signal flow of most oncogenes. We have therefore begun to develop Raf-1-based growth-inhibitory regimens, including antisense vectors and oligonucleotides, as well as vaccine trials in animal models with promising albeit preliminary results. This strategy runs counter to the prevailing dogma of immunology, which holds that altered intra-cellular proteins do not trigger attack of the mutant cell by the immune system.

7. A candidate transformation-associated sequence isolate from human nasopharyngeal carcinoma (NPC) cells. A novel transformation-associated sequence unrelated to any known oncogene has been cloned from human NPC cells by human Alu screening of an NPC/JB6 transfectant genomic library. This cloned sequence transfers transforming activity to JB6 mouse epidermal cells. A hybridizing mRNA of 1.7 kilobase (kb) is observed in NPC cells but not in mouse recipient cells. Sequencing of both the genomic clone and a recently isolated cDNA reveals an open reading frame unrelated to any known oncogene, promising a novel NPC oncogene. In addition, two independent NPC cell lines show the same expressed mutation in one of the "hot spots" of the tumor suppressor gene p53, thus appearing to activate it to an oncogene. Additional NPC samples and paired adjacent tissue from Taiwanese patients are being provided to us for examination of both the newly cloned NPC transforming gene and the p53 gene for transformation-relevant mutations.

8. Protein kinase C substrate is altered with respect to phosphorylation in JB6 mouse epidermal cells that have distinct sensitivities to tumor promotors. A putative C-kinase substrate of 80 kiloDaltons (kDa) has been found to be differentially phosphorylated in promotion-resistant (P-), -sensitive (P+), and neoplastically transformed JB6 mouse epidermal cells, with little or no phosphorylated 80-kDa phosphoprotein (pp80) seen in transformed cells. Western analysis indicates that p80 is regulated at the level of synthesis with little or no p80 protein detectable in transformed JB6 cells. A cDNA clone of p80 has recently been isolated by screening a JB6 P- library with p80 antibody. This p80 cDNA, when used as a probe, detects a 2.8-kb RNA that progressively decreases during the progression from P- to the transformed phenotype in certain cases but does not generalize to multiple independent cells of each phenotype. When compared with GenBank the sequence emerges as novel. The relationship between this locus and the inducible AP-1-regulated gene expression in promotion-sensitive JB6 cells is under investigation.

9. Progress in evaluating vaccine trials and disease progression of SAIDS. Three separate recombinant or peptide vaccines have been shown to protect macaques from an intravenous challenge with simian immunodeficiency virus (SIV) or type-D retroviruses (SRV-2). Macaques immunized with a vaccinia virus expressing the envelope (env) proteins of our molecular clone of SRV-2/WASHINGTON were protected against a homologous challenge with 10^6 infectious

particles of SRV-2. Four SIV peptides representing highly conserved and seroprevalent regions of HIV-1 gp120 and gp41 were mixed and used as immunogens in three rhesus macaques. The two animals with the highest neutralizing titers resisted a challenge of 100 infectious particles of SIV. In a third experiment, macaques have also been immunized with two doses of a vaccinia virus expressing the envelope proteins of our pathogenic molecular clone of SIV/Mne. After being boosted with 10 animal-infectious doses of SIV/Mne. A molecular clone derived from SIV/Mne-infected HuT 78 cells has been completely sequenced and shown to be pathogenic in macaques. We have followed the sequence variation in the env gene as a function of time after infection. Two regions of env within the variable regions V1 and V4 showed changes in up to 40% of their amino acids when AIDS became apparent. The region of SIV env that corresponds to the immunodominant V3 loop of HIV was conserved. Analysis of the biological properties of these variant env proteins will be useful in defining the mechanisms underlying progression to AIDS.

10. Resolution of functional interaction of HIV and lentivirus tat and tar genes in disease progression. The lentiviruses, equine infectious anemia virus (EIAV) and HIV, encode transcriptional trans-activating proteins (Tat) that bind to a specific hairpin structure at the 5′ end of nascent RNA (TAR) which positions them for interactions with the transcription apparatus. Thus, Tat proteins are predicted to contain an RNA-binding domain and an "activation" (or protein interaction) domain. The EIAV and HIV-1 Tat proteins (E-Tat and H-Tat, respectively) contain two conserved peptide motifs but are otherwise quite dissimilar and do not interact with the heterologous TAR element. We have addressed the problems of structure, function and mechanism of Tat proteins by exchanging domains between E-Tat and H-Tat and by tethering Tat sequences to an RNA operator via a bacteriophage RNA-binding protein. These strategies allowed us to define both the TAR-interaction and activation domains of each Tat protein. Both E-Tat and H-Tat appear to be simple, modular proteins in which the N-terminal half contains the activation domain and the C-terminal half is responsible for TAR binding. The activation domains of both proteins contain a highly conserved core sequence; the E-Tat activation domain consists of only 15 amino acids whereas the H-Tat activation domain is more complex and is composed of 47 amino acids. The activation domains of the two proteins are interchangeable and competition experiments suggest that they interact with a common cellular factor. The TAR-binding domains of both proteins contain a cluster of basic amino acids; in H-Tat this sequence of about 10 residues is sufficient for RNA binding, while E-Tat requires 26 residues for TAR binding. In E-Tat, this region may be highly structured to form an RNA-binding pocket.

11. Successful production of "test tube" offspring of non-domestic large felid species by in vitro fertilization. The empirical development of artificial methods for reproduction in the cat model has led to the first birth of large felid (tiger) cubs by in vitro fertilization. Improvements in reproductive physiology have occurred in the following areas: (1) in vitro fertilization and embryo development in vitro and in vivo; (2) artificial insemination (AI) via laparoscopic deposition of spermatozoa; and (3) oocyte rescue, maturation in vitro and the development of gene delivery techniques into embryos which will allow studying the mechanisms associated with transformation and inborn errors in early development. Progress to date has allowed (1) the routine production of embryos in domestic and nondomestic cats by in vitro fertilization; (2) the routine culture of embryos to the morula stage in vitro; (3) determining the biological competence of in vitro produced embryos from both domestic and nondomestic felid species; (4) the successful development of an efficient transabdominal AI approach; (5) in vitro maturation of immature, antral oocytes from both domestic and nondomestic

felid species and the fertilization and development of these oocytes *in vitro*; and (6) the discovery that the oocytes of various felid species have no mechanism for excluding penetration by heterologous or "foreign" spermatozoa.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05326-09 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
HLA Antigens: Structure, Function, and Disease Association

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

PI:	Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
Others:	William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
	James Goedert	Coordinator, AIDS Working Group	EEB	NCI
	Stephen J. O'Brien	Chief	LVC	NCI
	Patricia Martin	Visiting Fellow	LVC	NCI

COOPERATING UNITS *(if any)*
Cell Biology and Immunology, Stanford University, Stanford, CA (P. Parham); PRI/DynCorp, Frederick, MD (M. Carrington, M. O'Donnell, T. Miller)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	0.9	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.)*

Studies are being carried out to determine the influence of major histocompatibility complex (MHC) genes/gene products (HLA antigens) on disease progression and outcome in individuals infected with retroviruses. The cohorts infected with HIV-1 consist of 2 groups: homosexual men and hemophiliacs. The HLA phenotype of individuals in these cohorts was determined by two methods: serology, which identifies the allelic products of the genes at the various loci, and allele specific oligonucleotides (ASO), which identifies the various alleles at the DNA level. Certain HLA phenotypes, as determined by serologic methods, were elevated in frequency in 77 individuals with the diagnosis of Kaposi's sarcoma, relative to controls consisting of HIV-1 seropositive individuals without disease (462 homosexual men). In analysis of disease progression defined by years from seroconversion to decline of CD4+ cells below 20%, individuals with HLA-DR1 had more rapid disease progression relative to individuals without this phenotype, while CD4+ cell loss was less rapid in individuals with the HLA-DR7 antigens. HLA typing at the DNA level is capable of delineating more extensive allelic polymorphisms than those detected by serology. Using polymerase chain reaction technology, consensous primers, and ASO probes, the distribution of 8 alleles at the DQα locus and 13 alleles of DQβ were determined in the HIV-1-infected homosexual male populations. Several new techniques and strategies for HLA DNA typing have been developed. A method for delineating the various alleles (and potential new alleles) was developed using single-strand conformational polymorphism. Heteroduplex formation of amplified alleles was found to be capable of discriminating non-MHC identity not detected by serology. A technique for DNA HLA typing was developed using serum and plasma as source materials.

Project Description

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

Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
James Goedert	Coordinator, AIDS Working Group	EEB	NCI
Stephen J. O'Brien	Chief	LVC	NCI
Patricia Martin	Visiting Fellow	LVC	NCI

Objectives:

To determine the function, structure, and disease association of major histocompatibility complex (MHC) genes and/or their products. The studies are directed at elucidation of the phenotypic and genetic associations and potential genetic control of the immune response as it relates to disease processes and disease outcome. In order to accomplish these objectives, it is necessary to develop methods of HLA typing at the DNA level that are applicable to determining HLA alleles in individuals in populations where subtle differences in DNA sequences occur in different racial/ethnic groups. In addition, techniques that utilize non-tissue sources of DNA need to be developed.

Methods Employed:

The standard serologic microcytotoxicity techniques were used to detect 19 alleles at the A locus, 30 alleles at the B locus, 8 alleles at the C locus, 14 alleles at the DR locus, and 4 alleles at the DQ locus. Having assigned alleles to the patient population, analysis of phenotype frequencies in the various disease outcome categories and rates of disease progression was analyzed by χ^2 and non-parametric statistics. A large body of DNA sequence data is available on the MHC class II genes. Polymorphic regions in the second exon dictate allelic heterogeneity. Using consensous DNA primers, these polymorphic regions are amplified by polymerase chain reaction (PCR) technology. Oligonucleotides with DNA sequences for specific alleles are used as probes to identify the constitutive alleles of the individuals in a slot blot format.

In the single-strand conformation polymorphism technique, amplified DNA labeled with ^{32}P was denatured and electrophoresed on nondenaturing polyacrylamide gels allowing single strands to fold into their sequence-specific secondary structure. The gels are dried and exposed to x-ray film and the film is developed for analysis. Amplified ^{32}P-labeled DNA was also used in a heteroduplex assay to identify single to multiple base changes in allele sequences. The DNA was electrophoresed on denaturing polyacrylamide gels and analyzed by methods described above. Sera and plasma (0.5 to 1.0 ml) were centrifuged at high speed. Pelleted material was suspended in sodium dodecyl sulfate (SDS) proteinase K and allowed to stand overnight. After phenol-chloroform extraction, the aqueous fraction was concentrated and a portion used to amplify products of HLA class II loci. The individual alleles were identified by the techniques described above.

Major Findings:

The frequency of individual HLA antigens was determined in the various cohorts and was analyzed relative to disease diagnosis outcome and rate of disease progression. No significant differences were found comparing antigen frequencies in 460 HIV-1 seropositive with 130 seronegative homosexual men. Likewise, no differences were found in 250 HIV-1 seronegative compared to frequencies in 970 seropositive hemophiliacs. Furthermore, there were no significant differences in HLA antigen frequencies in the disease prevalent vs. the disease incident groups. We also examined the HLA antigen frequencies in the total study population and compared them to the published antigen frequencies of North American Caucasians (consisting of approximately 1,500 individuals). Again, no significant differences were found. These comparisons are indicative that the population under study is representative of the HLA gene pool insofar as can be identified by serologic techniques. With this background information we examined the HLA antigen frequencies to determine possible associations with specific disease outcome. Analysis of the HLA frequencies in 77 individuals with a diagnosis of Kaposi's sarcoma, a disease found only in HIV-1 homosexual men and not in HIV-1-positive hemophiliacs, revealed statistically significant differences in the following HLA antigen frequencies: HLA-B35, HLA-C4, DR1, and DQ1 compared to the frequencies of these antigens in the at-risk population. These same antigens remained increased in frequency when an additional 64 individuals with Kaposi's sarcoma with another AIDS diagnosis were added to the groups for analysis.

A large body of data indicates that the loss of circulating $CD4^+$ cells is indicative of disease progression. The association of one or more HLA antigens with loss of $CD4^+$ T cells was analyzed by non-parametric statistics with the parameter of time from seroconversion to decrease in $CD4^+$ cells below 20% compared to HLA phenotype. The maximum follow-up for 122 HIV-1-positive homosexual men was 9 years. $CD4^+$ cells declined more rapidly in individuals with HLA-A24 or HLA-DR1 compared to those without these antigens. Individuals with HLA-B57 or HLA-DR7 had significantly less rapid decline in $CD4^+$ T cells relative to the decline in individuals without this antigen.

The technique using single-strand conformational polymorphisms was developed to rapidly identify the 8 HLA-DQα and 14 DQβ alleles. This technique has proven useful in identifying polymorphisms heretofore not recognized by other DNA typing techniques. Alleles of HLA-DQβ2 were identified for the first time. The heteroduplex technique was used to confirm homozygosity in cells with indeterminant typing by serology and oligonucleotides. This technique was used to identify a recombinant within the HLA complex that was not recognized by other techniques. Isolation of DNA from serum or plasma PCR amplification and oligonucleotide typing was successful with as little as 0.5 ml volumes. Development of this technique will have a significant impact on our ability to type HIV-1-infected individuals who have died of AIDS, where serum samples remain the only potential source of DNA. HLA class I antigen, heretofore described as a product of activated T cells, was found to be a classical HLA gene product. Sequence analysis of this product identified this as a newly defined antigen in the black population.

Publications:

Madrigal JA, Belich MP, Little AM, Hildebrand WH, Mann DL, Parham P. The LA45 antigen of activated T cells is a product of classical HLA genes: the LA45 gene is HLA-Aw66. J Exp Med (In Press).

Mann DL, Murray C, O'Donnell M, Blattner WA, Goedert JJ. HLA antigen frequencies in HIV-1 related Kaposi's sarcoma. J AIDS 1990;3(suppl 1):51-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05328-09 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunologic Studies of the Human T-Cell Lymphoma Virus

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

PI:	Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
Others:	William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
	Patricia Martin	Visiting Fellow	LVC	NCI

COOPERATING UNITS *(if any)*
PRI/DynCorp, Frederick, MD (G. Hamlin-Green, K. Miller)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	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 human T-cell leukemia/lymphoma virus, HTLV-I, is thought to be directly associated with adult T-cell leukemia (ATL) and indirectly associated with other malignancies. More recently this virus has been shown to be associated with the neurologic disease, tropic spastic paraparesis (TSP). Studies are underway to examine the mechanism whereby the same virus appears to contribute to different types of diseases. Peripheral blood lymphocytes from HTLV-I seropositive individuals with the neurologic disease have been shown to undergo spontaneous proliferation when placed in culture. We have undertaken experiments to assess the nature of the lymphocyte population that is proliferating and the mechanism whereby this response is induced. Lymphocytes from HTLV-I-infected and noninfected individuals were tested prior to and after 6 days of culture for cell-surface markers that define activated lymphocyte subpopulations based on 3H-thymidine incorporation. Virus production was measured by p24 antigen capture assays. CD4+ lymphocytes appear activated, expressing the activation markers, HLA-DR and CD25, as 3H-thymidine incorporation increased and virus production was observed. Addition of autologous sera to the cultures increased the level of activation severalfold suggesting a mechanism for cell activation. Using antisera that identifies virus protein expression on the cell surface, subpopulations of cells producing virus were separated by flow cytometry and RNA and DNA was prepared from these cells. Diseases that might be associated with HTLV-II infection (a retrovirus related to HTLV-I) are unknown. A patient infected with HTLV-II was diagnosed as having large granulocytic leukemia by cell surface marker studies. The malignant cells from the peripheral blood were isolated by flow cytometry and DNA was prepared for viral integration studies.

Project Description

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

Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
Patricia Martin	Visiting Fellow	LVC	NCI

Objectives:

These studies were designed to examine the biologic effects of infection of human lymphocytes with the human T-cell leukemia/lymphoma virus (HTLV-I). A number of studies from our laboratory and other laboratories have demonstrated that HTLV-I infection alters the dynamics of the immune response. This alteration may be due to a proliferation of a population of cells infected with HTLV-I, or a population of cells that is reacting in response to the presence of HTLV-I infection. The latter may be direct stimulation of responsive cells and/or the induction of lymphokines which, in turn, effect the expansion of a cell population. The objectives of these studies are to examine differences in lymphocyte activation in individuals infected with HTLV-I with and without the leukemic and neurologic disease outcomes. The objectives of the HTLV-II studies are to determine the target cell for infection and for relevance of the infection to clonal expression of that particular cell type and malignant transformation.

Methods Employed:

Peripheral blood lymphocytes (PBLs) and sera were obtained from HTLV-I-infected individuals who were disease-free and individuals who had a diagnosis of tropic spastic paraparesis (TSP) and other individuals with the diagnosis of adult T-cell leukemia (ATL). The controls for these studies were HTLV-I seronegative individuals from the same geographic distribution as that of the individuals with the HTLV-I infection. Infection was determined by standard HTLV-I and/or HTLV-II serologic ELISA and Western blot assays. The cells were placed in culture at concentrations of 1×10^6/ml in media with the addition of autologous sera. Cells were examined by flow cytometry prior to and after 6 days of culture for cell-surface markers using combinations of antibodies that detect constituitive and activation surface markers and viral proteins. The subpopulation of cells of interest, based on viral expression and/or combinations of cell surface markers, were separated by flow cytometry. DNA and RNA were prepared from cells by standard procedures. ^{3}H-thymidine incorporation was measured in the cultured cells. Culture supernatants were tested for various cytokines, including IL-1α, IL-1β, IL-2, IL-4, IL-6, granulocyte-macrophage colony stimulating factor (GMCSF), and tumor necrosis factor (TNFα), using commercially available ELISA kits.

Major Findings:

PBLs from TSP patients contained subpopulations of both $CD4^+$ and $CD8^+$ populations that appeared to be activated based on the presence of the major

histocompatibility complex (MHC) class II molecules, HLA-DR. After culture, both the HTLV-I seropositive disease-free individuals and those with TSP demonstrated activation markers on the $CD4^+$ T cells. Lymphocytes from all infected individuals, except those with ATL, demonstrated spontaneous proliferation (as measured by ^{3}H-thymidine incorporation) that exceeded 3-10 times the background values that were found in HTLV-I-seronegative cultured lymphocytes. Addition of 1, 2, and 5% autologous sera increased the ^{3}H-thymidine incorporation in HTLV-I-infected individuals. The effect of the serum appeared to be dependent on two factors: the presence of antibody to HTLV-I and virus production by the cultured cells as reciprocal cultures of antibody-positive sera with HTLV-I negative cells and antibody-negative sera with HTLV-I-positive cells did not effect cell proliferation. PBLs from patients with ATL had lower to absent levels of spontaneous activities in culture with and without autologous sera. However, when sera from these patients were added to cells from seropositive disease-free individuals, the increased proliferation was observed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05367-07 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

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
	Melody E. Roelke	Guest Researcher	LVC	NCI

COOPERATING UNITS *(if any)* LCS, NIAAA, NIH, Bethesda, MD (D. Goldman); National Zoological Park, Washington, DC; (D. Wildt, M. Bush, L. Marker-Kraus); PRI/DynCorp, Frederick, MD (D. Gilbert, D. Janczewski); Department Ecological Behavior Biology, Minneapolis, MN (C. Packer); UCLA, Los Angeles, CA (R. Wayne); Victoria University of Wellington, New Zealand (S. Baker)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Genetics Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.2	0.7

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.)*

The monitor of the pattern and dynamics of molecular genetic variation in free-ranging populations has revealed much about the factors that influence population and species survival. Using the methods of molecular genetics (allozyme, two dimensional electrophoresis, restriction fragment length polymorphism of nuclear and mitochondrial DNA, and polymerase chain reaction) to monitor sequence variation), we have discovered several components that influence stability of natural populations. Historic inbreeding has been deduced in endangered populations of cheetahs, lions, Florida panthers and black-footed ferrets. The consequences of these inbreeding events included inbreeding depression as reflected in congenital defects and physiological impairment to reproduction. Further, as inbreeding reduces genetic diversity, these events increase susceptibility and vulnerability of populations to infectious disease vectors that occasionally evolve mechanisms to abrogate immune differences. Finally, efficacious application of the molecular clock hypothesis has resolved divergence timing and phylogenetic placement of species of orangutan, giant panda, and other species of carnivores and primates.

Project Description

Names, 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
Melody E. Roelke	Guest Researcher	LVC	NCI

Objectives:

(1) 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 the natural history of interacting gene systems that drive development and carcinogenesis. (2) Development of molecular procedures for assessing the genetic status of natural populations and for use in studying heritability of disease susceptibility, both congenital and etiologic. (3) The biologic resolution of adaptive strategies employed by rarely studied mammalian populations for defense against neoplastic and infectious 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 (2DE), (4) microcomplement fixation using heterologous rabbit antisera, (5) DNA hybridization, (6) high resolution cytogenetics procedures, (7) reverse fragment length polymorphism (RFLP) analysis of populations using nuclear and mitochondrial DNA (mtDNA) variants, (8) genetic fingerprints using minisatellite DNA families, and (9) statistical analysis of phylogenetic algorithms.

Major Findings:

1. Developing a high resolution genetic method for individualization and identification of human cell lines using DNA fingerprints. DNA fingerprints of 46 human cell lines were derived using minisatellite probes for hypervariable genetic loci. The incidence of 121 HaeIII DNA fragments among 33 cell lines derived from unrelated individuals was used to estimate allelic and genotypic frequencies for each fragment and for composite individual DNA fingerprints. We present a quantitative estimate of the extent of genetic difference between individuals, an estimate based on the percentage of restriction fragments at which they differ. The average percent difference (APD) among pairwise combinations from the population of 33 unrelated cell lines was 76.9%, compared with the APD in band sharing among cell lines derived from the same individual ($\leq$1.2%). Included in this survey were nine additional cell lines previously implicated as HeLa cell derivatives, and these lines were clearly confirmed as such by DNA fingerprints (APD $\leq$ 0.6%).

On the basis of fragment frequencies in the tested cell line population, a simple genetic model was developed to estimate the frequencies of each DNA fingerprint in the population. The median incidence was 2.9×10^{-17}, and the range was 2.4×10^{-21} to 6.6×10^{-15}. This value approximates the probability that a second cell line, selected at random from unrelated individuals, will match a given DNA fingerprint. Related calculations address the chance that any two DNA fingerprints would be identical among a large group of cell lines. This estimate is still very slight. For example, the chance of two or more common DNA fingerprints among 1 million distinct individuals is <.001. The procedure provides a straightforward, easily interpreted, and statistically robust method for identification and individualization of human cells.

2. Production and characterization of feline-specific DNA fingerprint probes for assessing parentage, genetic diversity, and degree of kinship in free-ranging felid populations. The application of hypervariable minisatellite genomic families to the reconstruction of population genetic structure holds great promise in describing the demographic history and future prospects of free-ranging populations. This potential has not yet been realized due to unforeseen empirical constraints associated with the use of heterologous species probes; to theoretical limitations on the power of the procedure to track genic heterozygosity and kinship; and to the absence of extensive field studies to test genetic predictions. We combine here the technical development of feline-specific variable number tandem repeat (VNTR) families of genetic loci with the long-term demographic and behavioral observations of lion populations of the Serengeti Ecosystem in East Africa. Minisatellite variation was used to quantify the extent of genetic variation in several populations that differed in their natural history and levels of inbreeding. Definitive parentage, both maternal and paternal, was assessed for 78 cubs born in 11 lion prides permitting the assessment of precise genealogical relationships among some 200 lions. The extent of DNA restriction fragment sharing between lions was empirically calibrated with the coefficient of relatedness (r) in two different populations that had distinct demographic histories. The results suggest that reliable estimates of relative genetic diversity, of parentage, and of individual relatedness can be achieved in free-ranging populations provided the minisatellite family is calibrated in established pedigrees for the species.

3. A genetic and adaptive basis for pride social organization in African lions. Because they are the only cat that displays cooperative behavior instinctively, lion social groups provide a valuable field opportunity to test competing hypotheses concerning the role of kinship in the evolutionary development of behavioral strategies. We have employed hypervariable minisatellite genomic families (also called VNTR) tailored for cat populations to determine parentage and the coefficient of relatedness, r, among over 200 lions under long-term observation in the Serengeti Ecosystem in East Africa. The combined genetic and behavioral observations lead to the following conclusions about the structure of lion prides: (1) Pride females are always closely related to each other but are distantly related to male coalitions that guard their territory and father their young. (2) Male coalition size varies in number from 2-10 males, but larger coalitions have a distinct advantage in successful pride takeovers. (3) Large male coalitions ($\geq$3) are nearly always first order relatives, usually brothers, while smaller

coalitions frequently are composed of unrelated males who have joined together after their eviction from different natal prides. (4) The variation of male reproductive success increases with coalition size since, in most cases (83%), paternity of all pride cubs was restricted to two males regardless of the resident coalition size. The genetic affirmation of behavioral inference provides support for a unifying hypothesis concerning the adaptive value of social organization in lions.

4. Calibration of molecular vs. geological clocks in primate and carnivore radiations. Numerous studies have used indices of genetic distance between species to reconstruct evolutionary relationships and to estimate divergence time. However, the empirical relationship between molecular based indices of genetic divergence and divergence time based on the fossil recorded is poorly known. To date, the results of empirical studies conflict and are difficult to compare because they differ widely in their choice of taxa, genetic techniques, or methods for calibrating rates of molecular evolution. We use a single methodology to analyze the relationship of molecular distance and divergence time in 86 taxa (72 carnivores and 14 primates). These taxa have divergence times ranging from 0.01 to 55 million years and provide a graded series of phylogenetic divergences such that the shape of the curve relating genetic distance and divergence time is often well defined. The techniques used to obtain genetic distance estimates include one- and two-dimensional protein electrophoresis (2DE), DNA hybridization, and microcomplement fixation. Our results suggest that estimates of molecular distance and divergence time are highly correlated. However, rates of molecular evolution are not constant; rather, they decline with increasing divergence time in a linear fashion. The rate of decline may differ according to technique and taxa. Moreover, in some cases, the variability in evolutionary rates changes with increasing divergence time such that the accuracy of nodes in a phylogenetic tree varies predictably with time.

5. Demonstration of species level difference between Bornean and Sumatran orangutans. The orangutan (Pongo pygmaeus), as currently recognized, includes two geographically separated subspecies: Pongo pygmaeus pygmaeus, which resides on Borneo, and P. p. abelii, which inhabits Sumatra. At present, there is no known route of gene flow between the two populations except through captive individuals which have been released back into the wild over the last several decades. The two subspecies are differentiated by morphological and behavioral characters, and they can be distinguished by a subspecies-specific pericentric chromosomal inversion. Nei-genetic distances were estimated between orangutan subspecies, gorillas, chimpanzees, and humans using 44 isozyme loci and using 458 soluble fibroblast proteins which were resolved by 2DE. Phenetic analysis of both data sets supports the following conclusions: the orangutan subspecies distances are approximately ten times closer to each other than they are to the African apes, and the orangutan subspecies are approximately as divergent as are the two chimpanzee species. Comparison of the genetic distances to genetic distance estimates done in the same laboratory under identical conditions reveals that the distance between Bornean vs. Sumatran orangutans is five to ten times the distance measured between several pairs of subspecies including lions, cheetahs, and tigers. Near species level molecular genetic distances between orangutan subspecies would support the separate management of Bornean and Sumatran orangutans as

evolutionary significant units. Evolutionary topologies were constructed from the distance data using both cladistic and phenetic methods. The majority of resulting trees affirmed previous molecular evolutionary studies that indicated that man and chimpanzee diverged from a common ancestor subsequent to the divergence of the gorilla from the common ancestor.

6. Demonstration that the endangered Florida panther subspecies (Felis concolor coryi) contains a genetic mixture of ancestral F. c. coryi and recently introduced pumas from a South American subspecies. The Florida panther (F. c. coryi) is a severely threatened relict population of puma or mountain lion whose historic range included much of the southeastern United States. The present population consists of 30 to 50 animals living in the Big Cypress Swamp-Everglades Ecosystem in southern Florida. Field observations indicated the presence of two distinct morphological phenotypes that are stratified between the two adjacent areas despite the occurrence of periodic migration between them. A comprehensive molecular genetic analysis using mitochondrial DNA and nuclear markers indicates the existence of two distinct genetic stocks concordant with the morphological phenotypes. One stock confined to the Big Cypress is derived from the historic ancestors of F. c. coryi. A second stock, found largely in the Everglades, is descended primarily from pumas that evolved in South or Central America, but were introduced (probably by man) into the Florida habitat very recently. The precarious genetic disposition of the few remaining Florida panthers may be benefitting from the introgression of genetic materials into the wild population.

Publications:

Bush M, Graves JAM, O'Brien SJ, Wildt DE. Dissociative anaesthesia (Telazol) in free-ranging male Koalas (Phascolarctos cinereus) and comparative data on selected marsupials in captivity. Australian Vet J (In Press).

Clegg MT, O'Brien SJ, eds. Molecular evolution, vol. 122. New York: Wiley-Liss, 1990;1-322.

Gilbert DA, Packer C, Pusey A, Wildt DE, Bush M, O'Brien SJ. Analytical DNA fingerprinting in lions: parentage, kinship, and genetic diversity. Science (In Press).

Gilbert DA, Reid YA, Gail MH, Pee D, White C, Hay RJ, O'Brien SJ. Application of DNA fingerprints for cell-line individualization. Am J Hum Genet 1990;47:499-514.

Janczewski DN, Goldman D, O'Brien SJ. Molecular genetic divergence of orangutan (Pongo pygaeus) subspecies based on isozyme and two-dimensional gel electrophoresis. J Hered 1990;81:375-87.

Miththapala S, Seidensticker J, Phillips LG, Goodrowe KL, Fernando SBU, Forman L, O'Brien SJ. Genetic variation in Sri Lanken leopards. Zoo Biol 1991;10:139-46.

O'Brien SJ. The genetic peril of the cheetah. In: Seidensticker J, ed. Great cats. Sydney: Weldon Owen Pty Ltd (In Press).

O'Brien SJ. Molecular evolution of the cats. In: Seidensticker J., ed. Great cats. Sydney: Weldon Owen Pty Ltd (In Press).

O'Brien SJ, Benveniste RE, Nash WG, Martenson JS, Eichelberger MA, Wildt DE, Bush M, Wayne RK, Goldman D. Molecular biology and evolutionary theory: the giant panda's closest relatives. In: Warren L, Koprowski H, eds. New perspectives on evolution. New York: Wiley-Liss, 1991;225-50.

O'Brien SJ, Mayr E. Bureaucratic mischief: recognizing endangered species and subspecies. Science 1991;251:1187-8.

O'Brien SJ, Roelke ME. What does geology have to do with panther conservation: response. Natl Geo Res 1991;7:117-9.

O'Brien SJ, Roelke ME, Yuhki N, Richards KW, Johnson WE, Franklin WL, Anderson AE, Bass OL Jr, Belden RC, Martenson JS. Genetic introgression within the Florida panther Felis concolor coryi. Natl Geo Res 1990;6:485-94.

Packer C, Gilbert DA, O'Brien SJ, Pusey AE. Kinship, cooperation and inbreeding in African Lions: a molecular genetic analysis. Nature (In Press).

Packer C, Pusey AE, Rowley H, Gilbert DA, Martenson JS, O'Brien SJ. Case study of a population bottleneck: lions of the Ngorongoro Crater. Conserv Biol 1991;5:1-12.

Wayne RK, Van Valkenburgh B, O'Brien SJ. Molecular distance and divergence time in carnivores and primates. Mol Biol Evol 1991;8:297-319.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05382-08 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

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:	Glenn A. Hegamyer	Health Science Officer	LVC	NCI
	Robert Hoover	Chief	EEB	NCI
	Alan Hildesheim	Epidemiology & Biostatistics Fellow	EEB	NCI

COOPERATING UNITS *(if any)*
PRI/DynCorp, Frederick, MD (Y. Sun); Department of Microbiology, University of North Carolina, Chapel Hill, NC (N. Raab-Traub); Georgetown University, Washington, DC (P. Woolley)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.7	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 aim of this research is to identify and characterize two classes of genes involved in multistage carcinogenesis. The first class includes genes associated with susceptibility to tumor promoter-induced neoplastic transformation. The second class includes genes that specify expression of tumor cell phenotype. Mouse JB6 promotion-resistant (P-), promotion-sensitive (P+), and tumorigenic (Tx) JB6-derived epidermal cell lines have been found to differ in the expression of several genes described in the accompanying project Z01CP05383-08 LVC, "Membrane Signal Transduction in Tumor Promotion." The changes in expression of these genes during the progression from P- to P+ to Tx phenotypes appear to be genetically controlled in these stable variant cell lines. We have recently discovered a stable change in a response to tumor promoters by a JB6-derived tumorigenic cell line. The change involves acquisition of a cell-killing response to activators of protein kinase C. The treated cells show the type of DNA damage that characterizes apoptosis (programmed cell death). A novel transformation-associated sequence unrelated to any known oncogene has been cloned from human nasopharyngeal carcinoma (NPC) cells by human Alu screening of an NPC/JB6 transfectant genomic library. A hybridizing mRNA of 1.7 Kilobase is observed in NPC cells but not in mouse recipient cells. A recently isolated NPC cDNA clone is being analyzed. In addition, two independent NPC cell lines show the same expressed mutation in one of the "hot spots" of the tumor suppressor gene, p53, thus appearing to activate it to an oncogene. Additional NPC samples from Taiwanese patients are being provided to us for examination of both the newly cloned NPC transformation-associated gene and the p53 gene for transformation-relevant mutations. Finally, we have learned that JB6 P+ cells can be transformed to a tumor phenotype by overexpression of activated v-H-ras, v-raf, or src, but not by any of several other oncogenes. The profiles of gene expression in these JB6/oncogene transfectant cell lines are being examined to increase our understanding of transformation pathways in the JB6 model.

Project Description

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

Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Glenn A. Hegamyer	Health Science Officer	LVC	NCI
Robert Hoover	Chief	EEB	NCI
Alan Hildesheim	Epidemiology & Biostatistics Fellow	EEB	NCI

Objectives:

The specific objectives of these studies are: (1) to elucidate the nature of genetically determined events that are causally related to preneoplastic progression in mice and humans, (2) to characterize the genes involved in tumor promoter-induced progression to the tumor cell phenotype in JB6 mouse epidermal cells, (3) to elucidate the structure and mode of activation and the regulation of expression of these promotion relevant genes to learn the functions of these genes and their products, (4) to clone and characterize a novel human transforming gene and its regulation in human tumors and during progression to tumor phenotype, and (5) to identify gene cooperation events that can be implicated in human carcinogenesis.

Methods Employed:

The following techniques are being utilized: (1) gene cloning techniques using sib selection, hybridization, and antibody binding search routines; (2) calcium-phosphate DNA transfection or lipofection followed by assay of sensitivity to promotion of anchorage-independence by the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA); (3) use of ligated dominant selectable markers; (4) generation of new recombinant DNA constructs; (5) restriction mapping; (6) sequencing the cloned genes using polymerase chain reaction (PCR) and the Maxam and Gilbert or dideoxy 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 and Tx genes; (9) RNase and S-1 protection to analyze size and sequence of RNA transcripts; (10) purification of nuclear and messenger RNA; (11) construction of genomic and cDNA libraries; (12) screening of libraries for P+-active sequences and for transforming sequences using JB6 recipient cells; (13) calcium phosphate DNA transfection followed by assay of anchorage-independent, transforming activity (without TPA); (14) deletion analysis to define minimum biologically active sequences; (15) PCR amplification of DNA or reverse-transcribed RNA sequences; (16) generation of site-directed mutants by PCR; and (17) in vitro transcription assays.

Major Findings:

1. Acquisition of a growth inhibitory response to phorbol ester involves DNA damage. TPA, a potent tumor promoter, has been shown to stimulate or inhibit cell growth depending on the cell type investigated. We recently found that RT101 cells, a transformed mouse JB6 epidermal cell line, acquired a

substantial inhibition response to TPA and to other protein kinase C (PKC) activators during conventional subcultivation. The growth of low passage RT101 cells was slightly inhibited by TPA in monolayer culture, but stimulated in soft agar. In high passage cells, however, the growth was greatly inhibited by TPA in both monolayer culture and in soft agar. Inhibition was dose-dependent, was directly correlated with tumor promoting and activating activities, and was found to be initially reversible. The increased sensitivity to TPA of late-passage RT101 cells could not be accounted for by differential induction of c-jun or c-fos, postulated to mediate many of the PKC-activated pathways, or to the differential antioxidant protection implicated in sensitivity to tumor necrosis factor α (TNFα)-mediated cell killing. The results suggest the possibility that early passage RT101 cells contained two subpopulations--one TPA-sensitive and one TPA-resistant population, with subcultivation selecting for the former. The response of late passage RT101 cells to C-kinase activators is characterized by the rapid generation of short double-stranded DNA fragments and extensive cell detachment. This response is reminiscent of programmed cell death or apoptosis, a finding of interest for cancer treatment.

2. Cloning of a transformation-associated sequence from the human nasopharyngeal carcinoma (NPC) cell line, CNE_2. NPC DNA transfers an anchorage-independent transforming activity (without tumor promoter) when transfected into mouse JB6 promotion-sensitive (P+) epidermal cells. DNA from primary NPC/JB6 clonal transfectants that is human Alu-positive has been used to generate secondary and tertiary transfectants. Human Alu screening of the genomic library of tertiary NPC/JB6 transfectants has led to the isolation of a 16 Kilobase (Kb), Alu-containing sequence whose nucleotide sequence differs from that of any of the oncogenes in GenBank. This cloned DNA, when cotransfected with pSV2 neo and G418 selected, transferred transforming activity to JB6 P+ cells that was six to eight times that in the neo-only background.

3. Genomic structure of the novel NPC transforming gene is preserved in the isolated clone. When high molecular weight DNA from CNE_2 cells was analyzed for the presence of a human Alu-negative EcoRI restriction fragment of 2.8 Kb, the 2.8 Kb diagnostic hybridizing fragment was found to be present. This hybridizing fragment was not seen in the DNA of JB6 mouse recipient cells, thus indicating that the NPC genetic sequence pre-existed in the NPC cells rather than being generated during transfection or cloning. In addition, DNA from nude mouse tumors induced after injection of CNE_2 or CNE_2/JB6 tertiary transfectant cells showed the same diagnostic 2.8-Kb hybridizing band, thus suggesting a causal role for this gene in neoplastic transformation. The preservation of a 3.0-Kb fragment from a separate region of the genomic clone has likewise been demonstrated.

4. Evidence for a 1.7-Kb NPC oncogene mRNA. Northern analysis of poly A^+ RNA from the NPC cell line CNE_2 and from transfection recipient JB6 Cl 41 mouse cells revealed an mRNA hybridizing to the subcloned 2.8-Kb Alu-negative EcoRI fragment of the 16-Kb genomic NPC clone that was 1.7 Kb in size. This 1.7-Kb mRNA was seen in human CNE_2 and in CNE_2-derived tumors but not in mouse JB6 poly A^+ RNA.

5. Molecular cloning and sequencing of a hybridizing cDNA from human NPC cells. The 2.8-Kb subclone (CNE2.8) of the NPC transformation sequence was used as a probe to screen a cDNA library generated from the C15 NPC tumor, an Epstein-Barr virus-positive, nude mice-carried NPC. Tertiary screening of the library yielded 12 positive clones. Nine of them had an insert with a size of 0.7 Kb. Four out of the nine clones were sequenced and all were verified to be identical, suggesting that there was only one CNE2.8-hybridizing sequence in the library. The potential AATAAA polyadenylation signal, followed by a poly A tail, was found in the 3'-end of these cDNA clones. Computer analysis revealed an open reading frame having no sequence homology to any known oncogenes, promising a novel oncogene in NPC.

6. A point mutation of the p53 gene in human NPC cell lines. The possible involvement of the p53 gene, a potent tumor suppressor gene, in human NPC cell lines was investigated by Southern analysis and reverse transcriptase-PCR. Results showed that the p53 gene expressed a normal sized mRNA in NPC cells, eliminating the possibility of gene rearrangements, deletions, or alternative splicing. Sequencing of both DNA and RNA, however, revealed the same point mutation in NPC cells from at least two different patients at one of the evolutionarily-conserved regions of the p53 gene. Southern analysis showed polymorphism that distinguished normal from NPC cells and NPC tumor biopsies. These data suggest that mutation of p53 can contribute, along with other factors, to the genesis of human NPC.

7. Transformation of mouse JB6 cells by cloned oncogenes. As part of an effort to ascertain the potential of mouse JB6 P+ cells to detect various oncogenic activities present in tumor DNA, a number of cloned oncogenes have been co-transfected with the geneticin resistance gene pSV2 neo singly and in pairs, and the transfected geneticin-resistant populations have been assayed for anchorage-independence and tumorigenicity. Oncogenes injected singly and found to induce anchorage-independence and tumorigenicity in nude mice include v-H-ras, v-Ki-ras, v-src, v-raf, and v-mos. Oncogenically inactive genes include c-fos, c-jun, v-myc, v-myb, epidermal growth factor receptor, and int-2. Expression of all of the above introduced genes was driven by retroviral long terminal repeat and was verified.

Publications:

Cao Y, Sun Y, Poirier S, Winterstein D, Hegamyer G, Malin S, Seed J, Colburn N. A novel transforming sequence from a nasopharyngeal carcinoma cell line. Mol Carcinog (In Press).

Garrity RR, Hegamyer GC, Seed JL, Colburn NH. Deletion mapping of tumor promotion susceptibility gene pro 1 implicates an RNA polymerase III transcription unit. Mol Carcinog 1991;3:243-50.

Sun Y, Poirier S, Cao Y, Hegamyer G, Colburn N. Isolation and characterization of a transformation-associated gene from human nasopharyngeal carcinoma cells. In: Rhim J, Dritschilo A, eds. Neoplastic transformation in human cell systems in vitro. Clifton: Humana Press (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05383-08 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

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:	Lori Bernstein	IRTA Fellow	LVC	NCI
	Robin Abramson	Staff Fellow	LVC	NCI
	Michael Birrer	Senior Investigator	NMOB	NCI
	Howard Young	Chief, Cell. and Mol. Immunol. Sect.	LEI	NCI
	Joost Oppenheim	Chief	LMI	NCI
	Fabriccio DeBenedetti	Visiting Fellow	LI	NCI
	Pat Steeg	Senior Staff Fellow	LP	NCI

COOPERATING UNITS (if any)

FUSO Pharmaceutical, Osaka, Japan (K. Nakamura); PRI/DynCorp, Frederick, MD (S. Simek); LMM, NIAID, NIH, Bethesda, MD (C. Kozak); The New Biologist, Bethesda, MD (E. Ben-Ari)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Cell Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	2.5	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 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 by protein kinase C (PKC) and epidermal growth factor (EGF) receptor kinase and kinase-regulated trans-activation of gene expression. A putative C-kinase substrate of 80-kDa has been found to be differentially phosphorylated in promotion-resistant (P-), -sensitive (P+), and neoplastically transformed JB6 mouse epidermal cells, with little or no phosphorylated 80-kDa phosphoprotein (pp80) seen in transformed cells. Western analysis indicates that p80 is regulated at the level of synthesis with little or no p80 protein detectable in transformed JB6 cells. A cDNA clone of p80 has recently been isolated by screening a JB6 P- library with p80 antibody. This p80 cDNA, when used as a probe, detects a 2.8-Kb RNA that progressively decreases during the progression from P- to transformed (Tx) phenotype in certain cases but does not generalize to multiple independent cells of each phenotype. When compared with GenBank the sequence emerges as novel. Recent studies on 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-inducible genes have focused on those regulated by the trans-acting transcriptional factor AP-1 (Jun/Fos Complex). The tumor promoters TPA and EGF induce AP-1-regulated gene expression in P+ but not P- JB6 cells. This suggests that AP-1-regulated gene expression may be required for tumor promoter-induced transformation. The mechanism of differential trans-activation and transformation by TPA appears to involve differential basal and induced levels of c-jun mRNA and Jun protein but does not involve differential induction of c-fos, fra, jun D, or jun B. A c-jun differential was not found after EGF treatment thus suggesting that TPA and EGF induce AP-1-dependent trans-activation of gene expression by different pathways. Finally, the phosphorylation of c-jun and fra-1 is differentially regulated in P- and P+ cells, suggesting a second promotion-relevant mode of regulation.

Project Description

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

Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Lori R. Bernstein	IRTA Fellow	LVC	NCI
Robin Abramson	Staff Fellow	LVC	NCI
Michael Birrer	Senior Investigator	NMOB	NCI
Howard Young	Chief, Cell. and Mol. Immunol. Sect.	LEI	NCI
Joost Oppenheim	Chief	LMI	NCI
Fabriccio DeBenedetti	Visiting Fellow	LI	NCI
Pat Steeg	Senior Staff Fellow	LP	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 (PKC)-catalyzed protein phosphorylation, and PKC-regulated trans-acting transcriptional factors AP-1, AP-2, etc. Tumor promoter-inducible AP-1-dependent gene expression is being analyzed for promotion relevance. An overall aim is to understand the nuclear gene regulation events triggered by activation of plasma membrane PKC. A PKC substrate of 80 kiloDaltons (kDa) that progressively decreases during the progression from preneoplastic to tumor cell phenotype is being analyzed. A parallel approach is being taken with epidermal growth factor (EGF), a transformation promoter whose receptor kinase also triggers a pathway leading to transcriptional activation.

Methods Employed:

(1) Assay of calcium-dependent, phospholipid-dependent protein kinase (C-kinase or PKC) activity, (2) assay of the effects of 12-O-tetradecanoyl-phorbol-13-acetate (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 PKC substrates or other transformation related proteins, (4) screening cDNA libraries using antibody or molecular probes, (5) assay of a PKC-regulated trans-acting factor function such as AP-1-dependent gene expression, (6) Northern analysis of mRNA levels, (7) nuclear run-off assays of transcription rate, and (8) gel shift assays of protein-DNA binding.

Major Findings:

1. A novel 97-kDa PKC in T cells. A mouse T lymphoblast cell line (Cl 9) showed induction of γ interferon production after treatment with phorbol ester. When Cl 9 cells were assayed under conditions known to autophosphorylate PKC (Ca^{++} phospholipid), instead of the expected 80-kDa Ca^{++}

dependent PKC phosphorylation, we observed only a 97-kDa phosphoprotein band whose phosphorylation was Ca^{++} independent. mRNA from Cl 9 cells hybridizes at moderate stringency with a PKC-ϵ clone. This PKC has been identified only as a minor subtype in brain tissue. Northern blot analysis, using subtype-specific molecular probes conducted under stringent wash conditions (60°C, 0.5 x SSC), demonstrated low levels of PKC-β mRNA expressed in Cl 9 cells. Immunoblotting experiments using specific PKC antisera showed PKC-β protein expression that was 10% as high as that seen in JB6 epidermal cells. The possibility that this Cl 9 kinase is a non-PKC kinase has been ruled out by the observation that the 97-kDa phosphoprotein is recognized by antisera specific for the catalytic site of all PKC's. Taken together, these data suggest that the 97-kDa protein is similar but not identical to PKC-ϵ and is the major PKC subtype expressed in Cl 9 T cells.

2. The novel PKC shows similar intracellular localization and unique substrate specificity when compared to other PKC subtypes. Cellular localization experiments revealed that upon autophosphorylation the majority of this novel PKC was present in the membrane fraction of Cl 9 cells. This result is similar to that seen with other PKC subtypes. The "in vitro" substrate specificity appears to be different for this PKC subtype when compared to PKC α,β,γ subtypes. Exogenous casein, but not histone, is phosphorylated.

3. Cloning of a cDNA for an 80-kDa phosphoprotein that is differentially expressed during preneoplastic progression of mouse JB6 cells: Sequencing and chromosomal localization. Peptide antiserum raised against an 87-kDa/pI 4.8 rat brain PKC substrate recognized an 80-kDa phosphoprotein and putative PKC substrate in JB6 mouse epidermal cells that decreases during progression from early preneoplastic to neoplastic phenotype. A JB6 mouse epidermal cDNA library was screened with peptide antiserum and a unique gene was isolated and sequenced. The p80 gene has been localized to chromosome 11 in mouse and linkage analysis places it near the end opposite the centromere. The p80 gene has been shown to be closely linked to two genes of interest. The first is metastasis suppressor gene NM23 (having a different sequence from p80) and the second is a tumor promotion susceptibility locus that distinguishes sensitive and resistance strains of mice. Cross-species hybridization, using the p80 cDNA as a molecular probe, shows homology and therefore conservation in human, primate, feline, equine, canine and avian DNA.

4. Characterization of the p80 mRNA and its expression during neoplastic transformation. When this p80 cDNA was used as a molecular probe against various mouse total cellular RNAs, a 2.8-kilobase mRNA was detected. The mRNA and protein encoded by this gene appears to be differentially expressed in certain mouse, rat, and human cell systems, such that the level decreases as the cells progress to a transformed phenotype. However, extensive analysis of many sets of early preneoplastic-late preneoplastic-neoplastic-metastatic-cells shows no consistent pattern of expression with respect to progression in any model system when multiple, independent lines of each phenotype were examined. Thus, it appears that while post-translational regulation of p80 may be transformation-relevant, its expression is not.

5. Further characterization of the phosphoprotein encoded by the novel p80 cloned cDNA. The p80 gene has been recombinantly cloned into a bacterial expression vector; the expressed protein will be used in in vitro phosphorylation experiments, along with purified protein kinases to demonstrate substrate specificity. Two peptides were generated from the predicted amino acid sequence of the p80 gene, and antisera are currently being used in immune precipitation and immunoblotting experiments to characterize intracellular localization and variation of phosphorylation status with physiologic conditions.

6. JB6 P+ and P- cells display differential TPA-induced accumulation of c-jun, but not jun D, c-fos, fra-1, or jun B mRNAs. Total RNA from JB6 P+ and P- cells treated with TPA was analyzed for relative levels of jun or fos mRNA by hybridization to specific cDNA probes. The basal levels of both c-jun and jun D mRNAs were higher in P+ than in P- cells (about fivefold and twofold for c-jun and jun D, respectively). TPA stimulated the accumulation of c-jun mRNA in both P+ and P- cells, but the P+ cells displayed higher absolute levels at all times examined. At the peak of TPA-induced mRNA accumulation (0.5 to 1 hour), the c-jun level in P+ cells was approximately threefold that in P- cells. In contrast, TPA-induced c-fos mRNA accumulation was slightly higher in P- cells at all time points, and no significant differences were observed in the TPA-stimulated peak levels of jun D, jun B or fra-1 mRNAs. Taken together, these data rule in the possibility that the defective promotion response and the inability of TPA to stimulate AP-1-dependent trans-activation in P- cells could be due, in part, to insufficient induction of c-jun mRNA, while ruling out defective TPA-stimulated c-fos mRNA accumulation, as an explanation for resistance.

7. No differences in EGF-stimulated accumulation of c-jun, jun B, jun D, c-fos, or fra-1 mRNAs in P+ and P- cells. Unlike the results observed with TPA, EGF treatment of P+ and P- cells stimulated the accumulation of c-jun, jun B, jun D, c-fos, and fra-1 mRNAs to the same or similar levels in P+ and P- cells. In the case of c-jun and c-fos, the degree of induction (fold induction) was considerably greater in response to EGF than to TPA in both P+ and P- cells. These data suggest that TPA and EGF are acting via different intracellular signalling mechanisms. In addition, the defective promotion and AP-1-dependent trans-activation responses in P- cells in response to EGF cannot be accounted for by differences at the level of induction of c-jun, jun B, jun D, c-fos or fra-1 mRNA accumulation.

8. P+ cells show higher basal and TPA-inducible levels of c-jun protein than do P- cells. Western immunoblotting of nuclear extracts from P+ and P- cells displayed 10-25% as high basal and induced levels of the Jun protein as the P+ cells. Maximum TPA-induced levels in the P- cells rarely exceeded the basal levels in the P+ cells. Furthermore, persistently high levels of Jun protein were observed in P+ cells, whereas in P- cells Jun protein levels returned to basal values by 48 hours. These data parallel those obtained for c-jun mRNA and are consistent with the hypothesis that threshold levels of c-Jun protein may be needed for trans-activation and promotion. Defective trans-activation and promotion responsiveness in P- cells may, in part, be explained by their inability to transcend this threshold or to display persistent induced synthesis of the protein.

9. Overexpression of c-jun in P- cells. A human c-jun expression construct driven by a Moloney long terminal repeat promoter was introduced into JB6 P- cells for the purpose of determining whether overexpression of c-jun was sufficient to confer promotion sensitivity. The clonal transfectants displayed 20-fold increases in c-jun mRNA levels. However, upon measuring c-Jun protein levels by both immunoprecipitation and Western analyses, no commensurate increase in c-Jun protein was observed, despite the increased levels of message. We therefore made a new battery of transfectants using an inducible c-Jun expression construct driven by a metallothione in MTIIA promoter. P- cells were transfected and displayed 20-fold to 50-fold increases in c-jun mRNA and 2-fold to 5-fold increases in c-Jun protein levels compared to untransfected controls, an increase sufficient to bring c-Jun levels up to those in TPA-treated parental P+ cells. No progression to P+ or transformed phenotype was observed in the P- recipients as measured by anchorage independent colony growth in soft agar. These data suggest that while elevation of c-Jun protein levels may be necessary for tumor promoter induced transformation in vitro, it is not sufficient to transform P- cells or to support promoter-induced transformation.

10. P+ cells preferentially display a novel, fast-migrating form of c-Jun protein. As shown previously, P+ cells display elevated basal and tumor promoter-induced levels of c-Jun protein compared to P- cells. In the course of these investigations, an electrophoretic gel was inadvertently overrun and the Jun protein, which previously ran as a single band, resolved into two bands. Reproducibly the upper band predominates in the P- cells, and the lower band predominates in the P+ cells and is preferentially induced by TPA and EGF. Others have recently demonstrated the existence of two forms of Jun: a phosphorylated form, which is inactive in trans-activation, and a dephosphorylated form which is generated upon TPA treatment and is active in trans-activation. We hypothesize that (1) the lower band (which we call fast-Jun) is dephosphorylated Jun and the upper band (slow Jun) is phosphorylated Jun; (2) slow Jun behaves as a competitive inhibitor of trans-activation by binding to AP-1 sites without inducing trans-activation, and thus the ratio of fast Jun to slow Jun can regulate net trans-activation activity; and (3) P- cells show resistance to promotion of transformation due to their relative excess of slow Jun and insufficiency of fast Jun, while P+ cells display promotion sensitivity due to the relative abundance of fast Jun. This hypothesis is currently being tested.

11. Overabundance of fast c-Jun in ras transformed cells and c-Jun transfectants. Increased abundance of the fast-migrating form of c-Jun (compared to levels of slow c-Jun) has recently been detected in P+ Cl 41 cells transformed by introduction of a human Harvey ras overexpression construct, suggesting that Jun, and in particular, the fast-migrating form of Jun, may play a role in signal transduction pathways leading to transformation by ras. It is likely not to be involved in all transformation pathways, however, as P+ cells transformed with other oncogenes, such as c-mos, c-src, and c-raf, do not have overabundant fast c-Jun protein. Interestingly, P- c-jun transfectants also have an elevated ratio of the fast and slow forms of Jun. Thus, while an elevated ratio of fast:slow may be necessary for promotion sensitivity, it is not sufficient since the Jun transfectants retain their P- phenotype.

12. Levels of Fra-1 and Jun B proteins in P+ cells and P- cells. Using polyclonal antisera directed against cellular Fra-1 protein, we observe TPA induction of levels of phosphorylated Fra-1 in P- cells but not in P+ cells. Levels of unphosphorylated Fra-1 are not inducible in either cell line and are close to equal. This observation is intriguing and its biological significance is yet to be determined. In contrast, both the phosphorylated and unphosphorylated forms of Jun B protein are inducible to equal extents in P+ and P- cells.

13. TGF-β acts as an antipromoter and TNFα as a promoter of transformation in JB6 cells. In collaborative studies on cytokines we have found that transforming growth factor β (TGFβ) inhibits the transformation promoting activity of phorbol esters synergistically with retinoids by a mechanism that may involve alteration of TGFβ receptors. In contrast, tumor necrosis factor-α transforms promotion sensitive JB6 cells by a response pathway that differs in part from that of TPA.

Publications:

Bernstein LR, Ben-Ari E, Simek S, Colburn NH. Gene regulation and genetic susceptibility to neoplastic transformation: AP-1 and p80 expression in JB6 cells. Environ Health Perspect (In Press).

Colburn NH. Stress responses and gene regulation: implications for tumor promotion and its prevention. In: Spatz L, Bloom A, eds. Reactive oxygen and disease. London: Oxford Press (In Press).

DeBenedetti F, Colburn NH, Oppenheim JJ, Faltynek CR. Tumor necrosis factor induces anchorage independent growth of two murine non-transformed cell lines. In: Oppenheim JJ, Powanda MC, Kluger MJ, Dinarello CA, eds. Molecular cellular biology of cytokines. New York: Wiley-Liss, 1990;275-80.

DeBenedetti F, Falk L, Ruscetti FW, Colburn NH, Faltynek CR, Oppenheim JJ. Synergistic inhibition of phorbol ester induced transformation of JB6 cells by transforming growth factor-β and retinoic acid. Cancer Res 1991;51:1158-64.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05384-08 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

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:	Hector N. Seuanez	Visiting Scientist	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	Stanley J. Cevario	Biologist	LVC	NCI

COOPERATING UNITS *(if any)*

PRI/DynCorp, Frederick, MD (W.Modi, M.Dean); Inst. Mol. Biol., Greece (N. Anagnou); Johns Hopkins Hospital, Baltimore, MD (B.Vogelstein, M.Levine); Rorer Biotech., Horesham, PA (M.Jaye); CEPH Center, Paris, France (J. Dausset); LDBA, NIDR (P. Killen); NICHD, Bethesda, MD (R.Klausner); Univ. Chicago, IL (R.Burke); Childrens Hospital, Philadelphia, PA (J.Tabas); Harvard Univ., Cambridge, MA (S.Karathanasis); NIMH, Bethesda, MD (T.Bonner)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Genetics Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.6	1.1

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 Human Gene Mapping project involves intramural and extramural collaborations with several groups of the scientific community. The Genetics Section has developed efficient facilities for establishing and maintaining this program through different approaches and methodologies. These include (1) the creation and maintenance of a hybrid cell panel, following hybridization of somatic cells of human and rodent origins and hybrid selection in special culture media; (2) biochemical and karyological characterizations of cell lines; (3) in situ hybridization of molecular clones to human metaphase chromosomes; (4) detection of restriction fragment length polymorphisms by screening human DNAs; and (5) linkage estimation using polymorphic human loci and placing genes on chromosome maps in the context of the Centre D'Etude du Polymorphisme Humain (CEPH) collaboration. Other techniques have also included the use of polymerase chain reaction amplification and electrophoretic analysis of RNA or protein binding products. Altogether these approaches have enabled us to map a number of disparate products such as proto-oncogenes, growth factors, tumor-related products, cell receptors, members of the immunoglobulin superfamily, and chemotactic substances.

The principal investigator serves in the human genome projects as an elected member of the Human Genome Organization as well as chairman of the International Committee on Comparative Gene Mapping. Status of ongoing collaborative projects are listed in tabular form in the Project Description of this report.

Project Description

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

Stephen J. O'Brien	Chief	LVC	NCI
Hector N. Seuanez	Visiting Scientist	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Stanley J. Cevario	Biologist	LVC	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) genes which code for growth factors, (3) genes coding for receptors for growth factors and for 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 sequences 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; (7) *in situ* hybridization to metaphase chromosomes; (8) restriction fragment length polymorphism (RFLP)

linkage analysis of human pedigrees and populations; and (9) polymerase chain reaction (PCR) techniques.

Major Findings:

1. Linkage mapping of the human polymorphic proteins identified by two dimensional electrophoresis. Nineteen polymorphic lymphocyte proteins were previously detected by two dimensional protein electrophoresis (2DE). In this report, we describe the genetic linkage mapping of six of these polymorphic proteins (NIAAA1-NIAAA6), the assignment and identification of a seventh (glyoxalase 1 on 6p21) and support for the mapping of an eighth (plastin or LCP1) to near the ESD locus on chromosome 13. NIAAA1-NIAAA6 were assigned, respectively, to 10q26, 16p13.3, 10q, 11p15, 3q, and 19q13. These genetic linkages were achieved by classic linkage analysis of 2DE protein charge polymorphisms to the panel of RFLPs previously typed in nine pedigrees in the Center D'Etude du Polymorphisme Humain (CEPH) collection.

2. Linkage mapping of the human granulocyte-macrophage colony-stimulating factor (CSF2) and interleukin-3 (IL3) genes. IL3 (encoded by the IL3 gene) and CSF2 (encoded by the CSF2 gene) are small secreted polypeptides that bind to specific cell surface receptors and regulate the growth, gene expression, and differentiation of many of the hematopoietic cell lineages, particularly non-lymphoid cells. The IL3 and CSF2 genes have been cloned and mapped to human chromosome bands 5q23-q31. Only 10 kilobases (kb) of DNA separate the two genes, suggesting that they have a common origin and/or regulation. We have cloned 70 kb of genomic DNA that include the IL3 and CSF2 genes, as well as flanking sequences, and report here a physical map of this region. Several unique-sequence DNA segments have been identified in this region, and one of these fragments detects two RFLPs in DNA from unrelated Caucasians. Segregation of these DNA polymorphisms was followed in the CEPH panel of 40 large three-generation pedigrees, and linkage was detected with 17 genetic markers previously typed in these families. Multipoint linkage analysis permits the placement of the region containing the IL3 and CSF2 structural genes on the recombination-genetic linkage map of chromosome 5q and thereby allows the role of these genes in leukemogenesis to be more critically examined.

3. Sequence and chromosomal location of the I-309 gene: Relationship to genes encoding a family of inflammatory cytokines. We previously reported the isolation and characterization of a cDNA clone, I-309, that encodes a small secreted protein produced by activated human T lymphocytes. This protein is structurally-related to a large number of recently identified proteins that are secreted upon cellular activation. In this report, we describe the isolation and characterization of the gene encoding I-309. The genomic organization is essentially identical to that found in genes encoding the structurally-similar proteins TCA-3, hJE/MCP-1, and mJE, strengthening the hypothesis that these genes are evolutionarily related. The region of the I-309 gene 5′ of the mRNA cap site exhibits extensive nucleotide sequence homology with the same region of the murine gene, TCA-3, providing additional evidence that I-309 and TCA-3 are likely to be homologs. Finally, panels of rodent-human somatic cell hybrids were used to map the I-309 gene to human chromosome 17. In conjunction with recent mapping data from other

laboratories, this result suggests the presence of a cluster of related genes on this chromosome.

4. Mapping of the gene encoding the α subunit of the stimulatory G-protein of adenylyl cyclase (GNAS) to 20q13.2-q13.3 in man by in situ hybridization. The signal-transducing G proteins are heterotrimers composed of three subunits: α, β, and γ. Multiple distinctive forms of the α, β, and γ subunits have been described, each encoded by a distinct gene. The α subunit of the stimulatory G protein ($G_s\alpha$) is the protein that is responsible for stimulation of catalytic activity of adenylyl cyclase, and may regulate activity of calcium channels. The human $G_s\alpha$ gene (GNAS) has been regionally localized to chromosome band 20q13.2-q13.3 by in situ hybridization. A human adrenal cDNA was used to construct a 1500-base pair probe, which was then labeled with tritium for in situ hybridization to human metaphase chromosomes. Localization of the GNAS gene to 20q13.2-q13.3 adds to a growing number of genes assigned to this region, including the gene encoding the signal effector enzyme phospholipase C-148. This regional assignment also localizes the locus of the human disorder Albright hereditary osteodystrophy, in which an inherited deficiency of $G_s\alpha$ protein results in pseudohypoparathyroidism type 1a.

5. Genetic mapping of the β1 GABA receptor gene to human chromosome 4 using a tetranucleotide repeat polymorphism. As more coding loci for functional human genes are described, there is a growing need to describe DNA polymorphisms in specific genes. By examining DNA sequences within the introns of the beta 1 subunit of the gamma aminobutyric acid receptor gene, GABARB1, we found a tetranucleotide repeat sequence (GATA). Amplification of this region using PCR revealed 7 alleles and a high degree of polymorphism (PIC = 0.75) in human populations. DNAs from the CEPH families were typed for the GABARB1 intron polymorphism and analyzed with respect to 20 linked markers on chromosome 4. The results permit placement of GABARB1 on the linkage map of chromosome 4 between D4S104 and ALB. These results affirm the value of sequence analysis of non-coding segments included within or adjacent to functional genes as a strategy to detect highly informative polymorphisms.

6. Chromosomal mapping of the human glutamate dehydrogenase (GLUD) genes to chromosomes 10q22.3-q23 and Xq22-q23. GLUD is an important mitochondrial enzyme that participates in neuronal transmission by catalyzing the deamination of L-glutamate, which serves as a potent excitatory neurotransmitter. The direct involvement of GLUD in the pathogenesis of certain human neurodegenerative disorders has been recently suggested. To investigate its possible role in the induction and progression of these disorders, we have initiated studies focusing on the chromosomal organization of the several members of the GLUD family and their functional status. In the present study, by using a panel of human x rodent somatic cell hybrids and in situ hybridization to metaphase chromosomes, we found that the members of the GLUD gene family are dispersed in the human genome. At least one functional split gene was mapped to chromosome 10q22.3-q23, an intronless pseudogene to chromosome Xq22-q23, while a third candidate novel gene was found not to be syntenic to either chromosome 10 or X.

7. BEK, a receptor for multiple members of the fibroblast growth factor (FGF) family, maps to human chromosome 10q25.3-q26. The gene for the FGF receptor, BEK, was assigned to human chromosome 10 by applying PCR techniques to DNAs from a panel of human-rodent somatic cell hybrids. The gene was further localized to the position 10q25.3-q26 by in situ hybridization. The BEK gene has a potential involvement in human cancer.

8. A nucleic acid binding protein gene coding for an iron responsive element (IRE-BP) is located on human chromosome 9. Three human mRNAs are regulated post-transcriptionally by iron via iron-responsive elements (IREs) contained in each mRNA. A cytoplasmic protein (IRE-BP) binds to these cis-acting elements and mediates the translational regulation of ferritin H- and L-chain mRNA and the iron-dependent stability of transferrin receptor mRNA. We have taken advantage of the different mobilities of the human and rodent IRE/IRE-BP complexes on non-denaturing polyacrylamide gels to determine the chromosomal localization of the gene encoding the IRE-BP. Utilizing a panel of 34 different human/rodent hybrid cell lines, we have assigned the IRE-BP gene to human chromosome 9. This new technique based on nucleic acid/protein interaction may allow determination of the chromosomal localization of other RNA- or DNA-binding proteins.

9. A gene that encodes a leukemia-associated protein (p18) maps to a region of human chromosome 1 that is frequently deleted in tumors of neuroectodermal origin. The cytosolic protein p18 which is expressed in increased amounts in acute leukemia cells is variably phosphorylated as a function of growth and differentiation. Proteins with identical amino acid sequences were independently found to be highly expressed in normal brain tissue and neuroendocrine tumor cells. Here we describe the mapping of the recently cloned p18 gene to chromosome 1p35-p36.1 by Southern blot analysis of human-rodent somatic cell hybrid DNA and by chromosome in situ hybridization using a p18 genomic probe. This region of the distal short arm of chromosome 1 is a frequent site of deletions or loss of heterozygosity in tumors derived from neural crest cells, particularly neuroblastomas and melanomas. The high levels of expression of p18 in brain and neuroendocrine tumor cells, its possible role in growth regulation and its chromosomal location in a region frequently deleted in neuroectodermal tumors suggest that this gene may be involved in common genetic events occurring in these tumors.

Publications:

Ferrari A, Seuanez HN, Hanash SM, Atweh GF. A gene that encodes for a leukemia associated phosphoprotein (p18) maps to a region of chromosome 1 that is frequently deleted in tumors of neuroectodermal origin. Genes Chromosomes Cancer (In Press).

Halverson D, Modi W, Dean M, Gelman EP, Dunn KJ, Clanton D, Oskarsson M, O'Brien SJ, Blair DG. An oncogene chromosome 8-9 gene fusion isolated following transfection of human ovarian carcinoma cell line DNA. Oncogene 1990;5:1085-9.

Miller MD, Wilson SD, Dorf ME, Seuanez HN, O'Brien SJ, Krangel MS. Sequence and chromosomal location of the I-309 gene: relationship to genes encoding a family of inflammatory cytokines. J Immunol 1990;145:2737-44.

Abbreviation	No. Genes	Collaboration*	STATUS Hybrid Panel	In situ Hybrid	RFLP	Publication
Colectin-1	1	L. Burns	Pending	Pending	Pending	Pending
Calpactin-1	1	L. Burns	Pending	Pending	Pending	Pending
Chromogranin A	1	M. Levine/JHU	Yes	Yes	Yes	Modi et al. (1989)
B-3 subunit GTP binding protein	2	M. Levine/JHU	Done	Done	No	Levine et al. (1990)
B-1 subunit GTP binding protein	1	M. Levine/JHU	Done	Done	No	Levine et al. (1990)
op18 protein	1	A. Ferrari	Yes	Yes	No	Ferrari et al. (In Press)
I-309 Cytokine	1	M. Kraugel	Yes	No	No	Miller et al. (1990)
Act-2	1	W. Leonard	Yes	Yes	No	Napolitano et al. (Submitted)
FGF-5	1	M. Jaye	Yes	No	No	Dionne et al. (1990)
ApoA1	1	S. Karathanasis	Yes	Yes	No	In Preparation
KGF25 OLIGO C KGF 97,9,F	5	M. Kelley	Yes	No	No	In Preparation
IRE-BP	1	M. Hentze	Yes	No	No	Hentze et al. (1989)
Hypothetical tumor suppressor	1	P. Haley/J. Lichy	Yes	Yes	Yes	In Preparation
Fibroblast growth factor receptor	1	M. Jaye/Rorer	Yes	Yes	Pending	Dionne et al. (Submitted)
α subunit, GTP binding protein	1	M. Levine/JHU	No	Yes	No	Levine, (Submitted)
BMPB	1	J. Tabas/Phila	Yes	No	Pending	In Preparation

*All at NIH except otherwise indicated.

Abbreviation	No. Genes	Collaboration*	STATUS Hybrid Panel	In situ Hybrid	RFLP	Publication
Muscarinic Receptors	5	T. Bonner	Yes	Done	Done Yes	In preparation
Interleukin-1 α subunit	1	K. Matsushima	Yes	Yes	Yes	Modi et al. (1988)
Monocyte derived neutrophile chem tactic factor	1	K. Matsushima	Yes	Yes	Yes	Modi et al. (1990)
Endonexin-II	1	M. Jaye/Rorer	Yes	Yes	No	Modi et al. (1989)
MLVI-2 (MoLV integration)	1	N. Anagnou/U. Wash P. Tsichlis	Yes	Yes	No	Anagnou et al. (1989)
Lambda HLM-25 (endogenous retrovirus)	1	N. Anagnou	Yes	Yes	No	Submitted, New Biologist
Glutamate dehydrogenase	2	N. Anagnou	Yes	Yes	Pending	Pending
ADH class III	2-3	D. Goldman	Yes	No	No	Rathna Giri et al. (1989)
T-cell receptor-Z	1	R. Klausner	Yes	Yes	Yes, VNTR	Weissmann et al. (1988)
PC-1-non erp	1	M. Dean	Pending	Pending	Yes	Pending
InTH	1	C. McGrath	Pending	Pending	Pending	Pending
NSI oncogene	1	H. Morse	Yes	No	No	Savage et al. (Submitted)
Glutamase	2	B. Mock	Yes	Yes	No	Mock et al. (1989)
OVC oncogene	1	D. Blair	Yes	Yes	Yes	Halverson et al. (1990)
Calbindin Vitamin D dependent Ca binding protein	1	Sylvia Christakos	Yes	Yes	Yes	Modi et al. (Submitted)
Lipocortin-1	1	L. Burns	Pending	Pending	Pending	Pending

*All at NIH except otherwise indicated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05385-08 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

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:	Naoya Yuhki	Visiting Associate	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	David E. Wildt	Special Volunteer	LVC	NCI

COOPERATING UNITS *(if any)*
PRI/DynCorp, Frederick, MD (W. S. Modi); H&W Cytogenetics Services, Inc., Lovettsville, VA (W. G. Nash); NIAID, NIH, Bethesda, MD (C. Kozak); NCBI, NLM, NIH, Bethesda, MD (D. Lipman)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.2	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 gene map of the domestic cat (Felis catus) has been constructed as a physical map of over 100 coding gene loci homologous to the human genes. The present map has been expanded to over 100 loci, including approximately 50 proto-oncogene loci, using a previously developed panel of cell hybrids. These markers have been used to define the syntenic segments of primitive chromosome associations between the human and feline genetic maps. Interspecies crosses between F. catus and leopard cat Prionailurus bengalensis and between F. catus and Leopardus geoffroyi have been initiated in order to produce backcross offspring to define a linkage map of domestic cat. To date, over 50 interspecies hybrid offspring have been born following artifical insemination; of these, 12 healthy females are maturing. Methods for in situ hybridization of feline metaphase chromosomes using biotin-labeled molecular clones have been attempted and improved. Procedures using human chromosome libraries for chromosome "painting," i.e., visualization of homologous chromosome segments between carnivores and primates, are in progress. To date nearly 35% of the human genome can be aligned band-for-band with chromosome segments of the cat genome. The combination of the three present methods (hybrid panel, heterologous species crosses, and chromosome painting) holds much promise in reconstruction of the historic phylogenetic genome exchanges.

Project Description

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

Stephen J. O'Brien	Chief	LVC	NCI
Naoya Yuhki	Visiting Associate	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
David E. Wildt	Special Volunteer	LVC	NCI

Objectives:

(1) The development and expansion of the genetic map of the domestic cat (Felis catus) with particular emphasis on molecular genetic loci involved in neoplastic transformation. (2) The understanding of the genomic and developmental organization of feline loci involved in cancer. (3) The description of the comparative structure of the cat genome relative to other felids, to other carnivores, and to other mammals; specifically, mouse and man. (4) The development of the gene delivery technologies for treatment of feline models of human inborn errors. (5) The assembly and organization of genetic data on gene mapping in the cat, related carnivores, and other mammals.

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 radio-immunoassay (RIA), reverse transcriptase assay, and viral cloning; (5) immunological assays, including RIA, cytotoxicity, fluorescent antibody procedures, immunoprecipitation, microcomplement fixation, and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques, including cDNA transcription in vitro, molecular cloning, gene splicing, DNA and RNA blotting; (7) in situ DNA hybridization to metaphase chromosomes; and (8) heterologous species genetic crosses.

Major Findings:

1. Constructing the gene map of the cat: Extensive conservation of linkage arrangement to the human genetic map. A research emphasis on the construction of the genetic map of the cat has been a major focus in our laboratory for several years. The cat gene map now consists of over 100 loci, including some 50 proto-oncogene loci. With the exception of a preliminary synteny map for the domestic dog, the only other carnivore gene-chromosome map was derived by the Russian geneticist, O. L. Serov, and his colleagues for the American mink (Mustela vision). In nearly all cases where they could be compared, the mink and cat linkages agree, thereby confirming the cytological indication of homology. When we first compared linkage maps of cat and man, we were struck

by the high degree of syntenic homology which existed between the two species, especially when we considered that cat and man were in different mammalian orders. In most cases, syntenic groups located on human chromosome arms were also syntenic in cats; and in the case of at least 5 human chromosomes (1, 6, 11, 12, and X), gene homologs from both arms are also syntenic in cats, suggesting conservation of large portions of these chromosomes. By contrast, the mouse gene map is three to four times more rearranged, compared with man, than is that of the cat.

The striking linkage homology between cat and man, combined with certain advantageous cytological characteristics of primate and feline chromosomal evolution, prompted us to search for cytological homology between syntenically homologous chromosomes in the two species. High-resolution, G-banded preparations of homologous chromosomes were carefully examined, and several regions of band-for-band homology were identified (HSA1p:FCAC1, HSA2p:FCAA3, HSA2q: FCAC1, HSA11:FCAD1, and HSA12:FCAB4). In all, we could align between 30 and 35% of the human karyotype band-for-band with the feline karyotype despite the passage of over 80 million years since these species shared a common ancestor.

2. Production of 9-20-cM contiguous linkage map of the domestic cat. In order to produce a high resolution linkage map of the cat, we have initiated an experimental protocol that is modeled after similar efforts in mouse genetics. Sexual crosses between distinct species of cats were produced by breeding and by artificial insemination. In one group, female domestic cats were inseminated artificially with semen from male leopard cats (Prionailurus bengalensis). In a second protocol, female domestic cats were bred with male Geoffroy's cats (Leopardus geoffroyi). At this writing, 55 F_1 offspring have been produced, and of these, 10 maturing F_1 females have survived. These F_1 females will be crossed by artificial insemination to domestic cat males to produce 100 to 200 offspring. The offspring will be typed for 200 anchor marker loci based upon syntenic homology and position in the gene map. The rationale for using interspecies crosses is to increase the incidence for restriction fragment length polymorphism variation between marker loci.

3. Development of a general strategy for producing and interpreting gene maps in animal species. The recent emphasis in human genome mapping has provided a stimulus for developing genetic maps in a number of domestic animal species. The two major uses of a gene map in animal species are: first, to provide a resource for genetic analysis and manipulation, particularly with regard to economically-important genetic characters; and second, as a component for dissecting the evolution of genome organization. Both of these goals produce valuable insight into the role of cellular genes in metabolic and infectious disease processes including neoplasia. Based on a long experience in building gene maps in man and cat, we have proposed a strategy for developing gene maps of a new species de novo. An important consideration is the inclusion of two equally important categories of anchor loci: Type I loci that encode coding genes and are conserved in sequence divergence among mammals and Type II loci that are highly polymorphic loci within the species to be mapped and are often (but not always) not associated with coding regions.

Publications:

Gardner MB, Kozak C, O'Brien SJ. The Lake Casitas wild mouse: evolving genetic resistance to retroviral disease. Trends Genet 1991;7:22-7.

O'Brien SJ. Mammalian genome mapping: lessons and prospects. Curr Opin Genet Dev (In Press).

O'Brien SJ, Graves JAM. Geneticists converge on divergent mammals: an overview of comparative mammalian genetics. Aust J Zool 1990;37:147-54.

O'Brien SJ, Graves JAM. Report of the committee on comparative gene mapping: human gene mapping 10.5. Cytogenet Cell Genet 1990;55:406-33.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
ZO1CP05389-08 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Reproductive Strategies in Animal Species Emphasizing Developmental Biology

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	Special Volunteer	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI

COOPERATING UNITS *(if any)*
Dept. of Animal Health, Natl. Zool. Park, Wash., DC (A. M. Donoghue, J. G. Howard, M. Barone, M. Bush); Veterinary Resources Branch, DRS, NIH, Bethesda, MD (P. M. Schmidt)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

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 identify biological factors influencing reproductive function and embryogenesis in felid species. Emphasis is placed on gamete interaction and embryo maturation in vitro and in vivo. Findings are relevant to (1) fundamental studies of fertilization mechanisms, gametogenesis, and early embryogenesis; (2) the propagation of genetically valuable laboratory animals and endangered species; and (3) developmental studies which eventually will allow delivering molecularly cloned genes into early-staged pre-implantation embryos. Areas of effort primarily focus on (1) in vitro fertilization and embryo development in vitro and in vivo; (2) artificial insemination via laparoscopic deposition of spermatozoa; and (3) oocyte rescue, maturation in vitro and the development of gene delivery techniques into embryos which will allow studying the mechanisms associated with transformation and inborn errors in early development. Current emphasis is applied to the gametes and embryos of the domestic cat with comparative studies conducted in a variety of nondomesticated Felidae species. To date, progress has allowed (1) comparison of factors which influence in vitro fertilization and embryo development in vitro in domestic cats and nondomestic felids (i.e., gas atmosphere, medium, protein sources, temperature); (2) development of suitable embryo recipients after fertilization and embryo production in vitro; (3) initiation of in vitro fertilization studies involving felid species producing large proportions of abnormal sperm and which reproduce poorly in captivity; (4) development of assays measuring permeation of domestic cat oocytes and embryos to cryoprotectants useful for developing embryo freezing protocols; and (5) continued success in the development of an efficient transabdominal artificial insemination approach in the domestic cat which has lead to comparative studies in nondomestic felids including production of domestic cat x leopard cat hybrids produced to develop a high resolution genetic linkage map of the domestic cat in order to further understand the genetic loci involved in neoplastic transformation as well as other disease states.

Project Description

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

Stephen J. O'Brien	Chief	LVC	NCI
David E. Wildt	Special Volunteer	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI

Objectives:

The specific objectives of these studies are to (1) describe the developmental biology of the domestic cat and related species in the Felidae family; (2) enhance our ability to routinely reproduce rare felid models; and (3) increase and integrate the reproductive-genetic data base, thereby permitting genetic manipulation in the cat and related species.

Methods Employed:

The following techniques were used: (1) hormonal induction of ovarian activity; (2) laparoscopic recovery of ovarian follicular oocytes; (3) electroejaculation and laboratory processing for sperm recovery and induction of capacitation; (4) in vitro maturation, fertilization and embryo culture; and (5) surgical technique (laparotomy versus laparoscopy) for artificial insemination (AI) and/or embryo transfer.

Major Findings:

1. Developmental potential of 2-cell cat embryos in vitro as influenced by the temperature and gas phase of the culture system. There are two primary reasons for sustaining in vitro-fertilized (IVF) felid embryos in culture until later developmental stages. First, access to more advanced embryos allows transfer of embryos directly to the uterus by less invasive approaches than surgical oviductal transfer. Second, understanding the fundamental nutritive and energy requirements for preimplantation of cat embryos provides comparative information for other carnivore taxa, most of which have never been studied. Although IVF and in vitro development to morulae can be achieved routinely in domestic cats, most of the embryos exhibit a developmental block and are unable to advance to blastocysts. This study examined the role of temperature and gas-phase composition on IVF and embryo development in domestic cats.

In the first study, oocytes were fertilized and cultured in 5% CO_2 in air at 37°, 38°, or 39°C. The second study evaluated the effects of 5% CO_2 in air versus 5% CO_2, 5% O_2, 90% N_2 versus 10% CO_2 in air. Neither fertilization (cleavage) nor development to the morula/blastocyst stage was influenced by temperature or gas treatments. Despite changing these culture conditions, oocyte cleavage averaged about 75%, and more than 80% of these 2-cell embryos proceeded to morulae in vitro. However, altering these specific conditions failed to overcome the in vitro morula-to-blastocyst developmental block.

These results, together with our earlier published findings concerning the effects of medium and protein supplement, demonstrate that successful IVF and early embryo cleavage in the domestic cat is remarkably resilient to substantial alterations in in vitro culture conditions. Within the limits already tested, it is now apparent that most conventional types of medium, protein supplement, temperature, and gaseous environment will support high rates of oocyte cleavage after IVF. Overcoming the late stage morula block, however, remains unresolved and will be a major area of research focus in FY 92.

2. In vitro capacitation of sperm as measured by penetration of heterologous oocytes. Progress in 1991 involved attempting to better understand the impact of teratospermia, commonly observed in felid species (and man) on the mechanism of fertilization. Previous studies demonstrated that fresh domestic cat oocytes can be bound and the zona pellucida (ZP) penetrated by heterologous felid sperm. However, relying on fresh cat oocytes is impractical for assessing sperm function and for making spontaneous sperm quality assessments. Oocyte collection also is laborious, and the freshness requirement prevents using this assay at other institutions with no access to domestic cat ovaries. A salt-stored ZP penetration assay is an attractive alternative and has proven utility in the hamster, rabbit, and human. Salt-stored ZP retains the ability to distinguish between capacitated and non-capacitated sperm, and a major advantage is that they can be banked to allow transport and/or later use. We have (1) developed a ZP penetration assay that relies on the use of cat follicular oocytes collected at ovariectomy, and (2) evaluated the ability of electroejaculated/washed domestic cat and leopard cat (Felis bengalensis) sperm to penetrate salt-stored versus fresh oocytes in the presence or absence of bovine serum albumin (BSA).

Oocytes were collected, matured in vitro, left fresh or salt-stored and then exposed to electroejaculated, swim-up processed sperm. Gametes were incubated in protein-free, modified Tyrode's solution or in the same medium containing BSA. Treatments were compared for % ZP penetration (< halfway vs. > halfway through ZP) as an index of sperm capacitation. There was no difference between species in the penetration of fresh ZP (domestic cat, 42.5%; leopard cat, 38.6%) and stored ZP (domestic cat, 32.4%; leopard cat, 27.6%). Sperm incubated in protein-free medium were less capable of ZP penetration (domestic cat, 14.6%; leopard cat, 7.9%) than sperm in BSA-containing medium (domestic cat, 60.3%; leopard cat, 58.4%). For both species, a large number of sperm remained in the outer half of the ZP in both the presence and absence of BSA. In the absence of BSA, <15 and 10% of the oocytes contained domestic cat and leopard cat sperm in the inner half of the ZP and/or the perivitelline space (PVS). When BSA was present for the domestic cat, 60.3% of the oocytes were > one-half ZP penetrated, and 44.3% contained sperm that had reached the PVS. When albumin was present for the leopard cat, 58.4% of the oocytes were > one-half ZP penetrated, and 35.4% contained PVS sperm. Regardless of whether the oocytes were fresh or salt-stored, the presence of BSA also increased the number of inner ZP-penetrated sperm (polyspermy) more than fivefold over non-protein controls.

These results indicate that albumin facilitates capacitation and/or the acrosome reaction in cat sperm. The relatively high incidence of poly-ZP

penetration may be normal in the cat since the level at which the block to polyspermy occurs is unknown. If the cat is like the rabbit, another induced ovulator, the block may be at the level of the plasma membrane. Overall, these results affirm that the domestic cat ZP apparently has no mechanism for excluding penetration by "foreign" felid sperm. In FY 91, studies emphasized the use of salt-stored cat oocyte ZP assay to further study the mechanisms of sperm function, especially for assessing capacitation kinetics and fertility potential in both the domestic cat and nondomestic felid species.

3. Development of a laparoscopic AI approach for producing domestic cat X leopard cat hybrids (see also Z01CP05385-07 LVC). A major objective of our program is to develop a high resolution genetic linkage map of the domestic cat to further our understanding of the genetic loci involved in neoplastic transformation and other disease conditions. To facilitate recognition of large numbers of DNA polymorphisms, hybrid felids are required. The objective is to produce a pedigree of backcross felids to test for linkage association of 200-250 gene markers for which molecular clones are available. To achieve this, there must be sufficient genetic/evolutionary distance between the parent species to provide the necessary genetic variation to allow tracking at least 95% of the marker genes. Offspring from the interspecies domestic cat X leopard cat hybridization will be raised to sexual maturity and backcrossed to domestic cat males to produce a desired combination of genetic markers for the study of linkage association. Natural breeding for hybrid production has not been possible because of behavioral incompatibility between mating pairs. Therefore, laparoscopic AI, a relatively minor surgical procedure, is being explored as a strategy for artificial propagation of other cat models useful for the study of human disease.

To date, a total of 26 laparoscopic AIs have been conducted using a technique whereby electroejaculated, processed leopard cat sperm were deposited transabdominally into the uterus of gonadotropin-treated, domestic cat females. Ultrasonic and/or radiographic examinations were performed 30 to 45 days later for determining pregnancy, and all pregnant females were allowed to develop to term. Pregnancy was diagnosed in 12 (46.2%) of the inseminated females and a total of 55 hybrid kittens were born.

These results indicate that: (1) Cross-species fertilization occurs following laparoscopic deposition of semen directly into the uterus. (2) The resulting embryos are biologically competent, as demonstrated by the production of live-born offspring. Production of hybrid females will continue, and when these animals reach sexual maturity at 9-12 months of age, each will be inseminated to begin producing the backcross young needed for the genetic linkage mapping studies.

Publications:

Donoghue AM, Johnston LA, Seal US, Armstrong DL, Tilson RL, Wolf P, Petrini K, Simmons LG, Gross T, Wildt DE. In vitro fertilization and embryo development in vitro and in vivo in the tiger (Panthera tigris). Biol Reprod 1990;43:733-47.

Howard JG, Brown JL, Wildt DE. Teratospermic and normospermic domestic cats: ejaculate traits, pituitary-gonadal hormones and improvement of sperm viability and morphology after swim-up processing. J Androl 1990;11:204-15.

Howard JG, Bush M, Wildt DE. Teratospermia in domestic cats compromises penetration of zona-free hamster ova and cat zona pellucidae. J Androl 1991;12:36-45.

Howard JG, Wildt DE. Ejaculate-hormonal traits in the leopard cat (Felis bengalensis) and sperm function as measured by in vitro penetration of zona-free hamster ova and zona-intact domestic cat oocytes. Mol Reprod Devel 1990;26:163-74.

Johnston LA, Miller AM, O'Brien SJ, Wildt DE. Culture medium and protein supplementation influence in vitro fertilization and embryo development in the domestic cat. J Exp Zool (In Press).

Johnston LA, Miller AM, O'Brien SJ, Wildt DE. Influence of temperature and gas atmosphere on in vitro fertilization and embryo development in the domestic cat. J Reprod Fertil (In Press).

Wildt DE. Fertilization in cats. In: Dunbar BS, O'Rand M, eds. A comparative overview of mammalian fertilization. New York: Plenum Press (In Press).

Wildt DE. Potential applications of IVF technology for species conservation. In: Bavister BD, Cummins J, Roldan ERS, eds. Fertilization in mammals. Norwell: Serono Symposium, 1990;349-64.

Wildt DE, Donoghue AM, Johnston LA, Schmidt PM, Howard JG. Species and genetic effects on the utility of biotechnology for conservation. Zool Soc London (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05414-08 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Characterization of Retroviruses (Type-D and SIVs) Isolated from Primates

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:	Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
	David Derse	Senior Staff Fellow	LVC	NCI

COOPERATING UNITS *(if any)*: University of Washington, Seattle, WA (W. Morton, M. Katze, J. Overbaugh, H. Ochs, C.-C. Tsai); PRI/DynCorp, Frederick, MD (L. Arthur, M. Gonda, L. Henderson); Henry M. Jackson Foundation, Rockville, MD (G. Eddy, M. Lewis)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Immunogenetics Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.6	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.)*

A molecular clone derived from simian immunodeficiency virus (SIV/Mne)-infected HuT 78 cells has been completely sequenced and shown to be 82% identical to HIV-2. This clone expresses all the structural and regulatory genes of SIV and is infectious and pathogenic. Two pig-tailed macaques (M. nemestrina) infected with this clone experienced marked $CD4^+$ depletion; one died 83 weeks after infection. These animals provide a model for following changes in the SIV envelope (env) gene as a function of time after infection. Two regions of env, within the variable regions V1 and V4, showed change in up to 40% of their amino acids when AIDS became apparent. The region of SIV env that corresponds to the immunodominant V3 loop of HIV was conserved. Overall, the rate of change in env was approximately 1%/year at the amino acid level, which is 10 to the sixth power-fold faster than the rate of mutation in cellular DNA. Analysis of the biological properties of these variant envelope proteins will be useful in defining the mechanisms underlying progression to AIDS.

Transmission of HIV from an infected pregnant woman to her infant is the most important mechanism of HIV infection in children. The mode of transmission of HIV from mother to fetus, the time of infection, and the mechanism(s) that protects approximately two-thirds of infants born to HIV-infected mothers are unknown. To facilitate a systematic study of maternal-fetal transmission, we have developed a nonhuman primate model by infecting pregnant M. nemestrina with a pathogenic, uncloned strain of SIV/Mne. Three animals infected during the third trimester delivered healthy infants. One of the three infants, a male born 31 days after his mother was infected, became virus-positive but failed to produce SIV-specific antibody. He died with overt AIDS and disseminated adenovirus infection at 6 months of age. This is the first reported maternal-fetal transmission with SIV and will serve as a useful model for determining factors influencing the frequency of transmission as well as for studies of the effectiveness of antiviral therapy.

Project Description

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

Raoul E. Benveniste	Medical Officer	LVC	NCI
Dean Mann	Chief, Immunogenetics Section	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
David Derse	Senior Staff Fellow	LVC	NCI

Objectives:

To characterize primate retroviruses, with emphasis on the type-D and lentivirus (simian immunodeficiency virus, SIV) classes. To determine, by molecular hybridization and immunological techniques, the homology between these viruses and other primate retroviruses.

New isolates will be characterized by examining their host range for various cells in vitro, by obtaining molecular clones, restriction enzyme maps, and DNA sequence data. In addition, viral proteins will be purified, amino acid sequences determined, and antisera to the individual proteins raised in rabbits in order to develop specific immunological reagents and to examine the extent of antigenic and molecular similarities to human AIDS viral isolates. The pathogenicity of these isolates in various primate species will be determined in order to develop a suitable animal model for AIDS. The effect of various genes on pathogenicity will be determined by performing site-directed mutagenesis and rechallenging primates with these new variants.

To determine the prevalence of these primate viruses in various primate colonies and in feral populations by examining sera for the presence of cross-reactive antibodies and peripheral blood lymphocytes (PBLs) for the presence of viruses.

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 primates were cocultivated with various cells and the supernatant assayed at intervals for reverse transcriptase activity. Cloned retroviral DNA was used as a probe for detection of related DNA sequences in primates. Viral proteins were purified by high pressure liquid chromatography. Antigens and antibodies were detected by radioimmunoassays, ELISA assays, and by Western immunoblot techniques.

Major Findings:

1. Sequence variation in the envelope genes of a molecular clone of SIV during progression to AIDS. Genetic diversity is a hallmark of the HIV genome, but it is not clear how much the emergence of HIV variants determines the progression to AIDS with each individual. The most extensive variation has been observed in the gene coding for the surface envelope glycoprotein,

gp120. This protein is likely to be a key target for the immune system, determines the cell tropesin of the virus and mediates cytopathicity in $CD4^+$ T lymphocytes.

Part of the difficulty in studying the effect of HIV variation on the progression of AIDS is the uncertainty in the sequence(s) of the infecting virus. We have therefore examined the changes in virus sequence in two macaques (M. nemestrina) that developed AIDS following inoculation with a molecular clone of SIV. One animal showed a marked drop in $CD4^+$ lymphocytes 1 year after infection but is still surviving, with low $CD4^+$ cells, 3 years after infection. The other animal was euthanized 83 weeks post injection with $CD4^+$ depletion and severe bleeding due to thermocytonemia.

In collaboration with J. Overbaugh, SIV envelope sequences were amplified by polymerase chain reaction (PCR) from PBLs obtained at various times after injection. The amplified region included five variable domains in the SIV/HIV-2 envelope gene (env) that correspond to the domains called V1 through V5 in the HIV env sequence. Multiple clones were prepared and sequenced. Two regions of env, within V1 and V4, showed changes in up to 40% of their amino acids when AIDS became apparent. Surprisingly, the V3 loop region, which is extremely variable among HIV isolates and has been described as a principal neutralizing epitope was conserved. Overall, the rate of change in env was approximately 1%/year at the amino acid level, which is one million times faster than the rate of mutation in primate cellular DNA. These SIV env variant sequences isolated from a macaque that developed AIDS following infection can now be incorporated into the parental SIV genome. Such chimeric viruses, with defined differences in envelopes, can be used to determine the immunogenic and pathogenic function of these variants that were selected for in the host.

2. Maternal-fetal transmission of SIV in macaques. With the virtual elimination of HIV transmission through blood transfusions in this country, transmission of HIV from an infected mother to her infant is the most important mechanism of HIV infection in children. The mode of transmission of HIV from mother to fetus and the time when infection occurs are unknown; approximately one-third of children born to HIV-infected mothers are, themselves, infected at birth. Although a possible protective effect of maternal antibody has been reported, effective measures to prevent infection of the offspring are lacking, and new avenues of therapy need to be explored, including treatment of mothers during pregnancy with antiviral drugs.

To facilitate a systematic study of maternal-fetal transmission, we have developed an animal model by infecting M. nemestrina with uncloned pathogenic SIV/Mne. M. nemestrina is an unusually SIV susceptible macaque species that rapidly develops a fatal immunorepressive disease characterized by anemia, generalized wasting, prolonged fever, lymphadenopathy, and opportunistic infections. These animals were inoculated intravenously during the third trimester with 10^5 infectious particles of SIV/Mne. All delivered healthy infants; one of the three, a male born 31 days after the mother was infected, became virus-positive. Although he had acquired SIV-specific maternal antibodies at birth, he failed to produce antibodies and died with simian AIDS and disseminated adenovirus (SV2U) infection at age six and one-half months.

The incidence of maternal-fetal transmission of SIV observed in pregnant M. nemestrina in this small study was one in three, similar to that observed in humans. Additional experiments are underway to confirm this observation and to study the effect of infecting mothers during the first and second trimesters on the incidence of infection in their offspring. This model of maternal-fetal transmission will allow us to study the effect of SIV on fetal development and of vaccines or drugs on the prevention of transmission.

3. Mucosal and intravenous infection of macaques with SIV: Evidence for virus infection in the absence of virus isolation or seroconversion. HIV-1 is transmitted both by heterosexual and homosexual contact; strong associations with HIV seropositivity and disease risk have been demonstrated for homosexual men who have engaged in anorectal sexual conduct or in receptive anal intercourse. Since intrarectal infection appears to be an important route of exposure to HIV and since the pathogenesis of SIV and HIV is similar, a study utilizing the rectal mucosal route of infection was designed.

In previous studies of macaques intravenously inoculated with SIV/Mne, all animals became infected, seroconverted, and developed simian AIDS. In order to determine whether infection or disease is influenced by changes in viral dose or route of administration, 12 M. nemestrina were infected intravenously or intrarectally with either 10^3, 10^2, or 10 infectious virus particles. The intravenously infected animals, regardless of dose, became infected and seroconverted; however, only one of the intrarectally infected animals that received the highest dose of virus seroconverted. All animals, regardless of the route or dose of virus exposure, were PCR-positive at various times after infection. Some macaques that were seronegative, virus isolation-negative, but PCR-positive, exhibited lymphadenopathy, anemia, and $CD4^+$ depletion.

The finding of PCR positive, but virus- and antibody-negative, intrarectally inoculated animals was unexpected. Seroconversion may require a certain threshold of virus replication; in the intrarectally-infected macaques, SIV may be sequenced in macrophages and may be latent for varying lengths of time. In humans, HIV provirus has been demonstrated by PCR in seronegative homosexual men, all of whom eventually seroconverted. The present study complements that observation and shows that seronegative and virus isolation-negative macaques can be clearly infected with SIV.

Publications:

Agy MB, Foy K, Gale MJ, Benveniste RE, Clark EA, Katze MG. Viral and cellular gene expression in $CD4^+$ human lymphoid cell lines infected by the simian immunodeficiency virus, SIV/Mne. Virology (In Press).

Alpers CE, Tsai CC, Hudkins KL, Kuller L, Benveniste R, Ward JM, Waas A, Clark EA, Morton WR. Focal segmental glomerulosclerosis in primates infected with a simian immunodeficiency virus. Lab Invest (In Press).

Gale MJ, Ledbetter JA, Schieven GL, Jonker M, Benveniste RE, Morton WR, Clark EA. CD4 and CD8 T cells from SIV-infected macaques have defective signaling responses after perturbation of either CD3 or CD2 receptors. J Immunol 1990;9: 849-58.

Krugner-Higby L, Kucera L, Lerche N, Sever J, Fucillo W, Allan J, Benveniste R. Serological survey for two simian retroviruses in macaques and African green monkeys. Lab Anim Sci 1990;40:24-8.

Kuller L, Morton WR, Benveniste RE, Tsai CC, Clark EA, Gale MJ, Hu SL, Thouless ME, Katze MG. Inoculation of Macaca fascicularis with simian immunodeficiency virus, SIV/mne: immunologic, serologic, and pathologic changes. J Med Primatol 1990;19:367-80.

Kuller L, Watanabe R, Morton WR, Tsai CC, Isahakia MA, Aman RA, Knott WB, Hill RW, Benveniste RE. Detection of serum antibodies to SIV, HTLV-I, and type D retroviruses in Ethiopian and Kenyan baboons. J Med Primatol (In Press).

Ochs HD, Morton WR, Tsai CC, Thouless ME, Zhu Q, Kuller L, Wu YP, Benveniste RE. Maternal-fetal transmission of SIV in macaques: disseminated adenovirus infection in a offspring with congenital SIV infection. J Med Primatol (In Press).

Overbaugh J, Rudensey LM, Papenhausen MD, Morton WR, Benveniste RE. Variation in env is confined to V1 an V4 during the progression to simian AIDS. Nature (In Press).

Willerford DM, Gale MJ, Benveniste RE, Clark EA, Gallatin WM. Simian immunodeficiency virus is restricted to a subset of blood $CD4^+$ lymphocytes which includes memory cells. J Immunol 1990;144:3779-83.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05417-07 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization and Expression of raf Oncogenes in Normal and Tumor Cells

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:	Jong-Eun Lee	Special Volunteer	LVC	NCI
	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

COOPERATING UNITS *(if any)*
PRI/DynCorp, Frederick, MD (T. Beck, S. Storm, M. Dean, G. Sithanandam)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.8	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 raf-1 gene is located at chromosome 3p25 near sites specifically altered in renal cell carcinoma, small cell lung carcinoma, and mixed parotid gland tumors, and is genetically linked to the von Hippel Lindau disease gene. A-raf-1 is located at Xp11.2-11.4 near the translocation breakpoint in synovial sarcoma t(X;18) and the loci for Wiskott-Aldrich and Norrie syndromes. Northern hybridizations to RNA from fetal and adult mouse tissues indicate that raf-1 is expressed in all tissues, although steady state levels vary about tenfold between tissues with the highest levels in the cerebellum, striated muscle, and fetal brain. A-raf is expressed preferentially in urogenital tissues with the highest levels in the ovary and epididymis. We have begun to characterize the promoters for the individual human raf genes. DNA sequencing of genomic clones, primer extension, and S1 nuclease have been used to identify the 5′ ends of raf-1 and A-raf RNA's. The raf-1 promoter has features of a housekeeping gene in that it is GC-rich (HTF-like), lacks consensus TATA or CAAT boxes, and has heterogeneous RNA start sites. The A-raf promoter displays features of a regulated gene in that it contains a TFIID binding site (TATA-box) and sequences identical to binding sites for several transcription factors. Fusion of a reporter gene to sequentially deleted sequences flanking exon 1 of both genes show positive and negative effects on the level of reporter gene expression. DNA sequences responsible for basal promoter activity have been defined by transient transfection assays, DNAse I footprinting, and gel retardation analyses.

Project Description

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

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Jong-Eun Lee	Special Volunteer	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

Objectives:

The specific objectives of these studies are: (1) to molecularly characterize raf genes in normal and tumor tissues, (2) to determine how these genes are regulated in normal and transformed cells, (3) to identify and characterize the genes and gene products that regulate raf expression in normal and transformed cells, and (4) to define the genetic mechanism(s) by which the raf family of proto-oncogenes become activated and cause neoplasia.

Methods Employed:

Standard recombinant DNA techniques were used to molecularly clone and sequence raf cDNA species and genomic DNA clones. DNA transfections, chloramphenicol acetyl transferase assays (CAT), DNAse footprinting, and gel retardation analyses were carried out using standard procedures.

Major Findings:

1. The raf-1 oncogene is located at chromosome 3p25, near a region specifically altered in the tumor tissue of patients with renal cell carcinoma (RCC) and small cell lung carcinoma (SCLC). In a study 84 SCLC's using restriction fragment length polymorphisms (RFLP) mapping to the raf-1 locus, all informative cases showed conversion to homozygosity at the raf-1 locus in the tumor sample. These results indicate that one allele of raf-1 is deleted in SCLC. Similar results have been obtained in RCC. Neither the RNA nor the protein product of the one retained raf-1 allele from SCLC cell lines appear altered as judged by Northern and Western blot analyses. Interestingly, immune complex kinase assays using extracts from SCLC cell lines demonstrated that the kinase activity of the Raf-1 protein appears to be constitutively activated. Whether this apparent activation results from genetic or epigenetic events is under investigation.

2. Genomic and cDNA cloning experiments have demonstrated that the human raf-1 gene is encoded by 17 exons that span at least 85 Kilobases (Kb) of DNA. The untranslated sequences from exon 1 (304 nucleotides) are displaced by a large intron of at least 35 Kb from the translated body of the gene encoded by exons 2-17 that span 40 Kb. The mRNA size is 3.4 and 3.1 Kb in human and mouse, respectively. The human raf-1 RNA contains 330 nucleotides of untranslated 5' sequences, a single large open reading frame (ORF) of 1944 nucleotides, and 764 nucleotides of untranslated 3' sequences. Both the mouse and human RNAs encode a protein of 648 amino acids with a predicted molecular weight of 73.0 kiloDaltons (kD).

3. The raf-1 gene is expressed in all tissues of the fetal (16 day) and adult mouse, as determined by Northern blot analyses, although the levels between tissues vary by as much as tenfold with the highest abundances detected in the cerebellum, striated muscle, and fetal brain. raf-1 RNAs are expressed at high levels in chemically-induced mouse lung carcinomas and lymphomas, in transformed murine and human tumor cell lines, and in chemically-induced preneoplastic nodules and carcinomas in rat liver. However, raf-1 gene expression is not altered by treatment of established cell lines with mitogens and growth inhibitors under conditions that modulate a variety of cell cycle regulated genes (e.g., fos, myc, and jun). These results demonstrate that raf-1 gene expression is ubiquitous in the mouse and is modulated at the level of transcription or post-transcriptional processing in a developmental, tissue specific, and tumor specific manner.

4. The promoter region of the human raf-1 gene has been identified by primer extension, S1 nuclease mapping, and nucleotide sequencing of genomic DNA clones. raf-1 RNA start sites were cell-type independent, as judged by primer extension assays of RNA from nine different human cell lines. However, an additional primer extension product of 230 nucleotides was evident with MCF-7 cell RNA. Consistent with its ubiquitous expression, the raf-1 promoter region has features of a housekeeping gene in that it is GC-rich (HTF-like), lacks TATA- and CAAT-boxes, contains heterogeneous RNA start sites, an octamer motif (ATTTCAT), and four potential binding sites for the transcription factor SP1.

5. To functionally characterize the human raf-1 promoter, we have constructed a series of 5′ and 3′ deletions of the raf-1 promoter region fused to a CAT reporter gene and assayed promoter activity by transient transfection into COS7 and HeLa cells. These studies have demonstrated that the parental construct containing 5.5 Kb of 5′ and 1 Kb of 3′ sequences flanking the raf-1 transcriptional start sites gave a 30-fold stimulation over promoterless CAT. Analysis of the deletion constructs indicated that cis-acting elements located 5′ and 3′ of the start sites modulate raf-1 promoter activity, and the minimal raf-1 promoter is located within a 340 base pairs (bp) fragment.

DNAse footprinting and gel retardation experiments have identified 3 regions within this 340 bp fragment that specifically bind nuclear proteins. Region 1 (nucleotides -96 to -77) shows sequence similarity to the HIP-1 binding site, and the binding of this factor can be blocked by oligonucleotides bearing the HIP-1 binding site. Region 2 (nucleotides +17 to +38) contains an inverted repeat containing a core sequence of 7 nucleotides that shows sequence similarity to a transcriptional activator sequence in exon 1 of dihydrofolate reductase (DHFR). Region 3 (nucleotide +120 to +140) does not show sequence similarity to any known transcription factor binding site. Thus, the minimal raf-1 promoter is structurally-similar to the minimal DHFR promotor in that they appear to share two transcription factor binding sites. However, the position of the HIP-1 binding site relative to the transcriptional start sites are different between the two promoters, which is interesting because HIP-1 is thought to direct the start site of transcription. In addition, the region 2 factor is an inverted repeat, suggesting that this factor binds as a homodimer in raf-1, yet in DHFR there is only one copy.

6. The human A-raf-1 locus has been regionally localized to Xp11.2-11.4, near the specific translocation in human synovial carcinoma t(X;18)(p11.2;q11.2), and the genes for Wiscott-Aldrich and Norrie syndromes. In addition, rare translocations of the X chromosome with autosomes have been reported, and X chromosome losses are a frequent occurrence in certain types of acute lymphocytic leukemia.

7. A near full-length human A-raf cDNA was isolated from the T-cell library, which is 2.46 Kb and contains a single long ORF of 1,818 nucleotides coding for a protein of 606 amino acids and a molecular weight of 67.5 kD. Southern blotting experiments and genomic cloning suggest that the A-raf gene is less than 37 Kb and that very short introns intersperse the coding sequences, at least in the 5′ half of the gene.

8. The expression of the 2.6 Kb A-raf RNA is more restricted in the adult mouse than is Raf-1 and shows a wider variation in its level of expression between different tissues. A-raf transcripts are predominantly expressed in urogenital tissues with the highest levels in the ovary and epididymis where A-raf transcripts are detected at levels approximately fivefold greater than raf-1. These results indicate tissue specific and developmental controls on A-raf gene expression.

9. The A-raf promoter was mapped by primer extension assays to a region of the A-raf gene that has features of a regulated eukaryotic promoter, in that it contains consensus sequences corresponding to the binding site for transcription factors associated with regulated genes. These include a TFIID binding site (TATA-box) at position -30, E2aE-C at -600, EF-1A/PEA-3 at -292; a glucocorticoid response element at position +49 (that is partially palindromic like conventional GRE elements); and an SV40-type core enhancer element located in intron 1.

The A-raf promoter region linked to the CAT gene in transient transfection assays gave a 30-fold stimulation of CAT activity over the promoterless construct in COS 7 cells. Analyses of deletion constructs demonstrated, in contrast to the results of the primer extension assays, that the minimal functional A-raf promoter is actually located within a 150 bp fragment located over 100 nucleotides downstream of the site mapped by primer extension. This 150 bp fragment is A+T-rich (35% G+C) and lacks a consensus TATA-box. Gel retardation assays have demonstrated that a nuclear factor associates specifically with this fragment.

10. We have constructed a series of mini-gene plasmids that express either normal or mutationally-altered forms of the raf-1 cDNA under the transcriptional control of either the raf-1 or A-raf promoter and the poly(A) addition site and 3′ flanking region from the normal human raf-1 gene. A truncation-activated version of raf-1 (BXB) transforms NIH/3T3 cells when driven by either the raf-1 or A-raf promoter. However, the A-raf-driven construct is consistent with the lower level of A-raf expression in NIH/3T3 cells.

Publications:

Beck TW, Kolch W, Heidecker G, Rapp UR. Raf-family serine/threonine protein kinases: gene structure, expression and protein structure. Biochim Biophys Acta (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05434-07 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Immunology of AIDS and AIDS-Related Diseases

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

PI:	Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
Others:	William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
	James J. Goedert	Coordinator, AIDS Working Group	EEB	NCI
	Robert J. Biggar	Medical Officer	EEB	NCI
	Raoul E. Benveniste	Medical Officer	LVC	NCI

COOPERATING UNITS *(if any)*: Department of Investigative Dermatology, Univ. of Vienna, Vienna, Austria (G. Stingl); PRI/DynCorp, Frederick, MD (G. Hamlin-Green, M. Cullen)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Immunogenetics Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	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.)*

Studies are being conducted to investigate the immunobiology of human immunodeficiency virus (HIV-1) infection. Cells (monocytes and T lymphocytes) that bear the CD4+ molecules are the primary targets for infection of HIV-1. In vitro, certain isolates preferentially infect one cell type or the other. Cell-free vs. cell to cell infection was compared using monocytotropic (BAL) and T cell tropic (HTLV-IIIB) strains. T cell infection was 10 to 15 times more efficient with HTLV-IIIB; however, virus production by the BAL-infected monocytes was increased by this order of magnitude resulting in equivalent levels of infection in T cells by cell to cell infection. Studies are underway to characterize the virus-like particle(s) seen in Kaposi's sarcoma tissue biopsies from individuals in a cohort of HIV-1, HIV-2, HTLV-I, and HTLV-II seronegative Greek individuals. Sera from these individuals, as well as from normal individuals, were tested by Western blot techniques for reactivity to nonhuman primate retroviruses. Reactions were observed in some instances to gag and transmembrane proteins of the type D viruses with sera from Kaposi's patients.

Studies are underway to investigate the nature of the antibody response to HIV-1 proteins in infected mothers and their infants. The antibody response in these individuals was varied with most frequent reactions of the IgG1 subclass with p24, gp120, and gp160. Other findings included the detection of IgA, IgD, and IgE antibodies to various viral proteins.

Project Description

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

Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
James J. Goedert	Coordinator, AIDS Working Group	EEB	NCI
Robert J. Biggar	Medical Officer	EEB	NCI
Raoul E. Benveniste	Medical Officer	LVC	NCI

Objectives:

To identify factors that predict and/or are associated with disease progression and outcome in HIV-1-infected individuals. To examine the cell to cell transmission of HIV-1 from different cell populations (monocyte/macrophage to T cells) where this interaction is a normal sequence of events in the immune response. To identify a putative infectious agent (other than HIV-1) that is associated with Kaposi's sarcoma (KS). To examine the type of antibody response to HIV-1 infection in relationship to disease progression.

Methods Employed:

Peripheral blood lymphocytes (PBLs), as well as sera, that are used in the study were obtained from individuals infected with HIV-1 and seronegative controls. The cell surface markers were enumerated using commercially available reagents that detect a variety of different cell surface structures associated with different types of lymphocytes. The studies were performed using the fluorescence activated cell sorter. In studies of cell to cell transmission of HIV-1, PBLs were obtained from HIV-1 seronegative individuals. The monocytes were isolated by surface adherence and infected with various isolates of HIV-1. After an appropriate time period and demonstration of productive infection in monocytes, monocyte-depleted lymphocytes stimulated with tetanus toxoid were exposed to the infected monocytes. Cell-free virus containing supernatants were also added to these lymphocytes. After 6 to 24 hours exposure, the T cells were removed and placed on noninfected monocytes for a period of 7 days. Virus production in these cultures was assessed by the production of the p24 HIV-1 gag protein using a commercially available antigen capture kit.

HIV-1 proteins, p24, p66, gp120 and gp160, produced by recombinant technology were obtained from a commercial source. These proteins were attached to beads in a noncovalent manner and tested for their reactivity with the sera obtained from HIV-1-infected mothers and their infants. In order to determine class and subclass specificity, a second fluoresceinated reagent was used that identifies specific classes and subclasses of immunoglobulins. The classes and subclasses tested for were IgA1, IgA2, IgG1, IgG2, IgG3, IgG4, IgD, IgE, and IgM. The tests were carried out on and analyzed by a fluorescence activated cell sorter.

A cohort of individuals from specific villages in Greece was identified that had an increase over the expected incidence of KS. These individuals were examined clinically and biopsies were taken from the KS lesions as well as from normal skin. PBLs were cultured for 7 and 14 days with phyto-hemagglutinin stimulation and media containing interleukin-2. The biopsy tissue and PBLs were cocultured with allogeneic monocytes and lymphocytes obtained from HIV-1 seronegative individuals. Supernatants from these various cultures were tested for reverse transcriptase activity using the Mn^{++} and Mg^{++} dependent substrates. The biopsies were examined by light and electron microscopy for structural characteristics and viral particles. The tissues were also tested by indirect immunofluorescence for cell surface markers. Sera from these individuals, as well as from HIV-1-infected individuals with and without KS, were tested for antibody activity to several different strains of nonhuman primate retroviruses in a Western blot assay.

Major Findings:

Primary infection of monocytes was about tenfold greater with the monocytotropic (BAL) strain relative to infection with the T cell tropic (HTLV-IIIB) strain. Culture fluid containing the quantity of virus produced in 24 hours (from BAL and IIIB) was added to T lymphocytes (cell-free virus infection). Monocyte-depleted T lymphocytes were exposed to the BAL- or IIIB-infected monocytes for 4 hours (cell to cell transmission). After exposure, the T lymphocytes were washed and cultured for 6 days. The level of infection measured by p24 production in the cell-free virus-exposed lymphocytes was more efficient with the HTLV-IIIB strain. With BAL, virus production by the monocytes was higher than with IIIB and T cell infection by the cell to cell contact equivalent.

Analysis of the nature of the antibody Ig class and subclass response to viral protein in HIV-1-infected mothers and their infants demonstrated different individual responses that did not correlate with HIV-1 transmission to the offspring. The unique finding was the presence of IgD antibodies to viral proteins in some individuals' sera. This is the first demonstration as far as we can determine that this class of immunoglobulins represents an antibody response to viral infection. IgG1 and IgG3 were the subclasses of IgG that were present most frequently in the population. No consistent or informative pattern of combination of class response was found.

KS is a known outcome of HIV-1 infection in homosexual males that does not occur in HIV-1-infected hemophiliacs. This suggests the possibility that another sexually transmissible agent is involved in this disease. We studied an isolated cohort of Greek individuals who have a clinical presentation of KS not unlike that seen in the HIV-infected individuals and share some features of classical KS. These individuals were HIV-1, HIV-2, HTLV-I, and HTLV-II seronegative. Sera from these individuals as well as from HIV-1 seropositive KS patients and HIV-1 seropositive individuals without KS were tested for reactivity to type D nonhuman primate retroviruses by Western blot. Sporadic positive reactions were observed with gag and transmembrane proteins from simian retroviruses 1, 2, 4, and 5, and a baboon type C virus. Lymphocytes as well as biopsies of KS lesions were cocultured with allogeneic lymphocytes and monocytes and tested for reverse transcriptase activity. No activity was

identified. Virus particles, considered to be structurally compatible with a retrovirus, were seen on electron microscopy adjacent to, and budding from, the KS cells.

Publications:

Harris PE, Strba-Cechova K, Rubinstein P, Mann D, King DW, Suciu-Foca N. Amplification of T cell blastogenic responses in healthy individuals and patients with acquired immundeficiency syndrome. J Clin Invest 1990;85:746-56.

Rappersberger K, Tschachler E, Zonzits E, Gillitzer R, Hatzakis A, Kaloterakis A, Mann DL, Popow-Kraupp T, Biggar RJ, Berger R, Stratigos J, Wolff K, Stingl G. Endemic Kaposi's sarcoma in human immunodeficiency virus type-1 seronegative persons: demonstration of retrovirus-like particles in cutaneous lesions. J Invest Dermatol 1990;95:371-81.

Stingl G, Rappersberger K, Tschachler E, Gartner S, Groh V, Mann DL, Wolff K, Popovic M. Langerhans cells in HIV-1-infected individuals. J Am Acad Dermatol 1990;95:371-81.

Tollerud DJ, Ildstad ST, Brown LM, Clark JW, Blattner WA, Mann DL, Neuland CY, Pankiw-Trost L, Hoover RN. T-cell subsets in healthy teenagers: transition to the adult phenotype. Clin Immunol Immunopathol 1990;56:88-96.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05528-05 LVC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Function and Mechanism of the HTLV-I and BLV Rex Proteins

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

PI: David Derse Senior Staff Fellow LVC NCI

Others: None

COOPERATING UNITS *(if any)*

PRI/DynCorp, Frederick, MD (L. Martarano); ABL-Basic Research Program, Frederick, MD (B. Felber, G. Pavlakis)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	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.)*

Human T-cell leukemia virus type I (HTLV-I) and bovine leukemia virus (BLV) are related retroviruses that both encode two regulatory proteins, termed Tax and Rex, that interact with specific cis-acting sequences to control virus transcription initiation and mRNA accumulation, respectively. The Rex protein is required for the cytoplasmic accumulation of viral mRNAs encoding structural proteins and is predicted to bind sequence elements at the 3′ end of the viral RNA. To better understand the molecular basis of Rex action, HTLV-I and BLV proviruses, Rex-deficient proviral mutants, and Rex expression plasmids were constructed. The effects of Rex mutations were analyzed in transfected cells by Northern blotting of RNA and Western immunoblotting of viral proteins. It was observed that Rex-deficient proviruses did not express viral structural proteins, but rather overexpressed the transcriptional activator protein, Tax. The partially-purified HTLV-I Rex protein, expressed in bacteria, was shown to bind to a structured RNA element *in vitro*. This cis-acting sequence encoded in the 3′ long terminal repeat is termed the RxRE. Finally, we have begun to analyze the transcription pattern of HTLV-I using the highly sensitive method of cDNA-polymerase chain reaction. Specific segments of cDNAs synthesized in cells transfected with the cloned HTLV-I provirus were sequenced.

Project Description

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

David Derse Senior Staff Fellow LVC NCI

Objectives:

(1) To construct molecular clones of human T-cell leukemia virus type I (HTLV-I) and bovine leukemia virus (BLV) from which to generate provirus mutants defective in regulatory gene expression, Tax and Rex (regulatory proteins) expression plasmids, and other subgenomic expression and test plasmids. (2) To examine the effects of Rex mutations and expression on the synthesis of viral RNA and proteins. (3) To characterize the in vitro binding of HTLV-I Rex protein to its RNA target. (4) To define the RNA species present in cells transfected with a cloned HTLV-I provirus and to characterize splice sites by nucleotide sequence analysis.

Methods Employed:

The following methods were employed: (1) cloning in bacteriophage lambda, (2) nucleotide sequencing, (3) recombinant plasmid construction, (4) transfection of mammalian cells, (5) Northern blotting, (6) Western immunoblotting, (7) bacterial expression of Rex and in vitro RNA-binding, and (8) cDNA-polymerase chain reaction (PCR).

Major Findings:

1. Pattern of HTLV-I gene expression in cells transfected with Rex-deficient proviruses and subgenomic virus expression plasmids. To better understand the roles of the HTLV-I Rex protein in virus expression, HeLa cells and HeLa-Rex cells (HeLa cells that constitutively produce Rex) were transfected with Rex^+ or Rex^- proviruses. Virus gene expression was analyzed by Northern blotting of RNA and by immunologic detection of viral proteins. In the absence of Rex there was a very low abundance of viral structural RNAs in the cytoplasm, but normal levels were observed in the nucleus; there was no detectable synthesis of viral structural proteins. However, the Tax mRNA and protein were overexpressed under these conditions. When Rex was supplied in trans, Tax was still overexpressed suggesting a cis mutation, but levels of structural proteins returned to normal. Thus, Rex acts to facilitate the transport of specific RNAs from the nucleus to the cytoplasm. When the proviral 3′ long terminal repeat (LTR) was replaced with a heterologous polyadenylation signal, viral RNAs could not be transported from nuclear to cytoplasmic compartments, even in the presence of Rex. Thus, Rex is a positive effector of RNA transport.

2. In vitro binding of HTLV-I Rex protein to its RNA element. The HTLV-I rex gene from the cloned provirus was subcloned into a bacterial expression vector, pUC12N. The bacterially-expressed protein was purified through several column steps and verified by reaction with anti-rex antiserum. A

segment of the viral LTR that encodes the Rex response element (RxRE) was cloned downstream of a T7 RNA polymerase promoter for the in vitro synthesis of radioactively labelled RxRE RNA. The in vitro binding of Rex to RxRE was examined by gel-shift analysis. We observed that HTLV-I Rex protein bound to RxRE RNA, as well as to RNA encoding the HIV-1 Rev-response element, but not to other RNAs including the antisense orientation of RxRE.

3. Identification and characterization of novel RNAs expressed in cells transfected with a cloned HTLV-I provirus. Transiently-expressed RNA was prepared from HeLa cells transfected with the HTLV-I provirus clone. This RNA was used to synthesize cDNA to be used as the template for PCR-amplification and nucleotide sequence analysis. This method of analysis allows the identification of spliced and processed RNAs that are present in very low abundance. A variety of PCR primers was selected to amplify specific regions across the genome. In addition to the four known splice sites used for env and tax/rex mRNAs, three additional splice acceptor sites were identified. One new splice site precedes the common env splice acceptor; the other two are present in the region preceding the common tax/rex splice acceptor site. The latter two sites could be used to generate two novel mRNAs containing open reading frames derived from the region between env and tax/rex. These open reading frames are joined by splicing to the initiation codons in the middle exon of the tax/rex mRNA. The putative protein products of these novel genes will be examined.

Publications:

Unge T, Solomin L, Mellini M, Derse D, Felber BK, Pavlakis GN. The Rex regulatory protein of HTLV-I binds specifically to its target site within the viral RNA. Proc Natl Acad Sci USA (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05529-05 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic and Molecular Organization of the MHC in the Domestic Cat

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

PI:	Naoya Yuhki	Visiting Associate	LVC	NCI
Others:	Stephen J. O'Brien	Chief	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary E. Eichelberger	Microbiologist	LVC	NCI

COOPERATING UNITS *(if any)*
PRI/DynCorp, Frederick, MD (C. A. Winkler)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.5	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.)*

The feline major histocompatibility complex (MHC) has been studied using molecular techniques as an approach to comparative genome organization of this important gene cluster. The MHC in most mammals consists of two classes of genes, classes I and II, which play special roles in presenting antigens to T-cell receptors. Sequence analysis of eight different domestic cat MHC (FLA) class I cDNA clones revealed strong conservation of invariant and variant amino acid residues in antigen-binding and T-cell recognition sites among human, mouse, and domestic cat. This evidence indicates that the domestic cat MHC class I molecule has a similar structure to human and mouse class I molecules, and its MHC class I molecules have highly polymorphic features. Further analysis revealed the participation of four major factors in the evolution of the feline MHC class I genes. These include: (1) a gradual accumulation of spontaneous mutational substitution, (2) negative selection for functional constraints of class I genes, (3) positive selection in favor of persistence of polymorphism, and (4) periodic intragenic and intergenic DNA recombination.

Sequence analysis of MHC class I molecules from two other feline species (cheetah and ocelot) showed that feline MHC class I molecules have highly mosaic structures in their entire coding regions. We identify at least 15 mosaic regions where each has at least two polymorphic sequence motifs. Some of these sequence motifs are conserved not only in Felidae MHC class I sequences but also in some of human, orangutan, and bovine MHC class I sequences. These data revealed that modern polymorphic sequence motifs found in mammalian MHC class I molecules are extremely ancient and shuffling of these motifs by DNA recombination plays a pivotal role in producing novel polymorphic MHC class I molecules in mammals.

Project Description

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

Naoya Yuhki	Visiting Associate	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary E. Eichelberger	Microbiologist	LVC	NCI

Objectives:

The major histocompatibility complex (MHC) genes encode two different classes of cell surface molecules which can present immunological peptides for T-cell receptors (class I and class II antigens). These molecules have highly polymorphic features. According to the X-ray crystallographic model of a human HLA-A2 class I molecule, the majority of polymorphic residues of this molecule are located on the site facing the putative antigen binding site in human and mouse class I molecules. This finding, together with the results that various HLA class I molecules have different binding capacities to immunological peptides, suggest that the polymorphism of MHC class I molecules reflects the capacity of each molecule to bind various spectrums of immunological peptides. Several theories, such as gene conversion, intra-exon shuffling and overdominant selection have been proposed to explain the extreme polymorphism of MHC molecules by analyzing human and mouse MHC genes or gene products. We used a different animal model, the domestic cat, to examine the mechanisms.

Methods Employed:

The following techniques were employed: (1) cDNA cloning, (2) reverse transcription-(RT)-polymerase chain reaction (PCR) DNA cloning, (3) DNA sequencing using the dideoxy nucleotide sequencing method, and (4) RNA and DNA blotting.

Major Findings:

1. DNA recombination and natural selection pressure sustains genetic diversity of feline MHC class I genes. Sequence comparisons of eight distinct MHC class I cDNA clones revealed that feline class I molecules have a remarkable similarity to human HLA genes in their organization of functional domains, as well as in the nonrandom partitioning of genetic variability according to the functional constraints ascribed to different regions of the MHC molecule. The distribution of the pattern of sequence polymorphism in the cat, as compared with genetic diversity of human and mouse class I genes, provides evidence for four coordinate factors that contribute to the origin and sustenance of abundant allele diversity that characterizes the MHC in the species. These include: (1) a gradual accumulation of spontaneous mutational substitution over evolutionary time, (2) selection against mutational divergence in regions of the class I molecule involved in T-cell receptor interaction and also in certain regions that interact with common features of

antigens, (3) positive selection pressure in favor of persistence of polymorphism and heterozygosity at 57 amino acid residues that comprise the antigen recognition site, and (4) periodic intragenic (interallelic) and intergenic recombination within the class I genes. We describe a highly conserved 23-base pair nucleotide sequence within the coding region of the first α-helix that separates two relatively polymorphic segments located in the α1 domain that may act as a template or for homologous recombination between class I alleles.

2. Exchanging polymorphic DNA sequence motifs in mammalian MHC class I genes: Ancient origins of MHC class I polymorphic domains. Sequence analysis of MHC class I cDNA clones from two diverged feline species (African cheetah and South American ocelot) were performed using methods of conventional cDNA cloning and RT-PCR DNA cloning. Comparisons of these sequences with published MHC class I sequences of domestic cat revealed Felidae-wide retention of polymorphic sequence motif in MHC class I molecules. MHC class I genes had highly mosaic structures with inter-species conserved sequence motifs in its entire coding region. At least 15 sites were found as units of mosaic structures and two polymorphic sequence motifs were identified in each site on average. Each polymorphism motif was apparent in the class I sequences of at least two or more feline species. Remarkably similar polymorphic sequence motifs encoding the first α-helix, which is involved in antigen and T-cell receptor recognition, were found in human classical and nonclassical class I sequences, also in orangutan and bovine class I genes. These observations suggest that: (1) these polymorphic sequence motifs are extremely ancient and probably existed before the separation of these mammalian species; and (2) shuffling of these motifs among classical, and also nonclassical, MHC class I genes play a major role to create novel molecules during evolution of the mammalian MHC.

3. Characterization of MHC class II DRA and DRB cDNA clones of domestic cat. To characterize MHC class II genes in the domestic cat, a spleen cDNA library was screened using a series of human MHC class II α and β cDNA clones as probes. Of human probes, only DRA and DRB probes successfully cross-hybridized with their homologous domestic cat cDNA clones. Sequence analysis of eight DRA- and seven DRB-like cDNA clones revealed that the domestic cat has at least one DRA and two DRB loci in its MHC. Feline DRA molecules have monomorphic amino acid sequences in α1 and α2 domains, although polymorphic sequences were found in leader and cytoplasmic regions. In contrast, feline DRB molecules have highly polymorphic sequences in a β1 domain which is involved in antigen and T-cell receptor recognition. Thus, the feline MHC has at least one monomorphic DRA and two polymorphic DRB loci like the human MHC. Multiple attempts to isolate feline DQA and DQB clones, such as locus-specific oligonucleotide screening and PCR amplification, were unsuccessful. Our results suggest that the domestic cat has highly diverged DQA and DQB from those of human and mouse sequences.

Publications:

Winkler C, Yuhki N, O'Brien SJ. Comparative analysis of FLA, the major histocompatibility complex of the Felidae. In: Clegg M, O'Brien SJ, eds. Molecular evolution: proceedings of the UCLA symposia on molecular and cellular biology, vol. 122. New York: Alan R Liss, 1990;29-50.

Yuhki N, O'Brien SJ. DNA recombination and natural selection pressure sustains genetic sequence diversity of the feline MHC class I genes. J Exp Med 1990;172:621-30.

Yuhki N, Winkler CA, O'Brien SJ. The MHC genes of the domestic cat. In: Skivastava R, Ram BP, Tyle P, eds. Immunogenetics of the major histocompatibility complex. Cambridge: VCH Publishers, 1991;348-67.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05531-05 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Functional Analysis of the Relationship Between raf and Other Growth Regulators

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:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
	Robert Bassin	Chief, Biochem. of Oncogenes Sect.	LTIB	NCI

COOPERATING UNITS *(if any)*
PRI/DynCorp, Frederick, MD (P. Lloyd, S. D. Showalter); Genetics Institute, Cambridge, MA (J. Knopf); Goedecke Ag, Freiburg, Germany (W. Kolch); Department of Pharmacology, University of California, San Diego, CA (M. Karin)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	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.)*

Cytoplasmic serine/threonine-specific kinases, such as raf and protein kinase C, play an integrative role in the transduction of signals from the cell membrane to the nucleus. To dissect raf-specific signaling pathways, we designed raf inhibition experiments employing monoclonal antibodies as well as expression of antisense RNA. The isolation of cells resistant to transformation by v-raf constituted a second strategy to identify factors which regulate raf-dependent signaling pathways. These cells, termed CHP25, express a functional v-raf oncogene. However, they are non-tumorigenic, do not form colonies in soft agar, and possess a flat morphology. CHP25 cells are resistant to transformation by sis, ras, and tyrosine- and serine/threonine-kinase encoding oncogenes suggesting that Raf functions downstream of most membrane-associated and cytoplasmic signal inducers. In contrast to v-raf-transformed cells in which the endogenous Raf-1 protein kinase is constitutively activated, v-Raf in CHP25 cells does not activate endogenous Raf-1 kinase. Since mitogen regulation of Raf-1 kinase in CHP25 cells is intact, we conclude that CHP25 cells are blocked at the level of Raf-1 substrate phosphorylation. Consistent with this interpretation, CHP25 cells show specific alterations of early gene induction. The serum induction of c-fos and junD, as well as the serum and 12-O-tetradecanoylphorbol-13-acetate (TPA) induction of junB and egr-1, are almost completely abolished. There is a downstream bypass to the Raf-1 block since v-fos can re-transform CHP25. However, v-myc and v-jun, c-jun, and junB are ineffective. We conclude that Raf-1 signaling is essential for transformation of NIH/3T3 cells by peripheral oncogenes and for regulation of a subset of early response genes by TPA and serum growth factors. The Raf-1 inhibition experiment points to jun as a substrate critical for raf oncogene transformation. Other substrates include a pp120 phosphoprotein which is currently being purified.

Project Description

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

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Robert Bassin	Chief, Biochem. of Oncogenes Sect.	LTIB	NCI

Objectives:

The goals of this study are to investigate the role and mutual interaction of Raf-1 and other growth regulators in growth factor signal transduction. The experimental strategy is based on specific inhibition of raf function by generation of mutant cells that block Raf-1 function. These cells are expected to lead us to the identification and isolation of a new suppressor gene(s) that is expected to control multiple oncogenes. The Raf-1 inhibitor analysis has pointed to several critical Raf-1 substrates including jun and a 120-kilodalton (kd) phosphoprotein. These potential substrate connections are being analyzed.

Methods Employed:

Standard DNA cloning methods were used to construct recombinant retroviruses. Analysis of transfected cell lines followed standard procedures. Flat revertants from v-raf double transformants have been derived as previously described. jun phosphorylation studies are being carried out in collaboration with the laboratory of Michael Karin, University of California, San Diego. The 120-kd phosphoprotein purification employs co-immune precipitation with Raf-1-specific antibody followed by column and gel purifications.

Major Findings:

A mutant cell was derived, called CHP25, which is resistant to transformation by a series of tyrosine kinase oncogenes, ras, raf, and mos. This block to v-raf function is dominant to functions present in control NIH/3T3 cells as fusion between CHP25 and NIH/3T3 did not restore v-raf transformation in the hybrid cells. v-raf kinase activity was demonstrated by immunocomplex kinase assays for v-raf from CHP25 and control transformed cells. Moreover, the v-raf gene from CHP25 cells was examined for the presence of mutations by sequencing of a polymerase chain reaction copy and found to be unaltered. We conclude that the mutant cell phenotype of CHP25 is not due to a dominant negative effect of a mutated v-raf gene. The function of endogenous Raf-1 in CHP25 is intact as far as receptor coupling is concerned. However, the block does affect the ability of v-raf to stimulate activating phosphorylations of Raf-1 that usually take place in v-raf transformed cells. We conclude from this that Raf-1 and v-raf are blocked in CHP25 cells at the level of substrate phosphorylation. A complete block in Raf-1 function is generally lethal for NIH/3T3 cells as we showed independently with antisense and dormant negative raf mutant experiments. Since CHP25 continues to proliferate, it seemed likely that either the block to Raf-1 function was leaky or the mutation had

pleiotropic effects including the activation of a downstream bypass for Raf-1 kinase. The first possibility appears most likely since CHP25 cells retain sensitivity to a Raf-1 antisense vector. A consequence of blocked Raf function is a block to induction of several early growth response genes by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and serum. These include c-fos, junD, junB and egr-1. These findings are consistent with transient transfection data (see Z01CP05684-01 LVC) that demonstrate transcriptional trans-activation of relevant reporter gene constructs by activated Raf-1 kinase and dependence of their serum and TPA induction on functional Raf-1. jun is an in vitro substrate for Raf-1 protein kinase. The in vitro phosphorylation sites overlap with those induced by ras oncogene-stimulation or growth factor-stimulation of NIH/3T3 cells. A block in Raf-1 function set by transfection of a transdominant negative Raf-1 kinase mutant eliminates the stimulatory jun phosphorylations induced by ras. These can be restored by introduction of activated Raf-1 kinase.

In addition to jun, phosphoproteins of 110-120 kd are candidates for Raf-1 phosphorylation. These proteins are currently being purified.

Publications:

Kolch W, Cleveland JL, Rapp UR. Role of oncogenes in the abrogation of growth factor requirements of hemopoietic cells. CRC Rev Cancer (In Press).

Kolch W, Heidecker G, Lloyd P, Rapp UR. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature 1991;349:426-8.

Patents:

Kolch W, Weissinger E, Mischak H, Troppmair J, Showalter SD, Lloyd P, Heidecker G, Rapp UR. US Patent 5683/74217/WTS/CLB: Monoclonal Antibodies Specific for raf Family Kinases, January 3, 1991.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05533-05 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

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

Domains Involved in Regulation of Raf Activity

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

PI:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Others:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
	Harald App	Visiting Fellow	LVC	NCI
	Joseph Bruder	IRTA Fellow	LVC	NCI
	Wayne Anderson	Senior Investigator	LCO	NCI
	Zoltan Olah	Visiting Fellow	LCO	NCI

COOPERATING UNITS (if any)

PRI/DynCorp, Frederick, MD (F.-M. Duh, P. Lloyd); ABL, Frederick, MD (D. Morrison)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Viral Pathology Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	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 protein products of the raf gene family are cytoplasmic serine/threonine protein kinases. They are active in the transmission of mitogenic signals from membrane-associated tyrosine kinase growth factor receptors to the nucleus. The Raf protein itself is phosphorylated, and its kinase is activated by several of the tyrosine kinases and other serine/threonine kinases. A series of point and deletion mutations have been used to identify the site of tyrosine phosphorylation and two of the major serine phosphorylation sites in the Raf kinase. They are Y230, S42, and S497/499. Several approaches to generate systems which produce activated Raf protein or the dominant negative raf mutant K375W conditionally have led to success. We have identified two point mutations responsible for the temperature-sensitive phenotype of the v-mil gene on an MH2 mutant stock. Both involve highly conserved residues found on all protein kinases. Incorporation of these mutations, either singly or in combination, into an activated raf background has conferred the temperature-sensitive phenotype. To study the effect of inactivating mutations, it is necessary to eliminate the endogenous Raf protein. To this end, we have generated vectors which express the antisense of the first 250 nucleotides of the raf mRNA. Constitutive expression of this antisense significantly lowers the number of colonies obtained after transfection compared to constructs expressing the sense sequence. Similar experiments have been done using other parts of the message with comparable results. Constructs which express these antisense RNAs, or the dominant negative raf mutant S375K under the control of either the heat-shock promoter or the glucocorticoid inducible MMTV-LTR, allow us to study their effects in a conditional system. Another approach to study the effect of wild-type and mutant Raf-1 on the signaling cascade is to analyze germinal vesicle breakdown in Xenopus oocytes following microinjection of the appropriate c-raf-1 mRNAs. To this end, we have obtained and sequenced a c-raf-1- homologous cDNA from Xenopus and have incorporated it, and several mutants, into an RNA expression vector.

Project Description

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

Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Harald App	Visiting Fellow	LVC	NCI
Joseph Bruder	IRTA Fellow	LVC	NCI
Wayne Anderson	Senior Investigator	LCO	NCI
Zoltan Olah	Visiting Fellow	LCO	NCI

Objectives:

Raf kinase functions as a link in the signal transduction chain relaying mitogenic stimuli from the cell surface to the nucleus. Oncogenically-activated raf genes have been shown to encode a constitutively active kinase and no longer depend on upstream signals. The goal of this study is to identify upstream activating ligands and downstream substrates of Raf kinase and to elucidate how Raf interacts with them. To these ends, we have established several systems expressing wild-type and mutant Raf kinases for in vivo and in vitro analyses. Retroviral and eukaryotic expression vectors will show which mutants have acquired transforming or suppressor activity in vivo, while Raf proteins produced in bacteria or baculovirus expression systems will be used for in vitro reconstitution assays.

Methods Employed:

Molecular cloning and site-directed- and linker-insertion mutagenesis were performed following standard protocols. Recombinant baculovirus expressing wild-type and mutant raf genes are grown in Spodoptera frugiperda cells following protocols established by M. Sumners. In vitro transcription and translation was done using commercially available kits following the suppliers' recommendations. Transfection of eukaryotic cells was done by the calcium-phosphate co-precipitation method. Polymerase chain reactions were performed according to the manufacturer's recommendations.

Major Findings:

1. The role of functional domains in the interaction of Raf with ligands and substrates. Raf proteins belong to the protein kinase family of oncogenes. Two domains can be identified based on homology to other kinases and on functional studies. The carboxy-terminal half constitutes the actual kinase, while the amino-terminal half has regulatory function. Removal of the latter domain results in constitutive activation of the kinase as demonstrated by the transforming activity of constructs containing truncation mutations. The mutants also show constitutive activity in transient assays analyzing the effect of Raf on transcription from promoters regulated by AP-1 or E1A. In contrast, several mutations in the carboxy-terminal half were shown to inactivate the Raf kinase. Mutations in the primary ATP binding site, changing the lysine residue to tryptophane, not only inactivated the kinase

activity but had a dominant negative effect on the activity of the endogenous Raf protein. This was demonstrated by lower yields of transfectants compared to wild-type raf constructs and by lower levels of transcription from AP-1 regulated promoters in transient assays. At least part of this phenomenon was also observed when constructs expressing whole or partial regulatory domain peptides were used, suggesting that the effect was due to a short circuit in the signal transduction pathway caused by the regulatory domains of kinase inactive Raf proteins binding all the activating signals without transducing them to Raf substrates. Alternatively, as the N-terminal domain has negative autoregulatory function, the dominant negative effect may result from down-regulation of endogenous Raf-1 kinase activity due to direct binding. A second class of mutations resulting in loss of Raf kinase activity involved a consensus substrate sequence for serine/threonine kinases that could either be autophosphorylated or a substrate for other Ser/Thr kinases such as protein kinase C (PKC). Changing the serine residue in this region to alanine or removing its positively charged environment on a truncated, i.e., normally activated, raf background resulted in oncogenically inactive protein without affecting the stability of the protein. This finding suggested that phosphorylation at this site is necessary for activation of Raf kinase.

A second site of serine phosphorylation was identified by comparing the phosphopeptide patterns of wild-type and mutant protein following in vivo labeling. The site identified is the serine, at position 42, that is located in a similar position as the pseudo-substrate site involved in the regulation of PKC. In vivo effects of this mutation have not been obvious when transfected into NIH/3T3 cells, possibly due to the presence of the endogenous wild-type protein.

To circumvent this problem, we have used antisense expression constructs that contain the very 5′ end of the raf message, which is not normally found on raf expression constructs. These antisense constructs were shown to both lower the colony yield when compared to the same amount of sense construct, as well as reduce the amount of Raf protein in cells that did arise after the transfection. Conditional expression of activated or mutant Raf proteins will allow us to analyze the immediate effects of activated and mutant forms of Raf in vivo. To this end, we have obtained a temperature-sensitive mutant of the MH2 virus and identified two point mutations in v-mil associated with this phenotype. Both exchanges are in highly conserved residues found in all protein kinases and were transferred to the raf gene either together or singly. One of the single exchanges exhibited the temperature-sensitive phenotype in fibroblasts. Germinal vesicle breakdown (GVBD) in Xenopus oocytes has also been shown to be regulated in a similar fashion as cell division in mammalian cells. Several other oncogenes have been shown to promote or interfere with GVBD when their mRNAs were microinjected into maturing oocytes. To analyze the role of Raf-1 in this process, we have created several reagents. We have sequenced the cDNA encoding a Xenopus homolog of c-raf and have transferred several of the mutations that we have analyzed in the mammalian system into this background.

2. Production of Raf protein for functional studies. While previously-generated bacterial and Sf9/baculovirus expression systems have allowed the production of large amounts of Raf protein, we have not been successful in

purifying large amounts of protein suitable for either crystallization or functional studies due to solubility problems with highly purified Raf protein. As a way around this problem, we have generated a glutathione transferase-Raf fusion construct in the hope that the glutathione transferase component will increase the solubility and facilitate the purification.

Publications:

Heidecker G, Huleihel M, Cleveland JL, Kolch W, Beck TW, Lloyd P, Pawson T, Rapp UR. Mutational activation of c-raf-1 and definition of the minimal transforming sequence. Mol Cell Biol 1990;10:2503-12.

Heidecker G, Kolch W, Morrison DK, Rapp UR. The role of raf-1 phosphorylation in signal transduction. Adv Cancer Res (In Press).

Kolch W, Heidecker G, Lloyd P, Rapp UR. Raf-1 protein kinase is required for growth of NIH3T3 cells induced by serum, TPA, and ras oncogenes. Nature 1991;349:426-9.

Smith MR, Heidecker G, Rapp UR, Kung H-S. Induction of transformation and DNA synthesis after microinjection of Raf protein. Mol Cell Biol 1990;10:3828-33.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05582-04 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Growth Modulation and Analysis of Chemically-Induced 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:	Ainsley Weston	Visiting Scientist	LHC	NCI
	Curtis Harris	Chief	LHC	NCI

COOPERATING UNITS (if any): PRI/DynCorp, Frederick, MD (T. W. Beck, S. M. Storm); St. Jude Children's Hospital, Memphis, TN (J. L. Cleveland)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Viral Pathology Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	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.)

We have developed a mouse model system in which rapid chemical induction of lung adenocarcinomas and T-cell lymphomas allows us to study lung carcinogenesis in vivo, and test potential countermeasures. Tumors are induced in NFS x AKR mice by transplacental injection of 1-ethyl-1-nitrosourea on day 16 of gestation. Tumor promotion by butylated hydroxytoluene nearly halves the latency period for these tumors and results in approximately 90 percent of animals succumbing within 5 to 14 weeks of age. Normal-sized Raf-1 protein and RNA are expressed at very high levels in all of the tumors and we have identified point mutations within exon 15 of the raf-1 gene in these tumors. For this reason we have begun to analyze the raf-1 gene in human tumor samples for point mutations. An analysis of 11 paired (normal versus tumor) samples of human DNA indicated that 4 lung tumor DNAs contained products with altered mobility in heteroduplex assays. Sequence analysis of 1 revealed a C to A transversion adjacent to the exon 14 splice donor. In addition, we have examined raf family RNAs in a variety of mouse tissues in order to define normal expression patterns.

Vaccination of carcinogen-treated mice with purified raf protein (patent pending) was previously shown to effectively eliminate the promoted phase of tumor development. However, the immunological basis for this tumor modulation is unclear, and we are implementing experiments using recombinant raf-expressing vaccinia virus and syngeneic cells expressing different forms of Raf-1 to delineate the host response. Hopefully this will lead to the identification of an immunogenic epitope(s) and enable us to design more effective anti-tumor vaccination regimens.

Project Description

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

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Ainsley Weston	Visiting Scientist	LHC	NCI
Curtis Harris	Chief	LHC	NCI

Objectives:

To utilize an animal model system which we have developed for the chemical induction of lung carcinomas relevant to human cancer in order to devise vaccination protocols for the delay, prevention and/or reversal of these neoplasms. To define oncogene, in particular raf-1, involvement in, and immune response to, these tumors in order to generate more effective anti-tumor regimens.

Methods Employed:

To induce lung adenocarcinomas and T-cell lymphomas in mice, the potent carcinogen, 1-ethyl-1-nitrosourea, was administered transplacentally to pregnant females. Tumor growth in the F_1 mice was accelerated by weekly intraperitoneal injection of butylated hydroxytoluene. Mice were sacrificed when moribund and tumors transplanted and grown in nude mice for molecular analysis. raf-expressing vaccinia viruses were generated according to standard procedures for in vivo recombination. Analysis of tumors and normal tissue was per standard molecular techniques for DNA (Southern), RNA (Northern) and protein (Western). Tumors were screened for ras mutations by polymerase chain reaction (PCR) amplification of genomic DNA which incorporated a diagnostic restriction site that allowed quick detection of mutant alleles. Analysis of the 3' end (kinase region) of raf in the tumors involved RNAse protection assays, PCR amplification of cDNAs followed by heteroduplex analysis, cloning, and sequence analysis.

Major Findings:

Several interesting observations were made from the oncogene expression data. As mentioned above, in every tumor examined, whether lung tumor or lymphoma, normal-sized (3.4 Kilobase [Kb]) raf-1 message was expressed at very high levels when compared to control tissues. Western analysis demonstrated that the protein levels did, in fact, correspond to the observed RNA levels. The other two active raf family members, A- and B-raf, were expressed at high levels in some tumors, but not all, and were not specific for either lung tumors or lymphomas. Most of the other oncogenes tested showed a somewhat random pattern of expression with high levels in some tumors and low levels in others. However, in every case, one of the myc family members (either c-, L-, or N-myc) was expressed at high levels. In addition, at least one member, and sometimes more than one, of the ras family (Ha-, Ki-, or N-ras) exhibited high transcript levels. Finally, all transcripts detected in Northern analysis

were of the expected normal size for each oncogene. Diagnostic digestion at codon 12 of PCR-amplified ras genes from 5 lung tumors, 5 lymphomas, 6 tumor-derived cell lines, and 1 second-round transfectant cell line revealed no Ha- or N-ras mutations and 2 Ki-ras mutations. One of the mutants was in a second-passage lymphoma, reaffirming previous RNAse protection data, and the other was in a cell line derived from a first-passage lung tumor. From these data and previous sequencing data from codon 13, it can be concluded that mutation activation of ras family members, at least at codons 12 or 13, does not play a major role in the generation of these tumors.

RNAse protection assays of 18 lung tumors using a probe for the 3′ end of raf-1 revealed the presence of an extra band, when compared to the completely protected fragment from an untreated F_1 mouse in 16 of the samples. The size of this fragment corresponds to an alteration in the region of the exon 14/15 junction. To determine the exact nature of this difference, we amplified a 435-base pair (bp) fragment from cDNAs which encompassed this area. Amplification products were then isolated and sequenced. To date, we have sequenced 8 clones from untreated F_1 mice and 16 clones from the tumors. All clones from the untreated mice have the same sequence and match that of normal mouse raf-1. Of the tumor clones, half are identical to the untreated mouse; however, in each case there is a mutant c-raf-1 allele present in the tumors. These mutations are all clustered within a 20 amino acid-region of the kinase domain, and each is a point mutation resulting in an amino acid change. We have transfected Raf-1 constructs containing these alterations into NIH/3T3 cells to gauge their transforming ability, and the first mutation examined (Isoleucine to Valine) is weakly transforming. To determine whether raf-1-transformed cells present antigen capable of inducing a cytotoxic response, NIH/3T3 cells harboring various raf constructs are to be irradiated and injected into syngeneic NFS/n mice to demonstrate the effectiveness of Raf-1 in eliciting an immune response when expressed in an oncogenically active form. By using cells which express various portions of Raf-1 as antigen, it should be possible to identify regions important for antigenicity and hopefully lead to determination of the epitope(s) responsible for an anti-tumor response. In addition, the effects of vaccination with raf-expressing vaccinia virus on rejection of live raf-transformed cells will be determined.

The human raf-1 oncogene is located at chromosome 3p25, near a region known to be specifically deleted in patients with small cell lung carcinoma (SCLC). Previously, we demonstrated that the raf-1 gene is located within the minimal region deleted in SCLC. This same chromosomal region (3p14-25) has also been found to be deleted in a variety of other human tumors including renal cell carcinoma, non-small cell lung cancer, breast cancer, and ovarian cancer. Therefore, it is important to determine whether the remaining raf-1 allele in these tumors is mutationally altered. Since the human raf-1 gene is very large (>90 Kb), we chose to focus on a region of the kinase domain that was previously demonstrated to be mutationally altered in mouse lung adenocarcinomas. We prepared oligonucleotide primers for sequences flanking a 600 bp region including exons 14 and 15. These were used to amplify this region by the PCR from paired of normal/tumor DNA samples obtained from 11 patients with lung cancer. The PCR products were heat denatured, allowed to reanneal by slow cooling, and analyzed by gel electrophoresis. The PCR products of four tumor DNAs showed a product with a decreased mobility that

were not observed in the normal tissue from the same patient, indicating that this was due to a mutational event in the tumor DNA as opposed to DNA polymorphism. The PCR products of one of these pairs have been analyzed by asymmetric PCR and subsequent DNA sequencing. The PCR product from this tumor in the region spanning exons 14-15 of raf-1 shows a C to A transversion adjacent to the exon 14 splice donor in one tumor allele that is not present in the normal DNA from the same patient. This mutation could presumably alter the normal splicing pathways of raf-1 or the normal controls on raf-1 transcription in the tumor of this patient.

Publications:

Propst F, Storm SM, Rapp UR. Oncogenes coding for protein serine/threonine kinases: raf and mos. In: Barbacid M, Kumar R, eds. Molecular basis of human cancer. Netherlands: Elsevier Press (In Press).

Patents:

Rapp UR. US Patent Pending: Anti-Tumor Vaccine.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05583-04 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transcriptional Regulatory Elements in Equine Infectious Anemia Virus

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

PI:	David Derse	Senior Staff Fellow	LVC	NCI
Others:	Magda Carvalho	Special Volunteer	LVC	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.7	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.)*

Equine infectious anemia virus (EIAV) is a lentivirus distantly related to the human and simian immunodeficiency viruses (HIV and SIV). Our aim is to identify and characterize the cis-acting elements that respond to both cellular and viral transcription factors to regulate EIAV gene expression. Our first aim was to define the cis-acting element that mediates the transcriptional activation by the viral Tat protein. We have cloned the EIAV promoter sequences located in the viral long terminal repeat (LTR) in a plasmid upstream of the chloramphenicol acetyltransferase (CAT) gene. Promoter activity was quantified by analysis of CAT mRNA or CAT enzyme activity. The EIAV Tat-response element (TAR) was shown to be located in the viral LTR between positions +1 and +25 with respect to the RNA start site. Insertion of EIAV TAR in place of the HIV-1 TAR element within the HIV-1 promoter conferred responsiveness to EIAV Tat. Mutagenesis of individual or clusters of nucleotides within the EIAV TAR sequence revealed that it functions as an RNA stem-loop structure similar to the TAR element of HIV-1. Mutations that disrupted the hairpin structure abolished trans-activation; compensatory mutations that restored the hairpin but altered the primary sequence restored activity. Mutagenesis of specific nucleotides in the loop region had varied effects, some positions were tolerant of substitutions whereas others impaired function. Thus, the EIAV Tat protein specifically recognizes a U-G base pair positioned between the helical stem and the loop regions of TAR RNA and may interact with several loop nucleotides. An analysis of cis-acting DNA sequence elements that bind cellular transcription factors has been initiated.

Project Description

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

David Derse	Senior Staff Fellow	LVC	NCI
Magda Carvalho	Special Volunteer	LVC	NCI

Objectives:

1. To identify the cis-acting element that is responsive to the viral transcriptional trans-activating (Tat) protein.

2. To characterize the structure and define the nucleotide determinants of the Tat-response element (TAR).

3. Identify and characterize the cis-acting DNA elements that bind cellular transcription factors that regulate equine infectious anemia virus (EIAV) expression.

Methods Employed:

The following methods were employed: (1) cDNA cloning, (2) nucleotide sequencing, (3) recombinant plasmid construction, (4) site-directed mutagenesis using polymerase chain reaction (PCR) methodology, (5) transfection of mammalian cells, (6) primer extension analysis of RNA and chloramphenicol acetyltransferase (CAT) assays, and (7) DNA footprinting using DNase digestion.

Major Findings:

1. Location of the EIAV Tat-response element. It was previously shown that EIAV encodes a transcriptional trans-acting protein called Tat. Sequences in the viral long terminal repeat (LTR) that are essential for Tat response were localized by deletion analyses to a region between -30 and +22 with respect to the RNA start site. To examine in greater detail what sequences comprise a minimal Tat-response element in EIAV, EIAV LTR fragments were inserted into heterologous promoters. A DNA fragment encoding nucleotides +1 to +25 of EIAV was inserted immediately downstream of the SV40 promoter RNA start site. This insertion conferred modest EIAV Tat responsiveness onto the SV40 promoter. The HIV-1 promoter is arranged like the EIAV promoter; i.e., the TAR element is downstream of the RNA start site. Hybrid promoters were constructed between EIAV and HIV-1 in which sequences upstream of the RNA start site from one virus were joined to downstream sequences of the other virus. These hybrid promoters were responsive only to the Tat protein that matches the cognate TAR sequence. Thus, the specificity of Tat activation was dependent on the TAR element, not on upstream promoter elements. Moreover, the minimal EIAV TAR element is contained between nucleotides +1 and +25 with respect to the RNA start site. Since this region is transcribed into RNA, it is possible that it acts as an RNA enhancer element.

2. Mutagenesis of the EIAV Tat-response element. The EIAV sequences at the 5′ end of its RNA are predicted to form a hairpin structure with an eight-base pair stem and a six-base loop. We have purified RNA from cells transfected with a plasmid containing the bacterial CAT gene controlled by the EIAV promoter in combination with plasmids that express EIAV Tat. The levels of CAT RNA increased approximately 40 times in response to Tat activation. Primer extension analysis used to quantify RNA levels revealed a secondary band due to pausing of reverse transcriptase at a stable secondary structure. Hence, it appeared that the 5′ end of EIAV RNA does form a stable stem-loop structure under these conditions. The functional importance of this secondary structure was tested by mutagenesis of the EIAV promoter, insertion into a CAT plasmid and transient transfections followed by CAT enzyme assays and RNA analyses. These experiments revealed that substitution of six bases in the stem of the hairpin such that base-pairing is prevented abolished TAR function. A compensatory six nucleotide change that restored the ability to form a stem structure but with a new primary sequence restored TAR function. Thus, the stem structure but not the sequence itself is essential for TAR recognition by Tat. The two base pairs in the stem abutting the loop (that is, the base pairs that close the loop) are non-Watson-Crick U-G base pairs. Mutagenesis revealed that only one of these U-G pairs at this position is essential for TAR function; substitution with a U-A or a C-G pair abolished activity. Substitutions of individual nucleotides within the loop region revealed that most changes and one in particular were deleterious; in contrast, there was a position at which changes led to increased activity. We conclude that the EIAV Tat protein recognizes and interacts with an RNA hairpin structure that contains a U-G base pair located between specific loop sequences and a helical stem region. The EIAV TAR element has structural and functional characteristics analogous to the HIV-1 TAR element, both of which may act as RNA enhancers.

3. Binding of cellular transcription factors to the EIAV LTR. The region upstream of the EIAV RNA start site that contains promoter-control elements that bind cellular transcription factors has not yet been characterized. The nucleotide sequence in this region has no homology to HIV-1 and consensus sequence motifs that bind known transcription factors are absent. However, previous deletion analyses and functional assays of the EIAV LTR have shown that a negative control element and enhancer sequences are present. In order to define these elements and to characterize the novel cellular proteins to which they bind, we have begun a physical analysis of DNA-protein interactions. Nuclear protein extracts have been prepared from several cell lines. The experimental conditions for analyzing DNA-protein complexes both by DNase footprinting and gel-retardation are being refined. Preliminary work has revealed specific sites within the EIAV promoter region that are bound by cellular proteins.

Publications:

Carvalho M, Derse D. Mutational analysis of the equine infectious anemia virus Tat-response element. J Virol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05584-04 LVC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Genomic Organization in Nonhuman Primates and Other Comparative Genetic Studies

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

PI:	Hector N. Seuanez	Visiting Scientist	LVC	NCI
Others:	Mary A. Eichelberger	Microbiologist	LVC	NCI
	Stephen J. O'Brien	Chief	LVC	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.6	0.3

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 comparative analysis of chromosome banding morphology and homologous linkage association holds much potential in the interpretation of human genome organization. Although cytogenetic description of most of the 203 living primate species has been presented, there have been less than a dozen syntenic maps developed. This project has as a goal the construction of a genetic map of the New World spider monkey, Ateles paniscus chamek (2n=34). Three panels of rodent X Ateles somatic cell hybrids were constructed and typed for 26 isozyme markers that were homologous to enzyme structural genes mapped in man and other primates. The derived syntenic groups were assigned to Ateles chromosomes by concordent occurrence with individual chromosome homologues. The genetic map of Ateles was interpreted in a phylogenetic context that is characterized by a high degree of chromosomal conservation during the primate radiation.

Project Description

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

Hector N. Seuanez	Visiting Scientist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI

Objectives:

The specific objectives of this project are (1) establishment of fibroblast cell lines from approximately 15 species of New World monkeys, (2) comparison of chromosomal banding patterns among these monkey species, and (3) gene assignment in Ateles paniscus by somatic cell hybridization.

Methods Employed:

The following techniques are being utilized: (1) primary fibroblasts are obtained by cultivating skin biopsies in tissue culture medium, (2) somatic cell fusion and tissue culture propagation of cell hybrids, (3) cytogenetic analysis of metaphase chromosomes, and (4) protein starch gel electrophoresis.

Major Findings:

1. Analysis of syntenic associations in the neotropical primate species, Ateles paniscus chamek (2n=34). This project has developed more than 80 clones and 20 subclones to establish syntenic associations of genes coding for some 26 isozymes. The following syntenic rearrangements have been observed: [PGD-ME1-AK1], [PEPC-MDH1-ACP1-GOT2-DIA4], [PEPB-NP-MPI-HEXA], [ACP2-LDHA], [HPRT-G6PD], [IDH2], [MDH2], [ADA], [APRT], [GUSB], [GPI], and [ESD]. Tentative chromosome assignments are now possible for some of these syntenic groups following the analysis of chromosome segregation in more than 20 cell lines. Tentative assignments are: [PGD-ME1-AK1] to chromosome 7; [PEPC-MDH1-ACP1-GOT2-DIA4] to chromosome 6; [PEPB] to the short arm of chromosome 2, and [NP-MPI-HEXA] to the long arm of chromosome 2; [ACP2-LDHA] to either chromosome 11 or 16; [IDH2] to chromosome 3; and [HPRT-G6PD] to the X chromosome.

A comparison between Ateles paniscus chamek (2n=34) and man (2n=46) indicates few chromosome similarities between species due to extensive evolutionary shuffling between them. However, these species have a very similar X chromosome. Moreover, there is a good correspondence between Ateles 2p and human 12q (both contain [PEPB]), and between Ateles 2q (containing [NP-MPI-HEXA]) and human chromosomes 14[NP]+15[MPI+HEXA]. Thus, comparative gene assignment has permitted the recognition of few chromosome homologies between these two distantly related primate species despite the fact that chromosome rearrangement has been prominent during the phyletic divergence of Ateles and man. The observed disruptions of several syntenic associations that are kept intact in man and other mammals (such as other primates and the domestic cat) is good evidence that Ateles is a karyotypically rearranged taxon.

2. Cytotaxonomy, biochemical and molecular characterization of the Platyrrhini species, Callimico goeldii. This species was considered to be intermediate between the Cebidae and the Callitrichidae, and included as a single representative of a separate family. Comparative cytogenetics, biochemical analysis of isozyme loci, and the analysis of LINE-1 (middle repetitive) elements in this species' genome were used for a genetic characterization and for including Callimico in the family Callitrichidae.

3. Identification of the pattern of DNA replication of the allocyclic X chromosome in the neotropical primate species, Cebus apella and Leontopithecus rosalia chrysomelas. Studies of late DNA replication in the chromosome complement of these species have been carried out with 5-bromodeoxyuridine and thymidine pulsing. The allocyclic X chromosome in these species' fibroblasts showed a pattern of replication that was identical to the human fibroblast pattern. Thus, while the human allocyclic X chromosome exhibits two patterns of DNA replication, which are tissue-specific (fibroblastic or lymphocytic), these two nonhuman primates have a single pattern, similar to that found in human fibroblasts.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05618-03 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Construction of Novel Retroviral Vectors Based on BLV and HTLV-I

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

PI: David Derse Senior Staff Fellow LVC NCI

Others: Luis Da Silva Special Volunteer LVC NCI

COOPERATING UNITS *(if any)*
PRI/DynCorp, Frederick, MD (L. Martarano); TNO Radiobiological Institute, Rijswijk, The Netherlands (J. L. Heeney)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.7	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.)*

Human T-cell leukemia virus type I (HTLV-I) and bovine leukemia virus (BLV) are related retroviruses associated with lymphoid malignancies in humans and cows, respectively. The biology of these viruses has been difficult to study due to their highly restricted expression; such phenomena as receptor-binding, RNA packaging signals, effects of mutations of structural genes, infectivity, etc., have not yet been addressed. To facilitate the study of these viruses, we have begun to generate recombinant viruses containing foreign genes controlled by strong promoters. Thus, the virus infectious cycle can be easily monitored by following the expression of the reporter gene product. We have already constructed a self-packaging, recombinant BLV which contains the NEO-resistance marker and requires complementation with viral regulatory genes only. In addition, a recombinant HTLV-I provirus was made containing the NEO-resistance marker that must be complemented with viral env and regulatory gene products. These recombinant viruses have several drawbacks for experimental use: first, cell clones expressing the selectable marker, NEO, take several weeks to identify by drug selection; second, the recombinant virus titers obtained have been low compared to parental virus. To overcome some of these problems, we have begun to construct a packaging-deficient HTLV-I helper virus to supply, in trans, the genes required for expression and packaging of the recombinant virus. Also, a new HTLV-I recombinant provirus containing the bacterial lacZ gene is being constructed; this will allow identification of cells expressing the lacZ gene in days rather than weeks.

Project Description

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

David Derse	Senior Staff Fellow	LVC	NCI
Luis Da Silva	Special Volunteer	LVC	NCI

Objectives:

(1) To construct a recombinant human T-cell leukemia virus type I (HTLV-I) provirus in which the 5′ long terminal repeat (LTR) and packaging signals are replaced with the cytomegalovirus (CMV) immediate-early promoter. (2) To examine the pattern of viral RNA and protein synthesis and the production of virions in cells transfected with the HTLV-I helper virus. (3) To construct a recombinant HTLV-I in which the structural and regulatory genes are replaced with the lacZ gene controlled by the herpes simplex virus (HSV) thymidine kinase (TK) promoter. (4) To examine the synthesis of lacZ in cells transfected with the HTLV-I/lacZ provirus and to determine whether it can be used to infect susceptible cells after complementation with helper virus.

Methods Employed:

The following techniques were employed: (1) recombinant plasmid construction, (2) transfections and infections of mammalian cells, (3) Northern and Southern blotting and polymerase chain reaction (PCR) analysis of RNA and DNA, and (4) immunoprecipitation and immunoblotting of viral proteins.

Major Findings:

1. Construction and expression of an HTLV-I helper virus. The cloned HTLV-I provirus, pCS-HTLV1, was used as the starting material to construct pCMV-HT1, an HTLV-I helper virus. The region immediately 5′ of the gag gene in pCS-HTLV1 was altered to introduce a BamHI restriction site. The provirus, lacking a 5′ LTR and primer-binding site, was cloned into a pUC19 plasmid downstream from a CMV promoter. Finally, a small fragment from the R region of the LTR that contains the first splice donor site, was inserted into the BamHI site between the promoter and the virus; this ensures the proper splicing to produce all virus mRNAs. pCMV-HT1 was transfected into HeLa cells to examine transient production of viral RNA and proteins. Northern blotting of RNA revealed that all virus mRNAs were synthesized at high levels and in normal ratios. The accumulation of all three viral RNAs in the cytoplasm indicated that the regulatory protein Rex was functional. Viral protein synthesis was assessed by immunological detection using HTLV-I-positive human serum and specific rabbit sera. The gag, env, and Tax proteins were detected in cellular extracts. In addition, gag and env proteins were detected in the growth medium of transfected cells indicating that virions are formed and released into the supernatant medium.

2. Construction of a recombinant HTLV-I provirus containing a lacZ gene. The bacterial lacZ gene has been joined to the HSV TK promoter. This cassette should allow appropriate expression of lacZ in a wide range of cell types. We are currently inserting this cassette into the pCS-HTLV1 provirus in much the same way as our previous construct, pHTLV-CMVNEO; i.e., HTLV-I Tax, Rex and env genes will be deleted and replaced with the reporter gene unit. The resulting provirus, pHTLV-LACZ, will be tested first for expression of the lacZ gene by transient transfection of cells in culture. Analysis of lacZ expression by an in situ method has been worked out and used in the lab for other promoter studies. Next, the ability of the combination of pCMV-HT1 and pHTLV-LACZ to produce infectious virions that confer lacZ expression via infection using the supernatants from cotransfected cells will be examined. These studies should establish the utility of this system for examining HTLV-I molecular genetics. The recombinant viruses described here should allow one to quantify virus infectivity independent of virus-controlled gene expression. These systems should help in defining specific receptors, and in analyzing the effects of antiviral agents.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05620-03 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Development of Vaccines and Antivirals Against Retrovirus Infection in Primates

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

PI:	Raoul Benveniste	Medical Officer	LVC	NCI
Others:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

COOPERATING UNITS *(if any)*

PRI/DynCorp., Frederick, MD (L. Henderson, P. Powell); Oncogene, Seattle, WA (S.-L. Hu); Univ. of Washington, Seattle, WA (W. Morton, C.-C. Tsai); Walter Reed Army Inst. of Research, Washington, DC (D. Burke, A. Shafferman); Henry M. Jackson Foundation, Rockville, MD (M. Lewis, G. Eddy)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Immunogenetics Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.4	0.6

CHECK APPROPRIATE BOX(ES)

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

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

Three separate recombinant or peptide vaccines have been shown to protect macaques from an intravenous challenge with simian immunodeficiency virus (SIV) or type D retrovirus (SRV-2). Macaques immunized with a vaccinia recombinant expressing the envelope proteins of our molecular clone of SRV-2/WASHINGTON (gp70 and gp22) were protected against a homologous challenge with 10 to the sixth power infectious particles of SRV-2. Vaccinated animals are not infected one year after challenge as determined by failure to isolate virus or lack of seroconversion to additional viral proteins. These animals are now being challenged with a heterologous strain (SRV-1). One of the potential difficulties in developing an HIV vaccine involves the large variation in the V-3 loop of the envelope protein gp120 among various isolates. Four peptides representing highly conserved and seroprevalent regions of gp120 and gp41 were mixed and used as immunogens in three rhesus macaques. The 2 animals with the highest neutralizing titers resisted a challenge of 100 infectious particles of SIV. One year after challenge, whole blood and lymph node cells were passaged to other macaques; there is no evidence of infection in the recipient animals by eight weeks after transfer. In a third experiment, macaques have also been immunized with two doses of a vaccinia virus recombined with and expressing the envelope proteins (gp120 and gp32) of our pathogenic molecular clone of SIV/Mne. After being boosted with gp160 protein expressed in baculovirus, these animals were challenged intravenously with 10 animal-infectious doses of SIV/Mne. Eight months later, these animals show no evidence of virus infection. These experiments are the first description of successful recombinant vaccines that protect against SIV infection and suggest that similar approaches may be directly applicable to HIV vaccine development. These studies have been facilitated by the use of well-defined stocks of virus that have been titered in various cell lines in vitro as well as in macaques.

Project Description

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

Raoul E. Benveniste	Medical Officer	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

Objectives:

To develop vaccines to protect against retrovirus infection in nonhuman primates to serve as a model for retroviral vaccines in man.

Methods Employed:

Molecular cloning, sequencing, and site-directed mutagenesis of various simian immunodeficiency viruses (SIV) in order to determine the molecular basis of pathogenicity. Isolation of single-cell clones of infected cells with unusual properties by using feeder layers of primary sheep choroid plexus cells in microtiter plates. Antibodies to viral proteins were detected by Western immunoblot techniques. Virus neutralization assays were performed by a rapid microtiter plate assay developed in this laboratory that measures appearance of syncytia after infection of cells by SIV retroviruses.

Major Findings:

1. Protection of macaques from SIV infection by immunization with a recombinant vaccinia virus expressing envelope glycoproteins. We have obtained a pathogenic molecular clone of SIV/Mne and, in collaboration with Dr. S.-L. Hu, constructed a recombinant vaccinia virus that expresses the envelope (gp120) and transmembrane (gp32) proteins. Four vaccinia-naive Macaca fascicularis were immunized at weeks 0 and 12 with 10^8 plaque-forming units. Although these animals developed readily-detectable antibodies to both envelope proteins, titers steadily decreased, and neutralizing antibodies were not detected. These animals were then boosted 62 and 70 weeks after the initial vaccinia immunization with partially purified gp160 produced in the baculovirus expression system. All four macaques then developed a marked increase in antibody titer and showed neutralizing activity in their sera. At week 74, these four animals and four controls were challenged with 10 animal-infectious doses (AID) of SIV/Mne. All four controls became readily infected, whereas there is no evidence of infection in the vaccinated macaques as determined by failure to isolate virus, lack of seroconversion to additional viral proteins, and failure to detect viral sequences by polymerase chain reaction.

2. Protection of macaques from SIV infection by immunization with conserved envelope peptides. Two small peptides in gp120 and two in gp41 have been previously identified by us as being highly conserved among the various HIV-1 isolates and as being equally seroprevalent in HIV-1-infected individuals and in SIV/Mne-infected macaques. Three rhesus macaques (M. mulatta) were immunized on days 0, 14, and 36 with a mixture containing Freund's adjuvant

and 4 μg of each peptide fused to β-galactosidase. One year later the animals received an additional 40 μg of each peptide and were subsequently challenged three weeks later with 100 AID of SIV/Mne. Three controls, which had only been immunized with β-galactosidase, were similarly challenged. All control macaques seroconverted and virus could be isolated continuously after infection. In marked contrast, two of the vaccinated animals did not seroconvert, and virus could not be isolated from peripheral blood lymphocytes (PBLs). Those two animals also had the highest neutralizing titers to SIV/Mne on the day of challenge. The third animal (with the lowest neutralizing titer) did not seroconvert, but virus could only be isolated from PBLs through week 16 post-challenge. Eighty-six weeks after challenge, 10 ml of whole blood and 20 x 10^6 lymph node cells from the three vaccinated animals and from one control were passaged to four naive rhesus monkeys. Although the recipient of blood from the control animal became infected, there is no evidence of infection (8 weeks after transfer) in the recipients of blood from the three vaccinated animals.

These studies are now being expanded in order to test each peptide individually. The three protected macaques will also be challenged with a heterologous strain of SIV in order to test the specificity of protection. In view of the highly conserved nature of these peptides, these studies suggest that similar vaccines might be developed that protect simultaneously against many HIV-1 strains.

3. Protection of macaques from infection by type D retrovirus (SRV-2). We have previously described a successful vaccine consisting of vaccinia virus recombined with and expressing the envelope proteins (gp70 and gp22) of the type D retrovirus SRV-2/WASHINGTON. This retrovirus causes simian AIDS and retroperitoneal fibromatosis in macaques and is a significant cause of morbidity and mortality in some primate colonies.

Animals immunized with this vaccinia construct resist a challenge with as much as 10^6 AID of virus. These macaques have now been challenged with a different serotype of type D virus, SRV-1, obtained from Dr. G. Fanning-Heidecker. Studies are underway to determine if the vaccine is group- or type-specific.

4. Production of vaccine challenge stocks of SIV. One of the components of a successful vaccine study is the availability of virus stocks that have been well-characterized and titered. We have produced large amounts of cell culture fluid from stocks of uncloned SIV/Mne and of single-cell and molecular clones of SIV. These stocks are clarified of cells, filtered (0.45μ), adjusted to 20% fetal calf serum, and frozen in liquid nitrogen in 1.0-4.0 ml aliquots. The stocks are also frozen as log dilutions (10^{-1} to 10^{-8}).

These stocks have been titered in several cell lines and PBLs in vitro and in various species of macaques (M. mulatta, M. nemestrina, and M. fascicularis) in vivo. The availability of these defined stocks permits challenges with the smallest dose that results in infection of all control animals; previous vaccine studies have failed because the challenge dose was either too high or too low.

Publications:

Benveniste RE, Hill RW, Eron LJ, Csaikl UM, Knott WB, Henderson LE, Sowder RC, Nagashima K, Gonda MA. Characterization of clones of HIV-1 infected HuT 78 cells defective in gag gene processing and of SIV clones producing large amounts of envelope glycoprotein. J Med Primatol 1990;19:351-66.

Henderson LE, Sowder RC, Copeland TD, Oroszlan S, Benveniste RE. GAG precursors of HIV and SIV are cleaved into six proteins found in mature virions. J Med Primatol 1990;19:411-9.

Shafferman A, Jahrling PB, Benveniste RE, Lewis MG, Phipps TJ, Eden-McCutchan F, Sadoff J, Eddy GA, Burke DS. Immunization of macaques with a vaccine of HIV-based, conserved SIV envelope peptides. Proc Natl Acad Sci USA (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05652-02 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Mutational Analysis of the Cystic Fibrosis Gene

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:	Marga Belle White	Special Volunteer	LVC	NCI
	Raleigh Boaze	Biological Laboratory Technician	LVC	NCI

COOPERATING UNITS *(if any)*: PRI/DynCorp, Frederick, MD (M. Dean); Boston University, Boston, MA (J. Amos); University of Utah, Salt Lake City, UT (M. Leppert); Hanneman University, Philadephia, PA (C. Kruegar)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Genetics Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	0.6	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 recent identification of the cystic fibrosis (CF) gene, the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Riordan JR et al., Science 1989;245:1066-73), revealed that the gene belongs to a family of membrane transport molecules that includes the multi-drug resistance genes. Approximately 70% of CF chromosomes contain a deletion of 3-base pairs resulting in the loss of a phenylalanine codon at amino acid position 508 (ΔF508). The focus of this project is to identify new mutations in this gene that comprise the remaining 30% of CFTR gene mutations. Using the polymerase chain reaction technique, the coding regions of the gene have been examined from patients that have at least one chromosome without the common CF mutation (ΔF508). This year we report the identification of five additional mutations in the CF gene (in addition to the eight reported last year). Most of the mutations were initially identified using an assay for single-stranded conformation polymorphisms. All mutations were subsequently characterized by direct sequencing of the amplified DNA and can be assayed by restriction enzyme digestion or allele-specific oligonucleotide hybridization.

The mutations fall into two classes: (1) insertions or deletions that introduce termination codons into the gene and are predicted to result in severely truncated protein products, and (2) point mutations in the putative membrane-spanning domains that replace charged amino acids with non-polar residues. In addition, we have cloned a region of the mouse CF gene homolog. Restriction mapping and Southern blotting experiments have indicated that this clone contains an exon that is highly homologous to exon 7 of the human CF gene. This exon was subcloned for use in homologous recombination/gene targeting experiments aimed at creating a disease model for CF.

Project Description

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

Stephen J. O'Brien	Chief	LVC	NCI
Marga Belle White	Special Volunteer	LVC	NCI
Raleigh Boaze	Biological Laboratory Technician	LVC	NCI

Objectives:

To find and characterize additional mutations in the human cystic fibrosis transmembrane conductance regulator (CFTR) gene, and to correlate these mutations to the functional domains of the protein and the clinical characteristics of the patients. Examination of the clinical manifestations of these mutations in the allelic combinations in which they occur should greatly increase our understanding of the function of the CFTR gene product and its role in the process of the disease. Understanding the mechanisms of action of the CFTR gene product should also help our understanding of the functionally important regions of other members of this gene family, such as the multi-drug resistance genes commonly activated in tumor cells.

Our second objective is to clone the mouse homolog of the CFTR gene for the purpose of aiding in the creation of a disease model for cystic fibrosis (CF). Such a model should provide a system for testing new drugs and therapies in the hopes of eventually developing a treatment or cure for the disease.

Methods Employed:

The following techniques were employed: (1) Polymerase chain reaction (PCR) amplification of exons of the CFTR gene using oligonucleotide primers based on the published cDNA sequence of this gene. (2) Analysis of these exons by denaturing these radiolabeled amplified products and electrophoresing them on non-denaturing polyacrylamide gels using the single-stranded conformation polymorphism (SSCP) technique of Orita M et al. (Genomics 1989;5:874-9). The PCR fragments enter the gel as single strands, folding back on themselves in a sequence-dependent manner. Mutations in the form of insertions, deletions, and even point mutations can be detected with this technique due to the fact that a single base change can alter the conformation of each of the strands. Fragments that have a mutation, therefore, will have a different mobility from the normal samples. (3) Mapping of the mutations within the PCR fragments using a variation of the SSCP technique. The PCR products are digested with the appropriate restriction enzyme, and the resulting restriction fragments are excised separately. Subsequent SSCP analysis on these individual restriction fragments indicates which fragment contains the mutation. This variation on SSCP allows for the resolution of several different nucleotide alterations in the same exon and simplifies the eventual DNA sequence analysis of the mutation. (4) Direct sequencing of the amplified products using the assymetric PCR technique (Gyllensten UB et al., Proc Natl Acad Sci USA 1988;85:7652-6) and dideoxy sequencing reactions (Sanger F et al., Proc Natl Acad Sci USA 1977;74:5463-7). (5) Designing assays for these new mutations

using either restriction enzyme digestion or allele-specific oligonucleotide hybridization.

Major Findings:

1. New CFTR gene mutations. Using an assay for the detection of the common CFTR gene mutation, we have identified 188 CF individuals that have at least one chromosome that does not contain the common ΔF508 mutation. Because CF is an autosomal recessive disease, these chromosomes are candidates for new CFTR gene mutations. Using PCR and the SSCP technique described above, we found the following new mutations in the CFTR gene:

Mutation	Exon	DNA Sequence Alteration	Amino Acid Alteration	Ethnic Origin
T729G	6A	T to G	His to Gln	Black
852del22	6A	22bp deletion	premature termination	?
1677delTA	10	TA deletion	premature termination	Russian
2522insC	13	C insertion	premature termination	Italian
C2683T	14A	C to T	premature termination	Caucasian

One of our frameshift mutations (1677delTA) occurs in a Soviet Georgian family with three children who died in the first weeks of life from either pneumonia or intestinal obstruction. Analysis of the PCR products from the parents demonstrated that they were both carriers of the same mutation (even though the parents themselves are unrelated). The alteration is a 2-base pair deletion in exon 10 that is predicted to result in premature termination of the protein product. Since this time, three other individuals from Bulgaria have been identified with this mutation in combination with the ΔF508 mutation. Thus, 1677delTA appears to be a severe mutation of Slavic origin. We have also identified a family in which the single affected child possesses a point mutation that creates a terminator codon in exon 14A (C2683T). Neither parent carries this mutation, and analysis with highly polymorphic markers indicates that the correct parental samples were obtained. Therefore, this is the first example of a spontaneous mutation in the CFTR gene. This class of mutations may be rare but will present additional complications for predicting the occurrence of CF by carrier screening or prenatal diagnosis.

2. Cloning the mouse cystic fibrosis gene homolog. We have screened two mouse genomic libraries (one from a C57/B16 mouse and the other from a Balb/c mouse) with the human CF gene cDNA. We isolated one clone (λCFMu6-11) from the C57/B16 library. A HindIII restriction fragment from this clone that hybridized to the human cDNA probe (indicating it contains CF exon sequences) was subcloned into the plasmid vector, pUC18. Subsequent DNA sequencing

experiments indicated that this subclone (pCFMuH1) contains a region that is homologous to exon 7 from the human CF gene. This mouse "CF gene" exon is 87% homologous to the human CF exon 7 at the DNA level and 83% homologous at the amino acid level. In addition, the intron sequences flanking the mouse exon contain sequences that conform to the acceptor/donor mRNA splice site rules. Six additional mouse CF clones have been isolated from the Balb/c genomic library. Preliminary mapping experiments indicate that three of these clones overlap the first clone.

Publications:

Dean M, Amos JA, Lynch J, Romeo G, Devoto M, Ward K, Halley D, Oostra B, Ferrari M, Weir BS, Finn PB, Collins F, Iannuzzi M. Prenatal diagnostics and linkage disequilibrium with cystic fibrosis for markers surrounding D7S8. Hum Genet 1991;85:275-8.

Dean M, White MB, Amos J, Gerrard B, Stewart C, Khaw KT, Leppert M. Multiple mutations are found in mildly affected cystic fibrosis patients. Cell 1991:61:863-70.

Iannuzzi MC, Stern RC, Collins DS, Tom Hon C, Hidaka N, Strong T, Decker L, Drumm ML, White MB, Gerrard B, Dean M. Two frameshift mutations in the cystic fibrosis gene. Am J Hum Genet 1991;48:227-31.

Ivaschenko TE, White MB, Dean M, Baranov, VS. A deletion of two nucleotides in exon 10 of the CFTR gene in a Soviet family with cystic fibrosis causing early death. Genomics 1991;10:298-9.

White MB, Krueger LJ, Holsclaw DS, Gerrard B, Stewart C, Quittell L, Dolganov G, Baranov V, Ivaschenko T, Kapranov NI, Sebastio G, Castiglione O, Dean M. Detection of three rare frameshift mutations in the cystic fibrosis gene in an African American (CF444delA), an Italian (CF2522insC), and a Soviet (CF3821delT). Genomics 1991;10:266-9.

Patents:

Dean M. US Patent 07/668.309: Detection of the Common Cystic Fibrosis Mutation, March 13, 1991.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05653-02 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Molecular Genetic Analysis of Homeobox Genes in the Domestic Cat

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

PI:	Naoya Yuhki	Visiting Associate	LVC	NCI
Others:	Ryuichi Masuda	Visiting Fellow	LVC	NCI
	Stephen J. O'Brien	Chief	LVC	NCI
	Stanley J. Cevario	Microbiologist	LVC	NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Genetics Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	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 feline homeobox-containing DNA sequence was isolated from the genomic library of the domestic cat using the mouse homeobox Hox-3.1 probe. The isolated homeobox was named cat HOX3A, according to the human homeobox nomenclature. The nucleotide sequence and the deduced amino acid sequence of cat HOX3A showed a higher similarity to those of cognate genes of human, mouse, and rat. Using a panel of rodent x domestic cat somatic cell hybrids, cat HOX3A was assigned to cat chromosome B4. Interestingly, it has been known that cat chromosome B4 shows syntenic homology with mouse chromosome 15 and human chromosome 12, where mouse Hox-3.1 and human HOX3A have been mapped, respectively. These data demonstrate evolutionary conservation of both HOX3A gene sequences and chromosomal locations during mammalian evolution.

Project Description

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

Naoya Yuhki	Visiting Associate	LVC	NCI
Ryuichi Masuda	Visiting Fellow	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI
Stanley J. Cevario	Microbiologist	LVC	NCI

Objectives:

The homeobox gene has a highly-conserved 183-base pair DNA sequence known as a homeobox domain, which is present in both invertebrates and vertebrates. The homeobox domain is considered to have a DNA-binding function and to play an important role during embryonic development. For the promotion of genetic and evolutionary studies of the feline families which have progressed in our laboratory, it is important to isolate and characterize homeobox genes from the cat genome. In this report, we summarize the results of molecular cloning and characterization of the cat homeobox genes.

Methods Employed:

The following techniques were employed: (1) Southern and Northern blotting, (2) molecular cloning, and (3) DNA sequencing using the dideoxy sequencing method.

Major Findings:

By screening the domestic cat genomic DNA library with the mouse homeobox Hox-3.1 probe, one clone was isolated with a molecular size of 3.8 kilobases (kb). As a result of restriction mapping of this clone, there were ten sites of main restriction enzymes, and a 1.2-kb PstI-PstI fragment included a sequence homologous to mouse Hox-3.1. The predicted homeobox present in this region was named cat HOX3A, according to the human homeobox nomenclature. The nucleotide sequence similarity of cat HOX3A was 96% to human HOX3A, 94% to mouse Hox-3.1, and 94% to rat R4. The deduced amino acid sequence (homeodomain) of cat HOX3A was identical to all homeodomains of these cognate genes. For chromosomal mapping of cat HOX3A, a panel of 21 (mouse x domestic cat) and 18 (Chinese hamster x domestic cat) somatic cell hybrid clones was examined by Southern blotting. According to the calculated discordance, cat HOX3A was assigned to cat chromosome B4, where three gene loci (TPI, triose phosphate isomerase-1; PEPB, peptidase B; LDHB, lactate dehydrogenase B) have been mapped. Mouse Hox-3.1 and human HOX3A are localized on mouse chromosome 15 and human chromosome 12, respectively. In comparison with other gene loci, there is a syntenic homology among human chromosome 12, mouse chromosome 15, and cat chromosome B4. These results demonstrate evolutionary conservation of both HOX3A gene sequences and chromosomal locations during mammalian evolution. RNA transcripts recognized by the cat HOX3A probe were found in all examined cat tissues. The ubiquitous expression of cat HOX3A contrasts with tissue-specific expression of mouse Hox-3.1.

Publications:

Masuda R, Yuhki N, O'Brien SJ. Molecular cloning, chromosomal assignment, nucleotide sequence, and expression of the feline homeobox HOX3A. Genomics (In Press).

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

PROJECT NUMBER
Z01CP05654-02 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Function of the Nef Protein of SIV/Mne

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

PI:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Others:	Raoul E. Benveniste	Medical Officer	LVC	NCI
	Richard Hill	Biological Laboratory Technician	LVC	NCI

COOPERATING UNITS *(if any)*

California Regional Primate Research Center, Davis, CA, (M. Marthas); PRI/DynCorp, Frederick, MD (W. Knott)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.6	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 function of the Nef protein encoded by HIV and simian immunodeficiency virus (SIV) is still unknown. Several reports propose that they are involved in viral latency due to a negative effect on viral transcription. Frequent inactivating mutations in the gene occurring during virus isolation suggest that the gene is not tolerated well during propagation in tissue culture. In order to investigate the function of this gene we have extended our screen of animals infected with different isolates of SIV to see whether the appearance of the anti-nef response has a prognostic value for disease progression as has been reported in the case of HIV and human AIDS.

Project Description

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

Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Raoul E. Benveniste	Medical Officer	LVC	NCI
Richard Hill	Biological Laboratory Technician	LVC	NCI

Objectives:

The nef gene is found in all HIV-1, HIV-2 and simian immunodeficiency virus (SIV) isolates. It is well conserved between different isolates, and is often inactivated through mutations during isolation of the virus on tissue culture cells. It shows homology to cellular G proteins; however, it is still debated whether the protein has guanysil triphosphate binding activity. A reported down-regulation effect on transcription from the HIV long terminal repeat is also not widely accepted. In humans infected with HIV-1, anti-Nef response is one of the earliest phenomena. We have generated several nef expression systems to elucidate the role of the Nef protein expressed by SIV/Mne.

Methods Employed:

Molecular cloning and site-directed mutagenesis were performed following standard protocols. Recombinant baculovirus expressing wild-type and mutant nef genes are grown in Spodoptera frugiperda cells following protocols established by M. Sumners. Transfection of eukaryotic cells was done by the calcium-phosphate co-precipitation method. Polymerase chain reactions were performed according to the manufacturer's recommendations.

Major Findings:

Anti-nef response in animals infected with SIV/Mac239 at the California Regional Primate and Research Center. Sera from animals infected with virus derived from the molecular clone, SIV/Mac239, and recombinants between this isolate and SIV/MacA1 were analyzed for presence of anti-Nef antibodies. None of the animals which had received virus derived from 239/A1 recombinants had seroconverted, suggesting that expression of the nef gene had been compromised in these constructs, although this was not obvious from their sequence. In contrast, 50% of the animals which had been inoculated with the SIV/Mac239 virus had mounted an antibody response. Latency for AIDS was inversely correlated with the time at which seroconversion was observed in this study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05655-02 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms Involved in Raf-1 Activation by Growth Factors

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:	Harald App	Visiting Fellow	LVC	NCI
	Zoltan Olah	Visiting Fellow	LCO	NCI

COOPERATING UNITS *(if any)*
Duke University Medical Center, Durham, NC (P. J. Blackshear, R. Lee)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.8	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 protein of the Raf-1 proto-oncogene is a protein serine/threonine kinase that is activated after stimulation of cells with insulin, epidermal growth factor, and other mitogens. Incubation with these mitogens leads to a shift in electrophoretic mobility, which is caused by an increased phosphate incorporation into the Raf-1 protein on serine and threonine residues. To investigate the mechanism of this activation, we used purified Raf-1 expressed in Escherichia coli as a substrate for a putative Raf-1 protein kinase. In three different insulin-sensitive cell types, insulin-activated Raf-1 kinase kinase was localized in the crude cytosolic fractions. The insulin-stimulated Raf-1 kinase kinase(s) phosphorylated the purified Raf-1 protein on multiple sites in vitro, as evidenced by tryptic peptide mapping. At least some of these phosphorylation sites appear to overlap with sites phosphorylated in response to serum in intact cells. The insulin-stimulated Raf-1 kinase kinase may play a role in mediating the phosphorylation and possibly the activation of the Raf-1 kinase by insulin and other growth factors.

Enzymatically active Raf-1 kinase was partially purified from baculovirus-expressed Raf-1 protein. This enables us to determine the activating phosphorylation site of the Raf-1 kinase and to search for in vitro substrates.

Receptor-mediated activation of Raf-1 protein kinase is followed by translocation of the normally-cytosolic enzyme to the perinuclear area and the nucleus. Raf-1 dependence of growth induction by three hematopoietic growth factors that we recently showed to regulate Raf-1 activity was tested with antisense oligonucleotides. All three receptors require Raf-1 for induction of DNA synthesis.

Project Description

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

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Harald App	Visiting Fellow	LVC	NCI
Zoltan Olah	Visiting Fellow	LCO	NCI

Objectives:

The objectives of these studies are: (1) to characterize the Raf-1 protein kinase with respect to the position in signal transduction and to identify the substrates and the pathways in which the enzyme operates, (2) to determine the self-phosphorylation site and the phosphorylation sites for other protein kinases on the Raf-1 molecule, (3) to identify putative ligands which may control Raf-1 protein kinase activity, and (4) to purify the active Raf-1 enzyme.

Methods Employed:

Immunoprecipitation, Western blotting, phosphoaminoacid analysis, peptide mapping and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, native electrophoresis, and purification of antisera were carried out according to published procedures. High performance liquid chromatography (HPLC), fast performance liquid chromatography, and liquid chromatography used for Raf-1 protein purification followed standard procedures.

Major Findings:

Insulin and other polypeptide growth factors stimulate a variety of cellular responses associated with growth and differentiation. Major cellular responses to these growth factors is the activation of protein tyrosine kinases and protein serine threonine kinases, such as ribosomal S 6 kinase and Raf-1 kinase. However, for most of the insulin- and growth factor-stimulated serine threonine kinases, the biochemical steps between receptor tyrosine kinase activation and serine threonine kinases are unknown. Insulin and epidermal growth factor (EGF) stimulation of H35 and HER14 cells causes an increased phosphate incorporation into the Raf protein on serine and threonine residues, but not on tyrosine residues, and to an activation of the Raf-1 kinase activity. An explanation might be that activated insulin or EGF-receptor could activate another serine threonine kinase(s) which could then phosphorylate and activate Raf-1 kinase.

We used insulin-stimulated HIR3.5 or H35 cell lines as a source for the identification and purification of the Raf-1 kinase kinase. As a substrate for the Raf-1 kinase kinase, Raf-1 protein was purified from Escherichia coli cells which had been transformed with the Raf-1 protein expression vector, pUSC, using a reverse phase HPLC chromatography technique.

Incubation of purified Raf-1 protein with Mg•ATP did not result in detectable phosphorylation of the protein, but Raf-1 was prominently phosphorylated when the protein was mixed with lysates from growth factor-stimulated cells. One other phosphorylated band in this *in vitro* kinase assay, with a Mr of 97,000, was identified as the probable Raf-1 kinase kinase. *In vitro* and *in vivo* (using Raf-1 high expressor cells) phosphorylated Raf-1 protein yielded very similar phosphopeptide maps, with more than ten spots being detected in both cases.

Because an *in vitro* phosphorylation of Raf-1 protein purified from *E. coli* does not lead to a typical shift in the mobility in SDS gels, nor to an increase in Raf-1 kinase activity, we attempted to purify active Raf-1 kinase from baculovirus-expressed Raf-1 protein (and several Raf-1 mutant forms) in *Spodoptera frugiperda* (SF 9) cells. Comparison of the phosphopeptide maps of baculovirus and bacterially expressed Raf-1 proteins can now be used to identify the shift-causing, activating phosphorylation site in the Raf-1 protein. Furthermore, active Raf-1 kinase can be used for determining the Raf-1 substrates *in vivo* and *in vitro* by comparing the phosphopeptide maps of the substrates.

Erythropoietin mediates the rapid phosphorylation of Raf-1 in the murine cell lines HCD-57 and FCD-P1/ER, which proliferate in response to this cytokine. Phosphorylation occurs at both serine and tyrosine residues, and as such is similar to the Raf-1 phosphorylation seen after interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor, and interleukin-2 stimulation in other murine cell lines. Such data suggest that these growth factors may share a common mechanism of Raf-1 phosphorylation. Furthermore, in association with Raf-1 phosphorylation, erythropoietin incudes a two- to threefold increase in Raf-1 kinase activity as measured in immune complex kinase assays *in vitro*. Finally, a c-*raf* antisense oligodeoxyribonucleotide, which specifically decreases intracellular Raf-1 levels, also substantially inhibits both erythropoietin and IL-3 directed-DNA synthesis. Together, these results provide evidence that activated Raf-1 is a necessary component of erythropoietin and IL-3 growth signaling pathways.

Publications:

App H, Hazan R, Zilberstein A, Ullrich A, Scholessinger J, Rapp UR. Epidermal growth factor (EGF) stimulates association and kinase activity of Raf-1 with the EGF receptor. Mol Cell Biol 1991;11:913-9.

Baccarini M, Sabatini DM, App H, Rapp UR, Stanley ER. Colony stimulation factor-1 (CSF-1) stimulates temperature dependent phosphorylation and activation of the RAF-1 proto-oncogene product. Embo J 1990;9:3649-57.

Blackshear PT, Haupt DM, App H, Rapp UR. Insulin activates the Raf-1 kinase. J Biol Chem 1990;265:12131-4.

Carroll MP, Clark-Lewis I, Rapp UR, May WS. Interleukin-3 and grandulocyte-macrophage colony stimulating factor mediate rapid phosphorylation and activation of cytosolic c-*raf*. J Biol Chem 1990;265:19812-7.

Carroll MP, McMahon M, Weich N, Spivak JL, Rapp UR, May WS. Erythropoietin induces raf-1 activation and raf-1 is required for erythropoietin-mediated proliferation. J Biol Chem (In Press).

Lee R, Rapp UR, Blackshear PJ. Evidence for a Raf-1 kinase activated by insulin and polypeptide growth factors. J Biol Chem (In Press).

Rapp UR. Growth factor regulation of raf-1 protein kinase. Oncogene (In Press).

Reed JC, Talwar HS, Cuddy MP, Baffy G, Williamson J, Rapp UR, Fisher, GJ. Mitochondrial protein p26 BCL-2 reduces growth factor requirements of NIH 3T3 fibroblasts. Exp Cell Res (In Press).

Reed JC, Yum S, Cuddy MP, Turner BC, Rapp UR. Differential regulation of Raf-1 protein kinase by ras and src oncogenes. Cell Growth Differ 1991;2:375-83.

Siegel JN, Klausner RD, Rapp UR, Samelson LE. T cell antigen receptor stimulation activates the c-RAF kinase exclusively via serine/threonine phosphorylation. J Biol Chem 1990;265:18472-80.

Turner B, Rapp UR, App H, Greene M, Dobashi K, Reed J. Interleukin 2 induces tyrosine phosphorylation and activation of P72-74 Raf-1 kinase in a T-cell line. Proc Natl Acad Sci USA 1991;88:1227-31.

Patent:

McMahon M, Weich N, Spivak JL, Rapp UR, May WS. US Patent Pending: Erythropoietin Induces raf-1 Activation and raf-1 is Required for Erythropoietin-Mediated Proliferation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05656-02 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: B-raf Protein Kinase: Structure, Function, Expression, and Activation In Vivo

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:	Zolton Olah	Visiting Fellow	LCO	NCI
	Wayne Anderson	Senior Investigator	LCO	NCI

COOPERATING UNITS *(if any)*: PRI/DynCorp, Frederick, MD (T. Beck, G. Sithanandam); ABL, Frederick, MD (D. Kaplan); University of Pennsylvania School of Medicine, Philadelphia, PA (J. Brugge); Section on Growth Factors, DIR, NICHHD (G. Guroff)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Viral Pathology Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.6	0.1

CHECK APPROPRIATE BOX(ES)

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

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

The B-raf gene was isolated from a human genomic library and its promoter region characterized. Moreover, a series of cDNA clones were isolated and sequenced. One of these contains the complete sequence of a 72/74 kiloDalton (kDa) B-raf kinase. There is a second form of the kinase, a 95 kDa phosphoprotein, that is currently being characterized. The rat pheochromocytoma cell line (PC12) expresses several growth and differentiation receptors, including those for epidermal growth factor (EGF), fibroblast growth factor (FGF), and nerve growth factor (NGF), and is extensively used as an in vitro model system to study the mechanisms of growth factor-induced differentiation and proliferation. Depending upon which receptor is activated, either proliferation or differentiation occurs. We have previously shown that a variety of ligands for transmembrane receptor tyrosine kinases regulate Raf-1 activity, including platelet-derived growth factor, colony stimulating factor, insulin, FGF, and EGF. PC12 cells express Raf-1 as well as B-Raf. We wanted to examine whether Raf kinase was involved in this signaling, and whether there was Raf isotype preference in the coupling to a proliferation versus differentiation receptor. Recent experiments demonstrate that stimulation of PC12 cells with EGF, FGF, or NGF activate B-Raf and lead to a shift in its electrophoretic mobility. This shift is caused by increased phosphate incorporation into the B-Raf protein on serine residue(s). B-Raf phosphorylation occurs within 1.5 minutes of NGF treatment and reaches a maximum level after 10 minutes. These results suggest that B-Raf may play a role in NGF-mediated signal transduction. In order to investigate the function of B-Raf in differentiation or proliferation signaling pathways, B-raf stimulation and inhibition experiments will be performed in PC12 cells employing (1) B-Raf-specific monoclonal antibodies, (2) expression of antisense RNA, and (3) dominant negative, as well as constitutively-activated, B-raf mutants.

Project Description

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

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Gunamani Sithanandam	IRTA Fellow	LVC	NCI
Zoltan Olah	Visiting Fellow	LCO	NCI
Wayne Anderson	Senior Investigator	LCO	NCI

Objectives:

The primary objectives of this project are: (1) to molecularly clone and characterize the human B-raf gene; (2) to identify and characterize the gene and gene products that regulate B-raf expression; (3) to isolate B-raf homologs from two experimental animal systems, mouse and Drosphila; (4) to localize the B-raf gene on human and mouse chromosomes and to identify restriction fragment length polymorphisms (RFLPs); (5) to analyze the structure function relationship of B-raf; and (6) to investigate the role of B-raf in cell differentiation.

Methods Employed:

Standard recombinant DNA techniques were used to molecularly clone and sequence B-raf cDNAs and genomic DNA clones. B-raf gene polymorphisms were analyzed using a panel of DNA from unrelated individuals. Protein analyses were done by immunoprecipitation, Western blotting, metabolic labeling, phosphoaminoacid analysis, and sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Major Findings:

1. A nearly full-length human B-raf cDNA clone has been isolated from a testes library. The 2.3-kilobase (Kb) B-raf cDNA contains a single open reading frame of 1,953 nucleotides coding for a protein of 651 amino acids and a molecular weight of 72.5 kiloDaltons (kDa). The amino terminal region of B-raf is very rich in serine residues (12/44). Overall B-raf shows a 54% homology to raf-1 and 52% to A-raf. In the kinase domain B-raf has a 76.4% homology with A-raf and 79% with raf-1.

2. Two new cDNA clones containing 5′ extensions relative to the original B-raf clones have been isolated. These clones have allowed us to isolate a genomic clone containing the 5′ end of B-raf gene, possibly exon-1, and B-raf promoter region. Characterization of this clone is in progress. During this process, we have also isolated a genomic clone containing a B-raf pseudogene.

3. The B-raf gene has been examined for polymorphic sequences with a battery of enzymes in a panel of DNAs from unrelated individuals. With Eco-RV, the probe detected 2 allelic fragments of 6 and 3 Kb having a frequency of 83% and 17%. The segregation of this Eco-RV polymorphism is now being tested in families. Preliminary results indicated an Eco-RI polymorphism within the

B-raf pseudogene. The human B-raf has been localized to chromosomal position 7q32. Application of the fluorescence in situ hybridization technique on metaphase cells using two single copy sequences, one from the B-raf pseudogene and one from B-raf, will allow us to distinguish the active B-raf gene locus from the pseudogene and precisely map human B-raf.

4. B-raf gene expression has been examined by Northern blot analysis in adult and fetal mouse tissues. Many adult tissues did not have a detectable amount of B-raf expression. High message levels of 10- and 13-Kb B-raf transcripts were observed in cerebrum and fetal brain. In addition to a 10-Kb transcript, alternate size transcripts of 2.6 and 4.5 Kb were observed. Murine myeloid FD cells transformed with the trk oncogene expressed high levels of B-raf transcripts.

5. Most of the work on Raf family kinases has been focused on the ubiquitous Raf-1 which plays a role in the flow of membrane-initiated proliferation signals. Very little work has been done on other isozymes. Since B-raf is highly expressed in neural tissues, we wanted to address whether B-raf would play a role in nerve growth factor (NGF) induced differentiation of PC12 cells. Preliminary results indicate that B-Raf kinase was activated in PC12 cells after stimulation with epideral growth factor, fibroblast growth factor, and NGF, and this stimulation was paralleled by increased B-Raf phosphorylation resulting in slower migration. The phosphoaminoacid analysis demonstrated that the serine residues were phosphorylated. B-Raf phosphorylation occurred within 1.5 minutes of NGF treatment, reached maximum levels after 10 minutes, declined thereafter, and returned to ground level within 120 minutes.

6. trk regulates expression of B-raf in NIH/3T3 cells. By using the immunocomplex kinase assay, we have shown that B-Raf is activated in trk-transformed NIH/3T3 cells. This suggests that ectopic expression of the nerve cell-specific NGF receptor leads to induction of components of its own signal transduction pathway. B-Raf inhibition experiments with B-raf-specific monoclonal antibodies, antisense RNA expression vectors, and B-raf-specific dominant negative mutants in PC12 cells should allow us to investigate the role of B-Raf in neuronal differentiation processes.

Publications:

Kiss Z, Rapp UR, Anderson WB. Differential regulation of the hydrolysis of phosphatidylcholine and phosphatidylethanolamine by ATP, phorbol ester, and oncogenes. Arch Biochem Biophys (In Press).

Mihaly A, Kuhnt U, Olah Z, Rapp UR. Immunohistochemical localization of raf protein kinase in dendritic spines and apparatuses of the rat cerebral cortex. Brain Res (In Press).

Mihaly A, Olah Z, Kuhnt U, Matthies H, Rapp UR, Joo F. Transient increase of raf-protein-kinase-like immunoreactivity in the dentate gyrus during long-term potentiation. Neurosci Lett 1990;116:45-50.

Olah Z, Komoly S, Nagashima N, Joo F, Rapp UR, Anderson WB. Cerebral ischemia induces transient intracellular redistribution and intranuclear translocation of the raf proto oncogene product in hippocampal pyramidal cells. Exp Brain Res (In Press).

Oshima M, Sithanandam G, Rapp UR, Guroff G. The phosphorylation and activation of B-raf by nerve growth factor in PC12 cells. J Biol Chem (In Press).

Sithanandam G, Kolch W, Duh FM, Rapp UR. Complete coding sequence of a human B-raf cDNA and detection of B-raf protein kinase with isozyme specific antibodies. Oncogene 1990;5:1775-80.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05678-01 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Developing High Resolution RFLPs for Human Genetic Analysis

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:	Raleigh Boaze	Biological Laboratory Technician	LVC	NCI
	Marga Belle White	Special Volunteer	LVC	NCI

COOPERATING UNITS *(if any)*
LCS, NIAAA, Bethesda, MD (D. Goldman); PRI/DynCorp, Frederick, MD (M. Dean)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.6	1.1

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 field of human genetics has been revolutionized by the development of a large collection of restriction fragment length polymorphisms (RFLPs). Even though there are now 2300 RFLPs characterized, there are only 500 polymorphisms in coding genes. Thus, there is a need to develop new methods to allow polymorphisms to be characterized in specific genes. We have characterized a number of polymorphisms in biologically important human genes using both standard approaches, as well as new methods that employ the polymerase chain reaction (PCR). Polymorphisms have been described in a gene that alters the morphology of papillomavirus transformed cells (p1596); and the immunoglobulin Fc receptor, which can also serve as a receptor for HIV. The PCR has been employed to amplify specific regions of genes, such as the introns or the 3′ untranslated region. The resulting DNA products are then assayed for variation by one of four approaches: (1) digestion with frequently cutting (4 base pair [bp] recognition site) enzymes; (2) analysis of simple sequence (microsatellite) loci on high resolution gels; (3) single-stranded conformation polymorphism (SSCP) analysis, a method for detecting single bp changes in DNA; or (4) heteroduplex analysis, a method developed by this project for detecting sequence variation. Using these approaches we have been able to identify and characterize polymorphisms in several different genes including the KIT oncogene, the insulin-like growth factor receptor 1, the dopamine D2 receptor, and the gamma aminobutyric acid receptor. All of these polymorphisms have been typed in the 40 large human pedigrees provided by the Centre D'Etude du Polymorphisme Humain for the construction of human genetic linkage maps. These methods should allow virtually any human gene to be developed as a genetic marker, and should greatly increase the possibility for understanding complex human disorders with at least a partial genetic basis.

Project Description

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

Stephen J. O'Brien	Chief	LVC	NCI
Raleigh Boaze	Biological Laboratory Technician	LVC	NCI
Marga Belle White	Special Volunteer	LVC	NCI

Objectives:

For many, if not all, human diseases the genetic characteristics of the individual play a role in the manifestation of the disorder. Even for diseases such as cancer and heart disease that are not strictly inherited, there are forms of the disease that are heritable or show evidence of the presence of susceptibility genes. Furthermore many diseases show distinct differences in frequency among different racial, ethnic, or geographic populations. The ability to separate the inherited component of complex human diseases has been hampered by the inability to detect variation in coding genes. The objective of this project is to develop and test methods that will allow virtually any human gene to be employed as a genetic marker, thereby allowing specific hypotheses to be tested in affected individuals and/or population disease cohorts.

Methods Employed:

1. Technical methods. The following techniques were employed: (1) detection and resolution of human restriction fragment length polymorphisms (RFLPs), using human DNA clones; (2) detection of polymorphism in defined gene segments using frequently cutting restriction enzymes; (3) analysis of microsatellite polymorphisms on high resolution gels; and (4) use of single-stranded conformation polymorphism (SSCP) analysis and heteroduplex analysis to detect variation in non-coding segments of genes.

2. Strategy. Approximately one in every 500 nucleotides in the human genome is estimated to be polymorphic. Standard RFLP analysis employs restriction enzymes with 5 and 6 base pair (bp) recognition sites, allowing only a fraction of the potentially variable sites to be assayed. Kreitman and Aguade showed in *Drosophila* that by employing multiple restriction enzymes with 4 bp recognition sites that allowed virtually all polymorphic sites to be identified, they could find at least 10 times more variable loci than with standard methods. This project represents an attempt to identify and develop methods that will allow similar approaches to be applied to human genes. To accomplish this we have begun amplifying non-coding segments of biologically important genes and assaying for variation using high resolution methods. We have focused on 3′ untranslated regions because these segments are typically at least 300 bp in length, are almost interrupted by introns, and the sequence is typically determined when the cDNA sequence of the gene is reported. For some genes the position and size of the introns are known, allowing these segments to be amplified as well. To detect variation we have employed a variety of strategies that include the characterization of dinucleotide and

tetranucleotide repeat loci, digestion of the polymerase chain reaction (PCR) product with frequently cutting (4 bp) restriction enzymes, and the detection of single bp changes by high resolution techniques.

Major Findings:

1. Detection of new polymorphisms. We have continued to identify polymorphisms in biologically important genes using standard Southern blotting methods. Table 1 shows the list of RFLPs described to date. The newly described RFLPs are in p1596, a gene that alters the morphology of human papillomavirus transformed cells, and the immunoglobulin gamma Fc receptor (FCRIII), an alternative receptor for HIV. Most of the RFLPs in Table 1 have been typed in the 40 large pedigrees of the Centre D'Etude du Polymorphisme Humain (CEPH) collection. This has allowed these genes to be placed on the genetic maps of the respective chromosomes.

In order to speed the collection of data by Southern blots we have employed an Accuflex robotic pipetter for the loading of agarose gels. The robot is capable of loading individual digested DNA samples from a microtiter tray onto agarose gels. We find that this increases both the speed and accuracy of this part of the process considerably.

2. Detection of RFLPs using the PCR. We are employing PCR to rapidly detect polymorphisms in specific genes. Table 2 shows the loci that are currently available for analysis. These RFLPs are detected in the introns or 3' untranslated regions of their respective genes. Many of these polymorphisms are detected by the presence or absence of specific restriction sites. Other polymorphisms can be detected by assaying for SSCP. SSCPs are detected on non-denaturing acrylamide gels, using denatured, radiolabelled PCR product. The technique is highly sensitive to the detection of nucleotide sequence variation. We have found that the D-5000 matrix from AT Biochem often produces a greater mobility shift than standard acrylamide.

A newly described class of polymorphisms is the microsatellite or simple sequence tandem repeat. These loci are composed of tandem copies of 2, 3, or 4 nucleotides. The number of repeat units can vary, creating a polymorphic system. Microsatellite loci are abundant and highly polymorphic, making them excellent genetic markers. We have begun employing CA/GT repeats in the tumor necrosis factor gene, and serotonin 1A receptor gene loci. The alleles are detected by resolving labelled PCR DNA on standard acrylamide sequencing gels. In addition, we have identified and characterized a tetranucleotide repeat locus in an intron of the gamma-aminobutyric acid receptor beta 1 gene on chromosome 4. This locus contains at least seven alleles and is highly informative. To permit further identification of such loci, we have synthesized a series of oligonucleotides corresponding to all of the known tri- and tetranucleotide repeat families. These oligonucleotides can be used to screen for microsatellites in genomic clones.

In order to permit the rapid typing of PCR-derived polymorphisms, we have begun to streamline and automate the generation of PCR products. We have programmed the Biomek 1000 to assemble PCR reactions in microtiter trays. The robot can dispense DNA, PCR cocktail and mineral oil into the trays, which can

be subsequently amplified. We are currently using a Biotherm oven to perform the PCR reactions. The oven is capable of simultaneously amplifying 3 trays, for a total of 288 samples. This strategy should enable us to efficiently generate data for PCR polymorphisms in our study group samples.

3. Development of a new method for detecting genetic variation: Heteroduplex polymorphisms. During PCR amplification of a sample that is heterozygous for a mutation or polymorphism, heteroduplex products are formed between the homologous strands of the two different alleles. These heteroduplex molecules contain a region of mismatch, and thus a segment of non-bp DNA. We have observed that such molecules often run aberrantly on acrylamide gels, and have tested the assay for the detection of point mutations. By mixing samples from several known alleles that differ at a single base, we were able to show that eight out of nine could be detected by this method. The heteroduplex method is very simple and can be applied to the same samples used for SSCP analysis. We are currently testing a strategy for mutation detection that combines the heteroduplex assay and SSCP.

Publications:

Bolos AM, Dean M, Lucas-Derse S, Ramsburg M, Brown GL, Goldman D. Population and pedigree studies reveal a lack of association between the dopamine D_2 receptor gene and alcoholism. JAMA 1990;264:3156-60.

Dean M, Gerrard B. Helpful hints for the detection of single-stranded conformation polymorphisms. BioTechniques 1991;10:332-3.

Dean M, Stewart C, Perry A, Stauffer D, Otterud B, White R, Leppert M. D7S448 detects a Hind III polymorphism located in the centromere region of chromosome 7. Nucleic Acids Res 1991;19:200.

Dean M, Stewart C, Perry A, Stauffer D, Otterud B, White R, Leppert M. D7S449 detects a Hind III polymorphism tightly linked to the MET gene on chromosome 7. Nucleic Acids Res 1991;18:7199.

Frolova EI, Dolganov GM, Mazo IA, Smirnov DV, Copeland P, Stewart C, O'Brien SJ, Dean M. Linkage mapping of the human CSF2 and IL3 genes. Proc Natl Acad Sci USA (In Press).

Poduslo SE, Dean M, Kolch U, O'Brien SJ. Detecting high-resolution polymorphisms in human coding loci by combining PCR and single-strand conformation polymorphism (SSCP) analysis. Am J Hum Genet (In Press).

Table 1

RFLPS CHARACTERIZED IN THE HUMAN GENETIC ANALYSIS LABORATORY

GENE	LOCATION	ENZYME	ALLELES	SIZE	FREQ.
CD3Z	1q22-25	Hinf I	>5 VNTR	5.5-6.5	
JUN	1p32-31	Kpn	1	10.5	.50
			2	9.0	.50
IL1A	2q13-21	Taq I	>5 VNTR	5.5-6.0	
		Taq I	1	9.0	.70
			2	7.0	.30
RAF1	3p24-25	Eco RI	1	15.0	.87
			2	12.0	.13
IL 8	4q13-21	Hind III	1	20.0	.60
			2	4.5	.40
ADH3	4q21-q23	Sac I	1	7.3	.83
			2	6.1	.17
IL-3/CSF2	5q23-31	Pst I	1	4.0	.75
			2	2.5	.25
		Bgl II	1	18.0	.88
			2	4.5	.12
I-20	7	Msp I	1	8.5	.83
			2	8.0	.17
D7S448	7cen	Hind III	1	6.0	.45
			2	5.8	.55
I-21	7q	Msp I	1	9.0	.72
			2	8.0	.21
D7S448	7cen	Hind III	1	6.0	.45
			2	3.3	.13
D7S426	7q31	Eco RV	1	12.0	.40
			2	9.5	.60
		Pvu II	1	9.0	.39
			2	6.0	.61
D7S424	7q31	Sac I	1	15.0	.39
			2	6.0	.61
CALB	8	Sac I	1	18.0	.62
			2	14.0	.38
OVC	9p	Pst I	1	6.6	.44
			2	5.0	.56
		Rsa I	1	1.7	.43
			2	1.2	.57
CD4	12pter -p12	Taq I	1	7.5	.80
			2	7.0	.20
RAR	17q21.1	Pst I	1	3.0	.18
			2	2.6	.82
RAFB		Msp I	1	12.0	.05
			1	9.0	.95
		Eco RV	1	7.0	.75
			2	3.5	.25

Table 2

POLYMORPHISMS DETECTED BY DIRECT AMPLIFICATION

	LOCATION	ENZYME	ALLELES	HETEROZYGOSITY	CEPH
KIT	4p11-q22	Hae III	2	28%	Y
GABRB1	4p13-12	(GATA)	7	48%	Y
TNF	6p21	(CA)	>13	>80%	N
CFTR	7q31	(CA)	4	>40%	N
CFTR	7q31	(GATT)	2	30%	N
D7S432	7q31	ins/del	2	15%	N
D7S433	7q31	(CA)	>6	>50%	N
DRD2	11q22-23	(SSCP)	2	47%	Y
IGFIR	15q25-ter	Sau 96	2	44%	Y

ENZYME refers to the restriction enzyme that detects the polymorphism; ins/del, insertion/deletion polymorphism; and (N) indicates the nucleotide repeat for microsatellite loci. CEPH indicates whether the polymorphism has been typed in all informative members of 40 CEPH pedigrees, and linked to adjacent markers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05679-01 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Identification of Human Genetic Loci Which Influence Susceptibility to HIV

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:	Raleigh Boaze	Biological Laboratory Technician	LVC	NCI
	James J. Goedert	Coordinator, AIDS Working Group	EEB	NCI
	Dean Mann	Chief, Immunogenetics Section	LVC	NCI
	William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
	J. Claiborne Stephens	Senior Staff Fellow	LVC	NCI

COOPERATING UNITS *(if any)*

LCS, NIAAA, Bethesda, MD (D. Goldman); City Clinic Annex, S.F., CA (G. Rutherford); Dept. of Statistics, NC State U., Raleigh, NC (B. Weir); Children's Hospital of Los Angeles, Div. of Pediatric Surgery, Los Angeles, CA (E. Gomperts); PRI/DynCorp, Frederick, MD (M. Dean, C. Winkler, M. Carrington)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	0.6	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.)*

Outcomes to both exposure to and infection by many viruses are highly variable in human populations. While the differential response may result from genetic differences in the virus, a possible interpretation of the epidemiological data is that there are genetic differences in the host population which influence both susceptibility to infection and disease outcomes following viral infection. The primary objective of this study is to identify those host genetic factors that regulate virus infection, disease progression, immune response and reactivity to therapeutics. We have established collaborations with well-defined epidemiologic disease cohorts at risk for AIDS and for Hepatitis B. Immortal B-cell lines from all members of the cohort were established and genomic DNA is screened using restriction fragment length polymorphism (RFLP) methodology (See Project #Z01CP05678-01 LVC). Distortion of allelic, genotypic or linkage equilibrium of linked human loci in clinically defined disease categories provides the signal to discover disease susceptibility/resistance loci in human populations. Approximately 250 RFLPs in both "candidate" genes and equivalently spaced anonymous RFLP markers are used in the screenings. A networked data base to monitor cell and DNA inventory, molecular reagents, accumulated data and genetic analysis has been established. Putative genetic disequilibrium in the two cohorts of homosexual men are being affirmed.

Project Description

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

Stephen J. O'Brien	Chief	LVC	NCI
Raleigh Boaze	Biological Laboratory Technician	LVC	NCI
James J. Goedert	Coordinator, AIDS Working Group	EEB	NCI
Dean Mann	Chief, Immunogenetics Section	LVC	NCI
William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
J. Claiborne Stephens	Senior Staff Fellow	LVC	NCI

Objectives:

A striking feature of human viral infections is the heterogeneity of host response to exposure, infection, and disease progression. While this differential response may depend on genetic differences in the viral pathogen, or the rate of undiscovered cofactors, a possible explanation is that there are genetic differences in human populations which regulate susceptibility or resistance to viral pathogens. The objective of this project is to identify those genetic factors which are operative in differential host response to two pathological human viruses, HIV and hepatitis B virus (HBV), using a combination of molecular biology, population genetic principles, and epidemiology.

Methods Employed:

1. Technical methods. The following techniques were employed: (1) immortalization of B-lymphocytes from fresh blood or from cryopreserved peripheral blood leukocytes (PBLs) by transformation with Epstein-Barr virus (EBV); (2) immunological assays (ELISA, western blot) for the detection of anti-HIV antibodies and HIV antigens in lymphocytes and sera or lymphoblastoid cell lines (LCL) and their supernatants; (3) resolution of established human restriction fragment length polymorphisms (RFLPs) using human DNA clones; (4) detection of genetic variation by polymerase chain reaction of gene segments; (5) electrophoretic resolution of polymorphic protein markers resolved using allozymes or two-dimensional gel electrophoresis (2DE); and (6) population genetic methods to detect distortion in genetic equilibria in natural populations.

2. Strategy. By analogy to murine and feline retroviral diseases, there are conceivably numerous genetic loci which can influence (positively or negatively) disease susceptibility. Human genetic loci which impact on AIDS sensitivity are likely to play a role in infectivity and disease progression. This project represents an attempt to use a combination of available human genetic technology, population genetic principles, and epidemiology to identify such genes in man. The strategy follows: the human gene map has grown in the last decade to include over 7,000 loci. Over 3,000 are defined by molecular clones and over 1,500 of these genes are polymorphic for RFLPs. In addition, nearly 50 polymorphic loci for proteins (resolved by isozyme and 2DE gels) have been reported. Thus, it is possible to take the human gene map

and to identify abundantly polymorphic loci at an average of 5- to 10-centiMorgan (cM) units along every chromosome (from 1 to X). The project involves collection of blood from AIDS or HBV cohorts in several locales with large numbers of patients in different clinical disease categories (e.g., HIV antibody-positive asymptomatic vs. AIDS patients). Patient B-cells are transformed with EBV and expanded for DNA extraction. Distortion of three population genetic parameters of polymorphic loci (allelic frequency, genotypic [Hardy-Weinberg] equilibrium, and linkage equilibria of paired loci) are interpreted as a signal for the occurrence of genes which impact on a patient's appearance in a particular clinical disease category. The gene markers include polymorphic DNA segments, allozymes, and 2DE variants. Included in this panel are clones of candidate loci (e.g., lymphokines, growth factors, receptors, genes of the immmune system, oncogenes). We are taking advantage of the thorough serological workups on AIDS patients by also noting exposure, symptoms, and presence of viruses for other diseases, such as cytomegalovirus, HTLV-I, HBV, and herpesvirus. The wealth of epidemiology background is being considered throughout in defining new disease categories for gene identification.

Major Findings:

1. Disease cohorts. Collaborations have been established with centers having well-characterized cohorts of HIV-exposed or -infected subjects. Specimens have been received from five homosexual cohorts or case-control study groups and two major hemophiliac cohorts described in Table 1. A pilot case-control study in collaboration with the Multicenter AIDS Cohort (MAC) group to determine genetic control of outcomes to HIV infection (Kaposi's sarcoma, pneumocystis carnii pneumonia, or no symptoms) is near completion. This study is to be extended to include an additional 1,000 seropositive individuals who have been HIV-infected for greater than 3 years. We are continuing a study of nearly 1,200 hemophiliacs with Drs. Goedert and Blattner of the NCI. In addition, we have recently formalized a collaboration with the Multicenter Hemophilia Growth and Development Study to study the relationships between genetics and HIV infection in child growth and development. These hemophiliac cohorts are useful in that in many cases HIV exposure from contaminated clotting factor lots can be documented.

The hepatitis study in collaboration with Drs. Palmer Beasley and Lu-Yu Hwang is a prospective, case-control study to determine the genetic contribution to differential responses to HBV vaccination, infection, chronic carrier status, and progression to hepatocellular carcinoma. We are continuing to receive cryopreserved PBLs from genetically unrelated cases and matched controls.

2. Cell transformation. While PBLs from most HIV-infected homosexual men readily transform using standard protocols, it is more difficult to establish LCLs from cryopreserved PBLs collected from HIV-infected and uninfected hemophiliacs. We are developing protocols to maximize the transformation efficiency of cryopreserved PBLs from hemophiliacs by comparing the efficiency of lymphoblastoid establishment using cyclosporine A or a monoclonal antibody against OKT3 to suppress the generation of EBV-specific cytotoxic T-cell clones.

3. Collection of human RFLP probes. To date we have collected 550 clones which detect human DNA polymorphisms. Each chromosome is represented by at least 12 probes, and the collection covers over 90% of the human genome at a resolution of 10 cM. All of these clones have been grown in bulk and are stored in duplicate as both a glycerol stock of transformed bacteria and as DNA. We have tested 260 of these clones, confirming their detection of RFLPs. We are detecting new polymorphisms using a variety of strategies such as single-stranded conformation polymorphisms, the presence or absence of restriction sites in 3' untranslated regions of specific genes, and variable number repeat polymorphisms in microsatellite or tandem repeat DNA (See Project #Z01CP05678-01 LVC).

4. Imformatics. We have developed a five-part computer data base system supported by a Novell Network consisting of 14 workstations. Inventory programs have been developed to track patient specimens from receipt through the development of cell lines. Inventories are also maintained for DNA probes with information on chromosome location, structure of the construct, and the frequency and size of the allele detected by the probe. HLA typings and the results of the fluorescein activated cell sorter are processed through the microcomputer network system and managed in SAS data bases at the Division of Computer Research and Technology (DCRT) at NIH. Genotypic data from the RFLP analysis is entered and processed on DCRT's mainframe using SAS and customized Fortran programs. Clinical data from collaborating centers are maintained in SAS data bases and interface with the network system. Updated data bases for all human cloned genes and RFLP probes are maintained by the Howard Hughes Medical Institute for each patient and are accessible by modem. The VAX computer system at the Frederick Cancer Research and Development Center has been utilized for Centre D' Etude du Polymorphism Humain-Human Polymorphism Study Center analysis.

5. Data analysis. Genotypic data from 465 homosexual men from the MAC and the DCG cohorts have been analyzed using computer algorithms to determine allele frequencies, Hardy-Weinberg equilibria, and linkage disequilibrium in different disease/progression groups. Statistically significant departures from expected population genetic parameters between different disease/categories groups, are being reanalyzed by confirmation in independent cohorts and using additional RFLP markers in the neighboring regions.

Publications:

Gardner MB, Kozak C, O'Brien SJ. The Lake Casitas wild mouse: evolving genetic resistance to retroviral disease. Trends Genet 1991;7:22-7.

Goldman D, O'Brien SJ. Comparative studies of proteins employing two dimensional electrophoresis. Methods Enzymol (In Press).

O'Brien SJ. Molecular evolution: plagues and genetic resistance. Curr Biol (In Press).

Table 1: Study Groups
Status - May 2, 1991

Location	Risk Group	No.	LCL Established	RFLP Genotype data	HLA	Collaborators
New Jersey/ Washington, DC	Homosexual					
	HIV (-)	134	117	111	127	J. Goedert
	HIV (+)	252	148	134	232	
	AIDS	165	69	61	151	W. Blattner
	KS	80	32	25	76	(NCI)
	PCP	63	29	27	53	
	OI	54	18	17	49	
	Lymphoma	12	18	6	9	
	---TOTAL---	386	265	245	359	
Multicenter	Homosexual					
	HIV (-)	0	0	0	0	J. Farr
	HIV (+)	324	243	215	254	R. Peters
	AIDS	184	125	114	146	C. Rinaldo
	KS	86	66	62	74	A. Saah
	PCP	125	78	72	95	A. Munoz
	OI	29	53	49	63	(Multicenter AIDS
	Lymphoma	9	7	6	6	Cohort Study)
	---TOTAL---	324	243	215	254	
Multicenter	Hemophiliac					
	HIV (-)	1003	139	64	445	J. Goedert
	HIV (+)	1069	95	53	554	(NCI)
	AIDS	167	8	6	64	Multicenter
	KS	2	0	0	2	
	PCP	108	6	5	38	
	OI	77	3	2	30	
	Lymphoma	7	0	0	2	
	---TOTAL---	2072	234	117	999	

Location	Risk Group	No.	LCL Established	Genotype data	HLA	Collaborators
San Francisco	Homosexual					G. Rutherford
	HIV (-)	25	17	19	20	
	HIV (+)	58	31	46	52	
	AIDS	15	8	12	15	
	---TOTAL---	83	48	65	72	
New Jersey	Drug Users					J. Goedert
	HIV (-)	87	71	68	0	
	HIV (+)	49	36	37	0	
	AIDS	0	0	0	0	
	---TOTAL---	136	107	105	0	
Multicenter	Hemophiliac					E. Gomperts
	HIV (+)	190		0	---	
	HIV (-)	108		0	---	
	Not tested	14	16	0	---	
	--TOTAL--	312		0	89	

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05680-01 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Progress Towards Mapping the Human Genome

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

PI:	J. Claiborne Stephens	Senior Staff Fellow	LVC	NCI
Others:	Stephen J. O'Brien	Chief	LVC	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	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.)*

Much of the data collected by the Human Gene Mapping Library (New Haven, CT) is relevant to the current status of the human gene map. However, there are many types of maps of human genetic information, and there is no universal coordinate system for comparison or quantification of the various mapping efforts. Furthermore, the goals set forth by the various agencies coordinating the Human Genome Project (HGP) have not been translated into targets that can be quantified. We developed a means of quantifying progress toward a complete, integrated map of the human genome. Current estimates of the total number of loci, the number of base pairs, and the relative lengths of chromosomes and their bands are very crude. The algorithms employed in our quantitative analysis were designed to be flexible for anticipated improvements in each of these parameters. Analyses of the current status of the HGP were summarized graphically as a wall chart and in more detail as an article in Science.

Project Description

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

J. Claiborne Stephens	Senior Staff Fellow	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI

Objectives:

The extensive research embodied by the Human Genome Project (HGP) will produce orders of magnitude more data than is currently contained in existing human genomic maps. It can also be anticipated that novel types of maps will arise, reflecting the different methodologies and interests that will almost certainly come out of the HGP. Given the potential for chaos, and the current proliferation of various types of maps, it is appropriate to search for commonalities among the different mapping efforts and their data representations that will allow integrated, quantitative summaries of the mapping effort at any point in time. In particular, quantification of the mapping activities should highlight regional variation throughout the genome, as well as the relative intensity of the various mapping efforts. For some types of maps, completeness can be defined, and in such cases, it is valuable to monitor each map's progress toward completion.

Methods Employed:

Data included in the study came from the databases of the Human Gene Mapping Library (HGML--New Haven, CT), the GenBank sequence database, and a large number of published linkage maps. The data maintained by the HGML included all loci and map locations from the Tenth International Human Gene Mapping Workshop (HGM10) and subsequent updates based upon articles published or in press through July 31, 1990. On this date, the HGML database contained relevant information for 6,652 loci (genes and anonymous DNA segments); 11,852 probes; 2,275 polymorphic loci; and links to GenBank records with 5,066,049 base-pairs of DNA sequence. Statistical methods of allocating this information to individual chromosome bands were developed as part of this project. Care was taken so that the methodology developed could be generalized to alternative coordinate systems, such as those anticipated to arise from molecularly-based physical maps.

Major Findings:

1. Construction of a conceptual framework for quantitative analysis of mapping. Most current mapping activities have maps that are based on intervals. For instance, cytogenetic maps give map locations that consist of one or more chromosome bands, and DNA sequences are given as lengths of base-pairs. Even linkage maps have an inherent uncertainty in the genetic distance between consecutive loci, so that each locus is in fact an interval. This common structure of genomic maps is largely due to uncertainty in current mapping methods. For example, most genes are smaller than the visible chromosome bands so that one could hope to pinpoint the map location of each

gene to a single band. However, at a microscopic scale, each gene is still, in fact, an interval. Since restriction sites, arbitrary clones, and other fragments of DNA are at different intervals that may overlap gene boundaries, any coordinate system attempting to accommodate these different types of mapping data simultaneously will be required to allow representation of all these data as intervals. Currently, the cytogenetic bands provide an internationally recognized coordinate system (860 bands at high resolution) that can accommodate a quantitative analysis of multiple types of mapping data. Ultimately the DNA sequence itself will provide a numerical coordinate system, although length polymorphism is a serious issue. Other coordinate systems in between these two extremes of scale will probably see widespread use (those based on molecular landmarks such as contigs, restriction site maps, or high resolution in situ hybridization), but it will not be a trivial task to convert representations of the different data types among these different coordinate systems. We have made some progress in coordinating linkage maps (centiMorgan [cM] scale and coordinates) and DNA sequences (base-pairs) with the cytogenetic coordinate system, but there are no universal conversion factors and very few common reference points.

2. Coordination of genetic linkage maps with cytogenetic maps. Although more than 2,200 polymorphisms have been characterized in the human genome, none of the three comprehensive linkage maps in existence has more than 600 loci represented. Each of the polymorphic loci has a known cytogenetic map location, which allows the coordination of all polymorphic loci simultaneously, even though this cannot yet be achieved on the linkage map. With this representation we identified loci whose order was especially well known, and charted the distribution of polymorphic loci relative to the identified loci. Since polymorphism is essential for the construction of linkage maps, this analysis reveals, to a first approximation, areas of the genome in which polymorphic markers are particularly dense, and hence could be incorporated into existing linkage maps to improve resolution. Conversely, the analysis reveals genomic regions from which additional polymorphic markers need to be identified.

3. Coordination of DNA sequences with cytogenetic maps. Records of DNA sequence data maintained by GenBank will often contain enough information to identify correspondence between the sequence record and a locus record maintained by HGML. In a collaborative effort with GenBank, corresponding entries were identified for 772 genes, enabling the coordination of over 5 million base-pairs of DNA sequence with the cytogenetic map. This represented over 80% of the available human DNA sequence data in GenBank at the time of our study.

4. Relative progress of mapping on a region-by-region basis. An algorithm for allocating mapping data (expressed as intervals) to arbitrary coordinate systems was developed to quantify the regional variability of each mapping activity. The algorithm was applied to six different types of mapping data--all loci, genes, sequenced genes, DNA base-pairs, probes, and polymorphic loci--and to the coordinate system given by the high resolution depiction of chromosomal bands in the International System for Human Cytogenetic Nomenclature (ISCN 1985). Although the actual lengths in this coordinate system are only poorly known, the ISCN depiction is an internationally

recognized standard with which any researcher can unambiguously indicate mapping coordinates for a given locus.

The logic of our allocation algorithm was to divide the map location of each locus into its fundamental intervals--the actual band lengths -- and allocate that locus proportionately. Thus, a locus mapped to chromosome 1 without regional localization has some probability of being in any band on the chromosome, so each band receives a proportional share of the locus. On the other hand, a locus mapped to a single band is allocated exclusively to that band. In this manner, the total number of loci allocated to each band is the sum of the proportional allocations from each locus.

As expected, the resulting analysis showed a great deal of variation by region for the various types of mapping activities. All types of activities were perceivably coordinated, as one might expect if interesting regions (e.g., immune function clusters, hemoglobins, cystic fibrosis) are dictating the research rather than brute force mapping of specific chromosomes.

5. Estimation of progress toward completion. The complete DNA sequence of one haploid human genome is a relatively straightforward goal of the HGP. Even so, there are caveats--the sequence will be composite, there will be many sites of polymorphism, and this reference sequence may contain many gaps (deletions) relative to other more common sequences. For many other types of mapping activity there may be no clear definition of "completeness." We have used a working definition of completeness of the cytogenetic map of genes as the identification of gene content of each chromosomal band. This definition is equivalent to the identification of all genes and their precise chromosomal map localizations. This definition leads to a quantitative method for assessing the completeness of the cytogenetic map--the ratio of the sum of the current allocations to the sum expected at completion. To calculate the latter, we can use an estimate of the number of genes in the human genome (100,000) and can approximate their distribution by assuming that genes are uniformly distributed in accordance with band length. The latter makes the same assumption made in the allocation algorithm, that is, genes are distributed proportionately by band length. The estimate of completion has two facets: it increases as genes are added to the map, and it increases as each gene's map localization is refined. The algorithm was used at three distinct levels: (1) globally, to provide the estimate that the gene map is about 0.52% complete; (2) for each chromosome, giving a range of 1.28% (chromosome 22) to 0.19% complete (chromosome 15); and (3) for each band, which suggests that bands 14q32.33 (immunoglobulin heavy chain cluster), 6p21.3 (HLA), and 11p15.5 (beta-hemoglobin cluster, insulin, Harvey-ras oncogene) are among the leaders in complete identification of gene content. These same bands are the highest in terms of DNA sequence completion (2.6%-3.5%). The algorithm as applied to completion of bands also suggests that many bands on chromosome 13 are largely unknown as far as their gene content. A locus-by-locus analysis of completion identified 170 genes, 72 fragile sites, and 340 anonymous DNA segments that had been mapped to a single chromosome band--the highest possible resolution under the cytogenetic coordinate system.

It is probable that several million nucleotide sites are polymorphic, which makes a comprehensive characterization of all of these as a "complete" linkage map unlikely. However, with a target goal of linkage map intervals being no more than 10 cM, we can define completion as the fraction of the current linkage map that is in intervals 10 cM or shorter. Using the linkage map from HGM10, we estimate 58.3% as the fraction in intervals $\leq$ 10 cM, whereas 29.2% is in intervals $\leq$ 5 cM and 5.7% is in intervals $\leq$ 2 cM.

6. Genomic organization. Two proposed hypotheses about biological differences between light and dark chromosome bands may be tested with our data. The proposition that functional genes tend to be clustered in the Giemsa light bands was tested by examining the distribution of genes. Over 59% of all genes were in fact in light bands, although the 99% confidence interval based on relative band lengths was only 48.1%-54.2%. Further support for this hypothesis comes from direct inspection of genes mapped to a single band; 115 (68%) of the genes mapped to a single band were mapped to light bands. Curiously, 89% of fragile sites and 78% of the anonymous DNA segments mapped to single bands were also in light bands, which suggests that there may be additional biases involved.

A second hypothesis states that the content of guanine and cytosine (GC) nucleotides of light bands is higher than that of dark bands. Our compilation of DNA sequence data mapped to single bands is relevant to this proposition. Although there is a slight trend in this direction (52% GC in light bands, 51% GC in dark bands); the considerable variation among bands of each type (43%-61% for 16 light bands, 40%-59% for 18 dark bands) suggests no such class distinction between light and dark bands.

Publications:

Stephens JC, Cavanaugh ML, Gradie MI, Mador ML, Kidd KK. Mapping the human genome: current status. Science 1990;250:237-44.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05681-01 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Estimation of Heterozygosity for Single Probe, Multilocus DNA Fingerprints

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

PI:	J. Claiborne Stephens	Senior Staff Fellow	LVC	NCI
Others:	Stephen J. O'Brien	Chief	LVC	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	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.)*

One of the most useful parameters that can be derived from population genetic data is the average heterozygosity (H), which has traditionally been employed to contrast levels of genetic variation among populations and to compare genetic variation among different loci. In spite of the increasing application of DNA fingerprinting to natural populations and to the genetic identification of humans, explicit methods for estimation of H from DNA fingerprinting data have not been developed. Contributing to this omission is the inability to determine relatively important genetic information for multilocus fingerprinting probes, such as the number of loci, the number of alleles, and the distribution of these alleles into specific loci. We have derived explicit calculations for the expected average heterozygosity and a maximum value for this estimate. These estimates are based upon the DNA restriction pattern matrices that are typical for fingerprinting studies of humans and natural populations. For several empirical datasets from our laboratory, these estimates are shown to be relatively close to each other. This observation, and consideration of the effect of missing alleles and alternate numbers of loci, suggest that the average heterozygosity can be accurately approximated using phenotypic DNA fingerprint patterns because this parameter is relatively insensitive to the lack of certain genetic information.

Project Description

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

J. Claiborne Stephens	Senior Staff Fellow	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI

Objectives:

DNA fingerprinting is ideal, at least theoretically, for a number of questions in population genetics or individual identification. This enormous potential is due to the ability to simultaneously screen genetic variability at multiple independent genomic loci with a single probe. Standard techniques of restriction enzyme digestion of genomic DNA and Southern blotting are used with such probes to produce restriction patterns that are largely individual-specific; hence, the analogy to fingerprinting. Characterization of this variation in conventional population genetic terms (polymorphism, heterozygosity) is usually not attempted because of a lack of understanding of the underlying genetics, such as the number of alleles and loci involved in a fingerprint. Instead, focus has been on phenotypic metrics (e.g., average band sharing or average band differences). We developed estimates of genetic polymorphism and heterozygosity that can be derived directly from conventional DNA fingerprinting data without requiring knowledge of the underlying genetic parameters.

Methods Employed:

Standard population genetic considerations allow us to: (1) estimate allele frequencies for each allele scored on a gel, (2) use these estimates to estimate the number of loci analyzed on a gel, (3) use the estimates of allele frequencies and number of loci to estimate average heterozygosity per locus and the percentage of polymorphic loci, and (4) derive a maximum estimate of average heterozygosity given the number of alleles and the number of monomorphic loci observed on a gel.

Major Findings:

Heterozygosity estimates for several dozen DNA fingerprinting datasets from this lab yield values that are reasonably close to the maximal values estimated from the same datasets. This observation suggests that the lack of certain genetic information (distribution of alleles into loci, number of loci) is not critical to the estimation of average heterozygosity from DNA fingerprinting. Furthermore, estimates of average heterozygosity seem to be highly correlated with the conventional measure of DNA fingerprint diversity within a population, the average percent difference. Use of our average heterozygosity estimate should enable comparison of different DNA fingerprinting probes and of different populations of organisms and should also facilitate the comparison of DNA fingerprinting studies to other types of genetic variation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP005682-01 LVC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Genetic and Molecular Characterization of Nuclear Mitochondrial DNA in Felids

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

PI:	Naoya Yuhki	Visiting Associate	LVC	NCI
Others:	Stephen J. O'Brien	Chief	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary E. Eichelberger	Microbiologist	LVC	NCI
	Stanley Cevario	Microbiologist	LVC	NCI

COOPERATING UNITS *(if any)*

PRI/DynCorp, Frederick, MD (J. V. Lopez)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.2	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 recently discovered a family of chromosomal mtDNA sequences, in several but not all felid species, that have a high degree of sequence homology to a large portion (8 kilobases [kb]) of the extrachromosomal mitochondrial (mt) DNA. This nuclear mitochondrial (Numt) DNA has been studied using molecular techniques as an approach to determining the molecular aspects of gene transposition, amplification and the mtDNA evolution. Fractionation of cytoplasmic and nuclear DNAs, and further purification of supercoiled mtDNAs revealed that these mtDNA sequences are located in a nuclear fraction. Chromosomal mapping using hybrid cell panels indicates that Numt is located on feline chromosome D2. Restriction enzyme mapping analysis of the cloned Numt DNA sequence suggested that the tandemly repeated structure of 7.8-kb Numt DNA is a single unit. Pulse field gel electrophoresis of high molecular weight DNAs digested with three, six-cutter restriction enzymes revealed two consistent Numt DNA bands (280 and 320 kb). These results suggest that Numt DNA is clustered to a single locus as an mtDNA fragment tandemly amplified approximately 40 times.

Sequence analysis of the Numt 7.8-kb clone confirmed the existence of genes for 12S and 16S ribosomal RNAs, transfer RNAs of serine, aspartate, isoleucine, and cytochrome oxidase I and II (COI, II) with the same gene order as mtDNA. However, the COII gene sequence was connected with possible D-loop regions and 12S rRNA sequences. Thus, Numt DNA contains approximately one half of the genome of intact mtDNA. On the border between COII and possible D-loop regions (direct five-time repeats of the motif, ACACAGT), direct repeat sequences were found. This evidence may suggest the involvement of this repeat sequence in the occurrence of Numt in the domestic cat.

Project Description

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

Naoya Yuhki	Visiting Associate	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary E. Eichelberger	Microbiologist	LVC	NCI
Stanley Cevario	Microbiologist	LVC	NCI

Objectives:

Mitochondrial (mt) DNAs are found as extrachromosomal small closed circular DNAs (16.5 kilobases [kb]) in mammals which encode 2 ribosomal RNAs (rRNas), 22 transfer RNAs (tRNAs), and 13 enzymes that involve electron transport and adenosine triphosphate synthesis. We have used an mtDNA clone to analyze the population and evolutionary structure of feline species. During these experiments, we found unexpected mtDNA fragments that ranged from 7 to 8 kb by Southern blot analysis. Preliminary experiments showed that this sequence is localized in a chromosomal DNA fraction and exists as multiple copies based upon the intensity of the signals of Southern blot analysis. Similar nuclear mtDNA sequences have been observed in lower eukaryotes including yeast, fungus, and insects. However, precise mechanisms for mtDNA transposition are still unknown.

To determine the molecular aspects of gene transposition into the nucleus and gene amplification of mtDNA, we analyzed this sequence using several molecular techniques.

Methods Employed:

The following techniques were employed: (1) DNA cloning, (2) DNA sequencing using the dideoxy nucleotide sequencing method, (3) polymerase chain reaction (PCR), (4) pulse field gel (PFG) electrophoresis, and (5) Southern blot analysis.

Major Findings:

1. Identification of an mt-like sequence in the nucleus. A nuclear location for this "heretical" mtDNA or nuclear mitochondrial DNA (Numt DNA) was concluded from the following evidence. (1) Southern blot analysis using a full-length cat mtDNA clone as a probe demonstrated that the Numt DNA was absent in DNA preparations containing supercoiled cytoplasmic DNA but was present in nuclear DNA. (2) The Numt DNA was detectable in genomic DNAs derived from somatic hybrid cell clones that contain feline chromosomes in a mouse or hamster background, although cat mtDNA was absent in these hybrid cell clones. (3) Numt DNA was concordant with feline chromosome D2 in a panel of rodent x cat somatic cell hybrids.

2. Occurrence of Numt DNA sequences in two feline lineages (domestic cat and panthera). Southern blot analysis and comparison of the size of detectable mtDNA bands in 32 feline species revealed that mtDNA sequences appear to be transposed into the nucleus in several species of Felidae (domestic cat, Sand cat, European wild cat, tiger, lion, jaguar and leopard). These species can be classified into two evolutionary feline lineages: domestic cat and panthera.

3. Isolation of an Numt DNA clone. To isolate Numt DNA clones, 6-10-kb EcoRI- digested genomic DNAs were used to construct a genomic DNA library. Screening of this library using purified puma mtDNA yielded a 7.8-kb Numt DNA clone. Comparison of the restriction enzyme map of this clone with the mtDNA map of the domestic cat indicated significant divergence in the restriction patterns. A sequence comparison between Numt DNA and mtDNA is currently in progress. This will be determined by characterizing another clone, C12, containing a cytoplasmic 12-kb mtDNA fragment that is partially homologous to Numt DNA by Southern blot analysis.

4. Organization of Numt DNA. The arrangement of Numt DNA in the nuclear genome is also being investigated. A tandemly repeated array of the 7.8-kb unit was predicted according to the pattern of three restriction enzyme sites of this clone. Secondly, PFG electrophoresis was performed on one individual cat DNA digested with a 6-bp restriction enzyme that does not recognize sequences within the Numt DNA clone. This analysis is expected to release the full-length nuclear locus that is comparable in the hybridization intensity of the Southern blot to the cytoplasmic mtDNA signal represented in 100 to 1,000 copies. Hybridization with Numt DNA probes have revealed two fragments migrating at about 280 and 320 kb in two enzyme digests and slightly higher in a third enzyme digest. These bands are more than one magnitude higher than the average mtDNA size found in animals (16.5 kb), supporting predicted repetitive structure. Experiments to clone contiguous multiple copies of the Numt DNA locus and possible flanking genomic DNA into cosmid vectors are underway.

5. Sequence analysis of Numt DNA. DNA sequencing of the Numt DNA has revealed homology with several mt gene sequences, including the cytochrome oxidase II (COII) subunit that appears to be continuous between both extreme 5′ and 3′ termini of the 7.8-kb Numt clone. Other DNA sequences derived from Numt DNA have identified genes for 12S and 16S rRNAs, COI, and the tRNA genes for serine, aspartate, and isoleucine, and possibly the D-loop. The order of these genes in Numt DNA appears to be colinear with the cytoplasmic mtDNA gene organization. Sequences at the junction between COII and the D-loop region revealed direct five-time repeats of the motif, ACACACGT. The fusion of the COII gene to the D-loop indicates the deletion of about one half of the intact mtDNA, which conforms to the size of the 7.8-kb Numt DNA clone.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05683-01 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Structure, Function, and Mechanism of Lentivirus Tat Proteins

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

PI:	David Derse	Senior Staff Fellow	LVC	NCI
Others:	Richard Carroll	IRTA Fellow	LVC	NCI

COOPERATING UNITS *(if any)*: Howard Hughes Medical Institute, University of California, San Francisco, CA (B. M. Peterlin); PRI/DynCorp, Frederick, MD (L. Martarano)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Genetics Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	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 lentiviruses, equine infectious anemia virus (EIAV) and the human immunodeficiency virus (HIV), encode transcriptional trans-activating proteins (Tat) that bind to a specific hairpin structure at the 5′ end of nascent RNA (TAR) which positions them for interactions with the transcription apparatus. Thus, Tat proteins are predicted to contain an RNA-binding domain and an "activation" (or protein interaction) domain. The EIAV and HIV-1 Tat proteins (E-Tat and H-Tat, respectively) contain two conserved peptide motifs but are otherwise quite dissimilar and do not interact with the heterologous TAR element. We have addressed the problems of structure, function and mechanism of Tat proteins by exchanging domains between E-Tat and H-Tat and by tethering Tat sequences to an RNA operator via a bacteriophage RNA-binding protein. These strategies allowed us to define both the TAR-interaction and activation domains of each Tat protein. Both E-Tat and H-Tat appear to be simple, modular proteins in which the N-terminal half contains the activation domain and the C-terminal half is responsible for TAR binding. The activation domains of both proteins contain a highly conserved core sequence; the E-Tat activation domain consists of only 15 amino acids, whereas the H-Tat activation domain is more complex and is composed of 47 amino acids. The activation domains of the two proteins are interchangeable and competition experiments suggest that they interact with a common cellular factor. The TAR-binding domains of both proteins contain a cluster of basic amino acids; in H-Tat this sequence of about 10 residues is sufficient for RNA binding, while E-Tat requires 26 residues for TAR binding. In E-Tat, this region may be highly structured to form an RNA-binding pocket.

Project Description

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

David Derse	Senior Staff Fellow	LVC	NCI
Richard Carroll	IRTA Fellow	LVC	NCI

Objectives:

1. To construct and test chimeric transcriptional trans-activating proteins (Tat) by exchanging regions between equine infectious anemia virus (EIAV) and HIV-1 Tat.

2. To construct and test fusion proteins containing portions of EIAV and HIV-1 Tat proteins joined to bacteriophage MS2 coat protein. Construct EIAV and HIV-1 promoters in which the Tat-response element (TAR) is replaced with an MS2 RNA operator.

3. To examine the molecular mechanism of Tat trans-activation by analyzing trans-dominant inhibition.

4. To express Tat proteins in bacterial expression systems for purification and *in vitro* analyses.

Methods Employed:

The following methods were employed: (1) radio-immunoprecipitation and immunoblotting of proteins, (2) nucleotide sequencing, (3) recombinant plasmid construction, (4) site-directed mutagenesis, (5) transfection of mammalian cells, (6) RNA analysis and chloramphenicol acetyltransferase (CAT) assays, and (7) bacterial expression and purification of viral proteins.

Major Findings:

1. Identification and characterization of Tat functional domains. HIV-1 and EIAV Tat proteins (H-Tat and E-Tat, respectively) were altered by site-directed mutagenesis to introduce compatible restriction enzyme sites separating specific peptide motifs. These sites were then used to exchange regions between H-Tat and E-Tat; the resulting chimeric Tat genes were cloned into eukaryotic expression vectors and their activities were tested on HIV-1 and EIAV promoters joined to the bacterial CAT gene. Most of the chimeric proteins lacked the ability to effectively trans-activate either promoter, although the Tat proteins were detected by radio-immunoprecipitation. Several hybrid proteins were functional and showed specificity for only one of the promoters. Analysis of these proteins allowed us to deduce that the TAR-binding domain of E-Tat is located at the C-terminus of the protein, is composed of 26 amino acids and contains a cluster of basic residues. Deletion or addition of amino acids at the C-terminus of E-Tat reduced trans-activation, suggesting that the RNA-binding domain is highly structured. In contrast, the cluster of basic amino acids in a similar position in H-Tat

appears to be sufficient for TAR-binding. We were also able to deduce that the "activation" domain of H-Tat is composed of 47 residues at the N-terminus of the protein. The chimeric protein that contains the N-terminal 47 residues of H-Tat joined to the C-terminal 26 residues of E-Tat specifically activated the EIAV promoter. Because an E-Tat protein deleted of N-terminal sequences such that only 15 residues immediately N-terminal of the E-Tat RNA-recognition domain were functional, we concluded that this 15 amino acid "core" region constitutes the E-Tat activation domain.

The analysis of Tat activation domains was pursued further by constructing proteins in which the N-terminal sequences of either E-Tat or H-Tat were joined to the bacteriophage MS2 coat protein; thus, the coat protein replaced the native TAR-binding domains. Hybrid promoters were constructed in which the TAR elements in the EIAV and HIV-1 long terminal repeats (LTRs) were replaced with the MS2 operator to which the MS2 coat protein binds. Fusion proteins containing either the 15 residue or 47 residue N-terminal sequences of E-Tat or H-Tat, respectively, joined to the coat protein, trans-activated the hybrid LTRs. This unequivocally defines the activation domains of these Tat proteins.

2. Trans-dominant inhibition of Tat activity. The domain-exchange experiments suggested that the activation domains of E-Tat and H-Tat were interchangeable and perhaps interact with a common cellular factor. This possibility was tested by examining the ability of wild-type or chimeric Tat proteins to inhibit the activity of one another. For example, E-Tat was shown to inhibit H-Tat activation of the HIV-1 promoter by approximately 50% when present at a tenfold excess over H-Tat; the converse was also observed. Furthermore, both E-Tat and H-Tat were effective inhibitors of trans-activation mediated by either EIAV or HIV-1 Tat-coat protein chimeras. These experiments revealed that the activation domains of both Tat proteins interact with the same cellular protein and suggest that Tat may act to attract a specific transcription factor to the transcription complex.

3. Bacterial expression, purification and in vitro analysis of EIAV Tat. EIAV Tat has been expressed as a glutathione S-transferase fusion protein in bacteria. The bacterially expressed protein has been purified to near homogeneity as assessed by sodium dodecyl sulfate gel electrophoresis and silver staining. A rabbit antiserum has been raised against a synthetic peptide representing a portion of E-Tat, and reacts with the native and bacterially expressed proteins. The purified protein is being used to study Tat binding to RNA in vitro and to elucidate interactions of Tat with cellular proteins.

Publications:

Carroll RC, Martarano L, Derse D. Identification of lentivirus Tat functional domains through generation of equine infectious anemia virus/human immunodeficiency virus type 1 Tat gene chimeras. J Virol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05684-01 LVC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
c-Raf-1 is Required for AP-1/Ets-Dependent Transcription

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:	Joseph Bruder	IRTA Fellow	LVC	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	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.)*

To investigate the role of c-Raf-1 in serum and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) activation of transcription through AP-1 and Ets binding sites, we utilized a dominant negative mutant, c-Raf-301, which has a single amino acid substitution, ARG to TRP, in the c-Raf-1 ATP binding site. Unlike c-Raf-1 and regulatory domain deletion mutants, e.g., c-Raf-BXB, c-Raf-301 did not transactivate transcription from reporter constructs containing AP-1 or Ets binding sites. c-Raf-301 did not transform NIH/3T3 cells, and interestingly, when overexpressed, blocked cell growth stimulated by serum and TPA reverted v-Raf and Ras-transformed cells to the untransformed phenotype. We have now tested whether induction of AP-1/Ets mediated transcription depends on Raf-1 function. Overexpression of the dominant negative mutant, c-Raf-301, blocked basal levels of transcription in HepG2 cells. Furthermore, c-Raf-301 blocked serum and TPA stimulation of these reporter constructs in NIH/3T3 cells. These results indicate that endogenous c-Raf-1 is required for basal levels of transcription in HepG2 cells and induced transcription in NIH/3T3 cells.

To identify the minimal region required for the dominant negative effect, we generated a series of deletion mutants in c-Raf-1. Analysis of these mutants revealed that the N-terminal 1-256 amino acids of c-Raf-1 are sufficient for the inhibitory effect. This region of c-Raf-1 contains conserved region 1, the cysteine finger region. We have previously postulated that the N-terminus of Raf-1 may negatively regulate Raf-1 kinase activity by intermolecular interactions. The finding that the N-terminal domain is sufficient to inhibit Raf-1 function, as judged by activation of transcription, is consistent with this model. Alternatively, this region may be necessary for interactions with an upstream regulator of c-Raf-1. Current work is aimed at distinguishing between these mechanisms of Raf-1 activation.

Project Description

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

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Joseph T. Bruder	IRTA Fellow	LVC	NCI

Objectives:

The present objective is to determine the mechanism by which the c-Raf-301 dominant negative mutant functions. We have determined that the N-terminal region was sufficient to confer the inhibitory effect on transcription. This region contains a cysteine finger motif which may function to negatively regulate Raf-1 kinase activity. The present aim of this study is to determine if the cysteine finger motif is essential for the inhibitory effect on transcription. Single amino acid changes in the cysteine finger domain in the background of the c-Raf-301 or the minimal blocking N-terminal domain mutant will be useful in determining the importance of this domain in transcriptional activation. The N-terminal region will be used to search for the ligand with which it interacts. Transfection of cells with a plasmid expressing the N-terminal region of c-Raf-1, and subsequent immunoprecipitation with specific antisera, will enable us to identify proteins in the cell which interact with this domain and likely regulate c-Raf-1 activity.

Methods Employed:

NIH/3T3 and HepG2 cells were transfected using a calcium phosphate transfection protocol. Forty-eight hours post transfection, cells were harvested and chloramphenicol acetyl transferase enzyme activity was measured. Deletion mutants in the c-Raf-1 gene were generated by standard recombinant DNA technology.

Major Findings:

1. The c-Raf-301 dominant negative mutant blocks basal levels of transcription in HepG2 cells, and serum and 12-tetradecanoyl-phorbol-13-acetate induced transcription in NIH/3T3 cells from reporter constructs driven by AP-1 or Ets binding motifs.

2. Deletion analysis of the Raf-1 protein revealed that the N-terminal domain is sufficient for the inhibitory effect on transcription. This finding supports the notion of the cysteine finger motif functioning to negatively regulate c-Raf-1 activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05685-01 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Raf-1 Activates Transcription from the HIV-Long Terminal Repeat (LTR)

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:	Joseph Bruder	IRTA Fellow	LVC	NCI
	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
	Peter Thompson	Senior Staff Fellow	LTVB	NCI
	Joseph Bolen	Chief, Biochemical Oncology Sect.	LTVB	NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Viral Pathology Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.8	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 transforming activity of c-Raf-1 can be activated by deletions or insertions in the N-terminal half of the protein. Using a cotransfection assay, we have demonstrated that c-Raf-1 kinase activates transcription from the HIV-LTR. Mutations that activate the transforming activity (e.g., c-Raf-BXB) also increase the transactivation potential of the Raf-1 protein. Activation of latent provirus occurs when cells are stimulated with physiological inducers or tumor promoters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA). We have begun experiments aimed at determining if c-Raf-1 is involved in TPA induction of the HIV-LTR. We have recently observed, in agreement with published data, that transcription from the HIV-LTR is activated by TPA. We explored the c-Raf-1 dependence of this induction utilizing a dominant negative mutant, c-Raf-301, which carries a single amino acid substitution (LYS-TRP) in the putative ATP binding site of Raf-1 kinase and found that it blocks TPA induction. The ability of c-Raf-301 to block TPA induction strongly suggests that c-Raf-1 is mediating TPA induction of the HIV-LTR. Clearly, c-Raf-1 is an important mediator of signal transduction from the membrane to the nucleus, and we have demonstrated that the HIV-LTR is a target for activated c-Raf. In many receptor systems where cell proliferation is the end result, triggering of the receptor is followed by hyper-phosphorylation of 100 percent of the c-Raf-1 molecules in the cell. Association of the HIV envelope with the CD4 receptor is an essential step in HIV infection. In the CD4 system, antibody cross-linking results in a transient phosphorylation of 1 to 5 percent of the c-Raf-1 molecules in the cell, and the mitogenic signalling is abortive. Thus, the role of c-Raf-1 in the initial stages of HIV infection may be different from its role in cellular proliferation.

Project Description

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

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Joseph T. Bruder	IRTA Fellow	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Peter Thompson	Senior Staff Fellow	LTVB	NCI
Joseph Bolen	Chief, Biochemical Oncology Sect.	LTVB	NCI

Objectives:

The present objective is to determine the specific nucleotides between positions -120 and -91, relative to the cap site, which are important for Raf-1 activation of transcription. Binding sites for at least three different transcription factors (NFkB, Ets, GM-CSFRF) are present in this region. We have generated a series of HIV-long terminal repeat (LTR) chloramphenicol acetyl transferase (CAT) constructs carrying point mutations in each of these binding sites to determine the importance of each motif for Raf-1 activation of transcription. After determining the specific sequence motif important for Raf-1 activation of transcription, we will determine if the protein that binds to this motif is modified by Raf-1 kinase. It will also be important to determine if Raf-1 modification of this putative substrate results in increased transcriptional activity.

Methods Employed:

NIH 3T3 cells were transfected using a calcium phosphate transfection protocol. Forty-eight hours post transfection cells were harvested and CAT enzyme activity was determined. Point mutations in the HIV-LTR were generated by polymerase chain reaction mutagenesis using a single mutagenic primer.

Major Findings:

1. Transcription from the HIV-LTR is activated by the Raf-1 oncogene.

2. Sequences in the HIV-LTR between positions -120 and -91, relative to the cap site, are important for Raf-1 activation of transcription.

3. Our results suggest that c-Raf-1 is involved in the induction of latent HIV provirus, since a dominant negative mutant, c-Raf-301, blocked 12-O-tetradecanoyl-phorbol-13-acetate induction of transcription from the HIV-LTR.

4. Raf-1 is phosphorylated on serine and tyrosine residues following CD4 cross-linking.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05686-01 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: v-raf Regulation of Lineage Commitment in Hemopoietic Cells

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:	Karen W. Muszynski	SRTP	LVC	NCI
	Jakob Troppmair	Visiting Associate	LVC	NCI

COOPERATING UNITS *(if any)*: Federal Food and Drug Administration, Division of Microbiology, Washington, DC (M. Principato)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Viral Pathology Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	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.)*

Current evidence suggests that the stimulation of growth factor receptors by their respective ligands affects lineage commitment in hemopoietic cells. It has also been shown that the Raf-1 kinase is phosphorylated in response to stimulation by a variety of hemopoietic growth factors including interleukin-2, interleukin-3, macrophage colony stimulating factor (CSF-1), and granulocyte-macrophage colony stimulating factor. This suggests a role for the raf gene product as a signal transduction molecule in pathways known to affect the growth and differentiation of hemopoietic cells. In addition, it has been shown that raf family genes are differentially expressed in cells committed to the macrophage and B-cell lineages. Experiments infecting mouse bone marrow cells with a v-myc/v-raf containing retrovirus generated B-cells and macrophages in which the presence of identical heavy chain gene rearrangements in both cell types suggested that cells previously committed to the B-cell lineage had subsequently differentiated into mature macrophages. Experiments were undertaken to analyze the role of each oncogene in the alteration of lineage commitment in these cells. Preliminary results suggest that the v-myc gene inhibits the expression of markers associated with commitment to the B-cell lineage while v-raf induces these cells to either differentiate into sIg+ B-cells or to switch their lineage commitment and differentiate along the macrophage lineage. In addition, the appearance of a cell surface marker associated with hemopoietic stem cells suggests that cells revert to a stem cell phenotype prior to differentiation along the macrophage lineage. To establish the extent to which these effects are related to raf gene function, reagents have been designed which will permit conditional expression of the v-raf gene in v-myc/v-raf-transformed bone marrow cells. A retroviral construct expressing a temperature-sensitive v-raf gene and a dexamethasone inducible dominant negative Raf-1 mutant will be used to evaluate the association between raf gene expression, lineage commitment and differentiation in hemopoietic cells.

Project Description

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

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Karen W. Muszynski	SRTP	LVC	NCI
Jakob Troppmair	Visiting Associate	LVC	NCI

Objectives:

The present objectives are: (1) to analyze the affect of v-raf on lineage commitment in v-myc/v-raf transformed B lineage cells that convert to macrophages, (2) to determine if the v-raf gene plays a role in the differentiation of hemopoietic cells, and (3) to evaluate the affect of different isotypes of the raf gene (A-raf, B-raf and c-raf-1) on the differentiation of hemopoietic cells.

Methods Employed:

The generation of cell lines expressing both the v-raf and v-myc genes was accomplished by retroviral infection of existing pre-B-cell lines by co-cultivation of cells with NIH/3T3 cell lines producing the appropriate retroviruses. A v-myc-transformed cell line in which a spontaneous mutation resulted in the expression of a v-raf gene was also analyzed for changes in phenotype. Northern and Western blot analysis were used to evaluate expression of the viral oncogenes. Changes in cell phenotype were assayed by flow cytometry analysis of cell surface markers. Two inducible constructs which allow conditional expression of the v-raf gene have been designed and will be used to further analyze the effect of raf gene expression in these cells.

Major Findings:

1. Infection of a v-raf-transformed pre-B-cell line with a retrovirus expressing the v-myc gene resulted in a 70% loss in the level of B220 expressed on the surface of these cells in comparison to the parent cell line. A small, transient population of variant cells in the culture also expressed Mac-1, a marker associated with differentiation along the macrophage lineage. These results suggest that while the expression of v-myc in a v-raf-transformed pre-B-cell can induce the expression of markers associated with the macrophage lineage in a small percentage of cells, its primary effect is to interfere with the expression of markers associated with commitment to the B-cell lineage.

2. The ability of a v-myc-transformed pre-B-cell line to induce foci on NIH/3T3 cells suggested that these cells were producing a transforming virus. Western blot analysis revealed that these cells were expressing a frameshift inactivated v-raf gene present in the original construct. Flow cytometry indicated the presence of cell surface antigens that were not expressed in the original v-myc-transformed pre-B-cell line. Five out of six subclones isolated from this culture became adherent and expressed the Mac-1 gene at

some point during passage. The kappa chain gene was also expressed, indicating the presence of the Ig receptor on the surface of between 9 and 35 percent of the cells in 3 of the 6 subclones. The phenotype of the subclones isolated from this culture suggests that expression of the v-raf gene in a v-myc-transformed pre-B-cell line induced differentiation of these cells along both the macrophage and B-cell lineages. In addition, these cells express a marker commonly associated with hemopoietic stem cells.

3. These results are consistent with the results of a previous experiment in which bone marrow cells infected with a retrovirus expressing the v-raf and v-myc genes generated clonally related B-cells and macrophages. In addition, these results suggest that these cells revert to a stem cell phenotype prior to expressing markers associated with macrophage differentiation.

4. Infection of the J3 cell line with a second v-raf gene, which included 220 base pairs not present in the spontaneously expressed J3 encoded v-raf gene, indicated that this small deletion has no effect on the phenotype of these cells.

Publications:

Baccarini M, Sabatini D, App H, Rapp U, Stanley E. Colony stimulating factor-1 stimulates temperature dependent phosphorylation and activation of the raf-1 proto-oncogene product. EMBO J 1990;9:3649-57.

Carroll M, Clark I, Rapp U, May W. Interleukin-3 and granulocyte-macrophage colony-stimulating factor mediates rapid phosphorylation and activation of cystolic c-raf. J Biol Chem 1990;265:19812-7.

Kurie J, Morse H, Principato MA, Wax J, Troppmair J, Rapp U, Potter M, Mushinski F. v-myc and v-raf act synergistically to induce B-cell tumors in pristane-primed adult BALB/C mice. Oncogene 1990;5:571-82.

Principato M, Cleveland J, Rapp U, Holmes K, Pierce J, Morse H, Klinken P. Transformation of murine bone marrow cells with combined v-raf/v-myc oncogenes yields clonally related mature B-cells and macrophages. Mol Cell Biol 1990;10:3562-8.

Storm S, Cleveland J, Rapp U. Expression of raf family proto-oncogenes in normal mouse tissues. Oncogene 1990;5:345-51.

Troppmair J, Potter M, Wax J, Rapp U. An altered v-raf is required in addition to v-myc in J3V1 virus for acceleration of murine plasmacytomagenesis. Proc Natl Acad Sci USA 1989;86:9941-5.

Turner B, Rapp U, App H, Greene M, Dobashi K, Reed J. Interleukin-2 induces tyrosine phosphorylation and activation of p72-74 raf-1 kinase in T-lymphocyte. Proc Natl Acad Sci USA 1991;88:1227-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05687-01 LVC

PERIOD COVERED: October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Role of ras in raf Coupling to Transmembrane Receptor Tyrosine Kinases

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:	Jakob Troppmair	Visiting Associate	LVC	NCI
	Stuart Aaronson	Chief	LCMB	NCI
	Timothy Flemming	Senior Staff Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*: Dana Farber Cancer Institute, Harvard Medical School, Boston, MA (G. M. Cooper); University of Pennsylvania, School of Medicine, Department of Pathology, Philadelphia, PA (J. C. Reed)

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Viral Pathology Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

CHECK APPROPRIATE BOX(ES)

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

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

The positioning of Raf-1 as an essential cytoplasmic signal transducer downstream of ras and membrane-associated tyrosine kinases was initially suggested by data obtained with ras revertant cell lines, microinjection of inhibitory ras antibodies, and more recently, established by use of antisense and dominant negative raf mutants. Further, support for such a position of Raf-1 in signal transduction comes from work demonstrating phosphorylation of Raf-1 protein, resulting in a mobility shift and increased kinase activity in cells stimulated with a variety of cytokines, as well as after transformation with oncogenic versions of growth factors, growth factor receptors, and tyrosine kinase oncogenes. We have further examined the role of ras in the coupling of Raf-1 to receptors with intrinsic tyrosine kinase activity by use of a dominant negative mutant of ras. Experiments with a dexamethasone-inducible dominant negative Ha-ras-Asn17 mutant demonstrate that (1) transfection of NIH3T3 cells with the Ha-ras-Asn17 mutant results in the inhibition of serum-induced proliferation, (2) serum induction of the Raf-1 shift is blocked in these cells, and (3) this block in the Raf-1 shift correlates with a block in the induction of Raf-1 kinase activity. Two further avenues were followed for characterization of a Ras/Raf coupling event. Raf-1 phosphorylation and kinase stimulation by Ras was examined under serum-free and low serum conditions. Activated Ras was found to depend on membrane signals for Raf-1 activation. Furthermore, a potential synergistic interaction was tested between ras and raf by cotransfection of NIH3T3 cells and assay for transforming activity. Wild-type Raf-1 alone is negative in this assay but greatly facilitates the ability of wild-type or activated Ras to induce transformation. We conclude that Ras controls the signal flow from activated receptors to Raf-1 and depends on Raf-1 for the transformation.

Project Description

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

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Jakob Troppmair	Visiting Associate	LVC	NCI
Stuart Aaronson	Chief	LCMB	NCI
Timothy Flemming	Senior Staff Fellow	LCMB	NCI

Objectives:

Previous work has established Raf-1 as an essential cytoplasmic mitogenic signal transducer functioning downstream of Ras. The objective of this study is to investigate how Ras works in the coupling of Raf-1 to transmembrane receptor tyrosine kinases. Using dominant negative mutants of ras, we will address the following questions: (1) Does the block of normal Ras function interfere with the activation of Raf-1? (2) Is Ras involved in bringing together the components of the receptor signaling complex? (3) Is the accumulation of active GTP-bound Ras that has been seen after mitogen stimulation involved in modulating Raf-1 kinase activity or the activity of kinases controlling Raf-1 kinase activity? Other work on Ras/Raf coupling includes analysis of the role of growth factor signals in this event and synergistic transformation assays using cotransfection of ras and raf genes into NIH3T3 cells.

Methods Employed:

Experimental strategy employs the establishment of transfectants stably expressing inducible dominant negative mutants of ras. Raf-1 protein will be analyzed after serum or growth factor stimulation for changes in intrinsic kinase activity and electrophoretic mobility, as well as phosphoaminoacid content using standard techniques established in the laboratory. Assembly of the receptor signaling complex will be analyzed in cells doubly-transfected with the epidermal growth factor receptor and a dominant negative mutant of ras. For analysis of growth factor dependence of Ras/Raf coupling the ability of Ras and control src oncogenes was examined for induction of Raf-1 activation under serum-free and low serum conditions. To test for a potential ras/raf connection in transformation, both genes were transfected into NIH3T3 cells and scored for their ability to induce transformed foci.

Major Findings:

1. Ras dependence of Raf-1 activation. Transfection of NIH3T3 cells with a dominant negative mutant of Ras Ha-ras-Asn17 results in the inhibition of serum-induced proliferation. Serum induction of the Raf-1 shift is blocked in these cells. This block in the Raf-1 shift correlates with a block in the induction of Raf-1 kinase activity.

2. Serum dependence of Ras/Raf coupling. We compared the effects of Ha-c-ras and v-src oncogenes on the regulation of p72-74 Raf-1 kinase in NIH3T3 cells. In both serum-starved and platelet-derived growth factor-treated v-src-transformed cells, the Raf-1 kinase was constitutively activated, displaying characteristic retarded mobility in electrophoretic gels and elevated activity in in vitro kinase assays. In contrast, the Raf-1 protein from quiescent ras-transformed cells did not exhibit constitutively shifted gel mobility or elevated kinase activity but did respond normally with regard to platelet-derived growth factor- and phorbol myristate acetate-induced changes in p72-74 Raf-1 phosphorylation and kinase activity. NIH3T3 cells transformed by Ras, however, contained elevated levels of p72-74 Raf-1 protein (as determined by immunoblotting), suggesting an indirect influence on this kinase. Quantitative differences in the levels and subcellular distribution of immunodetectable protein kinase C enzymes did not account for the difference between src- and ras-transformed NIH3T3 cells with regard to regulation of the Raf-1 kinase. These findings in serum-deprived NIH3T3 cells demonstrate that expression of a ras oncogene can be insufficient for full activation of the p72-74 Raf-1 kinase, implying necessity for an additional growth factor-mediated stimulus.

Publications:

Reed JC, Yum S, Cuddy MR, Turner BC, Rapp UR. Differential regulation of the p72-74 Raf-1 kinase in 3T3 fibroblasts expressing ras and src oncogenes. Cell Growth Differ (In Press).

ANNUAL REPORT OF

BIOLOGICAL CARCINOGENESIS BRANCH
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 to September 30, 1991

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; 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), conference grants (R13), academic research enhancement (AREA) awards (R15), first independent research support and transition (FIRST) awards (R29), outstanding investigator grant (OIG) awards (R35), the method to extend research in time (MERIT) awards (R37), program project grants (P01), research and development contracts (N01), and small business innovative research (SBIR) grants (R43/44). Currently, the Branch administers 414 research grants with an annual budget of approximately 92.38 million dollars. Administratively, the Branch is divided into seven functional entities which are shown in Figure I. The Branch scientific components are based primarily on the major subdivision of viruses by their type of nucleic acid core. Research programs on viruses with a DNA core which are involved in the induction of malignant transformation are included in the DNA Virus Studies components. The component designated DNA I deals with research on the two main groups of large DNA viruses, the herpesviruses and adenoviruses. The DNA II component supports research on the small DNA viruses, the polyoma, simian virus 40 (SV40), and papillomaviruses. Similarly, research dealing with RNA core viruses are covered by the RNA Virus Studies components. The component designated RNA I involves research concerning murine, feline, bovine, nonhuman primate, and human viruses. The RNA II component incorporates research involving avian tumor viruses, picornaviruses, hepatitis B virus, and other microbial agents. The Research Resources component arranges for the storage and distribution of research materials, helps oversee the various resource contracts, and maintains computerized information systems covering the

distribution of resources. The AIDS Virus Studies component was established to deal with the increasing concern about this public health problem. Originally, acquired immunodeficiency syndrome (AIDS) activities in the program were treated as special initiatives administered by the Branch Office. However, the high visibility of these efforts and the need for an accountable individual to expedite reports and responses to senior echelons dictated that a program director be assigned exclusively to these research oversight activities. Finally, the Office of the Branch Chief oversees and coordinates all of these activities, establishes program objectives and priorities, evaluates accomplishments, and interacts with the Division and Institute leadership.

The research resources payback system of the Branch has been described in previous reports. During this period, all traditional resource contracts were functioning in the payback mode. These include one for specialized testing services, and one for storage and distribution of frozen biological reagents. The payback system is 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 FY91 is estimated to be about 92.9 million dollars. It should be noted that the Branch now administers 20 program project grants at a level of 19.79 million dollars, 19 outstanding investigator awards at the level of 15.46 million dollars, and 33 merit awards at a level of 7.75 million dollars. Table II provides an estimate of the grant and contract support, respectively, in each of the Branch components and thus illustrates, in quantitative terms, the main areas of scientific endeavor. As can be seen from the table, except for the newer AIDS component, the four primary long-established Branch research components are well balanced in terms of their number of grants and funding level. This is not by design, but reflects the broad interests of the extramural community. Furthermore, approximately ten percent of the total number of grants currently in the program represent awards initially solicited by the Branch through requests for grant applications (RFAs). The Branch currently administers 414 grants and 3 contracts. Table III summarizes the specific targeted research activities initiated by the Biological Carcinogenesis Branch since 1982. 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 FY91, the Branch was again active in sponsoring a variety of research initiatives which reflected emerging areas of research opportunity. During this fiscal year, two workshops, one Institute-sponsored and one Branch-sponsored were held, and two RFAs were issued. The Institute-sponsored workshop was organized by the BCB, took place on October 29-30, 1990, and was entitled "Cancer Vaccines." This workshop was held partly in response to Congressional interest and partly because of advances in the scientific state-of-the-art. The workshop addressed the identification of relevant viral and/or tumor antigens; determination of the parameters needed for these

antigens to achieve effective immunogenicity; and determination of the immunological effector mechanism(s) responsible for induction by a vaccine of strong resistance against growth and metastasis of tumors. From remarks and discussions of participants at the consensus conference, as well as post-conference communications to NCI staff, it was apparent that a sufficient base of knowledge and technical skill exists to mandate a vigorous and broadly based cancer vaccine program at the NCI. An RFA concept entitled "Vaccines for Human Cancers of Viral Etiology" was presented to the DCE Board of Scientific Counselors for its consideration at the June 1991 meeting. The RFA emphasized basic and applied research on the identification of viral or viral-induced antigens which elicit protective immunity and which form the basis for vaccine preparation. The development of animal models to determine immune response to viral-induced tumors and to test the safety and efficacy of proposed vaccines, and development of prototype therapeutic vaccines for viruses whose malignant sequelae occur as the result of chronic infection were also important elements of the RFA. In addition, selected studies on newly discovered agents which have a strong potential for being involved in the etiology of viral-induced malignancies were encouraged. Specifically, applications were solicited for studies on the human papillomaviruses which are associated with human cervical cancer, for studies on Epstein-Barr viruses which have been associated with Hodgkin's and non-Hodgkin's lymphomas, particularly in immunosuppressed individuals, and for the newly identified hepatitis C virus and the consideration of its role in the etiology of liver cancer. Meritorious applications should receive funding in FY92.

A second workshop, held November 15, 1990, was entitled "Animal Models of Retrovirus-Associated Malignancies." This workshop was held partly in response to Congressional interest and partly because of advances in the scientific state-of-the-art. The workshop focused on the neoplastic potential of various retroviruses and the different molecular mechanisms by which viruses might be involved in the etiology of both lymphoid malignancies and sarcomas. Workshop participants also noted that little is known about the ability of the host to restrict the replication of retroviruses, and thus it is not known which host factors determine whether a virus replicates lytically or might be involved in the initiation or progression of neoplastic sequelae. The workshop participants concluded that studies on the pathogenesis of cancers of viral origin from animal models should result in additional information and knowledge relevant to human cancer. An RFA based on these recommendations was developed. At its June 1991 meeting, the DCE Board of Scientific Counselors considered the issuance of an RFA entitled "Domestic Animal Models for Retrovirus-Associated Human Cancers." Specific research goals of the RFA include the study of oncogenic mechanisms in domestic animal retroviruses; investigation of cancer etiology and viral pathogenesis from initial infection through the development of pre-neoplastic lesions and neoplastic sequelae with retroviruses of domestic animals; the role of RNA and DNA viral co-factors in cancer etiology in animal models; investigations to assess the role of the host immune system and host genetic factors in the control and limitation of virus replication, and the susceptibility or resistance of animals to oncogenic processes; and studies on the expression and regulation of viral and/or associated host cell genes in pre-neoplastic lesions and malignant tissues from retrovirus-infected domestic animals. It is anticipated that applications for this RFA will be received and reviewed in the Winter of 1992, with funding of meritorious applications in FY92.

As a result of previous Branch workshops held in 1989, four RFAs received concept approval by the Division of Cancer Etiology (DCE) Board of Scientific Counselors (BSC) during FY90 and were funded during this fiscal year. The first RFA was entitled "Viral Oncogenesis and Pathogenesis of Hepatocellular Carcinoma." Twenty-seven applications were received in response to this RFA, and six highly meritorious applications were subsequently funded, with a first-year total cost of $999,542. The grants address several important issues in hepatitis B virus (HBV)- and hepatitis C virus (HCV)-associated neoplasia and include: studies of a transgenic mouse model for immunopathogenesis of hepatocellular carcinoma (PHC), investigations of HBV as an insertional mutagen, studies of the oncogenic potential of the HBV X gene, co-carcinogenesis studies involving HBV and dietary carcinogens, studies on the replication and gene products of HCV, and investigations on the role of low-level HBV and HCV infection in patients with PHC.

A second RFA was entitled "New Approaches to Understanding Transformation by SV40 Virus, Polyomavirus, and Adenoviruses." Seventeen applications were received in response to this RFA and four highly meritorious applications were subsequently funded at a level of $748,859 for the first year. One grant will investigate the mechanism of action of small t-antigen (tAg) in transformation by SV40, and how this binding relates to transactivation by tAg. Another will examine the mechanism of E1A-mediated enhancer repression and the potential linkage of this function to stimulation of cellular DNA synthesis and immortalization of transformation, both of which map to overlapping regions of the gene for the E1A protein. A third grantee will study the binding of the adenovirus E1A protein to the p300 cellular protein using virus genetics and peptide binding/blocking experiments. The fourth investigator has developed a novel genetic system in yeast for detecting the interaction of two proteins and will apply this promising approach to identification of cellular proteins that interact with various viral oncoproteins.

A third RFA funded was entitled "Human T-cell Lymphotropic Viruses in Human Neoplasia." Twenty-three applications were received, and five were subsequently funded with a total first-year cost of $716,501. One applicant will utilize the transactivating potential of transforming viruses to detect viruses with transactivating ability in human cancers. A second applicant will attempt to determine the viral etiology of large granular lymphocytic leukemias. A third series of studies will focus on the events which occur following HTLV-1 and HTLV-2 infection in the severe combined immunodeficiency disease (SCID) mouse model. Two other investigators will determine the mechanisms involved in the induction of malignancy and neurological diseases by HTLV-1.

A fourth RFA was "Mechanisms of Viral-Induced AIDS-Associated Neoplasias." The goals of the initiative were to stimulate investigations in the extramural community on the role of HIV, viruses and viral genes, and cellular genes or cofactors in the initiation and progression of AIDS associated malignancies. Thirty-six applications responsive to the goals of the RFA were received, of which five were recommended for funding by program staff, with a total cost of $912,646 per year. These new scientific efforts focus on the role of B-cell growth factor in the proliferation of lymphomas, a primate animal model of Kaposi's sarcoma, the role of human cytomegalovirus as a viral cofactor in AIDS malignancies, and two projects focusing on possible mechanisms of induction of AIDS-associated lymphomas.

Research sponsored by the Branch has yielded a variety of fundamental information on biological carcinogenesis by studying human and 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 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 either through 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 interaction of viruses with a variety of environmental factors, such as 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.

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, in many cases, 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 general 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 40 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. In addition, a class of recessive oncogenes, called anti-oncogenes or suppressor oncogenes, has been discovered in which the absence of the oncogene product, rather than its presence, is responsible for transformation. One of the best understood examples of suppressor genes is the Rb gene of retinoblastoma, which appears to play an important role in the induction of retinoblastoma, as well as other commonly occurring cancers, such as cancers of the lung and colon. Loss or inactivation of both copies of the Rb gene in humans may predispose an individual to cancer. Other recessive oncogenes continue to be discovered, and their role in oncogenesis is currently being evaluated. Research highlights of the past year are presented here and, in greater detail, in the various section reports which follow.

DNA Viruses and Cancer

Studies of human and animal DNA tumor viruses provide basic information necessary for understanding how these viruses and their protein products convert a normal cell into a cancerous one. It appears that cancer may occur either through the direct effects of viral transforming genes, or perhaps

through the interaction of viruses with a variety of other factors, such as chemicals, radiation, alcohol and dietary components. Exciting new studies completed during the past year have added another possible mechanism for virally induced cancer, the inactivation of tumor suppressor genes by virally-coded protein products.

Human papillomavirus (HPV) types 16 and 18 are DNA tumor viruses that are associated with human cervical and anogenital cancers. Two HPV 16 and 18 gene products known as E6 and E7 have been shown to have transforming potential when introduced into epithelial cells. Last year investigators discovered that the E7 gene protein was capable of binding to the Rb tumor suppressor, but was not specific for carcinogenic transformation, since E7 protein from HPV types not generally associated with malignant progression also bound to Rb. This year, investigators have discovered that the HPV E6 protein binds with another tumor suppressor known as p53. This finding is exciting since the E6-p53 binding appears to be specific for HPV types 16 and 18, and is correlated to in vivo clinical behavior and in vitro transforming activity of HPVs. The role of the p53 tumor suppressor is also intriguing on its own, since p53 has been shown to bind to large T-antigen of SV40 and to the E1B protein of adenovirus type 5, suggesting that there may be a common cellular mechanism for transformation by small DNA tumor viruses.

Primary hepatocellular carcinoma, a form of liver cancer, is one of the most frequently occurring human cancers worldwide, and is responsible for more than 250,000 deaths annually. The greatest single risk factor for the development of this cancer is chronic infection with the hepatitis B virus (HBV). Little knowledge of the molecular mechanisms of hepatocarcinogenesis has been available until recently. The p53 tumor suppressor gene was studied at the DNA, RNA and protein level in 7 human hepatocellular carcinoma derived cell lines, and 6 of the 7 showed loss of expression of p53 or abnormalities in the protein. These observations suggest that alterations in p53 may be important events in the transformation of hepatocytes to the malignant phenotype. The likelihood that p53 mutations are important in hepatocarcinogenesis is increased by the finding, in an extramural and an intramural laboratory, that liver cancers from Africa and China showed similar mutations in codon 249 of the p53 protein. Such mutations may not only involve a loss of function in p53, but the existence of this "hot spot" at codon 249 implies an additional gain of function. It is possible that interaction of HBV-encoded proteins with mutant p53 provides some advantage to the growth of hepatocarcinoma. This mutant protein may lead to an increased understanding of the mechanism(s) of viral carcinogenesis if it can be shown to interact with HBV proteins.

A series of investigations carried out during the past year have focused on Epstein-Barr virus (EBV), a herpesvirus which infects both lymphoid and epithelial cells. Infection with EBV has been associated with several diseases including infectious mononucleosis, Burkitt's lymphoma (BL), B-cell lymphomas and nasopharyngeal carcinoma. Recent studies demonstrated that EBV genome sequences are found in approximately 10-15% of AIDS patients with non-Hodgkin's lymphomas and in every CNS B-cell lymphoma from AIDS brain autopsies at one clinical site, suggesting a possible role for the virus in the pathogenesis of AIDS. Understanding the mechanisms by which EBV induces both infectious diseases and neoplasia affecting two types of tissues, B-cells of the lymphoid system and epithelial cells, is important in designing strategies for the prevention and treatment of these EBV-related diseases.

Recently, EBV deletion mutants were discovered in clinical samples of mucosal tissue (which contains both epithelium and lymphoid tissue) from human pathological states and healthy individuals. The deletion mutants were shown to be lytic in epithelial cells and latent in lymphoid cells, and were stable, infectious, and transmissible. Studies of these mutants may contribute to our understanding of NPC, BL, and oral hairy leukoplakia in AIDS patients.

Adenoviruses are a group of medium-sized human DNA viruses that have been shown to transform cells in culture and cause cancer in rodents, including breast tumors of several types in rats which contain the entire adenovirus type 9 genome and require estrogen for development and maintenance. Adenoviruses serve as model systems for the characterization of the mechanisms involved in viral-induced neoplastic transformation and provide an opportunity to evaluate the immune response to tumors in animals. Research on these viruses has provided insight into mechanisms of gene regulation and viral-host interactions such as the synergistic action of the viral E1A gene product with cellular cAMP to activate transcription of the cellular oncogene c-fos. In addition, studies with cell cultures have demonstrated that a viral gene product can protect virus-infected cells from attack by tumor necrosis factor, a host defense. Further studies are needed in order to fully elucidate the mechanisms by which these viruses cause oncogenesis in vivo.

RNA Viruses (Retroviruses) and Cancer

Human retroviruses were discovered relatively recently and have been the focus of much attention among clinical scientists as well as virologists. The first human cancer virus, human T-cell lymphotropic virus, type 1 (HTLV-1), was isolated in 1980 and is recognized as the etiologic agent of human adult T-cell leukemias/lymphomas (ATL), a disease which occurs in southern Japan, central Africa and the Caribbean basin. The HTLV-2 virus was subsequently isolated in 1982 and was found to be immunologically similar to HTLV-1. It is found in humans with or without evidence of malignancy, and during the past year was found to be widely prevalent in intravenous drug abuser populations. Pioneering studies of these authentic human retroviruses provided the impetus and knowledge that led to the discovery of the human immunodeficiency viruses (HIV-1 and HIV-2) associated with AIDS.

The region of the HTLV genome termed the X region codes for at least three transacting proteins which regulate both virus and host cell gene expression. The expression of the X region genes has been proposed as an explanation for the unique cell transforming abilities of this group of retroviruses. Recently, scientists have documented that one of the X region regulatory genes, the tax gene, "turns on" other genes which activate T-cells, including the genes for interleukin-2 and the interleukin-2 receptor. They also have shown that the tax gene has the ability to participate in cancer induction, since three different lines of transgenic mice harboring the tax gene developed a variety of lesions including multiple mesenchymal tumors and neurofibromas. It was found that the neurofibromas secrete large quantities of interleukin-6 as well as nerve growth factor (NGF). This observation, that tax-1 protein may stimulate the production of NGF, could provide important new insights into the mechanism of HTLV-1-induced neurologic disease. Furthermore, and very important from a clinical standpoint, it has been shown that in humans infected with HTLV-1, the presence of serum antibodies against the tax protein is usually correlated with HTLV-1 infection during early life.

These tax antibodies, therefore, may serve as reliable markers for HTLV-1 infection during early life and possibly for susceptibility to lymphoma development.

Recent results raise the possibility that HTLV regulatory protein rex also contributes to the tumorigenic potential of HTLV-1 by cooperating with tax in the overexpression of cellular genes that alter the growth of CD4+ T-lymphocytes, which are the primary target cells of HTLV-1 transformation. It was found that rex increased the rate of IL2 promoted gene expression in a mitogenically stimulated cell line. Rex also increased the activity of the c-fos promoter to a lesser extent. Mitogenic activation of Jurkat cells was required to observe rex stimulation of the IL2 promoter and the effect was substantial only in the presence of tax. High levels of tax did not inhibit IL2 promoter activity. Rex may be the transactivator of transcription which acts via sites distinct from those responsive to the tax protein. This activity could be the result of an increase in the initiation of transcription or alternatively in more efficient elongation of messages.

In addition to the human retroviruses that have already been associated with cancer, there are some retroviruses of domestic animals which are associated with neoplasms and other diseases. Bovine leukemia virus has been demonstrated to induce lymphosarcomas in cows and avian sarcoma viruses induce solid tumors in chickens. Other domestic animal retroviruses, such as the equine infectious anemia virus, caprine arthritis encephalitis virus and bovine immunodeficiency virus, are associated with diseases of the blood and immunosuppressive diseases of the host animal. Studies of these domestic animal retroviruses continue to provide valuable insights into the mechanisms of cancer induction by viruses, and have shown that they represent valuable models for the evaluation of intervention strategies. These animal models have the advantage of permitting investigations of viruses and their oncogenic potential in the natural host. Thus, confounding factors involved in cross-species experiments have been avoided. The recent development of new biological reagents, such as monoclonal antibodies and cloned viral nucleic acids, has increased the potential for utilizing these domestic livestock animal systems for studies of the consequences of retroviral infections in humans.

Oncogenes, Growth Factors, and Growth Factor Receptors

The single most striking feature that defines a cancer cell is its ability to proliferate under conditions that normally would inhibit cell growth. In normal cells, growth is controlled by regulatory activities that take place at the cell surface. These surface activities are communicated through the cytoplasm of the cell to its nucleus. In this way, the synthesis of the unique proteins required for the start of DNA synthesis and subsequent cell division are regulated. If normal cells are grown in tissue culture and are subjected to either mechanical disruption or exposed to growth factors, the resulting changes in the molecular environment are recognized at the surface of the cell and transmitted through the cytoplasm to the nucleus, activating the molecular events which trigger cell division. Any of a variety of genetic changes that hamper the regulation of mitosis (cell division) may result in unrestrained growth and thus initiate malignancy. Certain viruses seem to play a role in keeping cells in an active mitotic state. Knowledge of the identity of their encoded proteins, and their interactions with various

cellular signal transduction factors, is important to understanding the fundamentals of biological carcinogenesis.

The myc protooncogene family, the cellular progenitor of the v-myc oncogene of avian leukosis virus, has been implicated in cell proliferation, differentiation, and neoplasia, but its mechanism of function at the molecular level is unknown. The myc oncogene is of considerable interest, since alterations in it appear to contribute to the development of lung cancer, Burkitt's lymphoma, and other human cancers. Although scientists have suspected that the myc oncogene protein product regulates gene expression, they were previously unable to find either a binding protein for it or specific DNA sequences to which it binds. A protein called Max (for myc-associated X) has recently been identified which may explain in part how the myc oncogene functions. The Max protein has been found to cooperate with the myc protein to form a DNA recognition site. Max specifically associated with c-myc, n-myc, and l-myc proteins, but not with a number of other proteins. Furthermore, the myc-Max complex bound to DNA in a sequence-specific manner under conditions where neither Max nor myc alone exhibited appreciable binding. The DNA-binding activity of the complex was dependent on both the dimerization domain and the basic region of c-myc. These results suggest that myc family proteins undergo a restricted set of interactions in the cell and may belong to the more general class of eukaryotic DNA-binding transcription factors. This finding opens the door to identifying the genes regulated by myc and determining myc's role in normal cells and in various disease states, including cancer. In addition, the method used for identifying Max may also be useful for identifying the binding partners for other gene regulatory proteins.

Over the last few years, tumor suppressor genes which are involved in controlling normal cell growth, have, with increasing frequency, been associated with a number of inherited and non-inherited types of cancer. Researchers have found changes in suppressor gene p53 in various types of human tumors and it has been strongly implicated in the development of colon cancer. However, in all of these instances it is believed that the gene was normal at the time of birth and became damaged at some point during the person's lifetime. If such a gene is indeed responsible for the inheritance of a predisposition to cancer, then, in those individuals who will eventually develop cancer, one of the two copies of this gene should be abnormal at birth in all of the cells of the body, even in those cells that remain normal. This assumption led to the study of the p53 gene in normal skin cells obtained from three generations of a cancer family. A small defect was discovered in exactly the same location within the p53 gene in four family members who developed cancer. This defect was present only in those individuals who had developed cancer and was not found in those who had escaped the disease. Neither was it present in individuals who had married into the family. Apparently, an inherited defect had occurred in the tumor suppressor gene p53 in this family. It is conceivable that this discovery could lead to the development of a diagnostic test which could identify some individuals at risk for developing cancer.

In humans, the translocation of chromosome 9 to 22 involves the aberrant expression of the abl oncogene. Chronic myelogenous leukemia (CML) develops as a result of the translocation of the c-abl locus and the subsequent aberrant production of the oncogenic $P212^{bcr/abl}$ fusion protein. Understanding

the mechanisms by which the abl oncogene affects various cell types has been hampered by a paucity of experimental systems that reproduce the range of biological effects associated with them. A mouse model system has now been developed for human CML. Expression of the CML-specific $P210^{bcr/abl}$ protein in the bone marrow of mice by a retroviral gene transfer/bone marrow transplantation approach leads to a variety of hematological malignancies, most prominently, a myeloproliferative syndrome with a striking resemblance to the chronic phase of human CML. Further analysis of mice with the CML-like syndrome has shown that the CML-like syndrome is, at least in some cases, a consequence of retroviral infection of the pluripotent hematopoietic stem cell. In addition, the disease is transplantable by transfer of bone marrow to syngeneic recipients, and clonally related acute leukemias of both lymphoid and myeloid origin have been observed in secondary transplant recipients, representing evolution of the disease to blast crisis. This CML-like syndrome induced by $P210^{bcr/abl}$ in mice closely resembles human CML in several fundamental aspects and thus should serve as a useful animal model for the studies of this human cancer.

Acquired Immunodeficiency Syndrome (AIDS)

Basic research in biological carcinogenesis has contributed greatly to our understanding of AIDS and the complications arising from HIV infection. The design of effective prevention strategies and therapies for HIV infection will ultimately depend on understanding the process of viral infection, proliferation, cell killing, impaired immune function, and eventual damage to major organ systems of the body. Basic research focuses on understanding the structure and function of the virus and its proteins, on cell culture experiments investigating the life cycle of HIV and its immunopathogenic effects on human cells, and on animal models in which the effects of HIV or its gene products can be evaluated in terms of their effects on living organisms.

As a consequence of their underlying immunodeficiency, patients with AIDS are at increased risk for the development of neoplastic sequelae, such as malignant non-Hodgkin's lymphomas (NHL) and Kaposi's sarcoma (KS). KS has been observed in at least one-third of individuals who die of AIDS. Approximately three percent of AIDS patients have developed NHL, representing a significant increase in the risk of this lymphoma when compared with the general population. Recent results indicate that the risk for the development of NHL is directly associated with degree of immunosuppression in persons infected with HIV-1, as it is in transplant recipients. This contrasts with the risk of KS, which can occur with less severe immune deficiency. There is strong epidemiologic evidence to indicate that the number of patients who develop AIDS-associated malignancies will increase and may emerge as an important long-term medical problem as patients' lives are prolonged with effective therapy for HIV and opportunistic infections. Ongoing research will emphasize its efforts to study the etiology and natural history of neoplasia in AIDS populations, since these cancers are becoming increasingly prevalent and are responsible for increased morbidity and mortality. Efforts to develop more effective methods of prevention, diagnosis and treatment of AIDS-related cancers will be expanded.

Unanswered questions with regard to AIDS-associated malignancies are the identification of the cells of origin and the etiologies of these

malignancies. Research during the coming year will focus on investigating the possibility that the AIDS immunosuppressed state allows for the selection of virus variants of Epstein-Barr virus, cytomegalovirus, human papillomavirus, hepatitis B virus and/or human T-lymphotropic viruses 1 and 2 which exhibit enhanced virulence or oncogenic potential. In other studies, investigators will attempt to clarify the molecular interactions between viral and cellular genes or proteins which might be involved in the initiation and progression of AIDS-associated malignancies. Direct and indirect processes by which multiple viral co-infections play a role in AIDS-associated neoplasia will also be expanded. Efforts will be directed toward the development and use of animal models to investigate the molecular basis of AIDS-induced neoplasia. Of particular interest will be the determination of how the pathogenesis and oncogenesis of the HIV virus is altered as a consequence of the immune status of the patient.

Kaposi's sarcoma (KS) is a rare tumor of blood vessels which is usually characterized by multiple skin nodules on the lower extremities of elderly men from the Mediterranean region. KS has gained importance because of the high incidence of this tumor in patients with HIV-1 infection and AIDS. Researchers have proposed that KS develops in AIDS patients due to stimulation of endothelial cell growth by the tat gene of HIV and continued growth stimulated by the growth factors released by these cells. Methods have been developed to maintain long-term cultures of KS cells through growth of primary KS cells in media (termed conditioned media) previously used for the long-term culture of human T-lymphocytes producing human leukemia viruses HTLV-1 or HTLV-2. Upon growth in conditioned media, KS cells release into the culture medium a number of biological factors which induce the AIDS-KS derived cells (as well as normal endothelial cells that line the blood vessels) to proliferate. These factors are biological growth promoting proteins (cytokines) which are released by the virus-infected T-lymphocytes, and are not products of the virus itself. While many of the cytokines were previously known, investigators have also detected the presence of a new cytokine. The most potent source of this new growth factor is a human T-lymphocyte cell line adapted to grow in a chemically defined growth medium. This was an important accomplishment because it permits the isolation, purification and characterization of the novel growth factor. A major goal of the current research effort is to produce large quantities of the novel factor, permitting scientists to better determine the protein's mode of action and its interaction with different cell types during the progression of KS disease.

The continued spread of AIDS underscores the urgent need to develop a safe and effective vaccine against HIV-1. Scientists are conducting research using several different experimental approaches. A killed virus preparation may not be ideal as an HIV vaccine because every virus particle must be inactivated to prevent inadvertent transmission of disease; this type of vaccine may also elicit a rather poor immune response since the most immunogenic viral proteins may be denatured during vaccine preparation. The use of "attenuated" or purposefully mutated HIV RNA-containing preparations is under evaluation; however, these types of vaccines present other potential safety problems, since virus mutations or the aid of a "helper" virus could render these potential vaccines infectious. These attenuated vaccines must be evaluated in cell culture experiments and animal models to exclude the possibility of accidental disease transmission. An important alternative approach to the development of an AIDS vaccine is the use of recombinant viral proteins,

especially virus envelope proteins produced in bacterial, insect cells or mammalian cell culture systems. The use of recombinant proteins offers several possible advantages, the most important of which is that large quantities of the vaccine antigens can be produced for evaluating the immune response and the efficacy of the vaccines in animal models. Research efforts will continue to develop a vaccine which will offer a solution to this major health problem.

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. 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 for their ultimate resolution.

FIGURE I

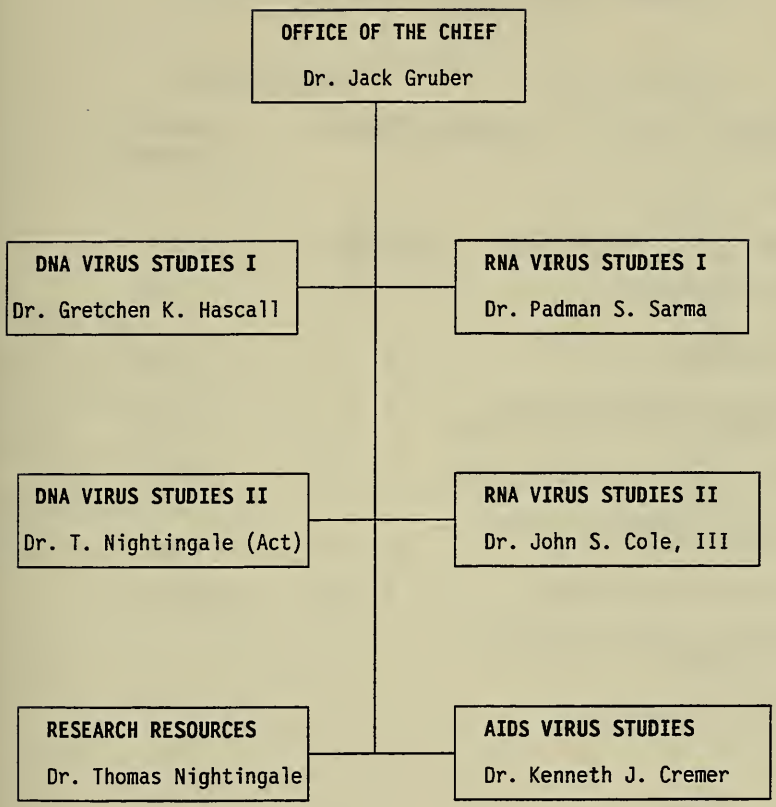

OFFICE OF THE CHIEF
Dr. Jack Gruber
DNA VIRUS STUDIES I
Dr. Gretchen K. Hascall
RNA VIRUS STUDIES I
Dr. Padman S. Sarma
DNA VIRUS STUDIES II
Dr. T. Nightingale (Act)
RNA VIRUS STUDIES II
Dr. John S. Cole, III
RESEARCH RESOURCES
Dr. Thomas Nightingale
AIDS VIRUS STUDIES
Dr. Kenneth J. Cremer

TABLE I

BIOLOGICAL CARCINOGENESIS BRANCH

Extramural Projects Active During FY 1991
(estimated)

	GRANTS/CONTRACTS	
	NUMBER	**DOLLARS** (Thousands)
Research Grants		
Traditional Project Grants (R01)	292	45,976
Conference Grants (R13)	10	13
Academic Research Enhancement Awards (AREA) (R15)	1	86
First Independent Research Support and Transition (FIRST) Awards (R29)	37	3,249
Outstanding Investigator Grants (R35)	19	15,461
Method to Extend Research in Time (MERIT) Awards (R37)	33	7,755
Program Project Grants (P01)	20	19,793
Small Business Innovative Research (SBIR) Grants (R43)	2	50
Research Resources Contracts (N01)	3	597
TOTAL	417	92,980

TABLE II

BIOLOGICAL CARCINOGENESIS BRANCH

Contracts and Grants Active During FY 1991

	FY 91 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	$ (Millions)	No. of Grants	$ (Millions)
DNA Virus Studies I	-	-	87	20.53
DNA Virus Studies II	-	-	98	21.16
RNA Virus Studies I	-	-	88	18.27
RNA Virus Studies II	-	-	95	22.37
AIDS Virus Studies	-	-	46	10.05
Research Resources	3	0.597	-	-
TOTAL	3	0.597	414	92.38

TABLE III

BIOLOGICAL CARCINOGENESIS BRANCH
Research Initiatives 1982-91

Title	Date Workshop	Date BSC Review	AWARDS 1ST YEAR FY	No.	Total Dollars
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	Sep 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	Jun 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	Oct 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	$ 594,667
NIH-NCI-DCE-85-21 (RFA) Studies on Novel Human Exogenous and Endogenous Retroviruses	Mar 85	May 85	86	4	$ 547,709

TABLE III (cont.)

BIOLOGICAL CARCINOGENESIS BRANCH
Research Initiatives 1982-91

Title	Date Workshop	Date BSC Review	AWARDS 1ST YEAR--- FY	No.	Total Dollars
NIH-NCI-DCE-86-07 (RFA) The Transformation Mechanisms of Human Polyomaviruses	Mar 85	Oct 85	87	6	$ 771,480
NIH-NCI-DCE-87-19 (RFA) Studies on Papillomavirus-Host Interactions	Feb 86	Oct 86	88	5	$ 777,796
NIH-NCI-DCE-87-18 (RFA) Studies of Functional Anti-Sense RNA in Oncogenic Viral Systems	Mar 86	Oct 86	88	3	$ 346,210
NIH-NCI-DCE-88-13 (RFA) Animal Models for Human Papillomavirus-Associated Neoplastic Diseases	Sep 87	Feb 88	89	2	$ 390,031
NIH-NCI-DCE-88-14 (RFA) Retrovirus Animal Models and HIV Pathogenesis	Oct 87	Feb 88	89	7	$1,293,221
NIH-NCI-DCE-89-08 (RFA) New Approaches to Studying EBV Oncogenesis	Apr 88	Oct 88	90	6	$ 824,391
NIH-NCI-DCE-90-08 (RFA) Viral Oncogenesis and Pathogenesis of Hepatocellular Carcinoma	Feb 89	Oct 89	91	6	$1,029,540*

TABLE III (cont.)

BIOLOGICAL CARCINOGENESIS BRANCH
Research Initiatives 1982-91

Title	Date Workshop	Date BSC Review	AWARDS 1ST YEAR FY	No.	Total Dollars
NIH-NCI-DCE-90-09 (RFA) New Approaches to Understanding Transformation by SV40 Virus, Polyomaviruses and Adenoviruses	Apr 89	Oct 89	91	4	$ 746,347*
NIH-NCI-DCE-90-10 (RFA) Human T-Cell Lymphotrophic Viruses in Human Neoplasia	May 89	Oct 89	91	5	$ 732,188*
NIH-NCI-DCE-90-15 (RFA) Mechanisms of Viral-Induced AIDS-Associated Neoplasia	Nov 89	Mar 90	91	5	$ 913,956*
(RFA) Vaccines for Human Cancers of Viral Etiology	Oct 90	Jun 91	92		$2,000,000**
(RFA) Domestic Animal Models for Retrovirus-Associated Human Cancers	Nov 90	Jun 91	92		$ 750,000**

*Estimated Funding
** Authorized Funding

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 herpesviruses and adenoviruses. In this component, extramural research is supported primarily by the grant mechanism. There are 87 research grants with an estimated total funding level of 20.53 million dollars. These include the traditional research grants, program project grants, conference grants, first independent research support and transition (FIRST) awards, method to extend research in time (MERIT) awards and outstanding investigator grant (OIG) awards. The major research emphasis lies in studies of the mechanism(s) of viral transformation, which include genome structure, gene function and expression 76%; and virus-cell interaction 24%. In terms of the viruses being studied, 20% involve herpes simplex virus (HSV), 36% involve Epstein-Barr virus (EBV), 3% involve cytomegalovirus (CMV), 12% involve other herpesviruses, and 29% involve adenoviruses.

Investigators supported by this program are attempting to elucidate the mechanism(s) of transformation of herpesviruses and adenoviruses using 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. Studies with a more biological orientation have investigated virus-host interactions in order to define the process of viral oncogenesis.

Herpesviruses

Most members of the herpesvirus family can transform cells in vitro and all of them can establish latent infections in man and animals. Many of the herpesviruses have been suspected of having a role in tumor induction in man, either directly or as cofactors. Because all herpesvirus infections result in life-long latent infections, reactivation of these viruses during immunosuppressive therapies is a cause of morbidity and mortality both in cancer patients and transplant recipients.

Epstein-Barr virus is a lymphotropic herpesvirus which has been associated with several disease entities including infectious mononucleosis, Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC) and other oropharyngeal tumors, as well as with B-cell lymphomas in immunocompromised individuals. When EBV infects epithelial cells in vivo, the virus undergoes a complete replicative cycle, thus providing infectious virus for the transmission of EBV infection from one individual to another. By contrast, when EBV infects B-lymphocytes in vivo or in vitro, the infection is primarily latent. However, these latently infected B-cells are altered, and unlike normal lymphocytes, can proliferate indefinitely and can be cloned and grown as continuous cell lines. It is hypothesized that, in immunocompromised individuals, these latently infected cells can directly or indirectly give rise to B-cell lymphomas. Because of the difficulties involved in obtaining epithelial cells for in vitro investigations, studies on these latently-infected transformed B-cells have provided most of our knowledge of the molecular biology of EBV.

When EBV establishes latency in B-cells, viral gene expression is limited to a few transcripts, including the nuclear protein EBNA-1 which has a role in the regulation of expression of the EBNA family of latency genes. EBNA-1 is a direct DNA-binding protein, and both binding and transcription are dependent on the interaction of EBNA-1 with its cognate DNA recognition sequences. To better understand EBNA-1 function, the DNA-binding domain of EBNA-1 was characterized by deletional analysis and short-term cotransfection. These experiments identified a core DNA-binding domain in the carboxyl-terminal region between amino acids 493 and 584. Transactivation was shown to be more efficient when a nuclear localization signal was present, and the amino acid structure and location of that signal were identified. Additional studies determined that the active DNA-binding form of EBNA-1 was a dimer (21,23).

Another gene expressed in latent B-cells is BNLF-1, which encodes the latent membrane protein (LMP), one of the putative oncogene products of the virus. This gene was expressed from two different enhancer-promoter constructs in transgenic mice to determine its biological activity and possible contribution to oncogenesis. While transgenic mice expressing LMP in many tissues demonstrated poor viability, expression of LMP specifically in the epidermis induced a phenotype of hyperplastic dermatosis. Concomitant with the expression of LMP in this tissue (and in the esophagus) was an induction of the expression of a hyperproliferative keratin, K6, at aberrant locations within the epidermis. The epithelial hyperplastic phenotype caused by the LMP-encoding transgenes implies that the LMP plays a role in the acanthotic condition of the tongue epithelium in the human EBV- and HIV-associated syndrome oral hairy leukoplakia, as well as possibly predisposing the nasopharyngeal epithelium to carcinogenesis (39).

EBV DNA structure and gene expression were analyzed directly in tissue specimens from oral hairy leukoplakia (HLP). EBV actively replicates in these lesions, as shown by the analysis of viral termini, response of the infection to acyclovir, and the detection of masses of viral particles in infected cells. To investigate the strains of EBV present in this permissive infection, variation in the EBNA-2 coding sequences was investigated. Two distinct strains of EBV were identified which were distinguished by highly divergent sequences which encoded the EBNA-2 gene, a nuclear protein required for transformation of B-lymphocytes. The two forms of the protein, known as EBNA-2A or EBNA-2B, differed both in size and structure. Transformation of B-lymphocytes by the 2B variant was less efficient and the cell lines which could be established had a slower growth rate and saturation density. Duplicate Southern blots, prepared with HLP DNA digested with BamHI, were hybridized to equal amounts of probes which distinguish the EBNA-2A and -2B variants. Although the EBNA-2B variant is believed to be less prevalent, the EBNA-2B probe strongly hybridized to multiple BamHI H fragments in all specimens. The detection of EBV-2B strains in all of the HLP specimens suggests that the 2B variant is more common than previously thought and that perhaps 2B variants may be more likely to reactivate and replicate in an immunocompromised host. In most specimens, multiple strains of both the A and B subtype were detected. The strains detected on one side of the tongue could differ from the other and change over time. These data suggest that HLP represents continual infection and superinfection with replication of multiple strains of EBV (54,55).

The incidence and association of EBV with central nervous system lymphomas in AIDS patients has also been examined. Prior studies had reported a 30-50% association of EBV in systemic lymphoma in AIDS, but the assays, which used the EBV BamHI-W internal repeat DNA as a probe, had a poor signal to background ratio. A new non-isotopic in situ assay was developed using a digoxygenin-tagged riboprobe for an EBV-encoded small nuclear RNA (EBER); it gave readings clearly positive or negative, with no ambiguity. From 167 AIDS brain autopsies conducted, 17 cases of CNS lymphoma had been detected. Of the 15 samples available to investigators, all 15 were B-cell lymphomas and all were found to be EBV positive by the new assay, giving a 100% correlation between EBV and CNS lymphomas in AIDS (23).

A model for studying EBV-associated B-cell tumors has been developed. This model uses severe combined immunodeficiency (SCID) mice which are reconstituted by injection with human tonsil cells or with peripheral blood lymphocytes from EBV-seronegative donors. Subsequent injection of EBV results in the rapid development of aggressive, fatal lymphoproliferative disorders of human B-cell origin. These tumors are high-grade lymphoblastic lymphomas and carry the EBV genome. In addition to providing a useful model for studying the progressive changes that occur in B-cells during the process of tumor development, this model is also being used for early studies of development of a potential EBV vaccine, based on the major envelope glycoprotein of EBV, gp350. Mice have recently been injected with lymphocytes from an EBV-negative donor and then immunized with gp350. They will be challenged with EBV to determine if the potential vaccine will block EBV-induced immortalization. Similarly, mice are being injected with lymphocytes from an EBV-positive donor to determine if the vaccine will block the growth of existing immortalized cells (9,10,53).

EBV variants have been isolated from clinical specimens of mucosal tissue from human lesions and healthy individuals. These variants have deletions of the EBNA-2 gene; similar deletions in laboratory strains prevent transformation of lymphocytes. The wild-type deletion mutants were shown to be lytic in epithelial cells and latent in lymphoid cells. A study of one donor couple showed the mutant to be stable over several years, infectious, and transmissible. An EBNA-2 deletion mutant has also been discovered in breast milk and traced to trafficking by latently infected lymphoid cells. Further studies of these and other wild-type mutants from clinical samples of oral mucosa may contribute to our understanding of oncogenesis and host defense mechanisms in nasopharyngeal carcinoma, Burkitt's lymphoma, and in oral hairy leukoplakia in AIDS patients (66,67).

In contrast to EBV, herpes simplex virus (HSV) types 1 and 2 are associated primarily with infectious diseases. While HSV-2 has been suggested as a cofactor in cervical cancer, the primary importance of these herpesviruses to cancer patients is HSV reactivation during immunosuppressive therapy. Such reactivations tend to become disseminated infections and are a significant cause of morbidity and mortality. Thus the mechanisms of HSV infection, maintenance of latency, and reactivation are important to cancer research.

Significant research is being done on the role of HSV glycoproteins in establishing infection. HSV-1 encodes 8 different glycoprotein species, but mutants with 5 of these glycoproteins deleted grew well in a variety of non-polarized cells in culture. The glycoproteins mediate viral attachment to

a receptor on the cell surface. Recent work showed that HSV is different from all other known viruses in that it can attach to two different cellular receptors: glycoprotein C was required for the entry of the virus from the apical surface of polarized MDCK cells, whereas other glycoproteins enabled the virus to attach to a receptor on the basal surface. When the gene encoding glycoprotein C was deleted, the virus could rely on other glycoproteins to enter cells, but by way of a different receptor. Since most cells in the body are polarized, these supplementary glycoproteins would facilitate entry of HSV into cells (59).

However, one HSV glycoprotein, glycoprotein D, showed an inhibitory effect on viral entry into cells. When cells were transformed to express only the HSV gene for glycoprotein D, they were resistant to HSV infection. This was shown to occur at the level of viral penetration into the cells, not at the level of virus adsorption to the cells, and was more pronounced for HSV-1 than HSV-2 strains. To investigate why a virus would evolve a function which blocks superinfection, mutant viruses were selected which overcame this resistance to infection; the mutation mapped to a single amino acid change in glycoprotein D itself. Cells infected with this mutant released 90 percent less virus and the cytoplasm contained many more unenveloped capsids than cells infected with the wild type parent. This suggested that a deficient glycoprotein D allowed the nascent viral capsids to fuse with their intracellular transport vesicles and be released prematurely into the cytoplasm, whereas native glycoprotein D allowed the transport vesicles containing virus to be transported intact to the cell membrane (59,68).

Research on HSV genes has suggested some promising lines of therapeutic research. One recent discovery is of a protease encoded by HSV and used to cleave a capsid protein related to the packaging of DNA into capsids. This protease will become a major target in antiviral drug development. Another is the construction of two genetically engineered HSV strains as models for HSV vaccines. The strains R7017 and R7020 were tested in owl monkeys previously shown to model herpetic diseases of immunocompromised patients and neonates. In contrast to the lethal disease seen in monkeys receiving 100-1,000 plaque-forming units (pfu) of wild-type virus, inoculation of $\geq 10^6$ pfu of recombinant viruses produced local lesions and viral shedding but not disseminated disease. Latent recombinant viruses were recovered from some ganglia innervating the sites of inoculation. Immunosuppression by total lymphoid irradiation did not significantly alter the tolerance to R7020 when applied before inoculation and did not cause clinical reactivation when applied to latently infected animals. Moreover, the virus was not transmitted from immunosuppressed infected females to normal male cage mates (59).

Adenoviruses

Adenoviruses are a group of medium sized DNA viruses whose oncogenicity has been demonstrated by their ability to transform epithelial cells in vitro and to induce tumors in newborn rodents in vivo. Human adenovirus type 9 (Ad9) is a member of the subgroup D adenoviruses, which were classified as non-oncogenic since they do not induce tumors following injection into newborn hamsters, but have subsequently been found to transform established rat fibroblast cell lines and to cause mammary tumors in female rats following subcutaneous inoculation in newborn rats. Ad9 possesses several interesting traits which set it apart from other oncogenic adenoviruses. First, a rodent

infected with an oncogenic human adenovirus such as adenovirus type 12 generally develops a sarcomatous tumor at the site of virus injection; Ad9 deviates from this principle by targeting exclusively breast tissue for tumorigenesis. Second, the breast tumors elicited by Ad9 require estrogen for development and maintenance; male rats do not develop tumors following injection with Ad9 unless they have been castrated and injected repeatedly with high doses of estrogen. Finally, Ad9 does not appear able to transform primary rodent cells in culture. The underlying cause for these unusual distinctions and the fact that Ad9 causes a breast tumor (fibroadenoma) in an animal model system that is common in women make it an attractive virus for tumor studies. Recent studies with this system have provided several new observations: (a) these breast tumors fall into three general histological categories -- fibroadenoma, phyllodes-like tumor, and solid sarcoma; (b) they are derived from mammary fibroblasts (benign tumors) or myoepithelial cells (malignant tumors) and do not change type with time; (c) they require estrogen for both initiation and maintenance; (d) tumor cells contain single or multiple integrated copies of the entire Ad9 viral genome; and (e) the tumor cells express detectable messenger RNAs for estrogen receptors, Ad9 E1a, and E4 genes, but not those for E1b or E3 genes. Studies are underway to test the possibility that a cellular oncogene contributes to Ad9 tumorigenesis (56,65).

Products of the adenovirus E1A gene can act synergistically with cAMP to activate transcription of several viral early genes and the cellular oncogene c-fos. Transcription factor AP-1 binding activity is also induced by the combined action of E1A and cAMP. Extensive analysis of E1A has demonstrated that this region contains genetic information necessary for transformation and also plays an important role in the replicative cycle of the virus where it serves as a regulator of viral and host cell gene transcription. There are two major E1A mRNAs, coding proteins of 243 and 289 amino acids which differ only by an internal 46 amino acid region that is unique to the 289 amino acid protein and is required for transcriptional activation. In contrast, significant induction of c-fos mRNA and AP-1 binding activity was observed in cells expressing either of these E1A proteins. These data suggest that both of these E1A proteins interact functionally with the cAMP signaling system to activate transcription of a cellular gene and AP-1 binding activity, and that the mechanism of this process is probably different from the mechanism of transcriptional activation of viral genes (65).

Group C adenoviruses encode a 14.7-kilodalton protein (14.7K protein) which has been shown to protect virus-infected fibroblasts from lysis by tumor necrosis factor (TNF). TNF is a cytokine which initially was shown to cause hemorrhagic necrosis of mouse sarcomas but recently has been demonstrated to have antiviral properties, preventing the replication of both DNA and RNA viruses and selectively lysing cells infected with pathogenic viruses, including adenovirus. Cells infected with group C adenoviruses that produce the 14.7K protein are not lysed by TNF, whereas cells infected with mutant viruses lacking the 14.7K protein are lysed. It has now been shown that adenoviruses of other groups are also protected from TNF-induced cytolysis.

Representative serotypes of groups A, B, D, and E produce a protein analogous to the 14.7K protein, as determined by deletion mutants and precipitation by antiserum to the 14.7K protein, and were protected from lysis by TNF. Although all known adenovirus serotypes infect epithelial cells, adenoviruses cause several diseases with various degrees of pathogenesis. This new finding

suggests that the 14.7K protein provides a function required for the in vivo cytotoxicity of many adenoviruses independent of the site of infection or degree of pathogenesis (16,85).

In summary, several new aspects of virus-host interactions in herpesviruses and adenovirus oncogenesis have been identified. In AIDS, less common variants of EBV have been shown to be associated with oral hairy leukoplakia, and a new assay has shown close correlation of EBV with CNS lymphomas. New data on a rat mammary tumor caused by an adenovirus suggests it may be a useful model for human mammary tumor studies. Finally, tumor vaccine studies appear more promising with the development of genetically engineered strains of HSV which appear protective in monkeys and the use of SCID mice as a model to test EBV envelope glycoproteins as vaccines.

DNA VIRUS STUDIES I

GRANTS ACTIVE DURING FY91

Investigator/Institution/Grant Number	Title
1. AURELIAN, Laure University of Maryland 2 RO1 CA 39691-04A1	Protein Kinase Activity and HSV-2 Transforming Potential
2. BABISS, Lee E. Rockefeller University 5 R29 CA 48707-03	Transformation Progression and Adenovirus 5 Gene Regulation
3. BERK, Arnold J. University of California (Los Angeles) 5 R37 CA 25235-13	Biosynthesis of Adenovirus Early RNAs
4. CALNEK, Bruce W. Cornell University (Ithaca) 5 RO1 CA 06709-28	Studies on the Avian Leukosis Complex
5. CARLIN, Cathleen R. St. Louis University 5 RO1 CA 49540-04	EGF Receptor Down-Regulation by Adenovirus
6. CHANG, Robert S. University of California (Davis) 5 RO1 CA 43051-03	Epstein-Barr Virus and Nasopharyngeal Carcinoma
7. CHINNADURAI, Govindaswamy St. Louis University 5 RO1 CA 31719-10	Genetic Analysis of Adenovirus 2 Early Genes
8. CHINNADURAI, Govindaswamy St. Louis University 2 RO1 CA 33616-12	Adenovirus LP Locus: Role in Oncogenic Transformation
9. COOPER, Neil R. Scripps Clinic and Research Foundation 5 RO1 CA 14692-18	Humoral Immunity to Viruses and Virus-Infected Cells
10. COOPER, Neil R. Scripps Clinic and Research Foundation 5 RO1 CA 52241-02	A Model for Epstein-Barr Virus Induced Lymphoma

11.	COURTNEY, Richard J. Louisiana State University Medical Center (Shreveport) 5 R01 CA 42460-07	Studies of Purified Herpes Simplex Virus Glycoproteins
12.	DESROSIERS, Ronald C. Harvard University 5 R01 CA 31363-10	Molecular Basis for Herpesvirus Saimiri Oncogenicity
13.	GALLOWAY, Denise A. Fred Hutchinson Cancer Research Center 5 R01 CA 26001-13	Herpesvirus Expression in Transformation and Latency
14.	GALLOWAY, Denise A. Fred Hutchinson Cancer Research Center 5 R01 CA 35568-08	Molecular Studies on Herpes- and Papillomavirus Proteins
15.	GAYNOR, Richard B. University of California (Los Angeles) 5 R01 CA 30981-10	Transcriptional Regulation by the Adenovirus E1A Protein
16.	GOODING, Linda R. Emory University 5 R01 CA 48219-04	Mechanism of Adenovirus-Induced TNF Resistance
17.	GOULIAN, Mehran University of California (San Diego) 2 R01 CA 11705-21A1	DNA Synthesis Studies
18.	GREEN, Maurice St. Louis University 2 R01 CA 29561-33A1	Biochemistry of Animal Virus Multiplication
19.	HARDWICK, Jan Marie Johns Hopkins University 5 R01 CA 43532-05	Epstein-Barr Virus: Regulation of Gene Functions
20.	HAYWARD, Gary S. Johns Hopkins University 5 R37 CA 22130-14	Structure and Regulation of Human Herpesvirus Genomes
21.	HAYWARD, Gary S. Johns Hopkins University 5 R01 CA 28473-11	Cellular Transformation by DNA of Human Herpesvirus
22.	HAYWARD, S. Diane Johns Hopkins University 5 R37 CA 30356-10	EBV Genome Expression: Localization of Specific Functions

23.	HAYWARD, S. Diane Johns Hopkins University 5 RO1 CA 42245-06	Regulation of Replication and Latency by EBV EBNA 1
24.	HOLMES, Edward W., Jr. Duke University 5 RO1 CA 47631-04	Retroviral Anti-Sense RNA: Cellular and Viral Responses
25.	HORWITZ, Marshall S. Yeshiva University 5 RO1 CA 11512-22	Adenovirus DNA Synthesis and Polypeptide Assembly
26.	HOWE, John G. Yale University 5 R29 CA 52754-02	Immortalization of B Lymphocytes by Epstein-Barr Virus
27.	HUANG, Eng-Shang University of North Carolina (Chapel Hill) 5 RO1 CA 21773-12	Cytomegaloviruses and Human Malignancy
28.	ISOM, Harriet C. Pennsylvania State University Hershey Medical Center 5 RO1 CA 23931-14	Regulation of Differentiation in Hepatocytes in Vitro
29.	IZANT, Jonathan G. Yale University 5 RO1 CA 47629-03	Enhancement and Modulation of Anti-Sense RNA Activity
30.	JARIWALLA, Raxit J. Linus Pauling Institute 5 RO1 CA 42467-03	Role of Transforming HSV-2 DNA Sequences
31.	JONES, Clinton J. University of Nebraska (Lincoln) 5 R29 CA 47872-05	Mechanistic Approaches to HSV-2 Induced Transformation
32.	KIEFF, Elliott D. Brigham & Women's Hospital (Boston) 5 R35 CA 47006-05	Molecular Biology of Epstein-Barr Virus Infection
33.	KLEIN, George Karolinska Instituet 5 RO1 CA 28380-09	EBNA and Other Viral Products in EBV Transformed Cells
34.	KLEIN, George Karolinska Instituet 2 RO1 CA 30264-10	Immune Effector Mechanisms in EBV-Carrying Patients

35. KLEIN, George
Karolinska Instituet
5 R01 CA 52225-02
EB-Viral Strategies in Latency Transformation and Immune Escape

36. KNIPE, David M.
Harvard University
5 R37 CA 26345-12
Genetics of Herpesvirus Transformation

37. KNUDSON, Jr., Alfred G.
American Association for Cancer Research
1 R13 CA 54994-01
Developmental Genetics of Childhood Cancer

38. LACY, Jill
Yale University
5 R29 CA 51449-02
Epstein-Barr Virus Transformation: Study of EBV-Induced Genes

39. LEVINE, Arnold J.
Princeton University
5 R01 CA 49271-03
The Proteins and Gene Functions of Epstein-Barr Virus

40. LUCHER, Lynne A.
Illinois State University
1 R15 CA 52091-01A1
Host Species Influence on Adenovirus Replication

41. MARTIN, Terence E.
University of Chicago
5 R01 CA 48189-03
Effects of HSV on Nuclear Structure and mRNA Processing

42. MATHEWS, Michael B.
Cold Spring Harbor Laboratory
5 P01 CA 13106-20
Cold Spring Harbor Laboratory Cancer Research Center

43. MEDVECZKY, Peter G.
University of South Florida
3 R01 CA 43264-06S1
Growth Factors and Herpesvirus Saimiri Induced Lymphomas

44. MILLER, I. George, Jr.
Yale University
5 R37 CA 12055-20
Studies of Epstein-Barr Virus

45. MILLER, I. George, Jr.
Yale University
5 R01 CA 52228-02
Mutants of Epstein-Barr Virus

46. MORAN, Elizabeth
Cold Spring Harbor Laboratory
1 R01 CA 53592-01A1
Protein Interactions and Repression Fuction of the E1A

47. MORAN, Elizabeth
Cold Spring Harbor Laboratory
1 R01 CA 55330-01
Cell Growth Control Functions of the E1A Oncogene

48. MOSS, Denis J.
Queensland Institute of Medical Research
(Brisbane)
5 R01 CA 52250-02
Role of T-Cell Epitopes in Tumor Surveillance

49. NEMEROW, Glen R.
Scripps Clinic and Research Foundation
5 R01 CA 36204-07
Infection of B Lymphocytes by Epstein-Barr Virus

50. NONOYAMA, Meihan
Tampa Bay Research Institute
10900 Roosevelt Boulevard
2 R01 CA 31949-08A3
Marek's Disease Transformation and Oncogenesis

51. O'DONNELL, Michael E.
Cornell University
Medical College
1 R01 CA 53525-01
Biochemical Action of EBNA1 on EBV Replication Origin

52. PADMANABHAN, Radha K.
University of Kansas
Medical Center
5 R01 CA 33099-07
Structure and Functional Analysis of Adenovirus Genomes

53. PAGANO, Joseph S.
University of North Carolina
(Chapel Hill)
5 P01 CA 19014-14
Viral Oncogenesis and Latency

54. PEARSON, Gary R.
Georgetown University
5 R01 CA 39617-08
Epstein-Barr Virus-Specific Antigens

55. RAAB-TRAUB, Nancy J.
University of North Carolina
(Chapel Hill)
5 R01 CA 32979-07
EBV Expression in Nasopharyngeal Carcinoma

56. RAAB-TRAUB, Nancy J.
University of North Carolina
(Chapel Hill)
5 R01 CA 52406-02
AIDS-Associated Leukoplakia: The Role of Epstein-Barr Virus

57. RASKA, Karel, Jr.
Robert Wood Johnson Medical School
(Piscataway)
5 R01 CA 21196-13
Adenovirus T and Suface Antigens and Tumorigenicity

58. RAYCHAUDHURI, Pradip
University of Illinois
1 R01 CA 55279-01
Transcription Control by the Nuclear Oncoprotein, E1A

59.	RICCIARDI, Robert P. Wistar Institute of Anatomy and Biology 5 RO1 CA 29797-10	Organization and Expression of Adenovirus Genes
60.	ROIZMAN, Bernard University of Chicago 5 R35 CA 47451-04	Molecular Biology of Herpes Simplex Viruses
61.	ROSENTHAL, Leonard J. Georgetown University Medical Center 5 RO1 CA 37259-05	Transformation by Human Cytomegalovirus Strain Towne mtrII and mtrIII
62.	ROSENTHAL, Leonard J. Georgetown University Medical Center 1 R13 CA 52490-01	15TH International Herpesvirus Workshop
63.	ROUHANDEH, H. Southern Illinois University 5 RO1 CA 38678-03	Transforming Sequences of Yaba Virus DNA
64.	SCHAFFER, Priscilla A. Dana-Farber Cancer Institute 5 R37 CA 20260-15	Immediate-Early Genes of HSV
65.	SCHNEIDER, Robert J. New York University Medical Center 5 RO1 CA 42357-05	Translational Regulation of Adenovirus Gene Expression
66.	SHENK, Thomas E. Princeton University 5 R37 CA 38965-07	Structure and Function of DNA Tumor Virus Genomes
67.	SIXBEY, John W. St. Jude Children's Research Hospital 2 RO1 CA 38877-07A1	Epstein-Barr Virus Expression in Normal Human Epithelium
68.	SIXBEY, John W. St. Jude Children's Research Hospital 5 RO1 CA 52258-02	Epstein-Barr Virus Infection of Mucosal Epithelium
69.	SPEAR, Patricia G. Northwestern University (Chicago) 5 R37 CA 21776-15	Herpesvirus Gene Expression in Transformed Cells
70.	SPECK, Samuel H. Dana-Farber Cancer Institute 5 RO1 CA 43143-06	Viral Transcription in EBV Transformed Human B-Cells

71.	SPECK, Samuel H. Dana-Farber Cancer Institute 5 RO1 CA 52004-02	Control of Epstein-Barr Virus Lytic Gene Expression During Latency
72.	SPECTOR, Deborah H. University of California (San Diego) 5 RO1 CA 34729-08	Human CMV, Cell-Related DNA, Oncogenes and Kaposi's Sarcoma
73.	STRAIR, Roger K. Yale University 5 R29 CA 49047-04	Isolation of a Human "E1A-Like" Factor
74.	TANAKA, Akiko Tampa Bay Research Institute 5 RO1 CA 50523-03	Marek's Disease Virus: Analysis of Latent Genes
75.	TEVETHIA, Satvir Pennsylvania State University Hershey Medical Center 5 PO1 CA 27503-12	DNA Viruses and Neoplasia
76.	THORLEY-LAWSON, David A. Tufts University 5 R37 CA 31893-10	Epstein-Barr Virus Membrane Antigen
77.	TIBBETTS, Clark J. Vanderbilt University 5 RO1 CA 34126-09	Adenovirus Genome Expression: Physical Mapping Studies
78.	VELICER, Leland F. Michigan State University (East Lansing) 5 RO1 CA 45479-03	Oncogenic Herpesvirus Secretory Glycoprotein Analysis
79.	WAGNER, Edward K. University of California (Irvine) 5 R37 CA 11861-22	Control of Viral RNA Synthesis in Herpesvirus Infection
80.	WANG, Frederick C.S. Brigham and Women's Hospital (Boston) 1 RO1 CA 52244-02	Molecular Genetic Analysis of Epstein-Barr Virus Growth Transformation
81.	WEISSMAN, Sherman Yale University 5 PO1 CA 16038-18	Program on the Molecular Basis of Viral Transformation
82.	WHITE, Eileen Rutgers University 1 RO1 CA 53370-01	Function of the Adenovirus E1B Oncogene

83.	WIGDAHL, Brian Pennsylvania State University Hershey Medical Center 5 R01 CA 34479-08	Latency and Transformation by Herpesviruses
84.	WILLIAMS, James F. Carnegie-Mellon University 5 R01 CA 21375-14	Genetic Analysis of Adenoviruses
85.	WILLIAMS, James F. Carnegie-Mellon University 5 R01 CA 32940-10	Type 12 Adenovirus Transformation-Defective Mutants
86.	WOLD, William S. St. Louis University 5 R01 CA 24710-13	Adenovirus 2 Coded Early Glycoprotein
87.	YATES, John L. Roswell Park Memorial Institute (Buffalo) 5 R01 CA 43122-05	The Functions of Epstein-Barr Virus Nuclear Antigen 1

SUMMARY REPORT

DNA VIRUS STUDIES II

The DNA Virus Studies II component of the Branch involves the investigation of the two major groups of mammalian small DNA tumor viruses: papillomaviruses and polyomaviruses. In the component, there are 98 research grants with an estimated total funding of 21.16 million dollars. These include traditional research grants (R01), program project grants (P01), conference grants (R13), outstanding investigator grants (R35), the method to extend research in time (MERIT) awards (R37), first independent research support and transition (FIRST) awards (R29), and Phase I small business innovative research (SBIR) grants (R43). The major research emphasis of this branch 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 determination of the role of these viruses in the etiology of human cancers. In terms of scientific areas, 27% deal with the structure and expression of viral genes; 28% deal with the biochemical properties and mechanisms of action of viral proteins; 27% deal with virus-host interactions; and 18% deal with the expression and function of cellular genes, including known proto-oncogenes that are involved in the transformation process. In terms of the viruses being studied, 33% concern human or animal papillomaviruses; 37% of the grants involve the simian virus 40 (SV40); 25% relate to human or mouse polyomaviruses; and 5% deal with viral or cellular oncogenes. Representative studies involving these groups of viruses are described below.

Papillomaviruses

Human papillomaviruses have been implicated in a variety of anogenital cancers. A specific subset of HPV DNAs are detected in approximately 90% of anogenital cancers and the viral genomes are integrated into host DNAs and are transcriptionally active in both tumors and cell lines. HPV DNAs detected in genital carcinomas induce immortality in cultures of human foreskin and cervical epithelial cells, but the immortal cells are not tumorigenic when transplanted into athymic mice. Both epidemiological and experimental studies suggest that HPVs are necessary but not sufficient for the development of genital cancers, and, therefore, additional factors are required for the progression to malignancy. One mechanism by which HPVs might contribute to the development of cancer is by altering the normal pattern of squamous differentiation in target cells of the genital tract. The epithelium that lines the genital tract consists of normally stratified squamous epithelium formed by continuous movement of cells from the basal to superficial layers, cell flattening, and expression of genes for structural proteins. Cancer is preceded by dysplastic alterations in normal squamous differentiation termed intraepithelial neoplasia. Such dysplastic lesions may persist, undergo regression, or progress in severity to an invasive carcinoma; therefore, intraepithelial neoplasia is a precancerous change with the potential for malignant progression. A subset of HPV types are detected in the majority of anogenital intraepithelial neoplasia, which suggests that these viruses contribute to dysplastic differentiation. The effect of HPV on squamous differentiation, normal human cervical and foreskin epithelial cells and cells immortalized by recombinant HPV DNAs were transplanted beneath a skin-muscle

flap in athymic mice. Xenografts containing normal cells formed well-differentiated stratified squamous epithelia 2 to 3 weeks after transplantation, but cell lines immortalized by four HPV types (HPV 16, HPV 18, HPV 31 and HPV 33) detected in anogenital cancer exhibited dysplastic morphology and molecular alterations in gene expression characteristic of intraepithelial neoplasia. Morphological alterations were accompanied by delayed commitment to terminal differentiation. Alterations in the pattern of HPV 18-immortalized cells developed dysplastic changes more rapidly than cells immortalized by HPV 16 DNA. These results suggest that a possible role of HPVs in the multistage carcinogenesis process is to immortalize a subpopulation of cells which irreversibly lose the ability to undergo terminal differentiation and, thus, are at increased risk for neoplastic progression (87).

Transformation to a dysplastic state occurs only in tissue levels that are still competent for cell division, the basal and parabasal cells, when a rare event occurs that disrupts the block to viral transcription and allows the high level transcription of the viral transforming genes E6 and E7. The most common lesion that can remove this transcription block appears to be integration of the viral DNA into the host genome in a manner that physically disrupts the E2 gene, thus preventing the production of an inhibitory protein encoded by it. The degree of cellular alterations seen in HPV-infected tissue varies from benign dysplastic disease, through three increasingly abnormal pathological stages (designated cervical intraepithelial neoplasia I (CIN), CIN II, and CIN III), to invasive carcinoma, depending on the extent to which the normal pattern of differentiation has been disrupted.

Progress this year has been made in elucidating the structure and function of HPV gene products including E6 and E7 proteins. Following their earlier findings of a strong transforming function associated with the HPV 18 E7 gene product, investigators examined the ability of the intact E6-E7 region of HPV 18, as well as individual E6 and E7 gene products, to immortalize human keratinocytes and alter their differentiation in vitro. They found that expression of the E6 and E7 open reading frames, under the control of its homologous promoter, resulted in high-frequency immortalization. Furthermore, by using a system that allowed for stratification of keratinocytes in vitro (a collagen matrix system), they observed that the morphological differentiation of those E6-E7 immortalized cells was altered such that parabasal cells extended throughout most of the epithelium, with abnormal nuclei present at the upper region. Examination of E6-E7-expressing cell lines in the collagen matrix at a later passage revealed that complete loss of morphological differentiation had occurred. E7 alone was a much less effective immortalizing agent than E6 and E7 together and acted only minimally to offer morphological differentiation in vitro. No such activities were found for E6 alone. High-frequency transformation of human epithelial cells thus appears to require expression of both E6 and E7 gene products (51).

To study the interaction of these transforming gene products and cellular factors in the transformation process, a series of protein binding experiments were conducted with HPV E6 and E7 in a mouse cell system that contains the wild-type tumor suppressor p53 protein. As in earlier studies, the HPV 16 E7 protein was capable of binding to the Rb protein, but did not bind to p53. When HPV 16 E6 was found to bind to p53, the investigators went on to determine if other papillomaviruses associated with genital tract lesions

could also form a complex with p53. Using a mix of E6 proteins from several HPV types and bovine papillomavirus type 1 (BPV-1), they determined that no HPV 6, HPV 11, or BPV-1 could be detected in complex with p53, and that HPV 18 was found at levels that were 50% of that of HPV 16 E6. These findings correlated with the in vivo clinical behavior and the in vitro transforming activity of these different papillomaviruses. Since the wild-type p53 protein has tumor suppressor properties and has also been found in association with large T- antigen and the E1B 55-kilodalton protein in cells transformed by SV40 and by adenovirus type 5, respectively, the investigators believe that this evidence indicates that the human papillomaviruses, the adenoviruses, and SV40 may effect similar pathways in transformation (81).

It has not yet been possible to develop a simple tissue culture system for HPV replication. Because of this difficulty, a number of investigators have used bovine papillomavirus (BPV) and cottontail rabbit papillomavirus (CRPV) as model systems in intact animals.

The bovine papillomavirus model has been useful in studying early events in the transformation process since the virus can stably transform fibroblasts in culture and establish its genome as a nuclear plasmid without integration. Under controlled conditions of passage and temperature, the viral DNA replicates as a stable circular episome replicating in synchrony with the cellular DNA. Utilizing this fibroblast culture system, investigators have been able to identify a series of BPV-1 genes and their functions. The E6 and E5 open reading frames (ORFs) produce proteins with known oncogenic properties, while the E1 ORF encodes for proteins which are important for replication and the E2 ORF encodes for a family of site-specific DNA binding proteins that affect both transcription control and replication. Progress has been made in defining both the gene products and their roles in replication. The E1 ORF is the largest of BPV-1, and has had multiple functions and protein products ascribed to it, with the most recent work showing two gene products to be present in transformed cells, with apparent molecular masses of 23 kDa and 68 kDa, respectively. The E2 proteins include a 48 kDa transactivator protein encoded by the entire ORF and two forms of a transrepressor (E2-TR and E8/E2), which may be independently expressed and result in either repression and/or activation by an as yet undefined mechanism. A further step in understanding the function of these gene products was the demonstration that the 68 kDa E1 protein forms a complex with the 48 kDa E2 transcription factor. This complex bound specifically to the viral origin of replication, which contains multiple binding sites for E2. Repressor proteins (E2-TR and E8/E2) encoded by the E2 open reading frame failed to complex with E1, suggesting that a site-specific region of E2 that participates in transactivation contained critical determinants for interaction with E1. The physical association between a replication protein and a transcriptional factor suggests that transcriptional activator proteins may function in targeting replication initiator proteins to their respective origins of replication (7, 8).

Cottontail rabbit papillomavirus (CRPV) induced benign tumors (papillomas) in rabbits which progress at a high frequency to cancers. There are important similarities between CRPV and human papillomaviruses associated with cancer development. Virus infection does not result in cancer directly. The malignancies only develop after a considerable period of time--months in rabbits and years in humans. In rabbits it has been directly shown that very

low levels of carcinogens which will not cause cancer on their own will result in cancers when virus is present. Indirect evidence in humans suggests that virus and co-carcinogens are also essential for cancer development. Some aspects of papillomavirus cell interaction can be studied in tissue culture. Along this line, investigators have developed a system to characterize potential viral transforming genes. A critical step here was the identification of sequences in the late region of the CRPV genome which are inhibitory to transformation. Taking this finding into account, they have identified three early (non structural) proteins which act as viral oncoproteins. Two of these proteins are products of the E6 gene. There is a protein representing the entire E6 protein or long E6 and a second protein designated short E6. These proteins have very different properties including the degree of phosphorylation and localization within the cell which suggests different functions. The third early protein they identified was the E7 protein. Since both E6 and E7 proteins have been identified in other cancer lines, the next step was to determine their mechanism of action in the CRPV system. This included investigation of interactions with cellular proteins, of complementation with known oncogenes and of effects of oncoproteins on cellular growth properties. A corroboration of tissue culture findings in whole animals is absolutely necessary. Toward this goal, they have been able to induce tumors in animals with viral DNA--an essential development, since papillomaviruses cannot be grown in tissue culture. Experiments along this line have shown that with in vivo systems in animals, the cooperation of all oncoproteins is required for papilloma formation (95,96).

Simian Virus 40 (SV40)

SV40 is a small DNA tumor virus which was isolated from monkeys and quickly became a convenient model system to study viral transformation of cells in culture and tumorigenesis in susceptible rodents. Previous research has 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 host cell. The polyomavirus group includes the mouse polyomavirus, which has served as an in vivo and in vitro model for virus transformation, and the human polyomaviruses, JC and BK. Polyomaviruses have many similarities with SV40. The SV40 and polyomavirus virions are morphologically identical and contain nearly the same amount of DNA. However, the viral genomes are organized differently, particularly with respect to the early gene region which encodes the transformation function. SV40 DNA encodes two tumor antigens, large T-antigen and small t-antigen. By contrast, polyomavirus DNA codes for three tumor antigens, large T-antigen, middle T-antigen, and small t-antigen. The relationship among the polyomavirus tumor antigens with respect 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 the middle T-antigen (which becomes membrane bound) is able to induce the transformed phenotype in previously immortalized cell lines.

Simian virus 40 (SV40) induces tumors in animals and transforms a variety of cell types in culture. Evidence from a number of sources has demonstrated that the viral early region which encodes the large and small tumor antigens carries the tumorigenic potential of the virus. In most cases, large T-antigen alone is capable of full transformation, with small t-antigen playing an ancillary role in some cell types or under certain assay conditions. T-

antigen is a multifunctional protein, consisting of a single polypeptide chain carrying multiple biochemical activities that act alone or in a concerted manner to control various aspects of viral infection and cellular behavior. The molecular mechanisms by which T-antigen induces cellular transformation are not known. However, two cellular proteins, the retinoblastoma (Rb) susceptibility gene product (p105) and p53, both with suspected roles as tumor suppressors, are known to complex with T-antigen. Mutants that produce T-antigens unable to form a complex with one or the other of these proteins are at least partially defective for transformation. In addition, T-antigen encodes an activity or activities capable of transactivating several viral and cellular promoters. Thus, T-antigen acts on multiple cellular targets to induce a variety of changes in cellular properties. Mutational analysis has revealed that a biochemical activity residing within the amino-terminal 121 amino acids of T-antigen is sufficient to induce the transformation of some cell lines, such as C3H10T1/2. The same domain of the molecule also encodes the transactivation function of T-antigen and the ability to complex with the Rb susceptibility gene product. However, the transformation of other cell lines, such as REF52, requires an additional activity that is affected by mutations in other portions of the molecule. It is therefore possible that more than two of the multiple activities of T-antigen may be involved in conferring the transformed phenotype (69).

Accounting the multiple functions of large T-antigen might be accomplished by assigning different functions to different domains of the molecule. Through the used of SV40 deletion and point mutations within the large T coding region, it has been possible to identify functional domains. The replication and transformation functions can be genetically separated, while other regions have been identified as relating to transport of T-antigen into the nucleus of a cell, binding to SV40 origin of replication, stimulation of cellular gene expression and ATPase activity. While attempting to correlate the ability of a set of seventeen SV40 large T-antigen point mutants to transform with the ability to replicate, bind the SV40 origin and bind p53, investigators found that all the mutants bound to p53 regardless of their ability to transform rat cells in culture. They also found that the ratio of p53 to large T did not correlate with transforming ability. While there is a body of evidence that indicates that the binding of large T-antigen to the proto-oncogene product p53 plays a critical role in transformation by SV40, these investigators concluded that transformation requires additional interactions with other as yet unidentified cellular factors (42).

Other work to define the molecular mechanisms by which SV40 T-antigen interacts with host cell proteins in the process of oncogenesis involves the use of temperature sensitive mutant forms of SV40 (tsA). These mutant forms allow viral replication in permissive murine cells at a permissive temperature, but fail to do so at a higher temperature. Cloning and sequencing of all known tsA mutants has revealed that the seventeen independently isolated mutants represented only eight distinct genotypes. The clustering of tsA mutations in a few "hot spots" in the amino acid sequence of T-antigen and the phenotypes of the mutants strongly suggests that those amino acids play crucial roles in organizing the structure of one or more functional domains. Most of the mutations were located in the highly conserved region of T-antigen that correlate with DNA binding, protein-protein interactions, or ATP binding. Seven of the eight groups of tsA mutants map within the p53 domain of T-antigen, while all of these mutants are temperature-sensitive for

transformation. Improved definition of the tsA mutants will allow more precise investigations of the relationships between the various domains of T-antigen and specific functions in DNA replication (88,89).

The recognition that the retinoblastoma (Rb) suppressor gene product binds to the large T-antigens of SV40, mouse polyomavirus, hamster polyomavirus, human JC virus and human BK virus, the E7 oncoprotein of human papillomaviruses and to E1A oncoprotein of adenoviruses suggests that this interaction is involved in transformation by these DNA tumor viruses and that there is a commonality of transformation mechanisms among several groups of small DNA tumor viruses.

An RFA entitled, "New Approaches to Understanding Transformation by SV40 Virus, Polyomavirus and Adenoviruses" was issued on February 16, 1990 with an application receipt date of August 24, 1990. A total of 17 applications were received, and reviewed by a Division of Extramural Activities Special Review Committee. Based on the scientific merit, program relevance and responsiveness to the RFA, four applications were funded. The four grants are a diverse mix, with one to investigate the mechanism of action of small t-(tAg) antigen in transformation by SV40, and how this binding relates to transactivation by tAg. Another will examine the mechanism of E1A-mediated enhancer repression and the potential linkage of this function to stimulation of cellular DNA synthesis and immortalization of transformation, both of which map to overlapping regions of the gene for the E1A protein. A third grantee will study the binding of the adenovirus E1A protein to the p300 cellular protein using virus genetics and peptide binding/blocking experiments. The fourth investigator has developed a novel genetic system in yeast for detecting the interaction of two proteins and will apply this promising approach to identification of cellular proteins that interact with various viral oncoproteins.

DNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY91

Investigator/Institution/Grant Number	Title
1. ALWINE, James C. University of Pennsylvania 5 R01 CA 28379-11	Regulation of DNA Tumor Virus Gene Expression
2. ANDROPHY, Elliot J. New England Medical Center Hospital 5 R01 CA 44174-05	Characterization of Papillomavirus E6 Proteins
3. BASILICO, Claudio New York University 5 P01 CA 16239-17	Biosynthesis in Normal and Virus Transformed Cells
4. BASILICO, Claudio New York University 5 R35 CA 42568-06	Viral and Cellular Gene Expression and Growth Regulation
5. BECKMANN, Anna M. Fred Hutchinson Cancer Research Center 5 R01 CA 47619-03	Pathobiology of Anogenital HPV Infection
6. BENJAMIN, Thomas L. Harvard Medical School 5 R35 CA 44343-05	Natural and Unnatural Roles of the Polyoma HR-T Gene
7. BOTCHAN, Michael R. University of California (Berkeley) 5 R37 CA 30490-11	Regulatory Interactions Between Tumor Viruses and Cells
8. BOTCHAN, Michael R. University of California (Berkeley) 5 R01 CA 42414-06	Bovine Papillomavirus - Model Systems
9. BRADLEY, Margaret K. Dana-Farber Cancer Institute 5 R01 CA 38069-06	Nucleotide Binding Properties of SV40 Large T Protein
10. BRAUN, Lundy A. Brown University 5 R29 CA 46617-04	Oncogenes and Growth Factors in Human Gynecologic Cancers

11.	BUTEL, Janet S. Baylor College of Medicine 5 RO1 CA 22555-14	Biological Properties of SV40 Early Proteins
12.	BUTEL, Janet S. Baylor College of Medicine 5 RO1 CA 25215-13	Tumor Viruses, Oncogenes and Mammary Epithelial Cells
13.	CARMICHAEL, Gordon G. University of Connecticut Health Center 5 RO1 CA 45382-05	Processing and Function of Polyoma RNA
14.	CARROLL, Robert B. New York University 5 RO1 CA 20802-15	Biochemical and Functional Properties of the SV40 T-Antigens
15.	CHERINGTON, Van Tufts University School of Medicine 5 R29 CA 44761-03	An Oncogene Sensitive Regulatory Event of Cellular Differentiation
16.	CHOW, Louise T. University of Rochester 5 RO1 CA 36200-08	Human Papillomavirus Gene Expression
17.	COLE, Charles N. Dartmouth College 5 RO1 CA 39259-07	The Molecular Biology of SV40 Large T-Antigen
18.	CONRAD, Susan E. Michigan State University 5 RO1 CA 37144-06	SV40-Induced Changes of Growth Regulation in Host Cells
19.	CONSIGLI, Richard A. Kansas State University 5 RO1 CA 07139-28	Studies in Polyoma Transformed Cells: Virion Proteins
20.	COWSERT, Lex M. ISIS Pharmaceuticals 1 R43 CA 52391-01	Oligonucleonotides as Inhibitors of Papillomavirus
21.	CRUM, Christopher P. University of Virginia (Charlottesville) 5 RO1 CA 47676-04	Pathology of Cervical Intraepithelial Neoplasia
22.	DAS, Gokul C. University of Texas Health Center at Tyler 5 R29 CA 47611-05	Regulation of Transcription in Polyoma Virus

23. DE BRITTON, Rosa C.
Gorgas Memorial Institute
of Tropical Medicine, Inc.
5 R01 CA 42042-05
Human Papillomavirus and Cervical Cancer in Panama

24. DIMAIO, Daniel C.
Yale University
5 R01 CA 37157-08
Analysis of Cell Transformation by Bovine Papillomavirus

25. ECKHART, Walter
Salk Institute for Biological Studies
5 R37 CA 13884-19
Viral Gene Functions and Regulation of Cell Growth

26. FARAS, Anthony J.
University of Minnesota
(St. Paul)
5 R01 CA 25462-12
Human Papillomaviruses and Malignant Diseases

27. FIELDS, Stanley
Research Foundation
of SUNY
1 R01 CA 54699-01
Yeast System to Detect Oncoprotein-Associated Proteins

28. FLUCK, Michele M.
Michigan State University
5 R01 CA 29270-10
Studies of the Integration of the Polyoma Virus Genome

29. FOLK, William R.
University of Missouri
5 R01 CA 38538-08
Mammalian Cell Transformation by Oncogenic Viruses

30. FOLK, William R.
University of Missouri
5 R01 CA 45033-04
Mechanism of Transformation by BK Virus

31. FRISQUE, Richard J.
Pennsylvania State University
(University Park)
5 R01 CA 38789-06
A Molecular Approach to the Unique Biology of JC Virus

32. FRISQUE, Richard J.
Pennsylvania State University
(University Park)
5 R01 CA 44970-04
Human Polyomaviruses: Oncogenic Potential and Mechanisms

33. GARCEA, Robert L.
Dana-Farber Cancer Institute
5 R01 CA 37667-07
Mechanisms in Polyomavirus Assembly

34. GREEN, Maurice
St. Louis University
5 R01 CA 28689-09
Biochemical Functions of Papillomavirus Oncogenes

35. GREEN, Maurice
St. Louis University
1 R01 CA 54703-01
Cellular Proteins Involved in Adenovirus E1A Repression

36. HANAHAN, Douglas
University of California
5 R01 CA 47632-05
Oncogenesis by Papillomavirus DNAs in Transgenic Mice

37. HANSEN, Ulla
Dana-Farber Cancer Institute
2 R01 CA 38038-05
Role for Transcription Factor LSF in Mammalian Cells

38. HARLOW, Edward E.
Massachusetts General Hospital
1 R01 CA 54696-01
Investigation of Cellular p300 protein

39. HARRISON, Stephen C.
Harvard University
5 R01 CA 13202-20
Structure and Assembly of Viruses

40. HEARING, Patrick
State University of New York
(Stony Brook)
5 R01 CA 44673-05
Analysis of a Polyomavirus Enhancer and Binding Protein

41. HOWETT, Mary K.
Pennsylvania State University
(Hershey Medical Center)
5 R01 CA 25305-11
Modulation of the Tumorigenicity of Transformed Cells

42. IMPERIALE, Michael J.
University of Michigan
(Ann Arbor)
5 R01 CA 19816-16
Role of SV40 Gene A in Cellular Transformation

43. JENSON, A. Bennett
Georgetown University
Medical School
5 R01 CA 50182-03
Antigenic Determinants of the Papillomavirus L1 Capsid Protein

44. KADISH, Anna S.
Albert Einstein College
of Medicine of Yeshiva
University
5 R01 CA 47630-03
Host Immunity to Genital Human Papillomavirus Infection

45. KASAMATSU, Harumi
University of California
(Los Angeles)
1 R01 CA 50574-02
Assembly and Morphogenesis of DNA Tumor Viruses

46. KELLY, Thomas J., Jr.
Johns Hopkins University
2 P01 CA 16519-17
Program on Molecular Biology of Viral Tumorigenesis

47. KELLY, Thomas J., Jr.
Johns Hopkins University
2 R01 CA 40414-07
Replication of the SV40 Genome

48. KHALILI, Kamel
Jefferson Medical College
Jefferson University
5 R29 CA 47996-04
Tissue Specific Transcription of JCV in Glial Cells

49. KREIDER, John W.
Pennsylvania State University
(Hershey Medical Center)
5 R01 CA 42011-05
Human Papillomaviruses in Cervical Cancer

50. KREIDER, John W.
Pennsylvania State University
(Hershey Medical Center)
5 R01 CA 47622-04
Studies on Papillomavirus Host Interaction

51. LAIMINS, Laimonis
University of Chicago
5 R01 CA 49670-03
HPV-18 Effects on Epithelial Cell Differentiation

52. LAMBERT, Paul F.
University of Wisconsin
1 R29 CA 55048-01
Coordinate Regulation in Transforming Papillomaviruses

53. LANCASTER, Wayne D.
Wayne State University
School of Medicine
5 R01 CA 32638-11
Role of Papillomavirus in Cervical Neoplasia

54. LANFORD, Robert E.
Southwest Foundation
for Biomedical Research
5 R01 CA 39390-08
SV40 T-Antigen: Model for Nuclear Transport of Protein

55. LEHMAN, John M.
Albany Medical College
of Union University
5 R01 CA 41608-05
Pathology of Neoplastic Transformation

56. LEVINE, Arnold J.
Princeton University
2 R37 CA 38757-07
Viral Transgene-Oncogene Induced Tumorigenesis

57. LIVINGSTON, David M.
Dana-Farber Cancer Institute
5 R01 CA 15751-18
Structure and Function of SV40 Non-Virion Proteins

58. LIVINGSTON, David M.
Dana-Farber Cancer Institute
2 R01 CA 24715-13
Isolation and Function of Small SV40 t-Antigen

59.	LIVINGSTON, David M. Dana-Farber Cancer Institute 5 R01 CA 49530-03	Repressor Control of SV40 Transformation
60.	LIVINGSTON, David M. Dana-Farber Cancer Institute 5 P01 CA 50661-03	Papovavirus Transforming Mechanisms
61.	LUSKY, Monica D. Cornell University Medical College 1 R01 CA 51127-02	Regulation of Bovine Papillomavirus Replication
62.	MANLEY, James L. Columbia University 5 R01 CA 46121-05	Mechanism of Alternative Splicing of SV40 Pre mRNA
63.	MAXWELL, Ian H. University of Colorado (Denver) 1 R01 CA 50285-02	Autonomous Parvovirus Vectors for Cancer Therapy
64.	MCDOUGALL, James K. Fred Hutchinson Cancer Research Center 1 R13 CA 55253-01	Tenth International Papillomavirus Workshop
65.	MUMBY, Marc C University of Texas SW Medical Center (Dallas) 1 R01 CA 54726-01	Protein Phosphatase 2A in Transformation by Polyoma/SV40
66.	NORKIN, Leonard C. University of Massachusetts 5 R01 CA 50532-03	Interaction of SV40 with MHC Class I Proteins
67.	OZER, Harvey L. University of Medicine and Dentistry of New Jersey (Newark) 7 R01 CA 23002-13	Host Functions Related to Tumor Virus Infection
68.	PALLAS, David C. Dana-Farber Cancer Institute 5 R29 CA 45285-05	The Role of Cellular Proteins in Polyoma Transformation
69.	PIPAS, James M. University of Pittsburgh 5 R37 CA 40586-07	Genetic Analysis of the SV40 Large Tumor Antigen
70.	PIRISI, Lucia A. University of South Carolina 5 R29 CA 48990-03	Papillomavirus Transformation of Human Keratinocytes

71. PIWNICA-WORMS, Helen M.
Tufts University
5 R29 CA 50767-03
Role of Tyrosine Kinases in Middle T-Antigen Transformers

72. PRIVES, Carol L.
Columbia University
2 R01 CA 26905-12
Polyoma and SV40 T-Antigen Roles in Viral DNA Synthesis

73. PRIVES, Carol L.
Columbia University
5 P01 CA 33620-08
Directed SV40 Mutation: Cell Molecular Consequences

74. RICCIARDI, Robert P.
Wistar Institute of Anatomy and Biology
5 R01 CA 44960-05
Role of BKV Enhancers in Virus Regulation and Cancer

75. ROBERTS, James M.
Fred Hutchinson Cancer Research Center
5 R29 CA 48718-03
Control of Viral Replication

76. ROBERTS, Thomas M.
Dana-Farber Cancer Institute
5 R01 CA 30002-10
Molecular Mechanisms of Polyoma-Induced Transformation

77. RUNDELL, Mary K.
Northwestern University
5 R01 CA 21327-14
Functions of the Simian Virus 40 Small T-Antigen

78. SCHAFFHAUSEN, Brian S.
Tufts University
2 R37 CA 34722-09
Products of the Transforming Genes of Polyomavirus

79. SCHLEGEL, Richard
Georgetown University
1 R01 CA 53371-01
Papillomavirus E5 Oncoprotein and Cell Transformation

80. SCHOOLNIK, Gary K.
Stanford University
5 R01 CA 43871-03
Cervical Neoplasia: Detection of HPV-16 Gene Products

81. SHENK, Thomas E.
Princeton University
2 P01 CA 41086-06
Viral and Cellular Oncogenes: Mechanism of Action

82. SIMMONS, Daniel T.
University of Delaware
5 R01 CA 36118-07
Structure and Function of the SV40 Tumor Antigen

83. SMITH, Alan E.
Integrated Genetics, Inc.
5 R01 CA 43186-05
Mutagenesis of Papovavirus Transforming Proteins

84. SMOTKIN, David
University of Utah
5 R29 CA 47127-04

Human Papillomavirus Gene Expression in Cervical Cancer

85. SNAPKA, Robert M.
Ohio State University
5 R29 CA 45208-05

Aberrant Papovavirus Replication after Genotoxic Damage

86. SOMPAYRAC, Lauren M.
University of Colorado (Boulder)
5 R01 CA 34072-07

SV40 Deletion Mutants: Oncogenic Proteins

87. STOLER, Mark H.
Cleveland Clinic Foundation
2 R01 CA 43629-06

Human Papillomavirus-Host Gene Expression in Neoplasia

88. TEGTMEYER, Peter J.
State University of New York (Stony Brook)
5 R37 CA 18808-17

Tumor Virus SV40: Protein Function and DNA Replication

89. TEGTMEYER, Peter J.
State University of New York (Stony Brook)
5 P01 CA 28146-11

Tumor Virus-Host Interactions

90. TEVETHIA, Mary J.
Pennsylvania State University (Hershey Medical Center)
5 R01 CA 24694-13

Mutagenesis of Specific Regions of the SV40 Genome

91. TEVETHIA, Satvir S.
Pennsylvania State University (Hershey Medical Center)
5 R37 CA 25000-14

Biology of SV40 Specific Transplantation Antigen

92. TJIAN, Robert T.
University of California (Berkeley)
5 R37 CA 25417-13

The SV40 Tumor Antigen

93. TUREK, Lubomir P.
University of Iowa
5 R01 CA 49912-02

Human Papillomavirus-16 Regulation in Cervical Cancer

94. WALTER, Gernot F.
University of California (San Diego)
2 R01 CA 36111-07

SV40 and Polyomavirus Transforming Proteins

95. WETTSTEIN, Felix O.
University of California
(Los Angeles)
5 R37 CA 18151-16

Analysis of the Shope-Papilloma Carcinoma System

96. WETTSTEIN, Felix O.
University of California
(Los Angeles)
5 R01 CA 50339-03

Immunology of Virus-Induced Rabbit Papillomas/Cancer

97. WILCZYNSKI, Sharon P.
City of Hope National
Medical Center
1 R29 CA 53005-02

Pathologic and Molecular Study of Human Papillomavirus in Cervical Cancer

98. YOUNG, Donald A.
University of Rochester
5 R01 CA 47650-03

Papilloma Virus Actions on Host Cell Gene Products

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 and bovine tumor viruses. In this program, extramural research is supported by several funding mechanisms: traditional research grants (R01), program project grants (P01), conference grants (R13), outstanding investigator awards (R35), and FIRST awards (R29). The overall effort consists of 88 grants, with a funding level of 18.27 million dollars. These grants involve studies in the mouse (70%), human (21%), feline (7%), and bovine (2%) model systems in the following areas: gene organization and expression, 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 retroviral viral vaccines.

Studies in the RNA Virus Studies I component are concerned with the elucidation of the molecular events associated with the viral conversion of normal cells to the malignant phenotype. Since the malignant phenotype is a stable inherited trait, 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 initiation and 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 homologs of viral oncogenes, in many instances, appear to be responsible for the in vitro conversion of normal cells in culture to the transformed phenotype, 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: local changes or mutations in genes involving base changes or small deletions which alter the functional properties of the gene product; 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, LTR) adjacent to cellular genes such that the level of gene expression is enhanced; gene amplification mechanisms which may increase the amounts of specific gene products; or changes in the activity of oncogene promoters by changing either the base sequence itself or by altering the 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. No other class of animal viruses exhibits such profound intimacy with the host genome. 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. Cancer induction occurs apparently through the activation and aberrant expression of oncogenes through the integration of viral genomes in their vicinity. In addition, retroviruses which use other/additional mechanisms for cancer induction have been recognized. The first of these are 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 and apparently induce cancer through the activation of oncogenes (i.e., a cell-derived oncogene). Sequences located within the env region appear to be responsible for their increased pathogenic properties. Recently, another group of viruses without oncogenes, the lymphocyte-transforming retroviruses (T-cell lymphotropic viruses, HTLV), have been recognized. These viruses also do not transduce oncogenes. They apparently bring about cellular transformation through a novel transactivation mechanism involving a trans-acting protein encoded by the Px region (now termed tax gene) of the viral genome. Extramural research involving all four types of retroviruses is being administered by this component of the Biological Carcinogenesis Branch.

Virus Studies

Highlights of studies on human retroviruses are as follows: Human T-cell leukemia virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia (ATL). More recently, HTLV-I has also been linked epidemiologically to a neurological disorder called HTLV-I-associated myelopathy (HAM) and tropical spastic paraparesis (TSP). The 3' region of the genome of HTLV-1 designated Px encodes a 40 kDa nuclear protein, tax that positively activates transcription from the viral transcriptional regulatory region. The mechanism of leukemogenesis by HTLV-1 is most likely mediated by the tax gene. Tax has been shown to transform mammalian cells both in vitro and in vivo, and appears to be the key determinant of HTLV-1 induced ATL. It has been proposed that transcriptional activation by tax of cellular genes, including those of the IL-2 receptor alpha chain, leads to polyclonal T-cell proliferation which ultimately results in leukemia. Three 21 base-pair repeat sequences in the viral LTR constitute the cis-element responsiveness for tax mediated trans-activation. Recently, it was found that tax does not bind to DNA directly, and trans-activation by tax is mediated via cellular factors that specifically bind the 21 bp repeats. Two cellular proteins of molecular sizes of 52 Kda and 46 Kda were found to interact specifically with this DNA sequence. Purified tax was found to bind directly to these cellular factors. This provides the first biochemical evidence indicating that the mechanism of action of tax is mediated by its direct interaction with the cellular transcription factors that bind to the 21 bp repeats. It was further shown that several distinct cellular transcription factors (including CREB and c-Jun) interact with the tax responsive region LTR, apparently conferring the viral regulatory sequence versatility to respond to different physiological and cellular control signals. The elucidation of the mechanism of action of

tax at the molecular level should provide a deeper insight into the initiation of the pathway which leads to adult T-cell leukemia and the fundamental mechanisms governing gene expression and cell growth in human T-lymphocytes (27).

The use of the transgenic mouse model has provided experimental evidence that the tax gene has the ability to participate in cancer induction. Three different lines of transgenic mice harboring the tax gene were produced. They developed a variety of lesions including multiple mesenchymal tumors and neurofibromas. It was found that the neurofibromas secreted large quantities of interleukin-6 as well as nerve growth factor (NGF). This observation, that tax-1 protein may stimulate the production of NGF, could provide important new insights into the mechanism of HTLV-1-induced neurologic disease. Of clinical importance is the observation that in humans infected with HTLV-1, the presence of serum antibodies against the tax protein is usually correlated with HTLV-1 infection during early life. These tax antibodies therefore may serve as reliable markers for HTLV-1 infection during early life and possibly for susceptibility to lymphoma development (32,38,53, AVS component, reference 9).

HTLV-2 which is related to HTLV-1, has been rarely associated with forms of leukemia related to hairy-cell leukemia, and has been reported to be prevalent among a significant proportion of intravenous drug abusers in the United States and Europe. In addition to the essential viral structure proteins, HTLV-1 and HTLV type 2 (HTLV-2) encode two regulatory proteins, designated tax and rex. The rex gene encodes two proteins of 21 and 27 Kda for HTLV-1 and 24 and 26 kDa for HTLV-2. Like tax, rex is localized to the nucleus. It has been determined that rex acts post-transcriptionally to increase the expression of mRNAs for virion proteins. Thus, rex is required for transport of unspliced and singly spliced messages from the nucleus to the cytoplasm, facilitating expression of the viral structural genes gag, pol and env. In HTLV-1, this action is mediated by rex via cis-acting element in the U3-R region of the 3' LTR. On the other hand, it was found that in the case of HTLV-2, these sequences are localized to the R-U5 region of the 5' LTR (9,67).

Recent results raise the possibility that HTLV regulatory protein rex also contributes to the tumorigenic potential of HTLV-1 by cooperating with tax in the overexpression of cellular genes that alter the growth of CD4+ T-lymphocytes, which are the primary target cells of HTLV-1 transformation. It was found that rex increased the rate of IL2 promoted gene expression in a mitogenically stimulated cell line. Rex also increased the activity of the c-fos promoter to a lesser extent. Mitogenic activation of Jurkat cells was required to observe rex stimulation of the IL2 promoter and the effect was substantial only in the presence of tax. High levels of tax did not inhibit IL2 promoter activity. Rex may be the transactivator of transcription which acts via sites distinct from those responsive to the tax protein. This activity could be the result of an increase in the initiation of transcription or more efficient elongation of messages alternately (30).

Retroviral infection can be blocked or neutralized by antibodies that bind to specific sites on the envelope glycoprotein. To map these sites associated with neutralization of HTLV-I infection, peptides containing amino acid sequences of the HTLV-I envelope were synthesized and used to immunize goats for the production of antibodies reactive to specific regions on the native

HTLV-I envelope protein. Antisera from these animals blocked HTLV-I induced syncytium formation and inhibited VSV/HTLV-I plaque formation. Among the different peptides used, only peptide SP-2 absorbed neutralizing antibodies in sera from goats immunized with peptides SP-2, 3 and 4A thus mapping an important neutralizing site of HTLV-1 envelope to amino acids 86-107. This mapping of the neutralizing region of the HTLV-1 envelope glycoprotein may be useful in the design of synthetic peptide based vaccines for blocking or ameliorating the pathogenic effects of HTLV-1 infection in humans (55).

A major consideration for vaccine development against HTLV-1 is an appropriate animal model for efficacy testing. HTLV-1 has been found to be capable of causing productive infections in rabbits. The HTLV-1 rabbit model is currently being evaluated for testing of HTLV-1 vaccine preparations and challenge strains. It is necessary to establish the value and reliability of the HTLV-1 rabbit model as an inexpensive alternative to the expensive primate for preliminary evaluations for various prototype vaccines. Recent studies utilizing diverse techniques to measure virus infections have provided irrefutable evidence that HTLV-1 chronically infects rabbits and establishes persistent infections for at least 22 months following infection (49).

The human genome contains many different types of endogenous proviruses and retrovirus-like elements. Some of these sequences are transcribed in a tissue-specific manner, although no human tissue has been found to be productively infected with an endogenous retrovirus. The function of endogenous retroviruses and retrovirus-like elements in human DNA is not known, but several possibilities have been suggested. Endogenous retroviruses may have played a role in evolution of the human genome, e.g., by serving as a source of genetic variation, by increasing the effective target size for possible lethal mutations, or by acting as insertional mutagens. In their present form, they may serve as a reservoir for recombination and may thereby contribute to the origin of pathogenic retroviruses. An unusual retrovirus-like element of feline leukemia virus (FeLV) related to the integrase-coding domain of the pol gene, has been isolated from human DNA. Nucleotide sequence analysis of Hs5 reveals that it is not an intact retrovirus, but contains only the 3' terminus of pol and a defective env gene without apparent LTR. This identification of a specific FeLV related retroviral element in the human genome emphasizes and demonstrates the prevalence of different types of "hidden" retroviral elements in the human genome and raises the possibility that one or more of these elements may be involved in the generation of new pathogenic retroviruses and the possible development of cancer through genetic recombination (45).

Murine leukemia viruses (MuLV) serve as important tools for understanding the mechanisms of leukemia induction in mice. Studies of this animal model continue in diverse grant supported studies. A fascinating aspect of mouse retrovirus biology is the striking difference in the incidence and latent period of disease induction and disease specificity of even very closely related replication-competent MuLV. The majority of oncogenic MuLV induce exclusively T-cell lymphomas, but some isolates induce tumors of B cells, myeloid cells, or erythroid cells. In most cases, viral integrations in critical regions of the chromosomes appear to perturb gene expression by placing a cellular proto-oncogene under the powerful influence of viral transcriptional signals. Aberrant gene expression presumably initiates a chain of events that leads to transformation of the infected cells. In many

cases, disease specificity is a property of the viral genome. Genetic studies have shown that multiple viral genes contribute to the pathogenic phenotype of non-defective C-type viruses. A particularly potent viral genetic determinant in many cases is the U3 region in the viral LTR, and, more specifically, the transcriptional enhancer of this region, which strongly influences not only the ability of a virus to induce disease, but also the latent period of disease induction, and, perhaps most surprisingly, the disease specificity. The enhancer region of non-defective MuLV is usually present as a direct repeat of a sequence 50-120 nucleotides in length and contains a variety of cellular transcription factors. Small differences in enhancer sequences can be sufficient to confer distinct biological properties to the virus. To study the contribution of individual protein-binding sites to viral pathogenicity, mutations were introduced into each of the known nuclear factor-binding sites in the enhancer region of the Moloney MuLV and these mutants were injected into newborn NFS mice. All viruses induced disease. Viruses with mutations in both copies of the leukemia virus factor a (LVa) site, or in just the promoter proximal copy of the glucocorticoid response element (GRE) had a latent period of disease specificity indistinguishable from that of the wild-type Moloney virus. Viruses with mutations in two or three of the GREs, in both copies of the leukemia virus factor b (LVb) site, in two of the four nuclear factor 1 (NF1) consensus motifs, or in both copies of the conserved viral core element showed a significant delay in latent period of disease induction. Strikingly, viruses with mutations in the core element induced primarily erythroleukemias, and mutations in the LVb site also resulted in a significant incidence of erythroleukemias (35).

The studies of Lenz and Wong also emphasize the importance of the viral enhancers' 3' LTR in disease induction. In the case of the highly leukemogenic SL3-3 strain of MuLV, pathogenicity studies have shown that a single base pair difference between the core elements of the U3 enhancers of SL3-3 and a non-leukemogenic Akv virus is extremely important. Mutation of the SL3-3 virus to introduce just this 1 bp change into the SL3-3 core drastically attenuated viral leukemogenicity (43,87).

An important question concerning the pathogenesis of the disease syndrome caused by mutant ts1 of the Moloney strain of MuLV, is whether the severe thymic atrophy the virus causes in ts1-infected mice is a direct effect of the ts1 virus or an indirect effect occurring secondary to neurodegenerative and wasting disease also observed in virus inoculated mice. Using site directed mutagenesis, hybrid viruses were created with modification in the enhancer region of the viral LTR. The substitution of the U3 region of ts1 with the corresponding U3 region of Cas-Br-E virus apparently changed the primary site of viral replication in the thymus. The reduced thymic viral titers corresponded with reduced thymic atrophy and cell death in mice paralyzed by ts1-Cas(NS). These results indicated that the marked thymic atrophy observed in ts1-infected mice is, in part, a direct consequence of high-level viral replication in the thymus. These findings make the ts1 model the only retrovirus, other than HIVs and SIVs, which possess the potential to kill both T-cells and neural cells. Therefore, the findings are novel as well as very important, and should pave the way to the eventual understanding of the molecular mechanism of how a retrovirus kills both T-cells and neural cells (87).

The studies of Kabat, Thomas, and Coffin, summarized below, establish the relative importance and participation of the retroviral envelope in the induction of certain types of cancers of mice. Erythroleukemia involving the transformation of nucleated precursors of erythrocytes is caused by the defective SSFV component of the Friend leukemia virus. Evidence has been obtained that the oncogenic membrane glycoprotein gp55 encoded by this virus interacts directly with erythropoietin receptors in the infected erythroblasts to cause unregulated proliferation of the cells. This gp55 is unique among oncogenic proteins in being encoded by a rearranged retroviral envelope gene rather than by a captured cell derived oncogene. Using recombinant DNA technology, retroviruses were produced which encoded the ligand erythropoietin (EpO), or the erythropoietin receptor (EpoR) or gp55. It was found that hematopoietic cells that are dependent on interleukin 3 for continued growth became growth factor independent after infection with retroviruses expressing EpoR and then subsequently grown in the presence of the ligand EpO. Also, superinfection of these cells (but not parental cells) either with EpO virus or with SSPV resulted in growth factor independence. The recombinant EpO virus resembled SSFV in being able to cause a disease in mice which resembled Friend erythroleukemia. These results have important implications for understanding signal transduction by erythropoietin receptor and the mechanisms by which oncogenic SSFV envelope protein is able to trigger cancer by abnormally stimulating this pathway of autocrine stimulation of cell growth (12,40,41,79).

In mouse strains with a high incidence of lymphomas, such as AKR, HRS, C58, and CWD, ecotropic viruses are expressed early in life and recombine with the endogenous polytropic and xenotropic sequences to form oncogenic recombinant MuLVs. The envelope genes are inherited from the endogenous polytropic viruses, but most AKR, HRS, and C58 recombinants inherit U3 region sequences from an endogenous xenotropic provirus Bxv-1. The Bxv-1-related U3 sequences have now been shown to encode functions that contribute to the leukemogenicity of AKR recombinants and facilitate the expression of virus sequences in the target thymocytes. Thus, the acquisition of U3 region sequences derived from the endogenous xenotropic provirus Bxv-1 appears to be an important step and the key determinant in the generation of leukemogenic recombinant viruses in AKR, HRS, C58, and some CWD mice. It was found that each of three lymphomas which appeared in CWD mice produced infectious xenotropic murine leukemia virus related to Bxv-1. These results establish that the virus progeny of Bxv-1 have the potential to donate pathogenic enhancer sequences to recombinant polytropic murine leukemia viruses (79).

In other similar studies, the formation of recombinant MCF viruses associated with spontaneous leukemia in AKR mice have been completed, and a parsimonious, but complete model for the virological events leading to AKR thymomas has been put forward. In this model, the recombination of ecotropic virus with the endogenous xenotropic virus Bxv-1 is likely to occur first, possibly in B-cells, and then a second recombination event with an endogenous polytropic gp70 template which may occur in the thymic epithelium. In addition to these events, somatic amplification of LTR sequences also occurs, probably to boost expression of the provirus. Although more complex models for formation of these recombinant viruses have been presented in the past, the parsimony of this model is consistent with strong biological selective pressures (12).

Mouse mammary tumor virus (MMTV) is a retrovirus which is associated with mammary cancer in mice. The cellular receptor which permits the entry of MMTV into mouse cells has been partially purified. It is a membrane-bound protein 60-65 kDa in both liver and mammary gland; however, it is 4-12 times more abundant in the latter tissue. Furthermore, it is elevated during pregnancy and can be induced in culture by sex steroids. This correlates well with the expression of the virus itself; i.e., the viral receptor is elevated when MMTV production is maximal. This coordination favors reinfection, thereby increasing the chance that the virus will integrate near an endogenous protooncogene (cancer-causing gene) and induce tumor formation. By better understanding the relationship between the virus and its receptor, strategies can be formulated to interfere with it and thwart carcinogenesis (6).

The lymphoid malignant diseases caused by FeLV are broad in spectrum and are referred to collectively as the leukemia-lymphosarcoma complex. These feline retroviruses have been classified into three envelope antigenic subgroups of A, B, and C. Whereas subgroup A and B viruses predominantly cause lymphosarcoma and/or immunodeficiency syndromes, subgroup C virus (e.g., FeLV-C Sarma) has been shown to be associated with a fatal aplastic anemia in infected cats. FeLV-C viruses occur rarely in nature. Aplastic anemia is a fatal disease caused by damage to the bone marrow, the organ responsible for producing blood cells. Neither the cause nor a successful treatment for most cases of aplastic anemia is known. Suppression of normal blood cell production is also a consistent feature in the progression of AIDS. The FeLV-C retrovirus induced aplastic anemia model is being studied to understand the ways in which retroviruses suppress bone marrow function. It was found that the anemia caused by this virus is mediated by the genes coding for one protein of the virus (the surface or envelope glycoprotein gp70). The studies have further localized the anemia causing sector of the surface protein to the initial 107 amino acids. This region is apparently responsible for the capacity of this virus to infect and destroy the precursors of red blood cells in the bone marrow (34).

T-lymphocytes recognize antigen on the surface of target cells through a T-cell antigen receptor. Specific antigen recognition also requires the engagement of an additional receptor, CD4 or CD8, with nonpolymorphic regions of the major histocompatibility molecules. Ironically, CD4 is also the receptor for the immunodeficiency virus, HIV. In recent studies, it was demonstrated that the surface receptors, CD4 and CD8, are not only required for efficient T-cell activation, but are essential for shaping the repertoire of T-cells during development. An exogenous CD8 gene was introduced into mouse embryos to generate transgenic mice which express an exogenous CD8 gene on all T-cells. Analysis of the T-lymphocytes within the thymus and the periphery of these mice have provided a model of T-cell development in which the interaction of either CD4 or CD8 with appropriate molecules on the surface of cells within the thymic epithelium appear to be the primary determinants in determining whether a cell will either be a helper cell or a killer cell within the cellular immune system (3).

A new technology has been developed to make human antibodies against viral antigens. The procedure involves using mRNA from any B-cell source (hybridoma, spleen cell, peripheral lymphocytes), PCR amplify the variable region genes, and clone these genes into a bacteriophage expression library. The library is then screened with an antigen of choice, as a probe, to

identify clones expressing antibodies directed against the antigen. Using this method it was possible to identify purely human Fab fragments directed against tetanus toxoid (as a model system). Thus, the technology offers an opportunity to make purely human antibodies against antigens of infectious agents. Since the antibodies are of pure human sequence, there is little chance for them to be significantly immunogenic in humans. Therefore, there is the possibility that such antibodies could be used to treat various diseases in humans, including cancer (72).

Oncogene Studies

Progress was made in studies of oncogenes which act through stimulation of cell growth as well as those whose inactivation leads to the removal of inhibitory controls of cell growth. Examples of research involving oncogenes and the interaction of retroviruses with oncogenes in the development of neoplasia are as follows: Individuals in Li-Fraumeni families have a strong predisposition for developing cancer and develop a variety of different forms of cancer, among which are breast cancer, sarcomas of the bone and other tissues, brain tumors and leukemia. Studies were conducted to identify the primary cause of their cancer predisposition. Over the last few years, tumor suppressor genes which are involved in controlling normal cell growth, have, with increasing frequency, been connected with a number of inherited and non-inherited types of cancer. Researchers have found changes in suppressor gene p53 in various types of human tumors and it has been strongly implicated in the development of colon cancer. However, in all of these instances it is believed that the gene was normal at the time of birth and became damaged at some point during the person's lifetime. If such a gene is indeed responsible for the inheritance of a predisposition to cancer in the Li-Fraumeni family, then in those individuals who will eventually develop cancer, one of the two copies of this gene should be abnormal at birth in all of the cells of the body, even in those cells that remain normal. This assumption led to the study of the p53 gene in normal skin cells obtained from three generations of the family. A small defect was discovered in exactly the same location within the p53 gene in four family members who developed cancer. This defect was present only in those individuals who had developed cancer and was not found in those who had escaped the disease. Neither was it present in individuals who had married into the family. Therefore, it is apparent an inherited defect has been identified in the tumor suppressor gene p53 in this Li-Fraumeni family. It is conceivable that this discovery could lead to the development of a diagnostic test which could identify some individuals at risk for developing cancer (8).

Unlike the well-studied murine retroviruses, FeLV infects a naturally outbreeding population. Such a population, in contrast to strains of laboratory animals, provides enormous genetic heterogeneity as a substrate for proviral integration. At least five oncogenes were identified on the basis of their transduction by FeLV (fes, fms, fgr, kit, and TPI), and four sequences identified as oncogenes in other retroviruses also appear in strains of FeLV (abl, sis, ras, and myc). Although FeLV-mediated induction of the leukemia-lymphosarcoma complex represents a valuable model for natural carcinogenesis in an outbreeding population, little is known about the molecular mechanism by which FeLV effects malignant change. Insertional mutagenesis of c-myc appears to play a role in the induction of T-cell lymphoma by FeLV, since proviral integration within or near c-myc is observed

with a frequency as high as 40% in such tumors. The role of insertional mutagenesis in the induction of other members of the leukemia-lymphosarcoma complex is unknown. It was recently shown that interruption of a host gene locus known as flvi-1 by FeLV proviral integration is associated with a particular kind of lymphoma. Examination of a bank of 21 naturally occurring FeLV-positive feline lymphomas revealed that exogenously derived FeLV proviral integration occurs at the flvi-1 locus in four independent tumors (19%). The four tumors in which flvi-1 is interrupted were classified as members of a phenotypic subgroup containing seven lymphomas. The small number of proviral integrations in tumors of this subgroup suggests that an early proviral integration event into flvi-1 can induce malignant change and play an important role in natural induction of lymphomas by FeLV (45).

Abl is a member of the protein tyrosine kinase family of oncogenes. The murine retrovirus, Abelson MuLV expresses an activated form of this oncogene; human patients with chronic myelogenous leukemia (CML) also express a related but distinct form of the oncogene. Recent work has focused on understanding the mechanism by which abl induces a malignant state of cells. To this end, a special series of Abelson virus mutants were used. These mutants induce a malignant state only when cells are maintained at a low temperature. When cells carrying such mutant oncogenes are grown at high temperatures, the oncogene is inactive and in most cases, the cells revert to a normal growth pattern. It was shown that an active abl oncogene is required at a specific point in the cell cycle, during the G1 phase. During this time, the cell is preparing to synthesize DNA prior to dividing and "deciding" whether to actually become a resting cell or continue growing. The result indicates that the abl oncogene perturbs the machinery involved in this decision process. In addition, these results show that the absence of the active oncogene leads to a special type of cell death. This type of cell death, called programmed cell death, is an active process, requiring specific cellular proteins. Thus, the central function of the abl oncogene appears to be the prevention of cellular death. This result implies that induction of leukemias by abl, including those associated with abl in man, involves stimulating cells to continue growing when they would normally die (64).

The introduction of mutations into the germ line of an organism is one of the most powerful genetic methods for determining the function of specific gene products. Recent advances in molecular biology now permit the introduction of mutations into virtually any gene for which a cloned DNA sequence is available. As mentioned earlier, the human abl oncogene has been implicated in at least two forms of cancer: CML and acute lymphocytic leukemia where the gene is activated by chromosomal translocations. Although much is known about the oncogenic potential of the abl gene, little is known about the function of the normal gene in development or in the life of the organism. The generation of defined mutations in the c-abl gene in the mammalian germ line could provide insights into the function of the gene product and the way in which mutations lead to malignant transformation. To this end, a mutation has been introduced into the abl gene of murine embryonic stem cells by homologous recombination between exogenously-added cloned abl DNA and the endogenous gene. These stem cells were then used to generate chimeric mice. In a key step, it was found that some of these chimeric mice have passed the mutation to their progeny. These mice carry the mutation as a normal heterozygous Mendelian allele of c-abl. Cells homozygous for this mutation have recently been analyzed and reveal developmental defects. A careful dissection of the

nature of these developmental changes should provide insight into the function of this gene during development, and the way in which alterations in this gene may lead to malignant transformation (3).

Whereas v-abl participates in the development of hemopoietic tumors such as pre B-cell tumors in mice and CML in humans, v-myc has been shown to participate in the induction of mouse plasmacytomas and in the development of Burkitt's lymphoma in humans. Chromosomal translocations affect the expression of these oncogenes and contribute to the formation of these blood-cell tumors. The effect of co-expression of v-abl and c-myc on tumor formation of mice has now been investigated. A retrovirus that expresses v-abl from a retroviral promoter and c-myc from a herpes simplex viral promoter was found to cause plasmacytomas in mice. Plasmacytomas are tumors of the B-cell lineage that secrete antibodies. It was also found that if mice are immunized with specific antigens and then infected with the v-abl/c-myc virus, 60% of the resulting tumors secrete antibodies to those specific antigens. This finding has several implications. First, it indicates that a B-lymphocyte which can be stimulated by exposure to an antigen is both the target for transformation by this virus and the precursor to the tumor cell. Second, it provides an alternative means to that of selection of hybridomas to generate large amounts of murine monoclonal antibodies to specific antigens. Third, and perhaps, most tantalizing, it provides a possible means of generating human monoclonal antibodies efficiently, because this virus, when packaged by an amphotropic helper virus, should infect human B-cells in culture. Studies are in progress to determine if the v-abl/c-myc virus transforms antibody-secreting human B-cells. If it does, the antibodies provided by such immortalized cells should be of significant therapeutic value (78).

In humans, the chromosome 9 to 22 translocation, involves the aberrant expression of the abl oncogene, CML develops as a result of the translocation of the c-abl locus and the subsequent aberrant production of the oncogenic $P212^{bcr/abl}$ fusion protein. Understanding the mechanisms by which the abl oncogene affects various cell types has been hampered by a paucity of experimental systems that reproduce the range of biological effects associated with them. A mouse model system has now been developed for human CML. Expression of the CML-specific $P210^{bcr/abl}$ protein in the bone marrow of mice by a retroviral gene transfer/bone marrow transplantation approach leads to a variety of hematological malignancies, most prominently, a myeloproliferative syndrome with a striking resemblance to the chronic phase of human CML. Further analysis of mice with the CML-like syndrome has shown that (1) the CML-like syndrome is, at least in some cases, a consequence of retroviral infection of the pluripotent hematopoietic stem cell, (2) the disease is transplantable by transfer of bone marrow to syngeneic recipients, and (3) clonally related acute leukemias of both lymphoid and myeloid origin have been observed in secondary transplant recipients, representing evolution of the disease to blast crisis. Thus, CML-like syndrome induced by $P210^{bcr/abl}$ in mice closely resembles human CML in several fundamental aspects and thus should serve as a useful animal model for the studies of this human cancer (4).

An experimental system for studies of the abl oncogene has now been developed in which murine hematopoietic stem cell populations are infected with either v-abl or bcr/abl retroviruses and are used to reconstitute lethally irradiated

mice. Irrespective of the form of activated abl, >90% of the animals reconstituted with such cells develop tumors. About 50% of them develop a myeloproliferative syndrome that shares several features with the chronic phase of chronic myelogenous leukemia; the remaining animals succumb to pre-B-cell lymphomas. The myeloproliferative syndrome is characterized by large numbers of clonally derived, infected myeloid cells. This model will now allow study of the mechanisms by which activated abl genes affect hematopoietic precursors in chronic myelogenous leukemia. Furthermore, the results demonstrate that introduction of an activated abl gene into the appropriate target cell, not the structure of the gene, is the major determinant in myeloid cell specificity (86).

The ligand for the c-kit proto-oncogene has now been identified. This proto-oncogene encodes a transmembrane tyrosine kinase receptor and is a member of the colony stimulating factor-1 (CSF-1)/platelet derived growth factor (PDGF)/kit receptor subfamily. The c-kit proto-oncogene is allelic with the murine white-spotting locus (W). W mutations affect melanogenesis, gametogenesis and hematopoiesis during development and in adult life. Cellular targets of W mutations in hematopoiesis include distinct cell populations in the erythroid and mast cell lineages as well as stem cells. In the absence of interleukin-3 (IL-3), mast cells derived from normal mice but not from W mutant mice can be maintained by co-culture with 3T3 fibroblasts. It had been proposed that fibroblasts produce the c-kit ligand. A 30kd protein, designated KL, has been purified to apparent homogeneity from conditioned medium of Balb/3T3 fibroblasts. KL stimulates the proliferation of normal bone marrow derived mast cells but not mast cells from W mice. Connective tissue-type mast cells derived from the peritoneal cavity of normal mice were found to express a high level of c-kit protein on their surface and to proliferate in response to KL. In combination with erythropoietin, KL was found to stimulate early erythroid progenitors (BFU-E) from fetal liver and spleen cells but not from fetal liver cells of W/W mice. The identification of the ligand for c-kit should facilitate the understanding of the role of the W locus in normal development of the mouse and the role of the kit oncogene in cancer (31).

The proto-oncogene int-1 plays an important role in mammary tumorigenesis. This oncogene is activated by proviral insertions of the MMTV. In normal mouse tissues, the gene is expressed in the embryonic neural tube, suggesting a developmental function, while in Drosophila the homolog of int-1 is the segment polarity gene, wingless. In Drosophila mutations of int-1 gene results in wingless offspring. In order to study the protein products of the human int-1, fibroblast cell lines were obtained which were infected with multiple copies of a retroviral vector expressing int-1 cDNA. By western blot analysis and immunoprecipitation, a 44 kD form of int-1 protein has been identified, which is secreted from these cells. The 44 kD species is distinct from the major intracellular forms of int-1 protein as judged by its slower mobility in SDS-polyacrylamide gels and by its longer half-life in pulse-chase experiments. Under normal growth conditions, little or none of the 44 kd protein is detectable in the cell culture medium; instead the majority is found associated with the extracellular matrix (ECM). The protein appears to bind heparin in vitro, suggesting that it might bind glycosaminoglycans in the ECM. These data support the view that int-1 protein may play a role in "cell to cell" communication over short distances (31).

RFA 90-CA-10 entitled "Human T-Cell Lymphotropic Viruses in Human Neoplasia," with set-aside funds of $800,000 per year for 5 years, was developed on the basis of a DCE workshop conducted in FY 89 to define the role of retroviruses in human cancers. Twenty-three applications responsive to the RFA were received and reviewed by an NCI ad hoc study section for the February 1991 NCAB. Five of these grants were funded on the basis of their scientific merit, program priority and relevance. Dr. Strair proposes to use an innovative assay for viral functions that depend on the ability of the transforming viruses to transactivate the expression of a reporter gene, E. coli b-galactosidase, attached to an adenovirus early promoter. The proposed studies are novel and represent a logical and systematic approach for detecting viruses with transactivating capacity in human cancers.
Dr. Loughran will investigate a potential viral etiology for large granular lymphocytic leukemia and thus will extend our understanding of the involvement of retroviruses in human disease. Studies of Dr. Chen propose to address the events that occur following infection with HTLV-1 and HTLV-2 viruses and will develop and use the SCID mouse model. Dr. Jay proposes to use the transgenic mouse approach to answer the question of tissue tropisms and differences in pathogenicity between HTLV-1 and HTLV-2 and decipher the mechanisms by which HTLV-1 induces cancers on one hand, and the neurologic disease tropical spastic paraparesis (TSP) on the other. Dr. Wigdahl will clarify the question of infection of neuronal cells with HTLV and address the key question of what confers the ability to HTLV-1 to cause cancer, on one hand, and neurologic disease on the other (10,39,48,77,84).

Thus, grants in the RNA Virus Studies I component have focused on the elucidation of the biology and diverse characteristics of human and animal retroviruses, on their interactions with host cells resulting in the transformation of normal cells to the malignant phenotype, and on the development of innovative technologies in these fields of inquiry. Recently, it was found that the tax protein binds to viral DNA via cellular factors. Evidence was obtained to suggest that transforming consequences of HTLV-1 infection of primary T-lymphocytes can be attributed to tax as well as to rex proteins encoded by the pX region of the viral genome. Studies of transgenic mice carrying the HTLV-1 tax gene suggested that the tax gene is capable of causing diverse mesenchymal tumors and neurofibromas. Studies of virus-host interactions provided information on the mechanisms of virus entry into host cells in a mouse mammary tumor virus. Clues were obtained on the mechanisms of viral carcinogenesis involving enhancer sequences in the viral LTRs and the viral envelope genes, and oncogenesis by oncogenes that have been implicated in the genesis of human chronic myelogenous leukemia (CML). A mouse model was developed for CML. A powerful transgenic mouse technique which allows the researcher to insert an altered gene in precise locations in the developing mouse embryos was applied to studies of the effect of the abl oncogene in normal development. The ligand for the c-kit proto-oncogene was identified. Members of a cancer-prone family were shown to suffer from mutations of the tumor suppressor gene p53 thus providing evidence on an etiologic association between this mutation and the development of cancers. A new in vitro technology was developed to make human antibodies against viral antigens.

These studies thus continue to unravel the participation of viral and cellular genes in the genesis of cancer, anemia, neurologic disorder and other diseases and provide insights into how the eventual control of these diseases might be achieved.

RNA VIRUS STUDIES I

GRANTS ACTIVE DURING FY91

	Investigator/Institution/Grant Number	Title
1.	ARLINGHAUS, Ralph B. University of Texas (Houston) 5 R01 CA 45125-05	Studies on Viral and mos Proteins
2.	ARLINGHAUS, Ralph B. University of Texas (Houston) 5 R01 CA 45217-05	A Temperature-Sensitive Retrovirus Splicing Mutant
3.	AXEL, Richard Columbia University 5 P01 CA 23767-13	Molecular Virology
4.	BALTIMORE, David Rockefeller University 5 R01 CA 51462-02	Malignancy and Normal Development in Pre-B Biomedical Lymphocytes
5.	BESMER, Peter Sloan-Kettering Institute for Cancer Research 5 R37 CA 32926-07	C-KIT and V-KIT: Normal Function and Oncogenic Activation
6.	BOLANDER, Franklyn F., Jr. University of South Carolina (Columbia) 5 R01 CA 42009-05	MMTV Regulation in Normal Mouse Mammary Epithelium
7.	BROWN, Martin J. Stanford University 5 R01 CA 03352-35	Biological Aspects of Carcinogenesis by Radiation
8.	CHANG, Esther H. Henry M. Jackson Foundation (Bethesda, Maryland) 5 R01 CA 45158-05	Oncogenes in Human Cancer Induction
9.	CHEN, Irvin S. Y. University of California (Los Angeles) 5 R37 CA 38597-07	Molecular Genetic Study of Human T-Cell Leukemia Virus
10.	CHEN, Irvin S. Y. University of California (Los Angeles) 1 R01 CA 54551-01	HTLV Pathogenesis in Vivo

11.	COCKERELL, Gary L. Colorado State University (Fort Collins) 5 R01 CA 43728-03	Latency and Leukemogenicity Bovine Leukemia Virus
12.	COFFIN, John M. Tufts University 5 P01 CA 24530-12	Molecular Genetics of Cancer
13.	COMPANS, Richard W. University of Alabama (Birmingham) 2 R01 CA 18611-16A1	Directional Transport of MuLV Glycoproteins
14.	COOPER, Geoffrey M. Dana-Farber Cancer Institute 5 R01 CA 18689-16	Infectious DNA for Endogenous RNA Tumor Virus Genes
15.	CROZEMARIE, Jacques Coordinating Council for Cancer Research (New York) 1 R13 CA 52478-01	Sixth International Symposium: Cancers and Environment: New Approaches
16.	CUNNINGHAM, James M. Brigham and Women's Hospital 5 R29 CA 47075-04	Isolation and Analysis of Murine Leukemia Virus Receptor
17.	DE FRANCO, Donald B. University of Pittsburgh 2 R01 CA 43037-04A1	Intracellular Mechanisms of Glucocorticoid Action
18.	DIAMOND, Don J. Beckman Research Institute of the City of Hope 5 R01 CA 52177-02	Analysis of Proteins Binding to the Human Gamma-Interferon Promoter
19.	DONOGHUE, Daniel J. University of California (San Diego) 5 R01 CA 34456-08	Expression of Retroviral Envelope Gene Fusion Proteins
20.	DUDLEY, Jacquelin P. University of Texas (Austin) 2 R01 CA 34780-07	Regulation of MMTV in T-Cell Tumors
21.	FAN, Hung Y. University of California (Irvine) 5 R01 CA 32455-11	Expression and Pathogenesis of Murine Leukemia Virus

22.	FLYER, David C. Pennsylvania State University (Hershey Medical Center) 5 RO1 CA 44633-06	Specificity of the CTL Response to Murine Leukemia Virus
23.	FUCCILLO, David A. SRA Technologies, Inc. (Rockville, MD) 1 R43 CA 54725-01	Developing a Hybridization Assay for HTLV-I and HTLV-II
24.	FOX, C. Fred Keystone Center 1 R13 CA 54780-01	Conference on Molecular Biology of Pathogenic Viruses
25.	GASPER, Peter W. Colorado State University (Fort Collins) 5 R29 CA 46371-04	Marrow Transplant Therapy for Retrovirus Infections
26.	GEIB, Roy W. Indiana University School of Medicine 5 R29 CA 47944-04	Analysis of a "Friend Virus-like" Disease
27.	GIAM, Chou-Zen Case Western Reserve University 7 RO1 CA 48709-03	Biochemical Mechanism of Trans-Activation in HTLV-I
28.	GOFF, Stephen P. Columbia University 5 R37 CA 30488-11	Construction and Analysis of Retrovirus Mutants
29.	HAAS, Martin University of California (San Diego) 5 RO1 CA 34151-09	Viral Malignant Lymphoma-genesis in X-Irradiated Mice
30.	HASELTINE, William A. Dana-Farber Cancer Institute 5 RO1 CA 36974-08	pX Region of HTLV I & II
31.	HAYWARD, William S. Sloan-Kettering Institute for Cancer Research 5 PO1 CA 16599-17	Mechanisms of Action of Viral and Non-Viral Oncogenes
32.	HINRICHS, Steven H. University of California (Davis) 7 R29 CA 49624-03	Effect of HTLV-1 TAT Expression in Transgenic Mice

33. HOLLAND, Christie A.
University of Massachusetts
Medical School
5 R01 CA 41510-05

Oncogenicity of Mink Cell Focus-Inducing Retroviruses

34. HOOVER, Edward A.
Colorado State University
(Fort Collins)
5 R01 CA 48594-04

Mechanisms of Retrovirus Induced Aplastic Anemia

35. HOPKINS, Nancy H.
Massachusetts Institute of Technology
5 R01 CA 19308-15

Studies on Endogenous and Other C-Type Viruses of Mice

36. HUNTER, Anthony R.
Salk Institute for Biological Studies
5 R35 CA 39780-07

Role of Protein Phosphorylation in Growth Control

37. JAENISCH, Rudolf
Whitehead Institute for Biomedical Research
5 R35 CA 44339-05

Retroviruses, Oncogenes and Mammalian Development

38. JAY, Gilbert
American Red Cross
(Rockville, MD)
1 R01 CA 51779-02

Neoplastic Disorders Induced by HTLV-1 in Transgenic Mice

39. JAY, Gilbert
American Red Cross
(Rockville, Maryland)
1 R01 CA 54564-01

Functional Analysis of the Human T-lymphotropic Viruses

40. KABAT, David
Oregon Health Sciences University
(Portland)
5 R01 CA 25810-13

Leukemogenic Membrane Glycoproteins: gp55s of SFFVs

41. KABAT, David
Oregon Health Sciences University
(Portland)
1 R01 CA 54149-01

Erythropoietin Receptors in Leukemia

42. KNEPPER, Janice E.
Villanova University
5 R29 CA 52853-02

A New Integration Locus for Mouse Mouse Mammary Tumor Virus

43. LENZ, John R.
Albert Einstein College of Medicine
Yeshiva University
5 R01 CA 44822-05

Leukemogenesis by Murine Retroviruses

44.	LERNER, Richard A. Research Institute of Scripps Clinic 5 P01 CA 27489-12	Consequences of Endogenous Retroviral Expression
45.	LEVY, Laura S. Tulane University School of Medicine 5 R01 CA 48801-02	Molecular Genetics of Feline Retroviruses
46.	LICHTMAN, Andrew H. Brigham and Women's Hospital 5 R29 CA 43651-05	In Vitro Models of Viral Leukemogenesis
47.	LILLY, Frank Albert Einstein College of Medicine Yeshiva University 2 R01 CA 19931-13A3	Mechanism of the H-2 Effect of Viral Leukemogenesis
48.	LOUGHRAN, Thomas P., Jr. Fred Hutchinson Cancer Research Center 1 R01 CA 54552-01	Human T-Cell Lymphotropic Viruses in Human Neoplasia
49.	MATHES, Lawrence E. Ohio State University (Columbus) 5 R01 CA 40714-06	Immunoprevention of HTLV Infection
50.	MERUELO, Daniel New York University 5 R37 CA 22247-14	Genetics of Resistance to Leukemia
51.	MERUELO, Daniel New York University 2 R01 CA 31346-09	Study of MuLV Sequences in the MHC: Cloning of Minor H Genes
52.	MURPHY, Edwin C., Jr. University of Texas (Houston) 5 R01 CA 34734-08	Molecular Mechanisms in MuSV-Ts110 RNA Splicing in vivo
53.	NERENBERG, Michael I. Research Institute of Scripps Clinic 5 R01 CA 50234-02	Mouse Models for HTLV-1 Induced Pathogenesis
54.	OVERBAUGH, Julie M. University of Washington 5 R01 CA 50180-02	Regulation of FeLV Expression and Pathogenesis
55.	PALKER, Thomas J. Duke University 5 R01 CA 40660-06	HTLV-1: Study of Host-Virus Interactions

56. PETERSON, David O.
Texas A and M University
5 R01 CA 32695-08

Genetic and Molecular Analysis of Steroid Responsiveness

57. PETERSON, David O.
Texas A and M University
5 R01 CA 48041-04

Mechanisms of Steroid Hormone-Regulated Transcription

58. PHARR, Pamela P.
Veterans Administration Medical Center
(Charleston)
5 R01 CA 50244-03

Retroviral Infection of Hemopoietic Stem Cells

9. PINTER, Abraham
Public Health Research Institute of the City of New York
5 R01 CA 42129-06

Biochemical and Genetic Studies of MuLV Envelope Proteins

60. POZZATTI, Rudy O.
American Red Cross
(Rockville)
1 R29 CA 52140-01A1

Transformation Properties of the HTLV-I Tax Gene

61. RACEVSKIS, Janis
Montefiore Medical Center
5 R01 CA 43864-05

MMTV Gene Products and Transformation

62. RADKE, Kathryn
University of California
(Davis)
5 R01 CA 46374-03

Target Cell Specificity of Bovine Leukemia Virus

63. REDDY, Premkumar E.
The Wistar Institute of Anatomy and Biology
5 R01 CA 47937-04

Transformation and Differentiation by v-abl and c-abl

64. ROSENBERG, Naomi E.
Tufts University
5 R01 CA 24220-13

Abelson Leukemia Virus Transformation

65. ROSENBERG, Naomi E.
Tufts University
5 R01 CA 33771-09

RNA Tumor Virus--Hematopoietic Cell Interaction

66. ROSENBLATT, Joseph D.
University of California
(Los Angeles)
5 R01 CA 52410-02

Pathogenesis of HTLV-1 Associated Myelopathy

67. ROSENBLATT, Joseph D.
University of California
(Los Angeles)
5 R01 CA 53632-02
Regulation of Gene Expression in HTLV Type I/II

68. ROTH, Monica J.
Robert Wood Johnson Medical School
(Piscataway, NJ)
5 R01 CA 49932-02
The Envelope Gene Products of Murine Leukemia Virus

69. ROY-BURMAN, Pradip
University of Southern California
(Los Angeles)
5 R01 CA 51485-02
Pathogenesis Mechanisms in Feline Leukemia

70. SEFTON, Bartholomew M.
Salk Institute for Biological Studies
5 R01 CA 42350-06
Thymoma Tyrosine Protein Kinase

71. SCHWARTZ, Richard C.
Michigan State University
5 R29 CA 45360-05
Synergy of Viral ras and myc in Lymphoid Transformation

72. SORGE, Joseph A.
Stratagene Cloning Systems, Inc.
(La Jolla)
5 R01 CA 36448-09
Gene Transfer and Expression Using Retrovirus

73. SPECK, Nancy A.
Dartmouth Medical School
5 R29 CA 51065-02
Genetics and Biochemistry of Viral Leukemogenesis

74. SRINIVAS, Ranga V.
University of Alabama
(Birmingham)
5 R01 CA 40440-07
Site-Specific Modification of SFFV Glycoproteins

75. STEFFEN, David L.
Baylor College of Medicine
5 R01 CA 30674-10
Analysis of Cellular Oncogenes in Virus-Induced Tumors

76. STEPHENS, Edward B.
University of Florida
5 R29 CA 47100-04
Molecular Engineering of Retroviral Vaccines

77. STRAIR, Roger K.
Yale University
1 R01 CA 54547-01
Detection of Viral Transactivators in Primary Cells

78. SUGDEN, Bill
University of Wisconsin
(Madison)
5 R01 CA 41302-06
Biological and Molecular Studies of A-MuLV Tumorigenesis

79. THOMAS, Christopher Y.
University of Virginia
5 R01 CA 32995-08

Molecular Genetics of Leukemia Viruses

80. TOMPKINS, Mary B.
North Carolina State University
5 R01 CA 43676-05

FeLV-Induced Alterations of Feline Hematopoietic Cells

81. VERMA, Inder M.
Salk Institute for Biological Studies
5 R35 CA 44360-04

Oncogenes, Proto-oncogenes and Retroviral Vectors

82. VOGT, Marguerite M.
Salk Institute for Biological Studies
5 R01 CA 13608-18

Viral Gene Functions Involved in Transformation

83. WEINBERG, Robert A.
Whitehead Institute for Biomedical Research
5 R35 CA 39826-07

Molecular Basis of Carcinogenesis

84. WIGDAHL, Brian
Pennsylvania State University (Hershey Medical Center)
1 R01 CA 54559-01

Pathogenesis of HTLV-I in the Developing Human Nervous System

85. WILSON, Michael C.
Research Institute of Scripps Clinic
5 R01 CA 33730-08

Regulation of Endogenous Retroviral Gene Expression

86. WITTE, Owen N.
University of California (Los Angeles)
5 R01 CA 27507-11

Transformation by Abelson Murine Leukemia Virus

87. WONG, Paul K.
University of Texas (Houston)
5 R01 CA 45124-06

Paralytogenesis Induced by MuLV Mutants

88. YOSHIMURA, Fayth K.
University of Washington
5 R01 CA 44166-06

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 95 research grants with an estimated total funding of 22.37 million dollars for FY91. Of these, approximately 80% are involved with studies of avian tumor viruses and 19% concern hepatitis B virus or other hepatitis viruses and their relationship to primary hepatocellular carcinoma. The remaining 1% deal with a variety of agents which are not as 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 the development and testing of hypotheses about the mechanism(s) of oncogenesis of viruses lacking oncogenes. In addition to 58 traditional R01 grants and 5 P01 program project grants, this component now includes 10 R35 outstanding investigator awards, 8 R37 method to extend research in time (MERIT) awards as well as 5 R13 conference grants, and 9 R29 first independent research support and transition (FIRST) awards.

Investigators whose grants are funded through the RNA Virus Studies II component have made significant progress in a number of areas this year. Two of these have been selected for inclusion into this year's annual report. These topics are retroviral oncogenes and hepatitis viruses. Progress in each of them will appear in the following paragraphs.

Retroviral Oncogenes

The myc protooncogene family, the cellular progenitor of the v-myc oncogene of avian leukosis virus, has been implicated in cell proliferation, differentiation, and neoplasia, but its mechanism of function at the molecular level is unknown. The carboxyl terminus of myc family proteins contains a basic region helix-loop-helix leucine zipper motif (bHLH-Zip), which has DNA-binding activity and has been predicted to mediate protein-protein interactions. The bHLH-Zip region of c-myc protein was used to screen a complementary DNA (cDNA) expression library, and a bHLH-Zip protein, termed Max, was identified. Max specifically associated with c-myc, n-myc, and l-myc proteins, but not with a number of other bHLH, bZip, or bHLH-Zip proteins. The interaction between Max and c-myc was dependent on the integrity of the c-myc HLH-Zip domain, but not on the basic region or other sequences outside the domain. Furthermore, the myc-Max complex bound to DNA in a sequence-specific manner under conditions where neither Max nor myc exhibited appreciable binding. The DNA-binding activity of the complex was dependent on both the dimerization domain and the basic region of c-myc. These results suggest that myc family proteins undergo a restricted set of interactions in the cell and may belong to the more general class of eukaryotic DNA-binding transcription factors.

A striking finding of the study was that Max interacts specifically with three members of the myc family of proteins. Numerous attempts to demonstrate heterodimer formation between myc and other bHLH, bZip, and bHLH-Zip proteins have not been successful. However, under the assay conditions utilized, Max

is capable of associating with c-myc, n-myc, and l-myc. Other proteins that contain related dimerization domains, including the bHLH-Zip proteins USF and AP-4, did not associate. Leucine zipper segments alone determine specificity in Fos-Jun association and act to organize the two proteins in a parallel array. Max and the myc proteins, however, all have HLH domains in addition to zipper regions, and the data show that the integrity of the HLH region is also important for heterodimer formation.

The fact that n-myc and l-myc as well as c-myc specifically associate with Max suggests that Max may serve to integrate the functions of these three proteins that are differentially expressed during development, differentiation, and neoplasia. If so, Max might be expected to be expressed in at least as many cell types as are myc family proteins. Initial experiments with Northern (RNA) blotting indicate that a 2.1-kb Max RNA is expressed in many cells and tissues at concentrations comparable to those of c-myc. In addition, low stringency Southern (DNA) blot analysis suggests that Max is highly conserved as a single gene or a small family of genes in vertebrate genomic DNA, but is absent from invertebrates that also lack myc homologs. These results are consistent with the possibility that Max, or a small number of Max-related proteins, interacts with myc family proteins to mediate their specific biological functions. Whether Max can also be oncogenically activated poses an interesting biological question, which remains to be resolved.

Important questions raised by this work concern the way in which the properties of myc and Max are altered through association. Whether the myc-Max heterocomplex has a specificity for DNA binding that is distinct from that of either of the homodimers is unknown. By analogy with MyoD and the E2A proteins, each member of a myc-Max complex might contribute half-site recognition in defining DNA-binding specificity. The myc-Max complex can be used directly to select a putative new binding sequence with the method for preferential binding and amplification of random sequences.

Another major question concerns the function of the myc-Max complex. It has been suggested that myc may function in transcription, DNA replication, or both. The characteristics of the myc-Max complex places these proteins in the same general class as bHLH transcription factors, but the results do not rule out other possible functions. The essential amino-terminal region of c-myc has been shown to act as a transcriptional activation domain when linked to yeast or prokaryotic DNA-binding domains. Whatever their function, the ability of these polypeptides to form multiprotein complexes suggests that the differential regulation of their relative concentrations could be an important determinant of Max-myc family associations, consequent DNA-binding specificities, and ultimately, the influence of myc on cell proliferation and behavior (22).

Thyroid and steroid hormone receptors are transcription factors that, upon binding cognate hormones, interact with specific DNA sequences and modulate the expression of target genes. These receptors therefore represent highly condensed signaling pathways, embracing in a single polypeptide a mechanism linking events originating in the extracellular environment directly to corresponding changes in gene expression.

Relevant for understanding the actions of these normal cell receptors are the properties of the v-erbA oncogene protein of avian erythroblastosis virus

(AEV). The v-erbA gene participates in the neoplastic transformation of cells by the AEV retrovirus and appears to be an imprecisely transduced copy of a host cell gene, c-erbA, which encodes a thyroid hormone receptor. The v-erbA protein has retroviral gag structural sequences fused to its N-terminus, and has sustained small N- and C-terminal deletions, as well as 13 internal amino acid changes, relative to the c-erbAα progenitor. In animal cells the v-erbA protein appears neither to respond to thyroid hormones nor to activate transcription of thyroid hormone-regulated target genes. Instead, the v-erbA protein binds to the thyroid hormone response element (TRE) sequences and precludes the action of bona fide thyroid hormone receptors at these sites. Thus, v-erbA may represent a novel oncogene that acts as a dominant inhibitor of the function of its normal cellular counterpart.

The glucocorticoid and estrogen receptors have been shown to function in the yeast Saccharomyces cerevisiae when expressed together with reporter genes linked to the appropriate hormone response elements. Although overall receptor function in yeast appears accurately to mimic receptor action in mammalian cells, detailed studies reveal that some aspects of signal transduction by the glucocorticoid receptor, such as the affinity for and response to various hormone ligands, appear to differ in yeast and animal cells. These studies imply that factors in addition to the hormone and receptor participate in the signal transduction pathway, and that a comparative analysis of hormone receptors in such phylogenetically diverse backgrounds as yeast and animal cells might facilitate the detection and characterization of those factors.

A functional analysis of the c- and v-erbA proteins in yeast was conducted. Unexpectedly, it was found that both c- and v-erbA proteins are transcriptional activators in S. cerevisiae and that transcriptional activation by the viral protein was strongly stimulated by thyroid hormones. The ability of v-erbA protein to function as a transcriptional repressor or an activator is determined by interaction with, or modification by, other cellular factors, and that this phenomenon may be relevant to understanding ligand regulation of the normal thyroid and steroid hormone receptors (61).

Additional studies of the src oncogene of the Rous sarcoma virus and of its cellular homologue have revealed novel properties of src homology regions 2 and 3 (SH2 and SH3). The products of the viral and cellular src genes appear to be composed of multiple functional domains. Highly conserved regions called src homology 2 and 3 (SH2 and SH3), comprising amino acid residues 88 to 250, are believed to modulate the protein-tyrosine kinase activity present in the carboxyl-terminal halves of the src proteins. To explore the functions of these regions more fully, 34 site-directed mutations were made in a transformation-competent c-src gene encoding phenylalanine in place of tyrosine 527 (Y527F c-src). Twenty of the new mutations change only one or two amino acids, and the remainder delete small or large portions of the SH2-SH3 region. These mutant alleles have been incorporated into a replication-competent Rous sarcoma virus vector to examine the biochemical and biological properties of the mutant proteins after infection of chicken embryo fibroblasts. Four classes of mutant proteins were observed: class 1, mutants with only slight differences from the parental gene products; class 2, mutant proteins with diminished transforming and specific kinase activities; class 3, mutant proteins with normal or enhanced specific kinase activity but impaired biological activity, often as a consequence of instability; and class 4,

mutant proteins with augmented biological and catalytic activities. In general, there was a strong correlation between total kinase activity (or amounts of intracellular phosphotyrosine-containing proteins) and transforming activity. Deletion mutations and some point mutations affecting residues 109 to 156 inhibited kinase and transforming functions, whereas deletions affecting residues 187 to 226 generally had positive effects on one or both of those functions, confirming that SH2-SH3 has complex regulatory properties. Five mutations that augmented the transforming and kinase activities of Y527F c-src [F172P, R175L, and deletions involving 198-205, 206-226, and 176-226] conferred transformation competence on an otherwise normal c-src gene, indicating that mutations in SH2 (like previously described lesions in SH3, the kinase domain, and a carboxyl-terminal inhibitory domain) can activate c-src (86).

Other observations on SH2 and SH3 have been made in studies of the crk oncogene of the CT10 virus. Although the oncogene product of CT10 virus does not itself phosphorylate proteins at tyrosine residues, it elevates phosphotyrosine in transformed cells. This oncoprotein contains SH2 and SH3 domains, which are conserved in several proteins involved in signal transduction, including nonreceptor tyrosine kinases. It also bound in vitro to phosphotyrosine-containing proteins from crk-transformed cells and from cells transformed by oncogenic tyrosine kinases. The association between the crk and src proteins was abolished by dephosphorylation of the latter. This suggests that the SH2 and SH3 regions function to regulate protein interactions in a phosphotyrosine-dependent manner (30).

A final study of SH2 domains in such diverse systems as the ras oncogene product, GTPase activating protein (GAP), and the EGF receptors, adds support to the important regulatory role of SH2 domains in a variety of systems.

Cytoplasmic proteins which regulate signal transduction or induce cellular transformation, including cytoplasmic protein-tyrosine kinases, the ras oncogene product, GTPase activating protein (GAP), phospholipase Cγ (PLCγ) and the v-crk oncoprotein, possess one or two copies of a conserved non-catalytic domain, src homology (SH) region 2. Direct evidence was recently provided that SH2 domains can mediate the interactions of these diverse signaling proteins with a related set of tyrosine phosphorylated ligands, including the epidermal growth factor receptor. In src-transformed cells, GAP forms heteromeric complexes, notably with a highly tyrosine phosphorylated 62kDa protein (p62). The stable association between GAP and p62 can be specifically reconstituted in vitro using a bacterial polypeptide containing only the N-terminal GAP SH2 domain. The efficient phosphorylation of p62 by the v-src or v-fps tyrosine kinases is in turn dependent on their SH2 domains, and correlates with their transforming activity. In lysates of EGF-stimulated cells, the N-terminal GAP SH2 domain undergoes an EGF-dependent association with both the EGF-receptor and p62. Bacterial polypeptides containing GAP or v-crk SH2 domains complex with similar tyrosine phosphorylated proteins from src-transformed or EGF-stimulated cells, but with different binding efficiencies. SH2 sequences therefore form autonomous domains which direct signaling proteins, such as GAP, to bind specific tyrosine phosphorylated polypeptides. By promoting the formation of these complexes, SH2 domains are ideally suited to regulate the activation of intracellular signaling pathways by growth factors (50).

Hepatitis Viruses

Significant progress has also been made in studies of the hepatitis B virus (HBV) and liver cancer in transgenic animals. The first of these provide evidence for an immune mechanism in the etiology of PHC linked to HBV.

The role of the immune response to hepatitis B virus-encoded antigens in the pathogenesis of liver cell injury has not been defined because of the absence of appropriate experimental models. HBV envelope transgenic mice were used to show that HBV-encoded antigens are expressed at the hepatocyte surface in a form recognizable by major histocompatibility complex (MHC) class I-restricted, $CD8^+$ cytotoxic T lymphocytes specific for a dominant T-cell epitope within the major envelope polypeptide and by envelope-specific antibodies. Both interactions led to the death of the hepatocyte in vivo, providing direct evidence that hepatocellular injury in human HBV infection may also be immunologically mediated. Together these observations establish the minimal requirements to a pathophysiologically relevant cytotoxic T-lymphocyte (CTL) response in vivo. Antiviral antibodies may also be able to destroy infected hepatocytes, a concept that has been, heretofore, considered unlikely. Since the experimental design favored the induction of an HBsAg group-specific immune response, further studies designed to examine the pathogenetic potential of antibody and CTLs specific for the remaining HBV-encoded antigens in multiple MHC backgrounds are needed to fully understand the relative ability of the response to each HBV antigen to cause liver cell injury in viral hepatitis (18).

A second series of investigations posit a direct role of a viral regulatory element, the x gene, in hepatocarcinogenesis. The exact role of hepatitis B virus (HBV) in the development of liver cancer is not known. The recent identification of a viral regulatory gene (HBx) suggests a possible direct involvement of HBV whereby the HBx protein acting as a transcriptional transactivator of viral genes may alter host gene expression leading to the development of hepatocellular carcinoma. This possibility was tested by placing the entire HBx gene under its own regulatory elements directly into the germ line of mice. Transgenic animals harboring this viral gene succumbed to progressive histopathological changes specifically in the liver, beginning with multifocal areas of altered hepatocytes, followed by the appearance of benign adenomas, and proceeding to the development of malignant carcinomas. While mice of both sexes were involved, males developed disease and died much earlier than females. This transgenic animal model appears ideal for defining the molecular events which follow the expression of the viral HBx gene and are responsible for the ultimate development of liver cancer.

While transgenic mice containing different HBV genes, including the entire viral genome, have previously been derived and analyzed by several laboratories, there has been little evidence to suggest a direct role of this virus in inducing liver cancer. In those constructs where the HBx gene was included, its expression was not detected. The uniqueness of this study was to introduce into the germ line of mice only the HBx transactivator gene with its own transcriptional control element. In doing so, heretofore undefined cis- or trans-regulatory elements which act to down-regulate the expression or function of the HBx gene may have been eliminated.

Because of the absence of a detectable inflammatory response and any evidence for liver injury, increased hepatic regeneration cannot be the mechanism underlying the development of HCC in transgenic mice used in this study. This latter mode of induction of HCC has previously been demonstrated in transgenic mice overexpressing the HBsAg through the use of the albumin regulatory element. This observation demonstrates that expression of the HBx gene alone will result in progressive morphological and, presumably, biochemical changes leading ultimately to the development of malignant liver cancer. Interestingly, even the expression of the HBxAg is not ubiquitous in all hepatocytes in the liver. It appears that expression of the HBx gene is restricted to a select subset of hepatocytes, most likely those at a specific differentiation state, thus resulting in multifocal rather than generalized disease in the liver.

These findings suggest the direct involvement of HBV in the development of liver cancer and argue against chronic hepatitis being an absolute prerequisite for the etiology of HCC in man. In addition, the transgenic mice described here will serve as an excellent model system for defining the molecular events subsequent to the expression of the HBx gene which are responsible for the progressive changes in the liver (36).

Another ongoing study of hepatocarcinogenesis seeks to determine other mechanisms which may be involved in its etiology. The p53 tumor suppressor gene was studied at the DNA, RNA and protein level in 7 human hepatocellular carcinoma derived cell lines, and 6 of the 7 showed loss of expression of p53 or abnormalities in the protein. By Southern blotting, the p53 gene was found to be partially deleted in Hep 3B and rearranged in SK Hep1 cells. Transcripts of the p53 gene were undetectable in Hep 3B, as well as in FOCUS cells which had no apparent deletion or rearrangement of p53 gene. Immunoprecipitation following [^{35}S]-methionine labeling of HCC cells demonstrated that p53 protein was absent in Hep 3B and FOCUS and reduced in concentration in PLC/PRF/5 cells. The synthesis of P53 by Mahlavu cells had a slower migration on SDS-polyacrylamide gels, suggesting it was an abnormal protein. In Huh 7 cells, p53 protein had a prolonged half-life leading to its accumulation in the nuclei. Increased levels of p53 protein were also found by Western immunoblotting. The p53 gene and its expression appeared to be unaltered in the hepatoblastoma derived Hep G2 cell line. The loss of p53 expression did not occur as a late in vitro event in the FOCUS cell line, since p53 protein was also undetectable at an early passage. The loss of p53 expression or the presence of abnormal forms of the protein are frequently associated with human hepatocellular carcinoma cell lines. These observations suggest that alterations in p53 may be important events in the transformation of hepatocytes to the malignant phenotype. The likelihood that p53 mutations are important in hepatocarcinogenesis is increased by the independent finding, in an extramural and an intramural laboratory, that liver cancers from Africa and China showed similar mutations in codon 249 of the p53 protein. Such mutations may not only involve a loss of function in p53, but the existence of this "hot spot" at codon 249 implies an additional gain of function. It is possible that interaction of HBV-encoded proteins with mutant p53 provides some advantage to the growth of hepatocarcinoma (55,90).

A basic phosphoprotein antigen defined by a monoclonal antibody (AF5) was found to be highly abundant in human hepatocellular carcinoma by Western immunoblotting. Under the same conditions, the AF5 antigen levels were low or

undetectable in normal liver extracts. The AF5 antibody was used to screen a cDNA expression library of a human hepatoma cell line (FOCUS). A 960 bp cDNA was isolated and found to be a partial cDNA encoding the human protein-tyrosine kinase substrate p36 also known as lipocortin II. The expression of p36 was highly abundant in hepatocellular carcinomas, detected both at the transcript and protein levels. Its expression was not induced significantly during rat liver regeneration following a partial hepatectomy. These results suggest that the induction of p36 expression is associated with malignant transformation rather than proliferation of hepatocytes. The p36 protein was previously shown to be phosphorylated upon transformation of normal fibroblasts by retroviral oncogenes without significant modulation of expression. These studies are the initial description of the association of increased p36 expression with malignant transformation (90).

The role of HBV in the course of patients with primary liver cancer who are negative for hepatitis B surface antigen has been debated. The polymerase chain reaction was used to evaluate 28 such patients for the presence of DNA and RNA sequences of the virus; 22 of these patients had associated cirrhosis. The patients were from areas with different prevalences of HBV infection (South Africa, Italy, France, and Japan). Antibodies to the surface and core antigens of HBV were detected in 10 of the 28 patients tested. HBV DNA sequences were detected in 17 of the 28 patients, including 8 of the 10 with HBV antibodies and 6 of 13 without HBV serologic markers. HBV RNA molecules were found in 4 of 5 tumors tested. These findings indicate that transcriptionally active HBV genomes are present in various geographic areas among patients with liver cancer who are negative for hepatitis B surface antigen. This observation is consistent with an etiologic role for the virus in the development of these tumors (90).

As a result of a previous workshop held in 1989, an RFA entitled "Viral Oncogenesis and Pathogenesis of Hepatocellular Carcinoma" received concept approval by the Division of Cancer Etiology (DCE) Board of Scientific Counselors (BSC) during FY90 and was funded during this fiscal year. Twenty-seven applications were received in response to this RFA, and six highly meritorious applications were subsequently funded, with a first-year total cost of $999,542. The grants address several important issues in hepatitis B virus (HBV)- and hepatitis C virus (HCV)-associated neoplasia and include: studies of a transgenic mouse model for immunopathogenesis of hepatocellular carcinoma (PHC), investigations of HBV as an insertional mutagen, studies of the oncogenic potential of the HBV X gene, co-carcinogenesis studies involving HBV and dietary carcinogens, studies on the replication and gene products of HCV, and investigations on the role of low-level HBV and HCV infection in patients with PHC.

In summary, characterization of oncogenes may uncover interesting new genes that are components of the growth regulatory system of the cell. Studies of hepadnaviruses are beginning to suggest mechanisms of hepatocarcinogenesis, and to identify compounds of potential preventive and/or therapeutic benefits.

RNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY91

Investigator/Institution/Grant Number	Title
1. ACS, George Mount Sinai School of Medicine 5 R01 CA 34818-06	Studies on the Replication and Oncogenicity of HBV
2. ANDERSON, Garth R. Roswell Park Medical Institute 5 R01 CA 48828-02	Anoxia Responsive VL30 Elements
3. BALUDA, Marcel A. University of California (Los Angeles) 5 R01 CA 10197-24	Tumor Induction by Avian Myeloblastosis Virus
4. BEEMON, Karen L. Johns Hopkins University 5 R01 CA 33199-08	Location and Function of M6A in Retrovirus RNAs
5. BEEMON, Karen L. Johns Hopkins University 5 R01 CA 48746-03	Retroviral Regulatory Sequences Within Coding Sequences
6. BISHOP, J. Michael University of California (San Francisco) 5 R35 CA 44338-05	Retroviruses and Cancer Genes
7. BOETTIGER, David E. University of Pennsylvania 5 R01 CA 16502-17	Genetic Analysis of RNA Tumor Viruses
8. BOETTIGER, David E. University of Pennsylvania 5 R01 CA 30383-10	Virus-Induced Myeloid Leukemia
9. BOETTIGER, David E. University of Pennsylvania 5 R01 CA 49866-03	Role of Integrin in Viral Transformation
10. BOS, Timothy J. Eastern Virginia Medical School (Norfolk) 5 R29 CA 51982-03	Analysis of Target Genes Activated by the jun Oncoprotein
11. BOSE, Henry R., Jr. University of Texas at Austin 5 R01 CA 33192-07A2	Transformation by Avian Reticuloendotheliosis Virus

12. BRUGGE, Joan S.
University of Pennsylvania
5 R37 CA 27951-12

Regulation of pp60-src and the Polyoma mT Protein Interaction

13. BURK, Robert D.
Albert Einstein College of Medicine of Yeshiva University
5 R01 CA 45476-03

Role of Hepatitis B Virus in Hepatocellular Carcinoma

14. BURR, John G.
University of Texas at Dallas
5 R29 CA 47098-03

Purification of Tyrosine Phosphoproteins

15. BUSS, Janice E.
La Jolla Cancer Research
5 R29 CA 42348-05

Attachment of Myristic Acid to p60-src

16. CARBON, John A.
University of California (Santa Barbara)
5 R01 CA 11034-23

Studies on Centromere Structure and Function

17. CASPAR, Donald L.
Brandeis University
5 R35 CA 47439-04

Switching in Virus and Membrane Assemblies

18. CHISARI, Francis V.
Scripps Clinic and Research Foundation
5 R37 CA 40489-07

Pathogenesis of Hepatitis B in Transgenic Mice

19. CHISARI, Francis V.
Scripps Clinic and Research Foundation
1 R01 CA 54560-01

Molecular Pathogenesis of Hepatocellular Carcinoma

20. COFFIN, John M.
Tufts University
5 R35 CA 44385-05

Molecular Biology of Retroviruses

21. DUESBERG, Peter H.
University of California (Berkeley)
5 R35 CA 39915-07

Retroviral onc Genes and Cellular Proto-onc Genes

22. EISENMAN, Robert N.
Fred Hutchinson Cancer Research Center
5 R37 CA 20525-15

Control Mechanisms in Avian Oncornavirus Replication

23. ESTES, Mary K.
Baylor College of Medicine
1 R13 AI 30488-01

Third International Symposium on Double-Stranded RNA Viruses

24.	FARAS, Anthony J. University of Minnesota 5 RO1 CA 18303-16	RNA-Directed DNA Polymerase and 70S RNA of Oncornaviruses
25.	FEITELSON, Mark A. Fox Chase Cancer Center 5 R29 CA 48656-04	Products of the X and Polymerase Genes of Hepadnaviruses
26.	GELINAS, Celine UMDNJ-Robert Wood Johnson Medical School 1 R29 CA 52048-01A1	Trans-Acting Function of the v-c-rel Oncoproteins
27.	GILMORE, Thomas D. Boston University 5 R29 CA 47763-03	Transformation of Cells by the v-rel Oncogene
28.	GRANDGENETT, Duane P. St. Louis University Medical Center 5 RO1 CA 16312-18	Avian Retrovirus DNA Synthesis and Integration
29.	HALPERN, Michael S. Wistar Institute of Anatomy and Biology 5 RO1 CA 31514-9	Endogenous Retrovirus as a Determinant of Tumor Immunity
30.	HANAFUSA, Hidesaburo Rockefeller University 5 R35 CA 44356-05	Analysis of Cell Transformation by Retrovirus
31.	HAYWARD, William S. Sloan-Kettering Institute for Cancer Research 5 RO1 CA 43250-05	Mechanisms of Viral and Non-Viral Oncogenesis
32.	HOLLINGER, F. Blaine Baylor College of Medicine 1 R13 AI 29473-01	Symposium on Viral Hepatitis and Liver Disease
33.	HUMPHRIES, Eric H. University of Texas Health Science Center (Dallas) 5 RO1 CA 32295-08	Characterization of the Avian Leukosis Virus-Induced Transformed Follicle
34.	HUMPHRIES, Eric H. University of Texas Health Science Center (Dallas) 5 RO1 CA 41450-06	Expression and Function of v-rel in Lymphoid Tissue
35.	HUNTER, Eric University of Alabama (Birmingham) 5 R37 CA 29884-11	Site Specific Mutagenesis in the Envelope Gene of Rous Sarcoma Virus

36. JAY, Gilbert
American Red Cross (Rockville)
5 R01 CA 51886-02

Role of the Hepatitis B Virus in Inducing Malignancies in Transgenic Mice

37. JOKLIK, Wolfgang K.
Duke University
5 P01 CA 30246-11

Regulatory Functions of Protein Nucleic Acid Interaction

38. JOVE, Richard
University of Michigan
5 R29 CA 47809-04

Mechanisms of Cell Transformation by the Viral *src* Gene

39. KNOWLES, Barbara B.
Wistar Institute of Anatomy and Biology
5 R01 CA 37225-07

Hepatitis Virus and Primary Hepatocellular Carcinoma Cells

40. KOPROWSKI, Hilary
Wistar Institute of Anatomy and Biology
5 P01 CA 21124-14

Virology and Genetics of Cancer

41. KUNG, Hsing-Jien
Case Western Reserve University
5 R37 CA 39207-07

Avian Erythroleukemia and c-*erb*B Activation

42. KUNG, Hsing-Jien
Case Western Reserve University
5 R01 CA 46613-04

Oncogene Activation in Avian B and T Lymphoma

43. LANFORD, Robert E.
Southwest Foundation for Biomedical Research
1 R01 CA 53246-01

Model Systems for Analysis of HBV Replication

44. LEIS, Jonathan P.
Case Western Reserve University
5 R01 CA 38046-08

Retroviral Proteins Involved in DNA Integration/Virion

45. LIANG, T. Jake
Massachusetts General Hospital
1 R01 CA 54524-01

HBV Variants and Hepatocellular Carcinoma

46. LINIAL, Maxine L.
Fred Hutchinson Cancer Research Center
5 R01 CA 18282-16

Retroviral Coded Functions

47. LIPSICK, Joseph S.
University of California (San Diego)
5 R01 CA 43592-05

Mechanism of Transformation by the v-*myb* Oncogene

48. MACARA, Ian G.
University of Rochester Medical Center
5 R01 CA 38888-07
Oncogenes and Control of Phosphoinositide Cycle/Kinase C

49. MAJORS, John E.
Washington University School of Medicine
5 R01 CA 38994-07
Analysis of Retroviral Transcriptional Regulation

50. MARTIN, G. Steven
University of California (Berkeley)
5 R37 CA 17542-16
Genetics of RNA Tumor Viruses

51. NEIMAN, Paul E.
Fred Hutchinson Cancer Research Center
5 R01 CA 20068-16
Molecular Mechanisms in Neoplasia

52. NEIMAN, Paul E.
Fred Hutchinson Cancer Research Center
5 P01 CA 28151-11
Retroviruses and Cancer

53. OGSTON, Charles W.
Rush-Presbyterian-St. Luke's Medical Center
1 R01 CA 54539-01
Woodchuck Hepatitis Virus Non-Structural Genes

54. OU, Jing-Hsiung J.
University of Southern California
1 R01 CA 54533-01
Mechanism of HBV and HCV-Induced Hepatocellular Oncogenesis

55. OZTURK, Mehmet
Massachusetts General Hospital
5 R29 CA 49832-03
Studies on Human Hepatoma Cell Surface Protein p50

56. PANGANIBAN, Antonito T.
University of Wisconsin
5 R01 CA 49741-02
Template Switching During Reverse Transcription

57. PARSONS, J. Thomas
University of Virginia (Charlottesville)
5 R01 CA 27578-11
Expression of Avian Retrovirus Transforming Genes

58. PARSONS, J. Thomas
University of Virginia (Charlottesville)
5 R01 CA 29243-11
Avian Sarcoma Virus Tumor Antigens

59. PARSONS, Sarah J.
University of Virginia
(Charlottesville)
5 RO1 CA 39438-07

Role of C-src in Retroviral Transformation

60. PEARSON, Gary R.
Georgetown University School
1 R13 CA 53079-01

Fourth International Symposium on EBV and Associated Diseases

61. PRIVALSKY, Martin L.
University of California
(Davis)
5 RO1 CA 38823-07

Characterization of v-erbB Oncogene Protein of AEV

62. PRIVALSKY, Martin L.
University of California
(Davis)
1 RO1 CA 53394-01

Mechanism of Action of the v-erbA Oncogene of AEV

63. REDDY, Premkumar E.
Wistar Institute of Anatomy and Biology
1 PO1 CA 52009-01A1

Viruses and Oncogenes in Hematopoietic Malignancies

64. RESH, Marilyn D.
Princeton University
7 RO1 CA 52405-02

Myristylation of Proteins in Transformed Cells

65. ROBINSON, Harriet L.
University of Massachusetts
Medical School
5 RO1 CA 23086-14

Retrovirus-Host Interactions

66. ROBINSON, Harriet L.
University of Massachusetts
Medical School
5 RO1 CA 27223-12

Avian Leukosis Viruses and Cancer

67. ROGLER, Charles E.
Albert Einstein College of
Medicine of Yeshiva University
5 RO1 CA 37232-08

WHV and HBV Associated Hepatocellular Carcinoma

68. ROHRSCHNEIDER, Larry R.
Fred Hutchinson Cancer Research Center
5 RO1 CA 20551-15

Mechanisms of Oncornavirus-Induced Transformation

69. RUECKERT, Roland R.
American Society for Virology
1 R13 AI 29979-01

International Congress of Virology - Berlin - American Society for Virology - Bloc Travel

70. SEEGER, Christoph
Cornell University
1 R13 AI 30384-01

Molecular Biology of Hepatitis B Viruses

71. SEFTON, Bartholomew M.
Salk Institute for Biological Studies
5 R01 CA 17289-16

Membranes and Viral Transformation

72. SELL, Stewart
University of Texas Health Science Center (Houston)
1 R01 CA 54526-01

Hepatic Co-Carcinogenesis in Transgenic Mice

73. SHAFRITZ, David A.
Albert Einstein College of Medicine of Yeshiva University
5 R01 CA 32605-10

Hepatitis B Virus - Chronic Hepatitis - Liver Cancer

74. SHALLOWAY, David I.
Pennsylvania State University (University Park)
5 R01 CA 32317-11

Role of pp60C-src Homolog of the RSV Oncogenic Protein

75. SHIH, Chiaho
University of Pennsylvania
5 R01 CA 48198-03

Dissection of the Life Cycle of Human Hepatitis B Virus

76. SHOWE, Louise C.
Wistar Institute of Anatomy and Biology
1 R01 CA 51918-01A1

Interactions of v-erbA/v-erbB/ BAND 3 in Erythroleukemia

77. SIDDIQUI, Aleem
University of Colorado Health Sciences Center
5 R01 CA 33135-07A2

Expression of Hepatitis B Virus Genes and Hepatoma

78. SKALKA, Anna M.
Institute for Cancer Research (Philadelphia)
5 R35 CA 47486-02

RNA Tumor Viruses - Control of DNA Integration and Gene Expression

79. SLAGLE, Betty L.
Baylor College of Medicine
1 R01 CA 54557-01

Hepatitis B Virus and Hepatocellular Carcinoma

80. SMITH, Ralph E.
Colorado State University
5 R01 CA 35984-08

Biochemistry of RNA Tumor Virus Replication

81. STAVNEZER, Edward
University of Cincinnati
5 R01 CA 43600-06

Origin, Structure and Biological Activity of SKVs

82. STOLTZFUS, Conrad M.
University of Iowa
5 R01 CA 28051-12

Avian Retrovirus RNA Metabolism

83. SUMMERS, Jesse W.
University of New Mexico
5 R35 CA 42542-06

Persistent Infections by Hepadnaviruses

84. TATTERSALL, Peter J.
Yale University School of Medicine
5 R01 CA 29303-11

Molecular Basis of Parvovirus Target Cell Specificity

85. TEMIN, Howard M.
University of Wisconsin (Madison)
5 P01 CA 22443-14

Molecular Biology and Genetics of Tumor Viruses

86. VARMUS, Harold E.
University of California (San Francisco)
5 R35 CA 39832-07

Molecular Analysis of Retroviruses and Oncogenes

87. VERDERAME, Michael F.
Pennsylvania State University (Hershey Medical Center)
5 R01 CA 52791-02

Genetic and Biochemical Regulation of pp60-v-src Activity

88. VOGT, Peter K.
University of Southern California
5 R35 CA 42564-06

Onc Genes in Virus and Cell

89. VOGT, Volker M.
Cornell University (Ithaca)
5 R37 CA 20081-15

Avian Retrovirus Structure and Assembly

90. WANDS, Jack R.
Massachusetts General Hospital
5 R01 CA 35711-08

Pathogenesis, Immunodiagnosis, and Therapy of Carcinoma

91. WANG, Lu-Hai
Mount Sinai School of Medicine
5 R01 CA 29339-12

Transforming Genes of Avian Sarcoma Viruses

92. WANG, Lu-Hai
Mount Sinai School of Medicine
5 R01 CA 49400-03

Expression and Function of Proto-oncogene C-src

93. WEBER, Michael J.
University of Virginia (Charlottesville)
5 R37 CA 39076-08

Signal Transmission by the src Oncogene

94. WEINTRAUB, Harold M.
Fred Hutchinson Cancer
Research Center
5 R35 CA 42506-06

Generation of Development
Mutants with Cloned DNA Vectors

95. WILLS, John W.
Louisiana State University
Medical School (Shreveport)
5 R29 CA 47482-05

Analysis of gag Domains Required
for Retrovirus Assembly

SUMMARY REPORT

AIDS VIRUS STUDIES

The AIDS Virus Studies component of the Branch supports research on the etiologic role of HIV in AIDS-associated neoplasms and in animal models of HIV infection for investigations of viral pathogenesis, HIV-associated neoplastic sequelae and experimental HIV vaccines. Currently, there are 46 research grants in the program with an estimated FY91 funding of 10.05 million dollars, including 41 traditional R01 research grants, 1 program project grant (P01), 1 outstanding investigator award (R35), 2 method to extend research in time (MERIT) awards (R37) and 1 first independent research support and transition (FIRST) award (R29). Research supported by the AIDS Virus Studies component focuses on the development of animal models for basic research in HIV and lentivirus pathogenesis (27%); the association of HIV with neoplastic sequelae in the setting of AIDS and the development of animal models for investigations of AIDS-associated malignancies (25%); the etiologic role of HIV in AIDS and other associated diseases (15%); basic laboratory research leading to the development and pre-clinical evaluation of experimental vaccines (15%); molecular mechanisms of HIV replication and gene expression (8%); viral mechanisms of immunopathogenesis, immune system dysfunction and lymphocyte depletion (7%); and the synthesis and biological evaluation of experimental antiviral agents (3%).

The heterogeneous clinical manifestations of primary HIV infection, such as fever, pharyngitis, lymphadenopathy, aseptic meningoencephalitis, peripheral neuropathy and erythematous maculopapular rash reflect an immunological response to rapid and widespread dissemination of HIV following infection. HIV has been isolated from peripheral blood mononuclear cells (PBMCs), cell free plasma, cerebrospinal fluid (CSF) and bone-marrow cells during primary infection. HIV infection and subsequent immune deficiency is characterized by the selective reduction in the number of CD4+ (helper/inducer) subset of T-lymphocytes and generalized suppression of immune system functions, leading to an increased incidence of fatal opportunistic infections and AIDS associated malignancies. The control of HIV infections and AIDS continues to present researchers and clinicians with many difficult problems. HIV, especially its surface envelope, is hypervariable, both between isolates from different patients and from the same patient at different times; recent data suggests that new virus isolates detected later in the infection may not be recognized by the patient's immune sera from an earlier stage of the HIV infection. HIV also has a broad tissue tropism and is able to evade the host immune system early in the infection and establish a latent infection. As a consequence of their underlying immunodeficiency, HIV infected patients are at an increased risk for developing cancers, such as Kaposi's sarcoma (KS) and non-Hodgkin's B-cell lymphomas (5,19,22,24,28,30,40,43,45).

Early in the AIDS epidemic, high grade non-Hodgkin's lymphoma (NHL) began to appear in individuals at risk for the development of AIDS. The first published report, in 1984, described a series of homosexual men with high grade B-cell lymphomas. In the intervening time, the type of lymphomas occurring in HIV-1-infected individuals has not changed significantly from that originally reported. However, the incidence of AIDS-associated cancers is expected to rise during the next several years as patients with AIDS survive for longer periods of time with improved treatment for HIV infections

and AIDS associated opportunistic infections. The AIDS-associated lymphomas have primarily been of intermediate or high grade histology and are equally divided into Burkitt's-like small non-cleaved, immunoblastic and large diffuse histiocytic categories. However, all these lymphomas have behaved biologically as high grade aggressive NHL, and there has been little progress in the development of successful therapy. Common characteristics of this class of NHL are very high rates of extranodal (85-97%) and central nervous system involvement (35%) (5,28).

Drs. Michael McGrath and Riccardo Dalla-Favera are both investigating the etiology of AIDS-associated lymphomas. Clinical features characteristic of AIDS-associated lymphomas include: 1) a 5- to 20-fold increase in the expected frequency of bone marrow and central nervous system involvement; 2) one-third of patients present with tumors in atypical extranodal sites such as rectum, liver, mouth, heart, and lung; 3) three-quarters of patients present with advanced disease, including fever, night sweats, and/or weight loss (symptoms which may also be associated with the underlying HIV-1 infection); 4) one-third of AIDS-NHL patients present with lymphoma as their initial manifestation of HIV-1 infection, one-third had pre-existing generalized lymphadenopathy, and approximately one-third had a diagnosis of AIDS prior to the development of lymphoma; and 5) the median survival of NHL patients with pre-existing AIDS is approximately two months, compared with a median survival of eight months for all those NHL patients without a pre-existing AIDS diagnosis. In contrast to non-AIDS associated NHL, durable long term remissions in AIDS-NHL is extremely rare. Dr. Dalla-Favera's research focuses on defining viral co-factors involved in AIDS-associated malignancies and the molecular analysis of the c-myc locus translocations in these neoplasms. Dr. Dalla-Favera is currently examining the presence of mutations of the H-, K-, and n-ras oncogenes, for mutations and deletions of the p53 gene and for loss of inactivation of the Rb gene in these AIDS neoplasms. Recent evidence indicates that as many as four different genetic lesions may be involved in the etiology of AIDS-NHL, indicating that both viral and cellular factors may be involved in human cancers. Dr. Michael McGrath is investigating whether AIDS-NHL represents outgrowths of viral or self antigen activated B-cells, to define the spectrum of immunoglobulin variable (V) region genes utilized by these lymphomas and to test whether subsets of lymphomas would utilize identical V regions. If viral determinants are found to drive the premalignant B-cell proliferation or common V region determinants are shared between different lymphomas, non-immunosuppressive therapeutic approaches to the prevention and possible treatment of AIDS-lymphomas could be devised. Results from these two basic research efforts on the etiology and growth properties of AIDS-NHL may provide insights to aid in more rapid diagnosis, prevention and treatment of these aggressive malignancies (5,28).

HIV infections are associated with an initial period of asymptomatic infection prior to the development of immunodeficiency and AIDS. Low levels of virus replication are detected early in infection, while increased levels of HIV are observed as the disease progresses. Multiple factors are involved in controlling the replication of HIV in vivo, including the host immune response to virus, the pathogenicity of viral strains present, and cofactors, such as cytokines and viral gene products that could accelerate or influence viral replication. Cellular factors that regulate the degree of viral RNA expression within infected cells may also contribute to the level of viral replication observed in HIV infected individuals. HIV DNA has been detected

in a higher proportion of cells than can be found to express HIV RNA or proteins, suggesting that latent infection of lymphocytes or monocytes may occur in vivo. Activation of viral expression from such latently infected cells might also contribute to the increased HIV replication seen in patients. HIV RNA transcription within an infected cell is regulated by both viral encoded trans-acting proteins as well as by cellular transcription factors. The HIV encoded tat protein is critical for high levels of HIV transcription and its presence is required for viral replication. Expression of the HIV tat protein results in a marked (50- to >100-fold) activation of gene expression directed by the viral long terminal repeat sequences (LTR); thus, tat acts as a strong positive feedback signal to the amplification of viral RNA. Although its precise mechanism of function has not been elucidated, tat has been shown to strongly induce HIV RNA transcription, at least in part, by facilitating transcriptional elongation of nascent RNA molecules. In addition to tat, other HIV proteins may also exert regulatory roles. The HIV rev protein facilitates the transport of unspliced HIV RNAs (virion RNA and viral RNAs encoding structural proteins and enzymatic activities) out of the nucleus of the infected cell and may thus modulate a transition between a regulatory phase of infection within a cell and a productive phase resulting in virion synthesis. The function of the HIV nef protein remains controversial, since several reports suggest that nef may exert a negative effect on HIV production, possibly mediated through the LTR, while other studies have failed to demonstrate a negative effect either on viral replication or on LTR activity. The HIV viral particle contains multiple copies of the vpr protein, the first HIV regulatory protein to be found in the virus particle. The vpr protein acts in trans to accelerate virus replication and cytopathic effect in T-cells, suggesting that it acts to facilitate the early steps of virus infection. These results and observations indicate that the HIV regulatory proteins can exert both positive and negative effects on HIV gene expression (1,16,36, DNA I component, reference 42).

Novel approaches and reagents to prevent the initial HIV infection or slow the course of infection are being developed. The CD4 lymphocyte receptor (CD4), the cellular protein to which HIV binds during the initiation of infection, was isolated by Dr. Richard Axel and expressed in an in vitro mammalian system in which a recombinant soluble form of CD4 (rsCD4) was secreted into tissue culture supernatants. Dr. Axel previously demonstrated that rsCD4 binds to the HIV envelope glycoprotein gp120, and thus is a potent inhibitor of HIV infection and virus-mediated cell fusion in vitro. Recently, Dr. Axel and his colleagues determined the molecular crystal structure of the CD4 molecule at 2.3 angstroms. The first two structural domains of the CD4 molecule are immunoglobulin-like, but exhibit significant departures from classical antibody structures. It was also demonstrated that the primary sites for HIV interaction are on loops that protrude from the first immunoglobulin-like domain. Moreover, the second major domain of the protein, which is intimately associated with the first, resembles constant domains of immunoglobulins. The availability of the precise molecular structure of this fragment of CD4 now permits a more rational design of agents to prevent the entry of the AIDS virus into susceptible cells (RNA I component, reference 3).

One important objective of the AIDS Virus Studies program is the development and utilization of appropriate animal models for investigations of AIDS and lentivirus pathogenesis. Lentiviruses which infect different animal species have been identified, including the equine infectious anemia virus, bovine

immunodeficiency virus, caprine arthritis encephalitis virus, simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV). Two viruses, SIV and FIV, have been convincingly linked to an AIDS-like syndrome, while the others cause varying degrees of immunodeficiency in the host. SIV induces a persistent infection in most species of Old World monkeys and great apes, but rarely causes disease in its African host species. Certain SIV strains isolated from African nonhuman primates, such as the sooty mangabey (SIV_{mac}), induce simian AIDS in Asian macaques, while other SIVs, such as the African green monkey isolate (SIV_{agm}), do not induce any AIDS-like disease. The immunodeficiency disease picture with most SIV isolates generally is more acute than that of human AIDS, with the intervening period of asymptomatic carriage between infection and development being short or absent. In domestic cats, FIV often produces a transient initial illness characterized by fever, lymphadenopathy and neutropenia, followed by a lengthy incubation period prior to the onset of feline AIDS (6,10,25,26,29,32,33,38,41, RNA I component, reference 35).

Domestic cats naturally infected with FIV develop an immune deficiency and an array of persistent diseases caused by secondary or opportunistic pathogens, similar to that observed in the human disease. Dr. Niels Pedersen has investigated the underlying immune dysfunction in experimentally infected, specific pathogen-free (SPF) cats. Cats infected with FIV for less than 10 months exhibited a decrease in the percentage of circulating CD4+ lymphocytes but retained normal numbers of CD4+ cells and normal in vitro lymphocyte proliferative responses to mitogens, chemicals which induce B- or T-cell specific proliferations. In contrast, animals infected for 25 months or longer with FIV exhibited a profound depression of response to the mitogens and a corresponding depletion of circulating CD4+ lymphocytes. These findings demonstrate that subtle but ultimately progressive immunologic abnormalities occur early in FIV infection, and that responses to mitogens, CD4+ cell percentages and CD4+/CD8+ ratios may serve as more sensitive indicators of these changes than absolute numbers of CD4+ cells. None of the FIV infected animals showed any outward signs of illness and disease; however, this is not unexpected since all animals were SPF (except for the experimental FIV infection) and were maintained in strict isolation. Field studies of naturally occurring FIV infection suggest that immunosuppressed cats suffer from the effects of common feline pathogens rather than members of the normal microbial flora (which are present in SPF animals). Thus, it is possible that some or all of the FIV-infected animals would not have remained asymptomatic for disease had they not been shielded from exogenous pathogens. Alternatively, CD4+ cell counts may not have declined to levels permissive for the development of FIV-AIDS. These considerations led Dr. Pedersen to question the role of pathogenic cofactors and the acceleration of FIV disease. In humans, activation of T-cells following contact with exogenous antigens (such as infectious disease agents) has been implicated as an important step in AIDS pathogenesis. Dr. Pedersen recently demonstrated that an underlying asymptomatic infection with another retrovirus, feline leukemia virus, greatly enhances expression and dissemination of FIV as well as the severity of FIV disease; additionally, cats co-infected with FIV and FeLV have a much higher incidence of neoplastic sequelae, such as lymphomas, than do animals infected with a single pathogen. The FIV immunodeficiency model will continue to be of great value for investigating the role of pathogenic cofactors in accelerating AIDS disease and lentivirus associated cancers (33).

The non-human primate lentiviruses closely parallel their human counterparts, HIV-1 and HIV-2, in genomic organization and gene homology, biological properties and tropism for CD4+ lymphocytes. Infection of rhesus monkeys with some SIV isolates results in a progressive immunodeficiency disease and fatal opportunistic infections, similar to human AIDS. Transcriptional regulatory sequences in the long-terminal repeats (LTR) of some murine and avian C-type retroviruses have been shown to be potent determinants of their pathogenicity. Despite striking differences in the regulation of viral gene expression between C-type oncoviruses and lentiviruses, transcriptional signals may play an important role in regulating viral replication and, hence, pathogenesis by immunodeficiency lentiviruses. Dr. Nancy Hopkins has used genetic techniques to identify viral genes that play an important role in shaping the disease-inducing phenotype of different SIV isolates. To determine the potential influence of SIV regulatory sequences on viral pathogenesis, Dr. Hopkins introduced a series of 5'-deletions and a point mutation into the viral sequences which define transcriptional enhancer regions. In vitro transcription activity of the mutated viral regulatory regions (termed viral U3 sequences) was analyzed by transient reporter-enzyme assays. Two distinct regions of the regulatory region were shown to contain important cis-acting sequences for positive regulation of transcriptional activity. In spite of a high degree of genetic relatedness between SIV and HIV, a negative regulatory element analogous to that identified in the U3 region from the HIV LTR was not found in the U3 region from the SIV isolate analyzed here. Thus, molecular analysis of viral regulatory sequences will be an important method of determining differences among pathogenic and non-pathogenic isolates of SIV. The SIV/macaque model will continue to be a useful model to study the role of viral regulatory genes in disease pathogenesis (RNA I component, reference 35).

Dr. Myron Essex and his collaborators have conducted molecular biological experiments to determine the function of SIV regulatory proteins and their potential role in pathogenesis. The genomes of SIVs isolated from rhesus macaques (SIV_{mac}) contain an open reading frame (ORF), termed vpr, which has a coding potential of 97 to 101 amino acid residues. A vpr-ORF-encoded protein of approximately 11 kDa was identified, and anti-vpr antibodies were detected in rhesus macaques infected with SIV_{mac}. These results provide clear evidence that the vpr-ORF is a coding gene of SIV_{mac}. The vpr protein, like the vpx protein which is encoded by another accessory gene of SIV_{mac}, was also found to be associated with viral particles, demonstrating that more than one accessory gene product can be present in the virions of this family of retroviruses. The presence of the vpr gene product in viral particles raises the possibility that this protein may have a role in early stages of the virus life cycle before de novo synthesis of viral proteins, such as activating cellular factors that support subsequent viral replication. It is known that the coding sequence of the vpr is one of the most conserved among the genes of HIV-1, HIV-2 and SIV_{mac}. However, the lack of such a conserved gene in the genome of SIV_{agm} (African green monkey isolate) is unusual; moreover, the absence of AIDS in SIV_{agm}-infected monkeys sharply contrasts with the presence of a vpr gene in HIV-1, HIV-2 and SIV_{mac} and the abilities of these viruses to cause AIDS in their respective hosts. Further studies using the SIV model may help to establish whether the vpr gene is indispensable for the induction of AIDS (9).

Prior to 1981, Kaposi's sarcoma (KS) was considered to be a rare tumor of blood vessels characterized by multiple skin nodules of the lower extremities. However, a sudden increase in KS cases was observed in San Francisco in the early 1980's in patients diagnosed with AIDS. The four forms of KS are based on the natural history of the disease. Classical KS occurs as a sporadic indolent disease among elderly men in eastern European and Mediterranean regions. Endemic KS occurs in equatorial Africa, occurring predominately in younger black men (25-40 years); however, the same disease in African children is predominantly lymphadenopathic and has a rapid downhill course. The third type of KS occurs in iatrogenically immunosuppressed individuals, including organ transplant recipients; while skin lesions are common, visceral disease and lymph node involvement is rare. KS tumors regress spontaneously in some patients when immunosuppressive treatment is reduced or terminated. The fourth and most recent form of KS is epidemic in patients with AIDS, and is one of the most common of the AIDS-related neoplasms. KS occurs almost exclusively in male homosexuals with AIDS, and frequently involves lymph nodes; more than 50% of patients with skin involvement also have disseminated disease in the gastrointestinal tract or other visceral organs. Only a few cases of cutaneous KS in AIDS patients have regressed spontaneously (22,42, 43,45, DNA II component, reference 4).

Recent evidence suggests that the rapid growth of KS cells and tumors involves cellular proliferation induced via autocrine mechanisms. In order to define the oncogenic potential of KS cells and determine if specific genes in the KS lesions are involved in uncontrolled cellular proliferation, Dr. Claudio Basilico searched for and characterized transforming DNA sequences from KS tumors that morphologically transform tissue culture cells and induce experimental tumors in rodents. Human DNA sequences, present in the in vitro DNA transformants, were cloned and identified as being related to the basic and acidic fibroblast growth factor (FGF) family of genes. The cloned human sequences, termed the k-fgf oncogene, encodes a Kaposi's derived growth factor that is secreted from transformed cells and transforms other cells through a mechanism of autocrine cell proliferation. K-fgf-transformed cells are highly tumorigenic when transplanted to immunocompetent allogeneic or syngeneic animals. To determine whether the K-fgf tumors could be prevented through active immunization with a k-fgf related antigen, BALB/c mice were immunized with a recombinant DNA produced protein corresponding to the mature, secreted form of K-fgf. Following vaccination, the mice were challenged with syngeneic K-fgf-transformed (positive cells) or H-ras-transformed cells (negative cells). Vaccinated animals exhibited a greater than 60% rate of protection against tumor induction, protection which was specific for K-fgf-transformed cells and correlated with the ability of the vaccinated mice to produce high titers of anti-K-fgf antibodies. The protection was specific for cells transformed by the K-fgf oncogene and thus did not result from a general boosting of the immune response. These experiments are important because they demonstrate that immunization with a single oncogene product can provide a substantial degree of protection against syngeneic tumor cells expressing the K-fgf oncogene (DNA II component, reference 4).

As the result of a previous Branch workshop, held in November 1989 and entitled "The Role of Human Immunodeficiency Virus and Other Viruses in Acquired Immunodeficiency Syndrome-Associated Malignancies," a BCB sponsored RFA was funded this fiscal year. The initiative, entitled "Molecular Mechanisms of Viral-Induced AIDS-Associated Neoplasia," had received concept

approval by the DCE Board of Scientific Counselors during FY90 for $1,000,000 per year. The goals of the initiative were to stimulate investigations in the extramural community on the role of HIV, viruses and viral genes, and cellular genes or cofactors in the initiation and progression of AIDS associated malignancies. Thirty-six applications responsive to the goals of the RFA were received, of which five were recommended for funding by program staff, with a total cost of $912,646 per year. These new scientific efforts focus on the role of B-cell growth factor in the proliferation of lymphomas, a primate animal model of Kaposi's sarcoma, the role of human cytomegalovirus as a viral cofactor in AIDS malignancies, and two projects focusing on possible mechanisms of induction of AIDS-associated lymphomas.

On November 15, 1990, the Branch sponsored a workshop entitled "Animal Models of Retrovirus-Associated Malignancies." Animal retroviruses isolated from many mammalian species have the potential to provide valuable basic information on the etiology and mechanism(s) of cancer induction by viruses. Mammalian retroviruses have been isolated from humans, monkeys, mice, cats, cows, goats, sheep, pigs and horses. In some virally infected animals, neoplastic and Kaposi's sarcoma-like lesions have been observed, supporting the hypothesis that retroviruses may be directly or indirectly involved in the development of malignancies. The workshop focused on the neoplastic potential of various retroviruses and the different molecular mechanisms by which viruses might be involved in the etiology of both lymphoid malignancies and sarcomas. Workshop participants noted that little is known about the ability of the host to restrict the replication of retroviruses, and thus it is not known which host factors determine whether a virus replicates lytically or might be involved in the initiation or progression of neoplastic sequelae. Some retroviruses, such as the equine infectious anemia virus, have not been shown to be involved in malignant sequelae, while other retroviruses, such as the feline leukemia viruses, are frequently involved in malignancies. The workshop participants concluded that studies on the pathogenesis of cancers of viral origin from animal models should result in additional information and knowledge relevant to human cancer. An RFA based on these recommendations was developed. At its June 1991 meeting, the DCE Board of Scientific Counselors approved the issuance of an RFA entitled "Domestic Animal Models for Retrovirus-Associated Human Cancers." Specific research goals of the RFA include: 1) study of the oncogenic mechanisms in domestic animal retroviruses; 2) investigation of cancer etiology and viral pathogenesis from initial infection through the development of pre-neoplastic lesions and neoplastic sequelae with retroviruses of domestic animals; 3) the role of RNA and DNA viral cofactors in cancer etiology in animal models; 4) investigations to assess the role of the host immune system and host genetic factors in the control and limitation of virus replication, and the susceptibility or resistance of animals to oncogenic processes; 5) studies on the expression and regulation of viral and/or associated host cell genes in pre-neoplastic lesions and malignant tissues from retrovirus-infected domestic animals. It is anticipated that applications for this RFA will be received and reviewed in the Winter of 1992, with funding of meritorious applications in FY92.

AIDS VIRUS STUDIES

GRANTS ACTIVE DURING FY91

Investigator/Institution/Grant Number	Title
1. BALDWIN, Albert S., Jr. University of North Carolina at Chapel Hill 1 R01 CA 52515-01	Inducible HIV and Class I MHC Enhancer Binding Proteins
2. BARKLIS, Eric Oregon Health Sciences University 2 R01 CA 47088-04	Core Assembly of the Human Immunodeficiency Virus
3. BOLOGNESI, Dani P. Duke University Medical Center 2 P01 CA43447-06	Pre-Clinical Studies on Prevention & Intervention in AIDS
4. CHAMPOUX, James J. University of Washington 5 R01 CA 51605-03	Plus-Strand Priming and Integration by M-MuLV and HIV
5. DALLA-FAVERA, Riccardo Columbia University 5 R37 CA 37295-08	AIDS-Associated Lympho- proliferative Disorders
6. DAVIS, William C. Washington State University 5 R01 CA 50141-03	Animal Models for Research on AIDS-Related Lentiviruses
7. DOUGHERTY, Joseph P. Robert Wood Johnson Medical School (Piscataway) 5 R29 CA 50777-03	Determination of Retrovirus Mutation Rates
8. ELDER, John H. Scripps Clinic and Research Foundation 5 R01 CA 43362-05	Development of a Synthetic Vaccine to Retroviruses
9. ESSEX, Myron E. Harvard University 5 R35 CA 39805-07	NCI Outstanding Investigator Grant
10. FINBERG, Robert W. Dana-Farber Cancer Institute 5 R01 CA 34979-08	Animal Models of AIDS
11. FORD, Richard J., Jr. University of Texas M.D. Anderson Cancer Center 5 R01 CA 52778-02	Role of Human Retroviruses in AIDS-Related Lymphomas

12.	GRANT, Christopher K. Pacific Northwest Research Foundation 5 R01 CA 43371-04	Anti-Idiotype Vaccines for Feline Leukemia Virus
13.	GREEN, William R. Dartmouth College 5 R01 CA 43475-06	Study on Development and Assessment of Retroviral Vaccines
14.	GREEN, William R. Dartmouth College 5 R01 CA 50157-02	The Pathogenesis of MAIDS and Specific T Cell Responses
15.	HALL, William W. North Shore University Hospital (Manhasset) 5 R01 CA 51012-02	Concomitant Infections with HIV and HTLV-1/HTLV-2
16.	HASELTINE, William A. Dana-Farber Cancer Institute 5 R01 CA 44460-05	Molecular Biology of the ART Gene of HTLV-III
17.	HIRSCH, Martin S. Massachusetts General Hospital 5 R01 CA 12464-21	Immune Reactivity and Oncogenic Virus Infections
18.	HIRSCH, Martin S. Massachusetts General Hospital 2 R01 CA 35020-09	Viruses, Acquired Immunodeficiency, and Kaposi's Sarcoma
19.	HIRSCH, Martin S. Massachusetts General Hospital 1 R01 CA 54741-01	HIV-CMV Interactions
20.	HOOVER, Edward A. Colorado State University 5 R01 CA 43216-06	Pathogenesis of Feline Leukemia Virus-Induced AIDS
21.	HUNTER, Eric Univ of Alabama at Birmingham 2 R37 CA 27834-11	Genetics of Primate 'D' Type Retroviruses
22.	JAY, Gilbert American Red Cross (Rockville) 5 R01 CA 53633-02	Role of the HIV-1 Regulatory Genes in Transgenic Mice
23.	KIESSLING, Ann A. The Faulkner Hospital, Inc. (Boston) 5 R01 CA 53899-02	Retroviruses Associated with Human Reproductive Tract

24.	KIPPS, Thomas J. University of California (San Diego) 1 RO1 CA 54755-01	Mechanisms of AIDS-Associated Lymphomagenesis
25.	LETVIN, Norman L. Harvard University 5 RO1 CA 50139-03	Immune Regulations in SIV Infections
26.	LI, Yen Harvard University 5 RO1 CA 50146-03	Diversity and Pathogenesis of SIVagm
27.	LOTZ, Martin K. University of California (San Diego) 5 RO1 CA 51406-02	TGF Beta and the Pathogenesis of HIV
28.	MCGRATH, Michael S. University of California (San Francisco) 1 RO1 CA 54743-01	Antigenic specificity and V region use of AIDS B NHL's
29.	MONTELARO, Ronald C. University of Pittsburgh 5 RO1 CA 49296-04	Gene Expression During Lentivirus Infections: EIAV
30.	NELSON, Jay A. Scripps Clinic and Research Foundation 5 RO1 CA 50151-03	A Transgenic Model for HIV/Opportunistic Interactions
31.	NEURATH, A. Robert New York Blood Center 5 RO1 CA 43315-05	Synthetic HIV-1 Env Protein Analogs for Future Vaccines
32.	PAYNE, Susan L. Case Western Reserve University 5 RO1 CA 50168-02	Lentiviral Diseases: EIAV Genome Structure
33.	PEDERSEN, Niels C. University of California (Davis) 5 RO1 CA 50179-03	Incidental Infectious Diseases as Cofactors in the Transmission and Progression of FIV Infection
34.	PITHA-ROWE, Paula M. Johns Hopkins University 5 RO1 CA 50158-03	Retrovirus-Induced Immunodeficiency: Role of Cytokines in Pathogenesis
35.	PRUSOFF, William H. Yale University 5 RO1 CA 05262-31	Iododeoxyuridine, Iodo-DNA and Biological Activity

36. RABSON, Arnold B.
Robert Wood Johnson Medical School (Piscataway)
1 R01 CA 55487-01
Effect of NFkB and rel-Family Proteins on HIV Expression

37. REDDY, E. Premkumar
The Wistar Institute
1 R01 CA 55492-01
Role of MYB in Transcriptional Regulation of HIV-1 and HTLV-1

38. ROTH, James A.
Iowa State University
5 R01 CA 50159-03
Bovine Lentivirus as a Model for HIV Infection

39. SCHOOLEY, Robert T.
University of Colorado Medical School
5 R01 CA 37461-08
Cell-Mediated Immune Response to Human Retroviruses

40. SHARMA, Surendra
Roger Williams General Hospital
1 R01 CA 54763-01
Pathogenetic Evaluation of AIDS-Associated Lymphomas

41. TOMPKINS, Wayne A. F.
North Carolina State University
1 R01 CA 54723-01
Pathogenesis of Feline Immunodeficiency Virus - AIDS

42. TSAI, Che-Chung
University of Washington
1 R01 CA 54757-01
Mechanisms of Viral Induced AIDS-Associated Neoplasia (RF)

43. VOGEL, Jonathan
American Red Cross (Rockville)
1 R01 CA 52408-01
Biological Studies of Kaposi's Sarcoma in Transgenic MI

44. VOLSKY, David J.
St. Luke's-Roosevelt Hospital Center
5 R01 CA 37465-07
Viral Etiology of AIDS: Role of HIV-Induced Membrane Injury in Cell Dysfunction

45. WONG-STAAL, Flossie
University of California (San Diego)
1 R01 CA 52412-01
HIV Tat Protein and Kaposi's Sarcoma Pathogenesis

46. YUEN, Pick-Hoong
University of Texas M.D. Anderson Cancer Center
5 R01 CA 52421-02
Kaposi's Sarcoma Associated with AIDS - A Mouse Model

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 the Research Resources component of the Branch.

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. Currently three research resources contracts are administered by the Branch with an estimated FY 1991 funding level of 0.60 million dollars. These contract activities include production, characterization and distribution of viral and anti-viral reagents; provision of specialized testing services for the examination of experimental materials; and storage, inventory and distribution of biological research reagents.

During this period, a solicitation was issued on April 20, 1990 requesting proposals for continuation of the repository contract for the storage and distribution of biological reagents. After evaluation of the proposals, a contract for operation of the repository was awarded to Quality Biotech, Inc. on February 15, 1991. The repository was relocated from Bethesda, Maryland to Camden, New Jersey on February 27-28, 1991.

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, a maximum of three assays were carried out on approximately 344 cultures from over 50 laboratories. In making these interspecies and intraspecies cell identifications, more than 780 procedures were performed using the following assays: immunofluorescent staining for species-specific surface antigens, isoenzyme analysis, and cytological analysis by means of chromosome banding (2).

During this period, 253 shipments of viral reagents and human specimens were made to domestic laboratories from the inventory of frozen biological

reagents. Appropriate demographic, clinical and characterization data were included with each shipment. In addition, 28 shipments of reagents and data were sent to foreign laboratories (1).

Relocation of the repository was accomplished over a two day period, with no major mechanical difficulties and no loss of samples. Through coordination of telephone requests between the former and new contractor, the repository was out of operation only for the actual days of the relocation. During the first month of operation by the new contractor, there were 23 domestic and one foreign shipments of viral reagents and human specimens (3).

Additionally, each year the Branch coordinates and prepares for publication an advertisement of resources and services available for cancer research from all programs within the Division of Cancer Etiology (DCE). This advertisement, which keeps the scientific community advised of currently available resources under the auspices of DCE and the contractor source of supply, is published in several major scientific journals each year.

All resource contracts administered by the Branch operate under a payback system which was implemented in 1981. Under this system, some of the costs of production, along with the costs for handling and distribution of research resources are collected from the recipient. The payback system 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 activities deemed to be no longer necessary.

RESEARCH RESOURCES

CONTRACTS ACTIVE DURING FY91

Investigator/Institution/Contract Number	Title
1. DONLEY, Elizabeth Microbiological Associates Inc. NO1 CP 61020	Repository for Storage and Distribution of Viruses, Viral Reagents and Human Sera
2. PETERSON, Ward D. Children's Hospital of Michigan (Detroit) NO1 CP 85645	Cell Culture Identification and Cytologic/Karyotypic Analysis
3. ROBISON, Alice K. Quality Biotech, Inc. NO1 CP 15665	Repository for Storage and Distribution of Biological Research Resources