Division of

CANCER ETIOLOGY

1985 Annual Report Volume I
October 1, 1984-September 30, 1985

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ANNUAL REPORT

DIVISION OF CANCER ETIOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

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ANNUAL REPORT

DIVISION OF CANCER ETIOLOGY

Richard H. Adamson, Ph.D., Director

October 1, 1984 through September 30, 1985

OVERVIEW

As part of a Division-wide reorganization begun last year, the Biological and the Chemical and Physical Carcinogenesis Intramural and Extramural Programs have been restructured with the creation of new programs. The biological carcinogenesis components of the Carcinogenesis Intramural Program (Laboratory of Viral Carcinogenesis, Laboratory of Molecular Oncology, Laboratory of Cellular and Molecular Biology and Laboratory of Molecular Virology) and the Extramural Biological Carcinogenesis Branch were incorporated into the new Biological Carcinogenesis Program. In addition, a new laboratory (Laboratory of Tumor Virus Biology) was created within the Biological Carcinogenesis Program and is located in Building 41.

Incorporated into the new Chemical and Physical Carcinogenesis Program are components of the Carcinogenesis Intramural Program consisting of the Laboratory of Biology, Laboratory of Human Carcinogenesis, Laboratory of Chemoprevention, Laboratory of Cellular Carcinogenesis and Tumor Promotion, Laboratory of Molecular Carcinogenesis, Laboratory of Comparative Carcinogenesis, Laboratory of Experimental Pathology and the Laboratory of Experimental Carcinogenesis (formerly the Laboratory of Carcinogen Metabolism). Also incorporated into the Chemical and Physical Carcinogenesis Program was the extramural Chemical and Physical Carcinogenesis Branch and the Low Level Radiation Effects Branch which was transferred to this Division from the Division of Cancer Treatment in June 1984.

The Field Studies and Statistics Program has also been reorganized and its name changed to the Epidemiology and Biostatistics Program. Moved into the Epidemiology and Biostatistics Program was the Extramural Programs Branch (formerly called the Special Programs Branch) which is responsible for the grant portfolio and other extramural activities in the field of epidemiology and biostatistics. In addition, a Radiation Epidemiology Branch was created last year from a section in the Environmental Epidemiology Branch. Thus the components of the Epidemiology and Biostatistics Program are now the Environmental Epidemiology Branch, the Clinical Epidemiology Branch, the Biostatistics Branch, the Radiation Epidemiology Branch and the Extramural Programs Branch.

It is anticipated that this Division-wide reorganization will enhance interaction between intramural scientists and the extramural community, facilitate the sharing of scarce resources and generally improve the management of research in the areas of biological carcinogenesis, chemical and physical carcinogenesis and cancer epidemiology. 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, particularly in the new area of "biochemical epidemiology." The current organizational chart for DCE is shown in Figure 1.

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

This year renovations in Building 41 for the Laboratory of Chemoprevention were completed as were renovations in Building 37 for the Laboratory of Experimental Carcinogenesis.

The past year has seen a continued reduction in overall contract support. This has been realized by gradual phase-out of contract-supported, investigator-initiated research in areas where grants provide adequate coverage, by reducing activities which provide materials and services, and by initiating various cost-recovery mechanisms. For example, in the Biological Carcinogenesis Branch six resource contracts are functioning in the cost-recovery, or "payback" mode. These include two for production of viral reagents, two for animal resources, one for specialized testing services and one for maintenance and distribution of stored frozen biological reagents. In the Chemical and Physical Carcinogenesis Branch, a payback system for the Chemical Carcinogen Reference Standard Repository and its associated contracts was introduced in April 1983. Reimbursement for full or partial costs of services has led to a more careful use of costly resource reagents and chemicals, with a subsequent reduced level of effort in several resource contracts or the termination of unnecessary activities. As support for research contracts has dropped, support for investigator-initiated research grants has continued to increase. In addition, the Cooperative Agreement is now being utilized as an additional instrument of support. The Biological Carcinogenesis Branch, in particular, has utilized this mechanism, and now administers 15 Cooperative Agreements at a level of $3.2 million dollars in the area of Acquired Immune Deficiency Syndrome (AIDS) research. 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. <u>Frederick Cancer Research Facility (FCRF)</u>

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

2. <u>The DCE Board of Scientific Counselors</u>

   Chartered in 1978, the Board is an advisory body whose members are drawn from the scientific community outside NIH. Chosen for their recognized expertise in chemical carcinogenesis, molecular biology, viral oncology, chemoprevention,

epidemiology, immunology, pathology and genetics, the members of the Board advise the Division Director on a wide variety of matters which concern the progress of the Division's programs.

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

The second cycle of site visits to the Division's entire intramural operation began with a site visit to the Environmental Epidemiology Branch on May 18-20, 1983. The Laboratory of Molecular Virology was site visited on December 2, 1983; the Laboratory of Biology on March 1984; and the Clinical Epidemiology Branch on April 12-13, 1984. The site visit to the Laboratory of Cellular and Molecular Biology occurred on July 17-19, 1984 and the Laboratory of Molecular Carcinogenesis was site visited on September 13-14, 1984. Four additional site visits to the Laboratory of Cellular Carcinogenesis and Tumor Promotion (February 21-22, 1985); the Laboratory of Chemoprevention (May 30-31, 1985); Laboratory of Molecular Oncology (June 13-14, 1985) and the Laboratory of Comparative Carcinogenesis (July 11-12, 1985) have also occurred. The Laboratory of Human Carcinogenesis will be site visited on November 21-22, 1985,

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

Several workshops, involving Board members as well as participants from the scientific community outside NIH, were held this year. As a consequence, new initiatives resulted in using the mechanism of a Request for Application (RFA) in the areas of human papillomaviruses; feline leukemia virus; development and assessment of retroviral vaccines; novel exogenous and endogenous human retroviruses; innovative approaches to chemoprevention; mutagens in human food; involuntary exposure to tobacco smoke; dietary markers for epidemiologic studies of cancer; obesity and cancer risk in women; and development, validation and application of biochemical markers of human exposure for use in epidemiologic studies.

In addition, an RFA for Cooperative Agreements for research in the area of HTLV types I and II which was originally published in 1984 was initiated and funded this year. A workshop on polyomaviruses held during the past year was chaired by a member of the DCE Board of Scientific Counselors and the recommendation of the participants was that the DCE issue an RFA to stimulate further study of the mechanisms by which these human viruses transform human cells and their possible role in the etiology of human cancer. Another workshop, on Neoplasia in Fish (Occurrence and Etiology), was held this year and was co-chaired by two members of the DCE Board of Scientific Counselors. An RFA entitled "Studies on the Etiology of Neoplasia in Poikilothermic Aquatic Animals: Finfish

and Shellfish" will soon be issued as a result of the recommendations of the workshop participants. The Division also co-sponsored a workshop with the International Agency for Research on Cancer entitled "Cyclic Nucleic Acid Adducts in Carcinogenesis." The purpose of the final discussion session was to identify future research needs and approaches to understanding the biological consequences of cyclic nucleic acid adduct formation. A Program Announcement will be issued to stimulate basic mechanistic studies of exocyclic nucleic acid derivatives.

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

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

FIGURE 1

DIVISION OF CANCER ETIOLOGY

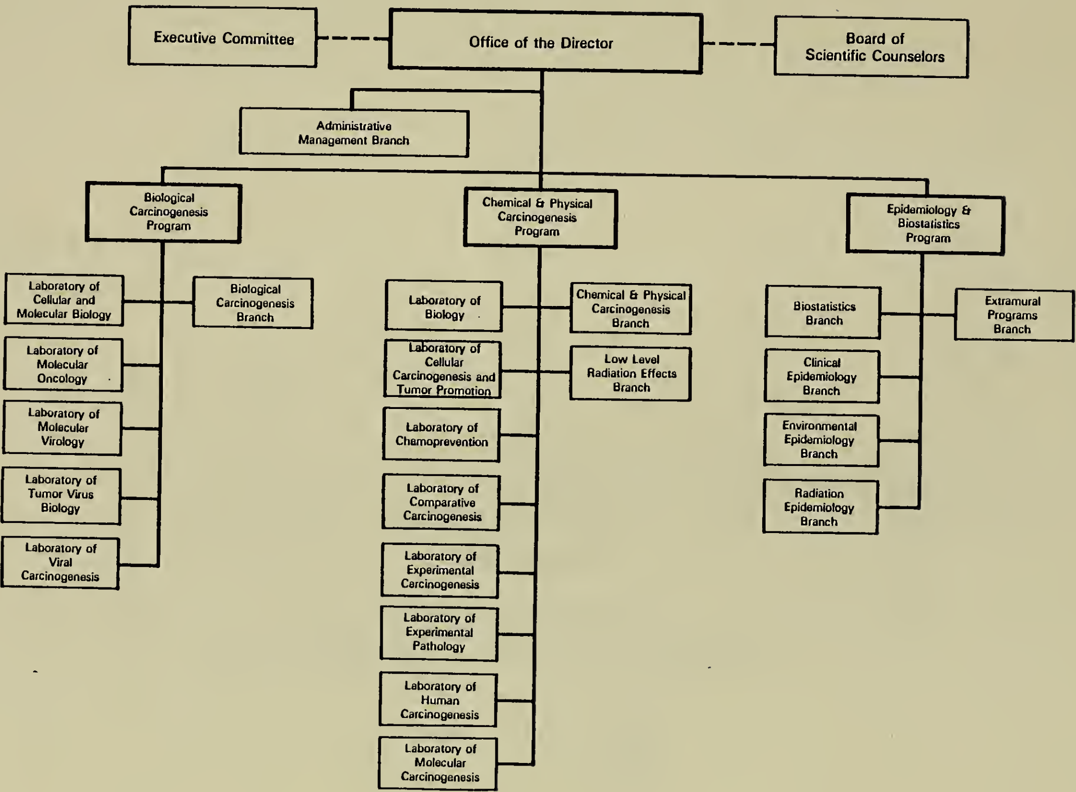


TABLE 1

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER ETIOLOGY

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

FY 1985 Estimate

	Office of the Division Director	Chemical and Physical Carcinogenesis	Biological Carcinogenesis	Epidemiology and Biostatistics	Total
Inhouse	6,098	17,900	12,008	7,633	43,639
Contracts	4,556	8,945	2,930	18,864	35,295
RFA	0	4,951	1,009	4,992	10,952
Cooperative Agreements	515	0	3,569	825	4,909
Research Project Grants	0	62,072	61,908	18,986	142,966
Total	11,169	93,868	81,424	51,300	237,761

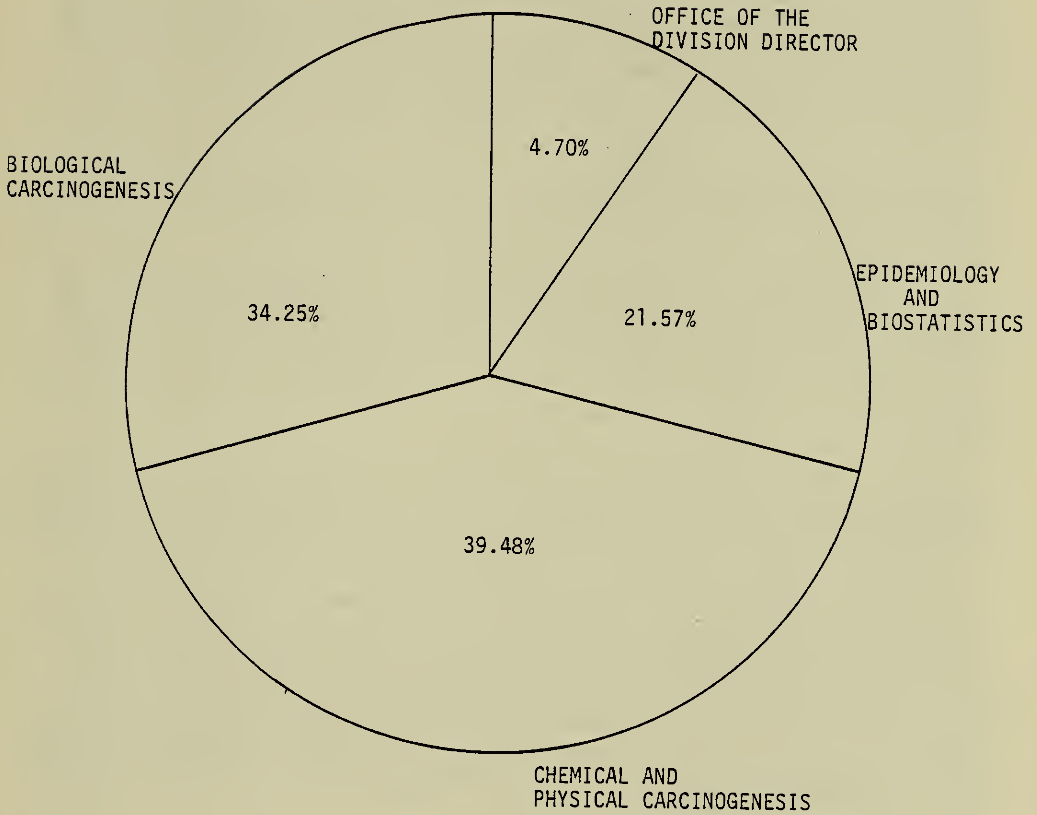
FIGURE 2

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER ETIOLOGY

Current Distribution of Funds

FY 1985 Estimate



SCIENTIFIC HIGHLIGHTS

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

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

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

Similarly, chemical carcinogens, both synthetic and naturally occurring, provide a diverse group of chemicals with cellular and tissue selectivities that influence transformation and progression from the normal to the malignant state. Studies in the area of chemical/physical carcinogenesis cover a broad range of approaches with emphasis on the mechanisms of action of chemical and physical agents. Investigators have focused on the effects of carcinogens on cell structure and function, the relationships between molecular structure and carcinogenic activity, enzyme systems associated with the metabolic activation of procarcinogens to carcinogenic forms, the effects of the binding of carcinogens to DNA, systems that repair DNA damage and on the possible activation of oncogenes by chemical carcinogens. Other relevant efforts include investigation of the role of various factors in the environment of the cell; for example, promotors, 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 various 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 micronutrients in the diet, alcohol consumption and smoking, special emphasis has been given to projects that may have more immediate health implications. Many studies deal with determining the presence of mutagens, carcinogens and natural anticarcinogens

in foods; other studies assess the carcinogenic components in cigarettes, and the influences of the total smoking experience. In addition, major studies on the viral etiology of cancer, occupational carcinogenesis, effects of low-level radiation, and environmental pollutants in air, water, and soil are under investigation.

Biological Carcinogenesis

Oncogenes

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

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

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

Despite advances in identifying cellular genes with transforming potenial, little is known about proto-oncogene function or how the altered counterparts of these genes disrupt normal growth regulation. Recently, however, studies of the *onc* gene of simian sarcoma virus (SSV), a primate transforming retrovirus, combined with investigations of platelet-derived growth factor (PDGF), a potent mitogen for connective tissue cells, have led to the discovery that the SSV transforming gene product and PDGF arise from the same or very closely related cellular genes. Thus the mechanism by which the SSV *onc* gene, v-*sis*, transforms cells may involve the constitutive expression of functions similar to those of PDGF.

During the past year it was demonstrated that expression of the normal human *sis*/PDGF-2 coding sequence induced cellular transformation. The human *sis* proto-oncogene was found to contain the coding sequence for one of two polypeptide chains present in preparations of biologically active PDGF. A human clone, c-*sis* clone 8, which contains all of the v-*sis*-related sequences present in human DNA, was transcriptionally inactive when transfected into NIH/3T3 cells. When placed under the control of a retrovirus LTR, the clone was transcribed at

levels comparable to those observed in cells transformed by SSV DNA. However, c-*sis* clone 8 DNA did not express detectable *sis*/PDGF-2 proteins and lacked biologic activity. A putative upstream exon was identified by its ability to detect the 4.2 kb *sis*-related transcript in certain human cells. When this sequence was inserted in the proper orientation between the LTR and c-*sis* clone 8, the chimeric molecule acquired high titered transforming activity, comparable to that of SSV DNA. Transformants containing this construct expressed human *sis*/PDGF-2 translational products. Thus the normal coding sequence for a human growth factor has transforming activity when expressed in an appropriate assay cell.

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

The v-*sis* transforming gene encodes the woolly monkey homolog of human platelet-derived growth factor polypeptide 2. After its synthesis on membrane bound polyribosomes, the glycosylated precursor dimerizes in the endoplasmic reticulum and travels through the Golgi apparatus. At the cell periphery, the precursor is processed to yield a dimer structurally analogous to biologically active PDGF. Small amounts of two incompletely processed forms are detectable in tissue culture fluids of SSV transformants. However, the vast majority remains cell associated. Thus this growth factor-related transforming gene product is not a classical secretory protein. These findings define possible cellular locations where the transforming activity of the *sis*/PDGF-2 protein may be exerted.

Analysis of human mammary tumors for transforming genes detectable by transfection analysis revealed the presence of an H-*ras* oncogene in the HS578T mammary carcinosarcoma line. The oncogene was cloned in biologically active form and the activating lesion identified as a single nucleotide substitution of adenine for guanine within the 12th codon. This results in substitution of aspartic acid for glycine at this position of the p21 coding sequence. Knowledge that this alteration creates a restriction site polymorphism for Msp I/Hpa II within the H-*ras* proto-oncogene made it possible to survey for the presence of the activated H-*ras* allele in normal cells as well as in clonally derived tumor cell lines of the same patient. The presence of unaltered H-*ras* alleles was demonstrated in normal HS578T cells. In contrast, every clonally derived HS578T tumor cell line analyzed contained an H-*ras* oncogene possessing the genetic this oncogene was the result of a somatic event, powerfully selected within all HS578T tumor cells. As such, the evidence strongly favors the concept that this oncogene played an important role in the development of the HS578T mammary carcinosarcoma.

A large series of urinary tract tumors were surveyed for *ras* oncogenes by DNA transfection and by molecular genetic analysis to determine their frequency in urothelial cells. H-*ras* oncogenes were detected in 2 of 38 tumors by transfection, molecularly cloned in biologically active form and shown to contain single base changes at codon 61 leading to substitutions

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

PA-1 is a novel cell line originally isolated from a patient with a metastatic ovarian germ line tumor for transfection studies. Early passage PA-1 cells were found to grow slowly in culture and form tumors in athymic mice at low efficiency and with long latent periods; the late passage cells grew rapidly and induced tumors with short latencies. A dominant transforming gene was detected in late passage PA-1 cells and was identified as a new isolate of the human N-ras locus. It contains a single G→A point mutation in the 12th codon, which results in a replacement of glycine with aspartic acid. DNA from early passage PA-1 cells do not yield foci in transfection assays; however, NIH/3T3 cells transfected with this DNA formed tumors in nude mice, although at a slower rate than cells transfected with late passage PA-1 DNA. Tumors induced by early passage PA-1 DNA transfection did not contain human N-ras DNA sequences, but this sequence was readily detectable in tumors induced by late passasge PA-1 DNA. Introduction of active N-ras transforming sequences into early passage PA-1 cells results in PA-1 cells which now rapidly form tumors in nude mice. These results suggest that early passasge PA-1 cells do not contain an activated N-ras sequence and that their inability to form tumors in nude mice is not due to a block in N-ras function.

As noted above, ras oncogenes are frequently activated in human tumors by mutations at position 12 or 61 in their coding sequences. To investigate how these subtle alterations exert such profound effects on the biologic activities of these genes, structural and conformational properties of human ras oncogene-encoded p21 proteins were studied. Striking differences were observed in the electrophoretic mobilities of the proteins under reducing and nonreducing conditions. These findings imply that intramolecular disulfide bonds affect native p21 conformation. The two activating lesions were shown to induce distinctly different alterations in p21 electrophoretic mobility unmasked only after reducing conditions. These results suggest that regions of the molecule containing such alterations are either not exposed or are under conformational constraints in the native p21 molecule. The opposing effects on protein mobility induced by the two activating lesions were confirmed using a recombinant gene containing both lesions. Its high titered transforming activity further established that the two lesions do not negatively complement one another with respect to transforming gene function. The findings of distinct alterations in electrophoretic mobilities of position 12- and 61-altered p21 molecules should be applicable to the immunologic diagnosis of ras oncogenes in human malignancies.

New studies of the transcriptional status of the fgr proto-oncogene in human tumors have revealed in general that certain lymphomas but not sarcomas or carcinomas expressed fgr-related mRNA. Further examination of lymphoid tumors demonstrated that expression of c-fgr was highly correlated with Epstein-Barr virus (EBV) infection. This correlation was extended to include cord and

peripheral blood lymphocytes established in culture by infection with EBV. Moreover, when EBV-negative Burkitt's cells were deliberately infected with the virus, fgr proto-oncogene transcripts were elevated 50-fold. These findings demonstrated that EBV infection was responsible for enhanced expression of the c-fgr gene, a member of the tyrosine-specific protein kinase gene family.

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

Recent investigations have begun to dissect the number and nature of genetic alterations associated with cancer cells. It has been possible to demonstrate that primary human epidermal keratinocytes acquired indefinite life span in culture but did not undergo malignant conversion in response to infection with a hybrid of adenovirus 12 and simian virus 40. The addition of Kirsten murine sarcoma virus, which contains a K-ras oncogene, to these cells induced morphological alterations associated with the acquisition of neoplastic properties. These findings demonstrate the malignant transformation of human primary epithelial cells in culture and support a multiple-step process for neoplastic conversion.

Other studies are also addressing the mechanisms by which cellular proto-oncogenes can become transduced, activated and expressed, and are attempting to delineate the pleiotropic molecular changes effected by these concerted neoplastic events. It has been found that the avian carcinoma virus, MH2, shares a transformation-specific sequence, myc, in common with other acute transforming retroviruses such as MC29, CMII, and OK10. A 5.2kb MH2 provirus DNA has been molecularly cloned and its complete genomic structure determined. In addition to Δgag, Δenv, and a c genetic region, shared in common with other nondefective retroviruses, a unique mht genetic element is present which appears to be a MH2 transformation-specific onc gene. Hybridizations with normal chicken cellular DNA, as well as with cloned chicken c-myc DNA, reveal that mht sequences are probably derived from a normal cellular gene, quite distinct from the c-myc proto-onc gene. Significantly, this avian mht region is 94% homologous to the murine raf oncogene at the deduced amino acid sequence level, the closest such homology determined thus far. Thus, it appears that the genome of the acute transforming retrovirus encodes two distinct genes having possible oncogenic functions, unlike the other avian retroviruses (MC29, OK10, and CMII), each of which carries a single hybrid Δgag-myc oncogene. Despite this fundamental difference, MH2 and MC29 viruses have similar oncogenic properties. Sequence analysis has revealed that MH2 contains two potential transforming genes, Δgag-mht and myc. The myc gene transforms primary cells by itself without the second potential oncogene, mht; and the Δgag-mht alone was without detectable transforming activity. In relation to MH2, it is significant that out of 19 known different viral oncogenes, 5 have been observed in viruses of different taxonomic groups. This observation suggests that the number of different cellular proto-onc genes most likely is limited, since unrelated taxonomic groups like avian MH2 and murine 3611 retroviruses have transduced

the same onc gene-specific sequences from vastly different cellular species. Furthermore, these observations suggest that specific deletions and linkages of the same proto-onc sequences integrated into different retroviral vector elements also effects differences in their oncogenic potential and transformation capabilities. Nucleic acid hybridization, heteroduplex and DNA sequence analysis indicates that the v-mht sequence captured by the MH2 virus is dispersed over 25 kb of the chicken genome. The chicken proto-mht contains 11 exons homologous to the v-mht sequence. Thus, it appears that the v-mht onc gene transduced a truncated version of the proto-mht gene; it lacks introns and possibly 5' coding squences. Because there is no sequence homology between the proto-mht and the retroviral helper sequences, the virus transduction probably occurred by legitimate recombination.

A synergistic transforming activity of v-raf and v-myc oncogenes has been demonstrated in vivo and in vitro. This provides the first example for synergistic action of independently active oncogenes in the transformation of lymphoid/hematopoietic and epithelial cells in vivo. The mechanism underlying synergism appears to involve constitutive expression of two independent signals for growth, a "competence" and a "progression" signal. V-myc would provide the former as deduced from the finding that v-myc expression in certain cells abrogates their requirement for specific growth factors, such as IL-2 and-3. V-raf has the potential to induce the latter since fibroblast cells transformed by v-raf produce TGF, a mitogen acting through the EGF receptor. A variant virus that predominantly induces carcinoma in mice has been isolated, providing the first example of a molecularly analyzed mammalian carcinoma-inducing virus.

Cells from tumors induced by the myc or raf + myc transducing viruses can readily be established in culture in regular medium, whereas culture of cells from raf oncogene-induced tumors requires the addition of IL-3. A function for myc in this synergism has been indicated in studies involving infection of a series of IL-2 and IL-3 dependent cell lines with the various viruses. These studies have demonstrated that expression of high levels of v-myc alone can abrogate the growth factor requirements of these cell lines and probably functions in the analogous manner in its synergistic action with raf in the development of hematopoietic/lymphoid tumors in vivo.

A prokaryotic vector has been developed which allows the expression of high levels of onc genes in E. coli. This vector (pJL6) has provided a means for studying both the chemistry of the proteins and for preparing appropriate immunological reagents for studying the expression of the oncogene product in transformed cells and tumor cell lines. This efficient expression vector contains several unique restriction enzyme sites 12 codons beyond the lambda phage cII gene initiation codon. A number of variants of this vector have been constructed containing several restriction enzyme sites suitable for cloning in a variety of viral and cellular onc genes. Thus far, fusion proteins of v-myc, first and third exon onc genes, v-myb, Ha-MuSV ras, v-mht, and c-sis are among the products expressed with this vector, in addition to specific portions of the HTLV-I envelope gene. These viral gene fragments were expressed as a 16,000 dalton portion of the N terminus of the p21E transmembrane fusion protein as well as a 13,000 dalton portion of the gp46c-terminal portion of the HTLV-I env gene product. In addition, a fusion protein representing amino acid sequences from the carboxy/terminus of the p42 LOR protein was prepared using the vector with the HTLV-I pX gene. These HTLV-specific proteins have been isolated, characterized and purified to homogeneity. Since these proteins

reacted with antibodies in patient sera, they have potential for use in diagnostic assays. Additionally, these proteins can be used to map epitopes recognized by monoclonal antibodies directed against these bacterially-expressed proteins.

Other studies have centered around the cellular ets gene to determine if a pattern of truncated normal genes in the transforming retrovirus can be extended to other onc genes. Consistent with this pattern, the cellular ets transcript is considerably larger than that DNA transduced by the E26 virus. In addition, it has been determined that the mammalian homolog of v-ets consists of two distinct domains, ets-1 and ets-2, located on different, yet homologous, chromosomes in cats, mice and humans. Using chromosome-specific probes it has been demonstrated that both loci, ets-1 and ets-2, are transcriptionally active and distinct from one another. Since the sequences homologous to ets-1 and ets-2 are colinear in chicken proto-ets, it is possible that they diverged and became functionally distinct prior to their evolutionary separation in mammalia. A number of human leukemias, such as the acute undifferentiated leukemias (AUL) and the acute myeloid leukemia with maturation (AML-M2) show specific chromosomal alterations involving chromosomes 11 and 21 which are known to contain the ets-1 and ets-2 loci. Specifically, it has been observed that there is a translocation of the ets-1 proto-onc gene locus from chromosome 11 to chromosome 4 in AUL patients presenting t(4;11)(q21;q23) translocations. Similarly, the c-ets-2 gene is entirely translocated from chromosome 21 to chromosome 8 in AML-M2 patients with the t(8;21)(q21;q22) translocation. This event seems to also affect the expression of the cets-2 gene in leukemic cells of AML-M2 patients.

A number of nontumorgenic human cell lines have been screened for their suitability as recipients for DNA transfection for use in measuring the transforming ability of cloned oncogenes. An SV40-transformed human cell line (SV80) and a cell hybrid between HeLa and a normal diploid fibroblast has identified cells which can be used and transfected for cloned selectable markers with high efficiency. Although introduction of viral mos or human ras did not induce morphologically-transformed foci, individual SV80 cells showed expression of the MSV-p30 containing polyprotein antigen and morphological alterations, and infectious virus could be rescued from the MSV-transfected cells. Tumors arose which contained functional mos, but the frequency was low, suggesting additional sequences; these SV80 cells have been analyzed in the nontumorigenic and tumorigenic hybrids and compared with the parental cells, to determine if their phenotypes could be correlated with respect to the tumorigenic potential of the cells. It was found that the level of ras and myc expression was constant among the various cells, but the levels of fos and myb expression show variations which correlate with the ability of cells to form tumors.

A series of studies is being continued with the transfected HOS cell. The HOS cell line, originally derived from a human osteosarcoma, is nontumorigenic but was transformed to anchorage-independent growth and tumorigenicity by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). It was previously shown that DNA from MNNG-HOS cells, but not HOS cells, would transform NIH/3T3 cells in DNA transfection assays. The MNNG-HOS transforming gene, designated met, has been cloned in several overlapping lambda clones totalling 40 kb of human sequence. This new oncogene shows no detectable homology with the known members of the ras oncogene family, nor with the oncogenes mos, myc, myb, src, erb, sis, rel or B-lym. No rearrangement or amplification can be detected in the met gene in MNNG-HOS cells when compared with HOS DNA or the DNA from several normal

and transformed human cells and cell lines. Cloned probes detect multiple species of polyadenylated RNA in HOS and MNNG-HOS cells, as well as in several human cell lines. A unique RNA species of 6.5 kb is present in NIH/3T3 cells transformed by *met*, and a corresponding RNA species is present in MNNG-HOS cells, but not *in* the HOS parent cell line. In collaborative studies it has been found that the *met* gene maps to chromosome 7 between 7p11.4 to 7qter.

A plasmid vector capable of expressing open reading frames (ORFs) has been constructed. This expression vector consists of the lambda p_L promoter, the first 13 codons of the lambda phage *cII* gene (which provides the translation start signal), and the major active portion of β-galactosidase gene (lacZ) from *E. coli*. The *cII-lacZ* genes are fused out of reading frames with each other. Small DNA fragments from genomic DNA of eukaryotes are cloned into the vector plasmid pWS50. The introduction of inserts with open reading frames restores complete translation of the lacZ gene. The resulting recombinants are detected by a simple colorimetric assay for β-galactosidase. High-level expression of the hybrid proteins is provided by transcription from the inducible p_L promoter. The hybrid proteins synthesized are readily distinguished from native *E. coli* proteins by their large size (>117,000 M_r) which may then be easily purified. In addition, these proteins are suitable for antibody production directed against determinants specified by the inserts. With this system it may be possible to conveniently map open reading frames from total genomic DNA.

Localization of Oncogenes on Chromosomes

Efforts to update the human gene map of proto-*onc* genes and other loci which impact on mammalian carcinogenesis are continuing. The combined application of principles and techniques of molecular biology and cell genetics has resulted in the identification and characterization of over 1000 human loci, a value which approaches the gene maps of Drosophila. Efforts are now being concentrated on somatic cell hybrid panels and in situ hybridizations to genes related to neoplastic processes including (1) cellular proto-oncogenes, (2) growth factors, (3) growth factor receptors, (4) endogenous retroviral families, (5) integration sites for retroviruses, and (6) restriction genes that delimit retrovirus replication in mammals. Within the last few years, the human gene map has experienced a large increase in the number of neoplasia loci that have been mapped to specific chromosomal positions. Twenty-seven specific human loci have been chromosomally mapped to date. Current studies are focused on improving understanding of the genomic organization of several genes: *ets*, *rel*, *raf*, *onc-D*, *fms*, DHFR and a Y chromosome gene family in man. The mammalian homolog to the *v-ets* oncogene of the avian E26 transforming virus has been shown to be encoded by two transcriptionally active, non-overlapping structural genes (*ets-1* and *ets-2*) located on human chromosomes 11 and 21, respectively. Over 30 endogenous retroviral loci have been chromosomally assigned using the hybrid panels. A hematological discorder, the $5q^-$ syndrome, has been shown to be hemizygous for the *fms* oncogene by genetic analysis. This proto-*onc*-gene was shown to be transcriptionally active in hematopoietic cells and has been shown to encode a specific receptor for macrophage colony-stimulating factor. The emerging human gene map continues to provide an unprecedented opportunity for molecular genetic analyses of the initiation and progression of neoplastic processes.

Two new human proto-oncogenes (c-*pks*-1 and c-*pks*-2) have been identified which are closely related to the oncogene, *raf*. The expression of c-*pks*-1 mRNA is elevated in peripheral blood mononuclear cells isolated from patients with

systemic lupus erythematosus and angioimmunoblastic lymphadenopathy with dysproteinemia, two diseases in which auto-antibodies are produced following the lymphoproliferative activation of B cells. Both diseases may ultimately progress to frank malignancies. Employing extensive analysis of mouse x human somatic cell hybrids, the c-pks-1 gene has been localized to the short arm of the X chromosome (Xpter-Xp11) and the other gene to chromosome 7 (7pter-7q22). Thus, the c-pks-1 gene represents the only known active proto-oncogene sequence on a sex chromosome.

Recent results indicate that proteins encoded by the cellular oncogene c-abl apparently help trigger chronic myelogenous leukemia, which affects about 10,000 new victims each year. Investigators have demonstrated that the appearance of the Philadelphia chromosome (involving the translocation of chromosome 9 with the c-abl oncogene to chromosome 22) triggers the abnormal activation of this c-abl oncogene in its new location on chromosome 22. As a result of this translocation, an altered mRNA and a structurally altered c-abl protein, p210 c-abl, are produced. This altered protein has a tyrosine kinase activity not detectable in the normal c-abl protein. It is hypothesized that this transforming protein may in turn cause a chain of abnormal chemical reactions in the growth cycle of the cell, making it malignant.

The molecular and functional characterization of the v-raf oncogene and its cellular homologs has continued as well as an investigation of their involvement in the etiology of human cancers. Four genes homologous to v-raf have now been identified in man: c-raf-1, c-raf-2, -raf, and -raf. C-raf-2 is a pseudogene in man located on chromosome 4. C-raf-1, the cellular homolog of v-raf, is an active gene located on chromosome 3p25, a site which is specifically altered in small cell lung carcinoma, ovarian carcinoma, and mixed salivary gland tumors. C-raf-1 specifies a protein of 648 amino acids with a calculated molecular weight of 74 kd. The complete c-raf-1 protein was expressed in E. coli using a complete human fetal liver cDNA cloned into an expression vector. The normal protein in mouse fibroblasts has been demonstrated by immunoblot analyses. The c-raf-1 gene is composed of 17 exons which span over 45 kb of DNA. Northern hybridization analyses with exon and intron specific c-raf-1 (genomic) probes has enabled the construction of a transcriptional map for the c-raf-1 locus and has demonstrated two alternatively spliced poly(A)+mRNAs. One of these mRNAs, containing the most 3' portion of c-raf-1, may be specifically associated with transformation. A second step in the oncogene activation of c-raf-1 appears to be truncation as all three transforming versions of c-raf-1 are amino-terminally truncated; v-raf with 37 kd, v-mil with 40 kd, and an LTR-inserted and activated c-raf-1 with 50 kd. There are additional raf-related sequences in mouse and human DNA. One of them, isolated from a mouse spleen cDNA library using v-raf, corresponds to an active gene, δ-raf, which is located on human chromosome 7 near the centromere. Another, γ-raf, was isolated from a human fetal liver cDNA library using δ-raf as probe. Homology between δ-raf and c-raf-1 is 69% for DNA and 74% for amino acid sequences. There are two δ-raf transcripts of 2.3 and 4.0 kb. The tissue-specific expression of c-raf-1, δ-raf, and γ-raf was investigated and it was found, that in contrast to c-raf-1 which is fairly ubiquitous in its expression, transcription of δ-raf and γ-raf shows tissue specificity.

Control of Gene Expression

Efforts to elucidate the signals associated with gene expression have continued, with particular emphasis on regulatory events which take place at the level of

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

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

In studies involving the isolation and characterization of cDNA clones derived from different class I genes, it was observed that class I antigens with different primary structures are expressed from the same class I gene by alternate RNA splicing. This novel finding suggests that there exist different functional subsets of these antigens and offers the opportunity to compare the products of hese cDNA clones in presenting tumor antigens to the cytotoxic T-cell. Such analyses would lead to a biochemical definition of immune surveillance.

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

Studies Related to HTLV and AIDS

A new area of intensive research activity has developed dealing with the HTLV family of retroviruses. HTLV-I and HTLV-II are associated with human

malignancies, and HTLV-III with AIDS. Research focusing on any one of these viruses has shed knowledge on the other two members of this group. Thus, comparisons of the similarities and differences among these viruses has the potential to increase our knowledge of three virally induced human malignancies. The linear viral genome of HTLV retroviruses has regions corresponding to the gag, pol and env genes of conventional retroviruses. Adjacent to the 3' end of the env gene is a region designated pX. While the functions of the gag, pol and env genes appear homologous to that of other retroviruses, the pX region does not appear to be homologous with other retroviruses. The viral genome is bracketed on each end by long terminal repeats (LTRs). The LTRs carry regulatory segments, known as promoters and enhancers, which activate transcription of viral genes into messenger RNA, the first step in protein synthesis.

Unlike some of the other retroviruses that can transform animal cells, HTLV-I and -II do not contain oncogenes derived from cellular protooncogenes and do not have to integrate adjacent to cellular oncogenes in order to activate uncontrolled cell growth. A novel system of cancer induction for HTLV-I and -II, and cell death caused by HTLV-III, has been proposed. It is theorized that HTLV-I and -II are able to cause uncontrolled cell growth, resulting in cancer, by producing a protein that turns on cellular genes responsible for cell division. In the case of HTLV-III, an analogous viral protein may turn off the cellular genes which stimulate cell growth and turn on those that cause cells to stop dividing, resulting in cell death. This protein, designated the 42 kd protein, is encoded in the pX region of the viral genome. It acts upon the enhancers present in the LTR regions and leads to increased transcription. This novel type of regulation has been called transactivation. Additional studies have shown that the 42 kd protein of HTLV-I was sufficient in itself to mediate the effect of transacting transcriptional regulation. Stable cell lines that expressed only this protein displayed a high level of trans-acting transcriptional regulation. The LTR of HTLV-II has also been found to be responsive to HTLV-I encoded trans-acting factors. However, the transcription unit of the HTLV-II LTR will only function in certain cell types, thus suggesting that cellular factors are required, in addition to virus trans-acting factors, for the trans-acting phenomenon.

The retrovirus responsible for the human AIDS has been variously termed human T-cell lymphotropic virus III (HTLV-III), lymphadenopathy associated virus (LAV), and AIDS-associated retrovirus (ARV). It was originally thought to be most closely related to the oncogenic HTLV-I which is responsible for a small proportion of human adult T-cell leukemia. However, a recent study of an AIDS retrovirus isolate using molecular hybridization and heteroduplex analysis has suggested that HTLV-III may be more closely related to th visna virus of sheep, a member of the lentivirus subfamily. The AIDS retrovirus isolate appeared to be less closely related to HTLV-I or to a variety of other retroviruses. The ability of the AIDS retrovirus to infect and to persist in brains of AIDS patients, its cytopathic effect on T4 lymphocytes in vitro, and its lack of overt oncogenic potential, suggests similarities with the cytopathic lentiviruses, some of which are neurotropic (visna, caprine arthritisencephalitis). However, the AIDS retrovirus is lymphotropic for T4 cells in a manner similar to HTLV-I and therefore its designation as human T-cell lymphotropic virus-III (HTLV-III) does reflect a biological relationship with the HTLV family of retroviruses. Thus, the evolutionary origin of HTLV-III remains unknown and is a subject of active research.

A number of studies have focused on the immunobiology of AIDS and AIDS-related diseases. The ratio of OKT-4-positive (helper) to OKT-8-positive (suppressor) cells in homosexual populations from the Washington, DC and New York areas has been determined. The total number of cells with these markers and the ratio of T-4 to T-8 (helper/suppressor ratios) were examined to attempt to correlate possible environmental or other risk factors. It was found that the Washington, DC male homosexual population had decreased numbers of OKT-4 cells in individuals who had homosexual contact with individuals in the New York, Los Angeles or San Francisco areas. All three of these areas are highly endemic for AIDS. Two other subgroups could be identified--those being the intermediate-risk group and a low-risk group. The intermediate risk group are those who had homosexual contact with the individuals in the Washington, DC high-risk group. The low-risk group did not have homosexual contact with either of the two groups.

There was a positive correlation of the low numbers of OKT-4+ cells in the individuals who had homosexual contact either directly or indirectly with individuals in the endemic areas. The sexual practices of these individuals were also related to the decrease in the OKT-4 population or helper/suppressor ratios. It was found that there was a positive correlation with increased numbers of homosexual partners as well as receptive anal intercourse. The sera from these individuals was studied for antibody to HTLV-III. Those individuals who had suppressed or decreased OKT-4-positive cells in their peripheral blood lymphocyte populations were generally positive for the HTLV-III retrovirus, showing a correlation of this retrovirus with the presence of one of the risk factors for this disease, i.e., the depression of the OKT-4-positive cells. Lymphocytes from individuals who are at risk for the disease (male homosexuals) and lymphocytes from a heterosexual male population were used to study possible models for the depletion of the OKT-4 positive lymphocytes. Lymphocytes were placed in culture with a pan T-cell stimulator (PHA) and acid stable and acid labile alpha interferon. Only those cultures of lymphocytes from AIDS patients and the male homosexual population with antibodies to HTLV-III showed depletion of the OKT-4 lymphocytes in vitro. These results suggest that antigen stimulation together with the presence of the HTLV-III and alpha interferon is necessary for the depletion of the OKT-4-positive cells. The depletion of OKT-4 lymphocytes suggested that the cell surface molecule bearing this epitope was a receptor for the HTLV-III retrovirus. Studies were performed to examine this possibility. Short-term incubation of the isolated banded retrovirus with the H9 cells demonstrated a rapid disappearance of epitopes on the OKT-4 molecule detected by antisera, OKT-4D, OKT-4F and OKT-4A. The epitope detected by the OKT-4 antisera disappeared after two to three days of exposure to the retrovirus. In all cells in which the retrovirus can be replicated, the OKT-4 molecule is absent. These results suggest that the specific receptor site for the HTLV-III retrovirus is on the distal portion of the OKT-4+ bearing molecule and that the molecule is internalized with the virus and that there is subsequent down-regulation of the expression of this molecule.

Recently, an HTLV-III type agent that naturally infects rhesus monkeys was detected. It is associated with the so-called simian AIDS (SAIDS). The virus was isolated from four monkeys with SAIDS using Hut 78 cells of human origin. Serum from these and other rhesus monkeys in the same cages precipitated proteins of a size identical to those of human HTLVIII. The sera of monkey origin could precipitate proteins of both the monkey virus and the human AIDS virus. Similarly, reference sera from AIDS patients precipitated the proteins of both the monkey virus and the human agent. By morphologic criteria, as well as reverse

transcriptase activity, the new monkey virus is clearly very closely related to the human agent. It is postulated that the human agent may have originated from some population of Old World monkeys or apes. These results are important, not only because they may elucidate the origin of the AIDS virus, but also because the rhesus monkey virus could provide a model for AIDS treatment and vaccine development. In another monkey colony, a macaque type D/Washington virus was isolated from an explant of retroperitoneal fibromatosis (RF) taken from a rhesus monkey with SAIDS.

To examine viral pathogenicity, four juvenile colony-born and four feral young adult Macaca nemestrina were inoculated with 2 X 10^6 viable viral particles (assayed by end-point dilution of infectivity in cell culture) from a 500X viral concentrate of filtered culture medium of dog thymus cells infected with the Macaque type D/Washington retrovirus. At 5 weeks, one colony-born juvenile macaque died with suggestive RF and with generalized lymphoid depletion. At 10 weeks, retroperitoneal nodules were palpated in a colony-born macaque that at laparotomy at 18 weeks were histologically diagnosed as RF. After 13 months, the macaque with RF and the one that died at 5 weeks are the only animals with viremia, as determined by isolation of Macaque type D/Washington virus from blood plasma, and are the only animals without seroconversion following virus inoculation. Eight matched control M. nemestrina, inoculated with filtered culture medium from uninfected dog thymus cells, have remained healthy.

Studies on DNA Viruses

The transforming genes of small DNA tumor viruses, such as SV40 and mouse polyoma, appear to be very different from the oncogenes of the RNA retroviruses. These DNA oncogenes are totally of viral origin and produce proteins called tumor or "T" antigens which are necessary to the normal replication and maturation of these viruses in lytic infections. In the SV40 system, the large tumor antigen gene, which alone can transform cells, has been extensively studied using biochemical, immunological, genetic and most recently transgenic methods. The polyoma middle T antigen has been identified as playing a central role in polyoma virus-induced oncogenesis. This protein is associated with a protein kinase activity which can be detected by in vitro phosphorylation of a tyrosine residue. This protein does not possess intrinsic protein kinase activity and is thought to associate with the cellular protein pp60c-src (the cellular homolog of the Rous sarcoma virus transforming gene). It is proposed that the polyoma virus middle T antigen protein kinase activity represents a property of the associated pp60c-src. The potential importance of this protein kinase activity in polyoma-mediated oncogenesis is suggested by the finding that viral mutants which are deficient in transforming potential also lack this associated kinase activity. Elevated levels of pp60c-src kinase activity have also been demonstrated in human tumor lines, particularly of neuroectodermal origin.

Transgenic mice have added a new dimension to the use of SV40 as a model for tumorigenesis. Using the new technique of macroinjection of recombinant DNA plasmids into fertilized mouse eggs, transgenic mice were produced which carry SV40 T-antigen genes integrated into the genome of every cell of the animals. A high percentage of these transgenic mice developed tumors within the choroid plexus. A line of these mice has been established in which the SV40 DNA is genetically transmitted to progeny. In this line, nearly every affected animal succumbed to choroid plexus brain tumors within 5 months after birth. Both SV40 T-antigen mRNA and protein were readily detected in affected tissues;

however, SV40 T-antigen gene expression was barely detectable in unaffected tissues or in susceptible tissues prior to overt pathology, suggesting that tumorigenesis depended upon activation of the SV40 genes. Cell lines could be readily established from tissues of transgenic mice; such cultured cells contained T-antigen. Examination of DNA extracted from tumor tissue or from cell lines derived from tumors revealed structural rearrangements and changes in DNA methylation of the SV40 DNA when compared to DNA from unaffected tissues in these same mice. The SV40 genes were frequently amplified in tumor tissue, further indicating that their expression is probably involved in tumorigenesis in transgenic mice. These mice may provide an excellent system to examine both tissue-specific tumorigenesis by SV40 large T-antigen and the selective expression of genes in developing eukaryotic organisms.

Studies on the role of the papillomaviruses in human carcinogenesis and on the molecular biology of this group of viruses are being expanded. Studies on the bovine papillomavirus type 1 have shown that the virus is able to transform rodent cells in tissue culture and as such provides a model for the systematic study of the molecular biology and genetics of this group of viruses. The viral genome remains as a stable multicopy plasmid in transformed cells and is partitioned faithfully to daughter cells. As such, cellular transformation and plasmid replication in the transformed cells provide models for studying the proliferative functions encoded by the papillomaviruses and latent viral infection by these viruses, respectively. A subgenomic segment, consisting of 69% of the genome, contains all of the necessary sequences required for transformation or for stable extrachromosomal plasmid maintenance and replication. This subgenomic transforming segment contains a non-coding region (NCR) consisting of a 1000 base region which sits 5' to a series of eight open reading frames (ORFs) which are transcribed in transformed cells. This NCR contains elements of a transcriptional promoter required for the expression of these open reading frames in transformed cells. It has also been shown that the NCR contains a transcriptional regulatory element with the properties of a transcriptional enhancer which is specifically transactivated by a specific viral gene product, the E2 gene product. Full-length cDNA cloning of the viral-specific messenger RNAs in transformed cells has permitted the structural and functional analyses of a subset of the viral RNAs in transformed cells. These studies have revealed that all of the RNAs are transcribed from a single strand and that they are polyadenylated at a common site. Multiple viral RNA species can be detected in transformed cells and are generated by differential splicing. Two independent species of cDNA clones contained in an Okayama and Berg expression vector are independently able to transform mouse C127 cells, indicating that BPV-1 contains two transforming genes. Mutational analysis in the wild-type viral DNA background reveals that the E6 and E5 ORFs must be intact in the respective cDNAs to effect transformation, suggesting that the gene products of the E5 and E6 ORFs are the transforming proteins of the bovine papillomavirus. Of principle interest in the future will be the identification of these putative transforming gene products within transformed cells as well as within productively infected cells. Mutational analysis indicates that expression of an intact E2 gene product is important for transformation as well as for stable plasmid maintenance. The E2 product is involved in the transactivation of a transcriptional regulatory element present within the NCR. Mutations in the E2 gene products affect the transcription of the early papillomavirus genes involved in plasmid maintenance and in transformation. Thus, the E2 gene product may be indirectly involved in transformation and extrachromosomal plasmid maintenance. Mutations within the E1 gene product have a minimal effect on transformation frequency but result in the integration of the viral DNA in the

transformed cells. Thus the E1 gene product is required for stable plasmid maintenance. Studies are also being directed at the expression of the E1 and E2 gene products in bacterial as well as mammalian cell vector systems in order to study these gene products and their potential interactions with the cis regulatory sequences of the bovine and papillomavirus.

The Papillomaviruses are associated with naturally-occurring tumors and experimentally-induced tumors in a variety of animal systems. In addition, evidence is mounting associating human papillomaviruses (HPV) with specific human carcinomas. The most compelling evidence involves the association of one group of specific HPVs with cutaneous carcinomas in patients with epidermodysplasia verruciformis and the association of another group of HPVs with cervical carcinoma. Specifically, HPV-16 and HPV-18 have been found in a high percentage of biopsy specimens of cervical carcinomas in several laboratories in Europe and the United States. A series of human carcinoma cell lines was investigated for the presence of HPV DNAs. Of eight cervical carcinoma cell lines examined, six have been shown to contain integrated HPV-16 or HPV-18 DNA. Of the six positive lines, five have been shown to be transcriptionally active for the papillomavirus seauences. The integration of HPV-16 DNA sequences and the expression of HPV-16 RNA in cervical carcinoma cell lines has been described. These integrated genomic sequences are now cloned and the cloning and expression of the cDNA copies of the viral transcripts in these cells is being carried out.

An observation which has further implicated HPV in an etiological relationship to cervical neoplasia was the detection of HPV DNA sequences in lymph nodes containing metastases. Samples of enlarged lymph nodes and the primary cervical carcinoma were obtained from thirteen patients. Those lymph nodes which contained metastases were invariably positive for the same HPV sequences present in the primary cancer. This result clearly showed that HPV genes are associated with malignant cells and that the sequences are maintained in these cells for the several generations required to form a metastatic lesion. Thus, the HPV genes are probably necessary to maintain the malignant phenotype. Similar results had been previously obtained with metastases from epidermodysplasia verruciformis patients.

Human papillomavirus-16 DNA has been transfected into NIH/3T3 cells. This achievement provides a useful model for studying the transforming functions of HPV 16 DNA. Data presented clearly establishes that the HPV 16 DNA is present within the cells, in multiple copies, and as with human cervical cancer is primarily integrated into the host DNA. The role of early gene expression in establishing the transformed state is under analysis. The E6 gene, which is considered to be a transforming gene for bovine papilloma, is of particular interest. Enzyme cleavage within HPV 16 DNA at a site close to the C-terminal end of E6 gene indicates a reduced in vivo tumorgenicity of the transformed 3T3 cells. Poly A+ RNA analysis also indicates that transformation is accompanied by expression primarily of early genes. The information obtained in the NIH/3T3 model for transformation is being used in a new approach for obtaining human cell malignant transformation. There is both experimental and clinical evidence to indicate that papillomaviruses can act as cocarcinogens with both physical and chemical carcinogen. Therefore, the plasmid containing the HPV 16 DNA has been transfected into a non-tumorigenic human fibroblast line that was originally transfected using an origin defective SV40 construct and into fibroblast and epithelial cells obtained from human foreskin. Southern blot analysis of the extracted DNA indicates that HPV sequences can persist in both fibroblast and epithelial cells.

Chemical and Physical Carcinogenesis:

In Vitro Studies on Human Tissue and Cells

Considerable progress has been made in the last few years in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. Chemically-defined media have been developed for long-term culture of human bronchus, colon, esophagus, and pancreatic duct. Primary cells cultures of human epithelial outgrowths have been obtained from many different types of human tissues. Isolated epithelial cells from human bronchus and esophagus can be transferred three or more times and can undergo more than 30 cell divisions. Human bronchial and esophageal cells can also be grown in serum-free culture medium. Morphological, biochemical and immunological cell markers have been used to identify these cells as unequivocally of epithelial origin. The availability of nontumorous epithelial tissues and cells that can be maintained in a controlled experimental setting offers an opportunity for the study of many important problems in biomedical research, including carcinogenesis. For example, the response of human bronchial epithelial cells (enhanced growth or differentiation) after either exposure to carcinogens and/or tumor promoters or DNA transfection by oncogenes is being actively investigated. Parallel investigations using epithelial tissues and cells from experimental animals allows investigators to study interspecies differences in response to carcinogens, cocarcinogens, and anticarcinogens.

Transforming growth factor-beta (TGF-B) isolated from human platelets was studied as the serum factor responsible for inducing cells to undergo squamous differentiation. Normal human bronchial epithelial (NHBE) cells were shown to have high affinity receptors for TFGF-B. TGF-B induced the following markers of terminal squamous differentiation in NHBE cells: 1) increase in Ca ionophore-induced formation of cross-linked envelopes; 2) increase in extracellular activity of plasminogen activator; 3) irreversible inhibition of DNA synthesis; 4) decrease in clonal growth rate; and 5) increase in cell area. The IgG fraction of anti-TGF-B antiserum prevented both the inhibition of DNA synthesis and the induction of differentiation by either TGF-B or blood-derived serum. Therefore, TGF-B is the primary differentiation-inducing factor in serum for NHBE cells.

Terminal squamous differentiation in the normal bronchial epithelial cells can be induced by blood-derived serum, platelet lysates, TGF-beta suspension in semisolid medium, confluence culture conditions or calcium ions (> 1 mM) and small amounts of serum. However, these inducers of differentiation do not have the same effects in either carcinoma cells or, as to be described later, oncogene-transformed cells which continue to grow and, in some cases, grow at a faster rate. These observations are consistent with the hypothesis that preneoplastic and neoplastic cells are resistant to endogenous and exogenous inducers of terminal differentiation and thus have a selective survival-growth advantage. These inducers of differentiation of NHBE cells are currently being characterized and their mechanisms of action defined. Information from such studies is being used in the design of in vitro carcinogenesis experiments in which these inducers of terminal differentiation are being used in a strategy to provide selective advantage to preneoplastic and neoplastic cells.

Epidemiological studies have established that exposure to asbestos fibers is the primary cause of mesothelioma in the industrialized world. Carcinogenesis studies with animals have shown that mesothelioma can be caused by intrapleural or intraperitoneal injections of asbestos. However, the long-term effects of asbestos fibers on human mesothelial cells in culture have not been reported previously. To study this important problem, methods to culture replicative normal mesothelial cells from adult human donors have been developed. The cells contain keratin and hyaluronic acid-mucin, exhibit long, branched microvilli, and retain the normal human karyotype to senescence. The mesothelial cells are 10 and 100 times more sensitive to the cytotoxicity of asbestos fibers than are bronchial epithelial or fibroblastic cells, respectively, from normal adult humans. Exposure of the mesothelial cells to amosite asbestos causes chromosomal rearrangements, including dicentrics. These aneuploid mesothelial cells have an extended population doubling potential of more than 35 divisions beyond the culture life span (30 doublings) of the control cells. One possible effect of asbestos exposure is that rearrangements of chromosomes may result in increased transcription of specific oncogenes or growth factors. Markedly increased production of TFG-β by a mesothelioma cell line has been detected; additional studies on growth factor requirements of normal mesothelial cells have determined that TGF-β is a potent mitogen for these cells.

The information gained from the study of rodent cells in vitro is of limited value in application to attempts to transform human cells. Cell survival and metabolic studies indicate that carcinogens are metabolized by human cells in vitro. Chemical carcinogen concentrations effective in inducing transformation in animal cells also increase the frequency of SCE and chromosome aberrations in human cells. Yet, only rarely are normal human cells converted to the malignant state after carcinogen exposure. These results are similar to those from experiments that utilized cells of other mammalian species such as dog, opossum, or monkey. Human cells possess control mechanisms that are responsible for a stable phenotype that cannot be altered readily by carcinogenic insults. For example, some data indicate that human cells have a capacity to efficiently carry out unscheduled DNA synthesis after exposure to ultraviolet light; other types of cells such as hamster can survive with unrepaired damage. Furthermore, the chromosomal defects found in human cells after carcinogen treatment are either less extensive or lack the specific defects associated with continued cell proliferation or loss of growth control commonly associated with malignant cells.

Fecapentaenes have been identified by Wilkins, Bruce and others as the major contributors to the mutagenicity found in human feces. Because these direct acting mutagens in Salmonella bacteria are potential carcinogens and may play a role in the etiology of human colonic carcinoma, a series of investigations to assess their pathobiological effects and mechanism of action has been initiated. Fecapentaene-12 has been shown in human fibroblasts to: (a) cause 6-thioguanine-resistant mutations and higher frequencies were found in xeroderma pigmentosum fibroblasts when compared to normal cells; (b) cause single strand DNA breaks; (c) enhance the frequency of sister chromatid exchanges; and (d) cause unscheduled DNA synthesis. In a bacterial plasmid assay, fecapentaene-12 causes: (1) mutations; (2) DNA-DNA cross-links; and (3) DNA fragmentation. Finally fecapentaene-12 transforms mouse Balb/3T3 cells. The effects of fecapentaene-12 in human colonic cells are being examined, the DNA adduct identified, and antibodies to these adducts for use in immunoassays to search for adducts in human populations are being prepared.

Other In Vitro Studies

Replicative culture systems have been established in selected serum-free media for primary and secondary cultures of mouse epidermal cells and rat tracheal epithelial cells. Clonal assays were used for the quantitative evaluation of factors that stimulate or inhibit epithelial cell growth. Studies on transformation and gene activation induced by carcinogens are under way.

Quantitative studies of mutation and transformation induced in mouse BALB/3T3 cells by exposure to alkylating agents for different periods of time showed an early maximal response for the induction of DNA single strand breaks (ssb) and of ouabain-resistance (oua^r) mutations, whereas a long exposure time is needed to reach maximal induction of transformation. In Chinese hamster ovary (CHO) cells, the same early time for maximal response was found for ssb and oua^r mutations, but the induction of 6thioguanine-resistance mutations increased progressively over a long exposure time. Analysis of repair of alkylated DNA adducts showed that O^6-alkylguanine was rapidly removed in BALB/3T3 cells but not at all in CHO cells; since both cell types show the same early maximal response pattern for oua^r mutations and ssb, the O^6-G adduct appears unlikely to be responsible for these effects.

In Vivo Studies

Recent results have shown that the physiological effects of carcinogenic divalent metals are often inhibited by the physiologically essential divalent metals, calcium, magnesium and zinc. Magnesium administered along with the carcinogenic metal inhibits lung adenoma formation in strain A mice by lead and nickel and sarcoma production in rats by nickel and cadmium. The inhibition by magnesium of carcinogenesis by metals appeared to result in part from the reduced uptake and retention of the carcinogenic metal in the target tissue. Calcium injection also inhibits lung adenoma induction in strain A mice by nickel and lead. However, high dietary calcium levels enhanced the carcinogenic activity of lead towards the kidneys of rats. In other studies the chemical binding of cadmium and of nickel to DNA was found to be inhibited by calcium, magnesium and zinc to an extent paralleling their inhibitory effects on cadmium and nickel carcinogenicity. These results indicate a frequent, but not universal, inhibition of metal carcinogenesis by the physiologically essential divalent metals. Such inhibition results in part from altered metabolism of the carcinogenic metal and may be associated with a decreased binding of the carcinogen to DNA. Further association between DNA and metal carcinogens was provided by studies showing a synergism between cadmium and azacytidine in inducing metallothionein in the livers of rats; in cell culture at least, metallothionein induction is dependent upon gene hypomethylation. In view of the widespread occurrence of metal carcinogens and of the great activity of some, investigations on their mode of action are important. The use of physiological metal antagonists may be expected to help specify the cellular sites of activity of the carcinogenic metals. The present studies will be expanded by examining the effects of essential metal deficiency on metal carcinogenesis.

Chromium (VI) has been identified as a human carcinogen from epidemiological studies. Chromium (VI) compounds have been shown to be tumorigenic in animals and mutagenic in various bacterial and mammalian cell systems. Although chromium (III), which is produced upon reduction of chromium (VI), is the form ultimately bound to cell DNA and protein, it produces no direct mutagenic and carcinogenic

effects. An uptake-reduction model has been used to explain the carcinogenicity of chromium (VI) which can enter the cell using the sulfate transport system. Chromium (III), which forms octahedral complexes, is assumed to be impermeable to the cell membrane. The reduction of chromium (VI) to its active form has been shown to be associated with the cytochrome P-450 electron transport system. The binding of chromium to DNA and protein may occur during the production of intermediate, labile oxidation states and ultimately produce chromium (III) complexes bound to cellular macromolecules. The interaction of chromium (III) with poly(dG-dC) was shown to inhibit the B to Z transition and resulted in the condensation of the polymer at high chromium/nucleotide ratios. At low chromium/nucleotide ratios chromium (III) was shown to enhance the ability of ethanol to induce the B to Z transition of poly(dG-dC). From these results it appears that the biological effects of chromium (III) will depend on its concentration in the nucleus. Thus, chromium (III) may interfere with gene expression and gene regulation through its ability to alter the B to Z transition and cause DNA condensation in active chromatin.

There is evidence that the use of oral contraceptive steroids by humans is associated with an increased incidence in benign liver neoplasms. In experimental studies in animals, results have been obtained which suggest that synthetic estrogen treatment following carcinogen exposure can enhance hepatic neoplasia. One study compared the enhancing activities of mestranol and ethinyl estradiol, analyzed dose-response effects and determined the incidence of hepatocellular carcinomas. In addition, the effect of the addition of betamethasone, a growth inhibitory corticosteroid for liver, was also determined. In diethylnitrosamine (DEN)-initiated female Sprague-Dawley rats, ethinyl estradiol and mestranol were shown to cause 3.5- and 4.4-fold increases, respectively, at 9 months in the number of gamma-glutamyl transpeptidase (GGT) lesions per liver and an increased incidence of hepatocellular carcinomas, while estradiol had no enhancing effect. A significant decrease in GGT lesion number, but not carcinoma incidence, was demonstrated by the addition of betamethasone to the mestranol-containing diet as compared to mestranol alone. The results confirm and extend previous studies and the results of others which indicate that synthetic estrogens can act as promoters of hepatocarcinogenesis.

Cyclosporine is a powerful new immunosuppressant in all species tested including humans, and is being used on patients undergoing organ transplants. A complication of cyclosporine therapy, as with conventional immunosuppressive agents, is the occurrence of lymphoproliferative disorders and lymphoreticular neoplasms. Recently, it was observed that dietary administration of cyclosporine to rats, in a dose comparable to clinical use, induced proliferative alterations of lymphoid tissues in the intestine and lymph nodes. Sequential morphological changes occurring in the lymphoid organs of rats on a cyclosporine diet for 8 to 10 weeks were examined and changes observed were correlated with changes in electrophoretic patterns of serum proteins. The lymphoid lesions induced in rats were shown to bear striking similarities to the post transplant lymphoproliferative lesions in patients receiving cyclosporine and in patients with AIDS.

Studies on Tumor Promotion

Studies in this area are carried out at the molecular level as well as in in vitro and in vivo model systems. The pathogenesis and promotion of tumors have been studied using liver initiation-promotion systems in mice and rats, skin

painting studies in mice, and an aged F344 rat liver model system. From these efforts and a review of those of other investigators, it can be concluded that tumor promotion can be an irreversible biological process which may require only a short period of exposure to the promoter for effective tumor promotion. For example, in the skin of SENCAR mice, it was demonstrated that after only two or four exposures to TPA (12-Otetradecanoyl-phorbol-13-acetate), effective skin tumor promotion occurred. In addition, the tumors promoted after only short-term exposure to TPA grew progressively and did not regress after exposure to TPA was terminated. In mouse liver, the tumor promoter di(2-ethylhexyl)phthalate (DEHP) was effective as a tumor promoter after only 28 days of exposure while phenobarbital was only effective after continuous exposure.

Two widely prescribed benzodiazepine tranquilizers, diazepam and oxazepam, have been shown to promote hepatocarcinogenesis in B6C3F1 mice initiated with DEN. This finding is of special interest in view of the fact that these compounds have been found to be ineffective as liver tumor promoters in the rat. The marked and unexplained differences among rodent species in susceptibility to the promoting effects of both these classes of compounds demonstrate the difficulty of extrapolating with confidence between even closely related species, let alone from rodents to man, and emphasize the importance of studies to define the mechanisms that effect tumor promotion and presumably are related to interspecies differences in susceptibility to promoting agents.

Studies on the long-term effects of polychlorinated biphenyls (PCBs) on tumor development in mice was undertaken as a result of an apparent promotive effect observed for Aroclor 1254, a mixture of PCBs, on liver tumors initiated by dimethylnitrosamine (DMN) in infant mice, even though the PCBs were administered to their pregnant mother and so were received only during the suckling period. Aroclor 1254 and other commercial PCB mixtures contain a variety of congeners, some of which are readily metabolized and excreted, while others, lacking molecular sites of accessibility to oxidative enzymes are retained in the body indefinitely, mainly in fat. The latter are generally not acutely toxic, but their actions during long-term storage are not known. In light of the general contamination of human bodies throughout the industrialized world with rather high levels of PCBs, and the readiness with which these may be transmitted to offspring in milk, a study of their effects on DMN carcinogenesis was initiated. A single initiating dose of DMN to newborn mice was followed by intragastric exposure to a single dose of PCBs. Neoplastic and preneoplastic lesions were measured and enumerated 4 or 7 months later, and at the same time amounts of individual PCB congeners in the bodies were quantified. The results confirmed that single doses of PCBs during the suckling period had significant, complex effects on tumor development, to some extent correlatable with retention in the bodies of two specific PCB congeners. A promoting effect of PCBs on lung tumors was demonstrated for the first time; organs other than liver must now be considered as potential targets for the action of these chemicals. This consequence of a single treatment with PCBs was concomitant with body levels of PCBs similar to those reported for the human population.

In vitro studies have shown that tumor promoters can induce terminal differentiation in one cell type and stimulate another subpopulation to proliferate. Therefore, the effects of tumor promoters, such as TPA, teleocidin B, and aplysiatoxin on growth and differentiation of NHBE cells have been investigated. Nanomolar quantities of TPA rapidly inhibit the clonal growth rate of NHBE cells and concomitantly induce terminal squamous differentiation, as measured by an increase

in cell surface area, progressive stratification of the squamous cells, enhanced plasminogen activator activity, and increased formation of cross-linked envelopes. Teleocidin B, aplysiatoxin, and 2,3,7,8-tetrachlorodibenzodioxin, tumor promoters with markedly different chemical structures from each other and TPA, also cause similar changes. In contrast, ten different human lung carcinoma cell lines were relatively resistant to TPA induction of terminal differentiation. Therefore, TPA may be useful for identifying preneoplastic human cells and for allowing the selective growth of these cells during in vitro carcinogenesis studies.

Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. Studies have indicated that basal cells are heterogeneous in response to phorbol esters in that some cells are induced to differentiate while others are stimulated to proliferate. This could form the cellular basis for selection. The molecular basis for the pharmacological heterogeneity is suggested by studies of the phorbol ester receptor in cultured keratinocytes. Multiple receptor classes are found in differentiating cultures indicating that maturation state may modify receptor affinity. Protein kinase C activation may be the major pathway which mediates phorbol ester responses in keratinocytes since exogenous diacylglycerols can mimic the effects of TPA. Furthermore, generation of endogenous diacylglycerols by exposure of cells to phospholipase C reproduces the biological effects of TPA. A consequence of phorbol ester-induced differentiation is the production of single strand DNA breaks as measured by alkaline elution. This effect is blocked by retinoic acid and does not occur in initiated cells which are resistant to TPA-induced differentiation. Interestingly, benzoyl peroxide, another skin tumor promoter, produces DNA strand breaks rapidly and directly, without inducing differentiation, and some initiated cell lines are also resistant to this effect of benzoyl peroxide. This differential effect of benzoyl peroxide on normal and initiated cells could form the basis for cell selection in tumor promotion by that agent.

As noted above, selective clonal expansion of preneoplastic ("initiated") cells is a basic tenet of tumor promotion. Examples of mechanisms that could lead to selective clonal expansion of "initiated" cells compared with normal cells include (a) resistance to either exogenous or endogenous inducers of terminal differentiation, (b) resistance of the preneoplastic and neoplastic cells to cytotoxic products of integrated viral genes, (c) enhanced expression in the preneoplastic cells of either cellular or integrated viral genes whose products stimulate cell division, (d) autocrine production of growth factors, (e) increased sensitivity of the initiated cell to growth factors, and (f) cell surface modifications, both antigenic and functional, that could cause aberrant intercellular communication, recognition, and adhesion.

There is now considerable evidence that protein kinase C is the major phorbol ester receptor. An impediment to biochemical and immunological analysis of the receptor has been that the published purification protocols are time consuming, afford low yields (0.5-5%), and are difficult to scale up. Taking advantage of new advances in column chromatography, purification protocols have now been developed that permit the rapid and efficient isolation of the receptor. Stabilization procedures have been identified that preserve receptor activity, which is otherwise quite labile once the receptor is in the purified state. The purified receptor has been used for preparation of polyclonal antibodies, which are suitable for immunoprecipitation and Western blot analysis. Efforts are ongoing to prepare monoclonal antibodies.

Studies on Oncogenes in Chemically-Induced Tumors

Activation and/or modification of cellular oncogenes is likely to be important in carcinogenesis. Although the vast majority of studies have utilized an inter-species assay, i.e., transfection of human tumor DNA into mouse NIH/3T3 cells, human epithelial cells are now being used as recipients of oncogene DNA transfected into the cells by a modified protoplast fusion method. Transfection of primary human bronchial cultures with plasmids carrying the vHa-*ras* oncogenic complementary DNA results in the following changes in the epithelial cells: altered cell growth properties, resistance to inducers of squamous differentiation, immortality, progression to anchorage independent growth, and tumorigenicity. The characterization of one of these recombinant cell lines (TBE-1) has established cells from clonal isolation at various stages of development for carcinogenic complementation studies with (1) other oncogenes and (2) chemical and physical carcinogens. TBE-1 has integrated vHa-*ras* into its genome and expresses transcripts that hybridize to Ha-specific structural gene and vHa-LTR probe DNA. TBE-1 cells express detectable levels of phosphorylated vHa-*ras* polypeptide, p21.

Infection of mouse epidermal cells with oncogenic retroviruses containing an activated *ras* gene indicate that expression of *ras* and subsequent synthesis of p21 provides a marked proliferative stimulus to basal cells. However, such cells respond to high Ca^{++} by cessation of proliferation. These cells do not terminally differentiate but appear to be blocked in some non-terminal state of differentiation. Interestingly, blocked cells appear to remain responsive to tumor promoters and do not synthesize pemphigus antigen, a marker of suprabasal differentiation in vivo. Exposure to TPA reactivates their proliferative activity. Thus an activated *ras* gene could serve to produce a conditionally initiated cell which could only display the tumorigenic phenotype after exposure to a tumor promoter.

The consistent demonstration of a specific dominant transforming gene, Ki-*ras*, in F344 rat renal mesenchymal tumors induced by a single exposure to an alkylating agent adds another category of chemically-induced neoplasm to the list of those that are known to be oncogene-associated, and because its histogenesis is well defined it should be possible to study the temporal sequence of expression of Ki-*ras* in relation to development of the neoplasm. As the result of the F344's use in standard bioassays for carcinogenic potential, and its common use in experimental oncology studies, abundant literature is available on the induction of neoplasms by a wide variety of chemical agents. Since the effects of chemicals in the animal model are inferred to predict human risk and act by mechanisms common to other species, the model system should reveal mechanisms relevant to human carcinogenesis.

Activation of proto-oncogenes is being studied in two other model systems: the hamster and the guinea pig. Activated oncogenes have been identified by foci formation after transfection of DNA into NIH/3T3 cells from a number of transformed hamster lines. A number of transformed foci contain hamster DNA as evidenced by presence of hamster intracisternal A particle sequences (IAP). Further analysis of these lines indicates that the hamster activated oncogenes are not related to *Hras*, *Kras*, *bas*, *myc*, *fos*, *mos*, *src*, or *abl*. In the case of one transformed line, the activated oncogene is closely linked to a hamster IAP sequence. An activated oncogene has been molecularly cloned from a guinea pig transformed line. This same oncogene is activated in four other independently isolated lines. Activation is closely associated with tumorigenic potential and was

independent of the initiating carcinogen. The biologically active cloned oncogene has weak homology with Hras. However, further analysis of DNA and RNA expression indicates that this is an oncogene that has not been described previously.

In other studies the transcription of six cellular oncogenes during the process of compensatory growth in rat liver after partial hepatectomy was examined. Polysomal poly$(A)^+$ RNA populations were obtained at various times after partial hepatectomy. The mRNAs corresponding to c-Ha-ras, C-Ki-ras and c-myc genes were shown to increase 2- to 10-fold with the highest increase for the c-Ki-ras gene transcript. Transcripts of c-abl and c-src were shown to be essentially unchanged and c-mos transcripts were virtually undetectable in either normal or regenerating rat liver. Changes in c-myc transcripts were shown to occur before DNA synthesis in liver regeneration after partial hepatectomy or CCl_4 injury. The elevation of c-myc and c-ras transcripts was shown to be sequential in that the highest levels of c-myc transcripts were detected 12 to 18 hrs. after partial hepatectomy, whereas the levels of c-Ha-ras and c-Ki-ras were maximal by 36 to 48 hrs. By 96 hrs. transcripts of all activated oncogenes returned to their basal levels. The results suggest a role of c-myc in triggering the S phase or in inducing hepatocytes to enter the cell cycle. c-Ha-ras and c-Ki-ras genes may play a role concomitant with or subsequent to DNA synthesis. The expression of the above six proto-oncogenes was examined during the course of hepatocarcinogenesis induced by a choline-deficient diet containing 0.1% ethionine. The abundance of c-Ki-ras, c-Ha-ras and c-myc transcripts in polysomal poly$(A)^+$ RNA from liver cells was shown to increase by 2 weeks after the start of the carcinogenic diet. c-Ki-ras and c-myc expression remained elevated during the 35 weeks of the diet, but c-Ha-ras transcripts were shown to only increase transiently. High levels of both c-Ki-ras and c-myc RNA were formed in a primary tumor sampled at 35 weeks after the carcinogenic diet was started. The abundance of c-src transcripts was shown to be unchanged throughout carcinogenesis and c-abl and c-mos transcripts could not be detected in either preneoplastic or neoplastic livers. Hepatocytes, oval cells and bile duct cells were isolated from normal and preneoplastic livers to determine which cell types within the liver contained the proto-oncogene transcripts. The results showed that proto-oncogenes are expressed differentially in these cell types during hepatocarcinogenesis and that the expression of c-Ki-ras and c-myc was high in oval cells throughout carcinogenesis. The results of these and other studies suggest that elevated c-myc expression is associated with hepatocarcinogenesis, whereas c-Ha-ras expression may be related to hepatocyte proliferation.

Studies on Gene Expression and Growth Control of Tumor Cells

Cancers of the human esophagus and lung represent major causes of death in certain populations of people throughout the world. In studies on these two organ systems, the pattern of expression has been characterized of the main markers of epithelial differentiation, namely keratins, involucrin, and cross-linked envelopes, during the course of embryonic development, post-natal maturation, and/or in neoplasia. The pattern of expression was found to be dependent on cell type, the stage of differentiation and/or development, and the extrinsic environment of the cell. Distinctive qualitative and quantitative differences in the spectrum of keratin proteins are found in the carcinomas compared to their nontransformed counterparts. Analysis of keratin protein patterns is a useful adjunct in defining the type of tumor present. Moreover, assessment of cross-linked envelope-forming capabilities and the presence of involucrin serve as specific markers for squamous differentiation, and the extent of envelope formation and involucrin staining correlates

well with the degree of squamous differentiation in the tumor with more well-differentiated squamous carcinomas forming more cross-linked envelopes or possessing involucrin. Human esophageal and lung carcinoma cells lines were established in cell culture to evaluate if their properties in vitro faithfully manifest those of the original tumor, thereby representing useful models of carcinogenesis in vitro. Moreover, the growth and differentiated properties of these carcinoma cells were compared to their nontransformed counterparts. Numerous morphological and biochemical differences are observed between normal and malignant epithelial cells in culture. Significant changes in the array of keratins and in the proportions of cells making cross-linked envelopes were found. The results obtained parallel findings with tumor masses indicating that the tumor cells in cell culture continue to maintain a program of gene expression reflective of that of the original tumor.

Studies with keratinocytes have indicated that initiation of carcinogenesis is associated with a change in normal differentiation. In order to understand this association at the molecular level, the regulation of specific differentiation products is being explored. The genes which code for the major keratins of proliferating and differentiating keratinocytes have been cloned. Their expression has been studied in keratinizing vaginal epithelium where a hormone-regulated, coordinated, sequential program related to proliferation and differentiation has been determined. Interestingly, the expression of these genes is markedly altered in skin treated with TPA and in carcinomas. These results are consistent with other evidence for a fundamental derangement of differentiation in promoter-treated epidermis and in malignant epidermal cells. On the basis of this expression data, an assay was developed using antisera that are monospecific for individual keratin subunits that can distinguish between benign and malignant tumors. Sequence analyses of keratin cDNAs have revealed unique structural aspects of keratins expressed at different differentiation-states that may change the properties and functions of the filaments that they form. Isolation of a genomic fragment of one of the differentiation keratins has been completed and the gene structure has been characterized by sequence analysis in comparison to the cDNA.

Vitamin A is essential for the normal growth and differentiation of epithelial cells. In tracheobronchial epithelium, normally a mucus secreting tissue, deficiency of vitamin A causes squamous metaplasia. This lesion is similar to that observed in vivo in tracheas treated with benzo[a]pyrene and occurs in organ cultures of tracheas obtained from hamsters kept for 4 weeks on a vitamin A-deficient diet and cultured in a vitamin A-depleted, chemically-defined medium. Retinoids added to the medium can prevent the development of the lesions and restore mucociliary differentiation. It was of interest to identify and characterize keratin subunits in vitamin A-deficient tracheas, as part of ongoing efforts to understand the mode of action of vitamin A in normal epithelial differentiation and its involvement in anticarcinogenesis. The present studies employed a tracheal organ culture system and have reproduced the in vivo phenomenon of squamous metaplasia during culturing under vitamin A-free conditions as well as after carcinogen treatment. The squamous metaplasia induced by vitamin A deficiency, both in vivo and in vitro, was accompanied by an overall increase in keratin synthesis. Vitamin A-deficient tracheas were shown by immunoblot analysis to contain keratins of 50, 48, 46.5 Kd detected with the antibody AE_1, and 58, 56 and 52 Kd detected with AE_3. These proteins were either absent or present in much less quantity in control tracheas. In deficient tracheas 60 Kd keratin was found to be located specifically in squamous suprabasal cells, and 55 and 50 Kd keratin proteins were found in a greatly expanded basal cell compartment. Following

carcinogen exposure, the appearance of 60 Kd keratin and the enhanced expression of 50 and 55 Kd keratins preceded the squamoid metaplastic response as detected morphologically. Both the keratin changes and the morphological changes were prevented by retinoid treatment.

The condition of vitamin A deficiency causes a marked (up to 95%) decrease in the incorporation of mannose into glycoproteins in vivo. Accompanying this decrease in protein mannosylation were an accumulation of free mannose and a decrease in guanosine diphosphate mannose and in dolichylphosphate mannose in severely deficient livers in vivo. Reduced food intake was shown to be primarily responsible for the depletion of guanosine diphosphate mannose in severely vitamin A-deficient hamsters (6 weeks on a vitamin A-deficient diet). Recent data demonstrate that accumulation of dolichylphosphate occurs in hamsters kept for only 4 weeks on a vitamin A-depleted diet, i.e., much before any other symptoms of deficiency become manifest. In these animals liver guanosine diphosphate mannose levels were above normal, while the amount of free mannose and of mannose incorporated into dolichylphyosphate mannose, oligosaccharide lipids and glycoproteins was within normal ranges. Therefore, accumulation of unglycosylated dolichylphosphate seems to be the earliest measurable effect of vitamin A deficiency and it likely explains the profound effect of the vitamin on glycosylation reactions.

Studies on cellular differentiation and transformation have evaluated total cellular protein patterns using computer-based two-dimensional gel electrophoresis analysis. Studies on Ca^{++} and TPA-induced differentiation of mouse epidermal cells have identified 11 proteins that were changed in the same direction for both differentiating agents. These results suggest that a common program of protein synthesis is induced by both Ca^{++} and TPA, and that these proteins are involved in epidermal differentiation. Chemical transformation of Syrian hamster fetal cells (HFC) was also analyzed by the two-dimensional gel electrophoresis technique. Common qualitative changes were observed in seven neoplastic HFC lines. It has been possible to identify polypeptide changes that are closely associated with both early morphological changes in the transformation process and the acquisition of tumorigenicity. Studies employing a chemically-induced rat hepatocarcinogenesis model have led to identification of a set of proteins common to both the preneoplastic and neoplastic stages that appear to be under coordinated regulation during the evolution of the neoplastic process.

In Syrian hamster fetal cells, early polypeptide changes were determined in a series of 20 $NaHSO_3$ induced morphologic transformed colonies two days after isolation. Polypeptides 1 and 2 were always shifted and polypeptide 5 was missing. Polypeptide 3 was present in 7 of the colonies, and polypeptide 4 was present in 14 of the clones. Some clones had neither 3 and 4, both 3 and 4, or only 3 or 4. Thus polypeptide changes in 1, 2, and 5 are associated with early steps in the transformation process and related to morphologic changes, whereas polypeptides 3 and 4 appear to occur later and are more closely associated with acquisition of tumorigenicity.

During the course of hepatocarcinogenesis one of the most important processes in the neoplastic transformation of the liver is the development of focal lesions of proliferative hepatocytes shortly after initiation. After further carcinogen treatment or after promotion, these lesions enlarge to form grossly visible hyperplastic nodules. These nodules have two options: the majority (90-98%) "redifferentiate" back to normal appearing liver while a few persist, enlarge

further, and may serve as sites for the formation of the ultimate hepatocellular carcinomas. Since the hyperplastic nodule serves as a critical point in the formation of cancer a study concerning the biochemical nature of these nodules was recently initiated. Two-dimensional electrophoretic separation of silver stained polypeptides from normal rat liver tissue and from neoplastic hyperplastic nodules was carried out. Approximately 1100-1200 polypeptides were readily visible on each electrophoretogram. Although patterns for normal and neoplastic tissue were very similar, numerous qualitative and quantitative polypeptide differences were readily detected. Comparison of 750-1000 membrane and 500-800 cytosolic polypeptides from preneoplastic and neoplastic nodules revealed that roughly 4-8% of the membrane and 610% of the cytosolic polypeptides were undergoing quantitative changes of at least fourfold during hepatocarcinogenesis. Polypeptides which showed significant modulation occurred at all pH and molecular weight regions. Twenty-one membrane-associated and 10 cytosolic polypeptides were downregulated, while 14 membrane and 6 cytosolic polypeptides were up-regulated during hepatocarcinogenesis. In all but three polypeptides, the direction and magnitude of change were the same in both preneoplastic and neoplastic nodules.

The HL60 cell line, a continuously proliferating suspension cell culture, originally derived from a patient with acute promyelocytic leukemia, is widely used as an in vitro model for studying cellular differentiation along the myeloid/monocyte pathways. Treatment of HL60 cells with compounds such as retinoic acid, hypoxanthine, actinomycin D, butyrate, dimethyl sulfoxide and hexamethylene bisacetamide results in a greater than 90% commitment of the cells to differentiate, both functionally and morphologically, into mature granulocytes. Exposure of HL60 cells to phorbol esters, such as TPA, results in an irreversible commitment to monocyte differentiation, characterized by the shift from suspension to adherent cells, the acquisition of macrophage associated surface markers, monocyte specific esterases and the cessation of cell growth associated with terminal differentiation.

Treatment of HL60 cells with difluoromethyl ornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), results in a cessation of growth without committing the cells to differentiate. However, DFMO does not prevent differentiation of HL60 cells into monocytes when TPA is subsequently added, demonstrating that TPA-induced monocytic differentiation is independent of the decrease in cell proliferation also associated with TPA treatment. The expression of two of the HL60-associated oncogenes, c-myc and N-ras, was investigated both during terminal differentiation and during growth inhibition by DFMO that is independent of terminal differentiation. The c-myc oncogene is present in multiple copies in this cell line as well as in the original cell isolate and is highly responsive to treatment with retinoic acid or dimethylsulfoxide. C-myc transcripts are reduced 80-90% in HL60 cells treated with these granulocyte differentiating compounds when compared to untreated controls. The N-ras oncogene has been reported to be the transformation-specific gene in HL60 as assayed by the transfection of NIH/3T3 cells.

The 8 to 10-fold decrease in c-myc transcripts was associated with differentiation of the promyelocytes into mature monocytes. C-myc regulation also appears to be an early event of HL60 cells as evidenced by a burst of c-myc synthesis within the first hour of TPA treatment, followed by the rapid decline in the level of c-myc-specific transcripts. This response was similar to that observed in mitogen stimulated B cells, T cells, and normal fibroblasts although the HL60 induced level was neither as dramatic nor as long as that reported for normal cells. Although this response was reportedly associated with the cell cycle regulation

of proliferation in normal cells, the level of c-myc-specific transcripts was found invariant during the HL60 cell cycle. In addition, unlike the mitogenic response of other agents, or normal resting cells, TPA-induced differentiation of HL60 cells resulted in no cellular proliferation or significant change in DNA synthesis when compared to control or DFMO-treated cells during the first 24 hours, again supporting the association of c-myc with the differentiation state of the cell.

The asialoglycoprotein receptor (ASGPR) is a hepatocyte cell surface receptor which binds and internalizes serum glycoproteins with galactoseterminal carbohydrate chains. Recent experiments have demonstrated that cell-surface binding activity and cell-surface receptor proteins are decreased or absent in fetal, regenerating, phenobarbital-treated and neoplastic liver, while binding activity is increased in livers of pregnant dams. This phenomenon was exploited to isolate preneoplastic foci in carcinogen-treated rat livers. To verify this phenomenon the genetic regulation of the ASGPR was investigated by isolating poly(A)RNA from fetal, neonatal, regenerating, phenobarbital-treated and chemicallyinduced neoplastic Fischer rat livers. When compared to adult liver, the amount of ASGPR specific transcripts showed no change at 1, 3, 12, 24 and 64 hour post 70% hepatectomy; in fetal, neonatal and livers of pregnant dams; in livers of phenobarbital treated rats; and in chemicallyinduced preneoplastic and neoplastic liver samples. These data suggest that, in vivo, the ASGPR is regulated post-transcriptionally, possibly at the cell membrane. Interestingly, no transcripts could be detected in a Morris hepatoma cell line, 7777, despite being able to detect the ASGPR gene by Southern blot analysis. This indicates that in tissue culture, the ASGPR gene could be regulated at the transcriptional level. In addition, preliminary data indicates that freshly isolated hepatocytes, when placed into tissue culture, have signficiantly lower ASGPR specific transcripts within approximately 2 hours.

Regulatory signals involved in the control of mouse mammary tumor virus (MMTV) transcription by glucocorticoids have been examined in detail in two independent expression systems. In the first, fusions between the v-ras gene of HaMuSV, the MMTV LTR, and an enhancer element from the HaMuSV LTR have allowed us to monitor steroid-inducible transcription from the MMTV LTR by a rapid transfection assay. Efficient transfection of NIH/ 3T3 cells to the transformed phenotype occurs only when glucocorticoids are present in the medium. In the second assay system, the CAT from the bacterial Tn9 transposon has been placed under control of the MMTV promoter; in this system, the elaboration of CAT enzyme activity was shown to be inducible by glucocorticoids during transient expression after acute DNA-mediated transfection. Using these assays, deletion analysis by molecular techniques has localized sites sufficient to confer hormone sensitivity on the MMTV LTR to between 100 and 200 nucleotides 6' to the MMTV cap site. In both of these assay systems, an increase in the uninduced, constitutive expression from the MMTV LTR was observed after deletion of the hormone responsive sequences, suggesting that the mechanism of hormone action may be more complex than a simple induction effect. It is now apparent that the glucocorticoid regulatory element can regulate the activity of an exogenous enhancer introduced into the fusion chimeras. A model is suggested in which the hormone regulatory sequence is actually composed of more than one element, a positive activator sequence, and another previously uncharacterized element that regulates the activity of the positive element.

Studies on Carcinogen Metabolism and Interaction with DNA

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

Xenobiotics such as drugs and carcinogens as well as endobiotics such as steroids and fatty acids are metabolized by the mixed function oxidase systems. Cytochrome P-450 is the key component of mixed function oxidases and the type and quantity of specific forms of cytochrome P-450 determine the disposition of a particular substrate. Monoclonal antibodies (MAbs) are specific probes for particular isoenzymes. Myeloma cells were hybridized with spleen cells of mice immunized with purified human placenta mitochondrial cytrochrome P-450. Twenty-five independent hybridomas producing MAbs to the human placenta mitochondrial cytochrome P-450 were obtained. 3-Methylcholanthrene-inducible cytochromes P-450 of rats are also inducible in human placenta by smoking, and this induction has been measured with MAbs.

The multiplicity of cytochromes P-450 was examined with monoclonal antibodies (MAbs) to 3-methylcholanthrene (MC)-induced rat liver cytochrome P-450. A semi-quantitative, direct radioimmunoassay (RIA) has been developed to measure cytochrome P-450 in the microsomes from various tissues in animals that are untreated, or treated with MC. The amounts of cytochrome P-450 in different tissues and species, including human samples such as placentas and lymphocytes, were examined by competitive RIA. Individual differences have been observed by this method, which is more reliable than measurements of enzyme activity. Placentas from women who smoked cigarettes contained greater amounts of cytochrome P-450 with the MAb-specific epitope than placentas from nonsmokers. The amount of MAb-specific cytochrome P-450 in human peripheral lymphocytes increased after treatment with benz(a)anthracene. RIAs with multiple MAbs have also been used to detect epitope-specific cytochromes P-450 in animal livers, with the goal of classifying various tissues with respect to MAb-specific cytochromes P-450. Much higher levels of cytochrome P-450 recognized by MAb 1-7-1 were observed in MC-treated rats and C57Bl/6 mice than in untreated rats, MC-treated DBA/2 mice and guinea pigs. These analyses provide an approach to the study of cytochrome P-450 multiplicity that is complementary to enzymatic and structural studies. RIA methods will aid in defining the epitope-specific cytochrome P-450 content in different tissues, species, and strains of laboratory animals, and in understanding the diversity of the cytochromes P-450 and their role in individual susceptibility to carcinogenesis.

Polycyclic aromatic hydrocarbons induce oxidative and conjugative enzymes in responsive animals and in human cells. These oxidative enzymes (cytochrome P-450s) are thought to generate active intermediates from procarcinogens and thus initiate chemical carcinogenesis. This hypothesis will be investigated by directly introducing molecularly cloned full length P-450 cDNAs into different

cells and analyzing the extent of binding of the carcinogen to the cellular macromolecules, and the incidence of tumors by challenging with appropriate carcinogens. The infectious eukaryotic expression vector, vaccinia virus, was previously shown to express enzymatically active foreign proteins that are transported to the appropriate subcellular site. The mouse P1-450 and P3-450 genes have been introduced into the recombination vector and these genes will be transferred into the vaccinia virus to generate the infectious recombinant vaccinia virus.

As noted above, the cytochrome P-450s, though functionally related in catalyzing the oxidation of endogenous and foreign compounds, comprise a family of enzymes that differ from one another in primary structures, substrate specificities, antigenic characteristics and spectral properties as well as in their induction response to various xenobiotics. Research directed at both the protein and nucleic acid sequence levels has clearly shown the existence of other closely related isozymes with extensive homology to previously known forms. The basis for this microheterogeneity is thought to be primarily genetic, with each slightly variant cytochrome P-450 being coded by a separate gene, or an allelic form of a gene which is itself a member of a gene family. The genetic localization of two cytochrome P-450 gene families, the NADPH-cytochrome P-450 oxidoreductase gene and the epoxide hydratase gene, is being studied. A cDNA probe to a major rat phenobarbital-induced cytochrome P-450 mRNA (P-450 PB) and a cDNA probe to a major rat pregnenolone-16 alpha-carbonitrile-induced species (P-450 PCN) was used to detect cytochrome P-450 sequences. These gene families were shown to be genetically divergent from each other and showed no cross-hybridization. Using mouse x Chinese hamster somatic cell hybrids (EBS cell lines), all distinguishable P-450 PCN sequences were found to map to chromosome 6, whereas all P-450 PB sequences were located on chromosome 7. The data presented support the proposition that the region of the Coh locus on chromosome 7 is the site of the cytochrome P-450 PB gene family. NADPH-cytochrome P-450 oxidoreductase which appears to be encoded in many vertebrate species by a single gene, was found to be located on chromosome 6. The data presented showed that the Eph-1 locus on chromosome 1 is the site of at least one microsomal epoxide hydratase gene.

It is known that genetic polymorphisms contribute in large part to the large inter-individual differences observed in the metabolism of foreign compounds, such as drugs, by humans. Debrisoquine 4-hydroxylase activity is considered to be a prototype for genetic polymorphism in oxidative drug metabolism in humans; about 10% of caucasian populations exhibit a poor metabolizer phenotype, and the clearance of at least 14 other drugs has been shown to be deficient in patients exhibiting this phenotype. Prior to conducting studies on the human debisoquine 4-hydroxylase enzyme, a minor cytochrome P-450 was purified from Sprague-Dawley rat liver using debrisoquine 4-hydroxylase activity as an assay. This cytochrome P-450 (designated P-450_{UT-H}) was characterized by gel electrophoresis, peptide mapping and immunochemical analysis. Antibodies prepared to the rat cytochrome P-450_{UT-H} were found to inhibit the oxidation of debrisoquine and sparteine, encainide and propranolol, three other drugs suggested to be associated with this phenotype, in human liver microsomes. The oxidation of seven other cytochrome P-450 substrates was not inhibited by the antibodies. The antibody was shown to recognize a single polypeptide of MW 51,000 following polyacrylamide gel electrophoresis and immunochemical staining of human liver microsomes. When liver microsomes from 44 organ donors were examined, the intensity of the stained band was found to be significantly correlated with debrisoquine 4-hydroxylase activity. By the immunoprecipitation of in vitro translation products of total liver RNA,

it was calculated that the level of translatable mRNA coding for the debrisoquine-hydroxylating cytochrome P-450 is about an order of magnitude less in human liver than in rat liver. The availability of these antibodies will provide a biochemical basis for further basic and clinical studies on the role of a particular cytochrome P-450 polymorphism in humans.

The metabolism of procarcinogens of several chemical classes which are considered to be important in the etiology of human cancer is being systematically examined. Procarcinogens of several chemical classes can be activated enzymatically to electrophilic reactants that bind covalently to DNA in cultured human tissues. The studies of activation and deactivation of representative procarcinogens have revealed that the metabolic pathways and the predominant adducts formed with DNA are generally similar between humans and experimental animals. Wide quantitative inter-individual differences (50- to 150-fold) are found in humans and other outbred animal species. When the metabolic capabilities of specimens from different levels of biological organization are compared, the profile of benzo[a]pyrene metabolites is similar in cultured tissues and cells, but subcellular fractions, e.g., microsomes, produce a qualitative and quantitative aberrant pattern.

The metabolic processing of several other chemical carcinogens by human tissues has been studied. Two distinct phenotypes, slow and fast metabolizers, were observed for both metabolic activation and detoxification of the model carcinogen, 2-acetylaminofluorene, in human liver microsomes from 28 individuals. It was observed that individuals who were fast activators of the carcinogen were, in most cases, also fast detoxifiers of the chemical. However, different phenotype pattens exist, suggesting that fast activators of a toxin need not also be phenotyped as fast detoxifiers. Studies in this area may help understand whether certain individuals were predisposed to a higher rate of chemically-induced cancer.

Whether or not they require metabolic activation, most chemical carcinogens bind covalently to DNA and these reactions are considered to be critical in the initiation of carcinogenesis. Antibodies specific for carcinogen-DNA adducts have probed the nature, extent, and consequences of in vitro and in vivo DNA modification. DNAs substituted with 2-acetylaminofluorene (AAF), benzo[a]pyrene (BP), or cis-diamine-dichloroplatinum II (cis-DDP) were analyzed by quantitative immunoassays able to detect one adduct in one hundred million nucleotides, and by immunohistochemical procedures developed to localize adducts in situ. In hepatic DNA of rats fed a carcinogenic dose of AAF for 4 weeks, adduct accumulation reached a plateau at 2-3 weeks and adducts were shown by immunohistochemistry to be primarily localized in the periportal areas. During 4 subsequent weeks on control diet, adduct removal was biphasic. A computer-derived pharmacokinetic model consistent with these data proposed that adducts are formed into two genomic compartments, one from which adducts are removed rapidly and another from which they are removed slowly. In contrast to the high levels of AAF adducts formed in rat liver DNA, at least 50-fold lower adduct quantities were formed in the DNA of mouse epidermis and cultured mouse epidermal cells exposed to initiating doses of BP. When activated forms of both carcinogens were utilized in the keratinocyte focus assay, N-acetoxy-AAF yielded more adducts per molar concentration than the BP derivative but no differentiation-altered foci formed in N-acetoxy-AAF treated cultures. Nucleated peripheral blood cell DNA was obtained from cancer patients at multiple times during courses of cis-DDP therapy, and a total of 223 samples were analyzed. Of these, 23 untreated control samples were negative, and 46% of the 200 samples from patients receiving cis-DDP were positive. Adduct accumulation, in positive patients, occurred as a function of total cumulative

dose, suggesting relatively slow adduct removal. Disease response data on 47 patients indicated that individuals with adduct levels greater than 200 attomoles/γg DNA have a very high (65%) rate of complete response to therapy. Parallel experiments in animal models have demonstrated that the same adduct forms in kidney, gonads, and tumor of rats and mice in direct relation to dose.

A method of detecting the in vivo formation of carcinogen-DNA adducts that does not require the use of radiolabelled test compounds has recently been developed. The procedure involves the incorporation of (^{32}P) orthophosphate into carcinogen-nucleotide adducts obtained by digesting DNA from experimental animals that had been treated with the test compound. The labelled adducts are then resolved by thin layer chromatography and the radioactivity detected by autoradiography and scientillation counting. A modification of the technique was applied to the study of the adducts formed in liver DNA of adult CD-1 mice given a single dose of a series of alkenylbenzenes, plus allylbenzene and isosafrole. The long-term persistence of safrole-DNA adducts in mouse liver and the influence of the sulfotransferase inhibitor, pentachlorophenol, on safrole-DNA adduct formation was also investigated. The known hepatocarcinogens, safrole, estragole and methyleugenol were shown to exhibit the strongest binding to mouse liver DNA when analyzed 24 hrs. after administration of the test compounds. Several related compounds (allylbenzene, anethole, myristicin, parsley apiol, dill apiol and elemicin), which have not been shown thus far to be carcinogenic in rodent bioassays, were found bound to mouse liver DNA at 3- to 200-fold lower levels. No binding was detected for eugenol. Low binding to mouse liver DNA was also observed for the weak hepatocarcinogen, isosafrole. The main adducts observed appeared to be guanine derivatives. Adducts formed by reaction of 1'-acetoxysafrole with mouse liver DNA in vitro were determined to be chromatographically identical to safrole-DNA adducts found in vivo. The pretreatment of mice with pentachlorophenol was shown to inhibit the binding of safrole to mouse liver DNA. This provides further evidence that the metabolic activation of the allylbenzenes proceeds by the formation of 1'-hydroxy derivatives as proximate carcinogens and 1'-sulfooxy derivatives as ultimate carcinogens.

Studies on the roles of fetal and maternal metabolism of carcinogens in transplacental carcinogenesis were continued as were invesigations of whether maternal and fetal genotypes governing responsiveness to induction of MFO enzymes play a determining role in sensitivity to transplacental carcinogenesis. In a pharmacogenetic model system involving genetic crosses of C57BL/6 (induction responsive) and DBA/2 (nonresponsive) mice, sensitivity to tumorigenesis by subcutaneous MC, and to toxic effects by polycyclic aromatic hydrocarbons in several systems, has been found to correlate positively with responsiveness to induction. The importance of such responsiveness in fetuses is not well understood, although ontogenetic appearance of activating enzymes is often postulated to be a limiting factor in fetal susceptibility to tumorigenesis. Therefore a transplacental dose response assessment with MC was carried out in the C57/DBA model, using both responsive (C57BL/6 x DBA/2)F_1 and nonresponsive DBA/2 mothers. Two clear results were obtained. Fetuses which were responsive to induction of aryl hydrocarbon metabolism developed 2 to 3-fold more lung and liver tumors than did nonresponsive littermates at almost every MC dose, confirming that this characteristic is indeed an important determinant of fetal susceptibility. Secondly, fetuses of nonresponsive mothers developed many more tumors after the same dose of MC than did fetuses of responsive dams; the fetus, like other distal target organs, is probably protected by enzyme induction in the mother.

Studies on Growth Factors and Lymphokines

The peptide growth factor called transforming growth factor-beta (TGF-B) has been characterized and purified to homogeneity. Although this peptide was named for its ability to cooperate with members of the epidermal growth factor family to induce phenotypic transformation and anchorage-independent growth of non-neoplastic fibroblasts, it has recently been shown that TGF-B can inhibit the anchorage-independent growth of certain tumor cells. This bifunctional character of TGF-B is best shown in experiments using fibroblasts transfected with the myc gene; in these cells TGF-B can either stimulate or inhibit the anchorage-independent growth of the cells depending on the complete set of other growth factors operant on the cells.

Human platelets are a major storage site for TGF-B; they contain 100 to 1000-fold more of this peptide than do other cells which have been examined to date. TGF-B can be purified from platelets in a two-step procedure that involves sequential gel filtration in the absence and then presence of denaturant. Structural studies on homogeneous TGF-B show that it is composed of two 12,500-dalton subunits which are held together by disulfide bonds. Platelets also contain smaller amounts of an EGF-like peptide which can synergize with platelet TGF-B to induce growth of NRK fibroblasts in soft agar. Mechanistically, these two peptides interact; incubation of purified TGF-B with NRK cells specifically increases the number of receptors for epidermal growth factor.

The complete amino acid sequences for human TGF-alpha and TGF-B have been deduced from the cDNA nucleotide sequence for each of these peptides cloned in collaboration with Genentech, Inc. Each of these peptides is synthesized as a part of a larger precursor molecule, and the messenger RNAs encoding each of these peptides are considerably larger than expected. TGF-alpha, a single-chain peptide of 50 amino acids, has been expressed in milligram quantities in E. coli; the recombinant peptide is fully active biologically. TGF-B is a homodimer with each chain composed of 112 amino acids containing 9 cysteine residues; it has not yet been expressed in a biologically active form.

An important area for potential application of peptide growth factors is in the enhancement of wound healing. Despite the need for rapid healing in the treatment of severe burns, trauma, diabetic and decubitus ulcers, and other conditions, there is no practical way at present to accelerate wound healing with pharmacological agents. It has recently been shown that subcutaneous injection of TGF-B into the back of rats enhances wound healing, as measured by increased accumulation of total protein, collagen, and DNA. Nanogram amounts of TGF-B cause a pronounced fibrotic reaction when injected subcutaneously into newborn mice. In other studies with newborn mice, it has been shown that human TGF-alpha is as potent as human EGF or mouse EGF in eliciting a physiological eyelid-opening response. All of the above studies indicate that transforming growth factors have potential clinical utility for enhancement of specific growth of cells.

Lymphokine preparations prepared from antigen or mitogen stimulated lymphocytes contain a potent anticarcinogenic activity which is the property of a new lymphokine termed leukoregulin. Furthermore, leukoregulin induces specific target cell plasma membrane changes accompanying its natural killer cell sensitizing and tumor cell proliferation inhibitory activities. The membrane changes can be rapidly detected by flow cytometric analysis of both light scatter and membrane

permeability changes, the latter being followed by the uptake or by the loss of intracellular fluorescent molecules such as fluorescein or propidium iodide, respectively. The same changes are observed in target cells during the course of natural killer cell cytotoxicity. This suggests that leukoregulin may be an intrinsic mediator or element of natural lymphoid cell cytotoxicity and occupy a central role in immunological homeostasis.

Epidemiology and Biostatistics

Continued emphasis was given this year to case-control and cohort studies aimed at evaluating key hypotheses in cancer etiology. Case-control studies of selected cancers have been undertaken when high-risk communities are identified on the cancer maps or when major testable hypotheses and special resources become available. Whenever possible, laboratory procedures are incorporated into the epidemiologic studies to better clarify exposures, preclinical responses, and mechanisms of carcinogenesis.

Biochemical Epidemiology

Multidisciplinary projects combining epidemiologic and experimental approaches have been emphasized whenever possible to evaluate the influence not only of oncogenic viruses, but also of dietary and metabolic factors, host susceptibility, air and water pollutants, and a wide variety of other risk factors that are likely to escape detection unless laboratory probes are integrated with epidemiologic investigations. This approach, sometimes called biochemical or molecular epidemiology, has only recently been developed in cancer epidemiology. The laboratory parameters allow investigations to define past exposures and subclinical or preclinical response to initiators, promoters, and inhibitors of carcinogenesis, and to evaluate host-environmental interactions. The Program is seeking ways to effectively utilize this approach to clarify carcinogenic risks associated with nutritional influences or specific environmental agents that can be detected in tissues or body fluids. Opportunities are also being sought to assess specific host factors that influence susceptibility to cancer, including endocrine function, immunocompetence, and genetic markers including oncogenes. Of special interest are techniques to detect and quantify exposure to particular carcinogens or their metabolites in vivo through chemical analyses, mutagenesis assays, or immunologic detection techniques. It is now possible to measure the interaction of certain agents with cellular target molecules, for example, through adduct formation with proteins and nucleic acids, excretion levels of excised adducts, and markers of altered gene expression. Collaborative studies with the NCI Laboratory of Human Carcinogenesis are being developed to investigate these mechanisms in lung cancer, and studies with the NCI Laboratory of Experimental Carcinogenesis and the Division of Cancer Treatment are underway to clarify the role of fecal mutagens in the development of colorectal cancer. Collaborations with other intramural laboratories are ongoing in viral carcinogenesis, especially to evaluate the role of retroviruses and papillomaviruses in human cancer.

Diet, Nutrition and Cancer

Studies were further intensified this year as evidence accumulates to suggest that dietary factors contribute to a large though uncertain fraction of human cancer. Several studies have utilized geographic areas in the United States (e.g., north/south differentials for large bowel cancer) and migrant groups (e.g., Japanese- and Norwegian-Americans) whose cancer risks may be altered by

changing dietary habits. A role for dietary fat in colon cancer has been suggested, and a case-control study was begun to clarify the role of dietary patterns, in conjunction with laboratory measurements of blood and feces for lipids, fiber micronutrients and mutagenicity in several bacterial systems. Dietary fat may also alter the risk of breast cancer, perhaps by increasing estrogen production. A case-control study of young Asian-American women with breast cancer has been started to clarify the role of nutritional and hormonal factors and their interactions. Fat intake and hormonal levels may also contribute to renal cell cancers, since a case-control study in Minnesota revealed that obesity was a risk factor in females, but not males. Women whose body mass index was in the highest 5% percent had a risk of 6-fold compared to those in the lowest 25%.

Evidence is mounting that a low intake of certain food groups may contribute to certain cancers. A case-control study of esophageal cancer in black males implicated a broad nutritional deficiency in addition to alcohol intake. A case-control study of oral cancer in southern women pointed to deficiencies in fruits and vegetables, resulting in low intake of micronutrients such as vitamin C and carotene. In a case-control interview study of lung cancer in New Jersey, males in the lowest quartile of carotenoid intake had increased risk compared to those in the highest quartile after adjusting for smoking. No reduction in lung cancer risk was associated with retinol or total vitamin A consumption. Vegetables afforded even more protection then the carotenoid index, particularly the consumption of dark yellow-orange vegetables, which have a high content of alpha and beta-carotene relative to other carotenoids. The effect of vegetable intake was greatest for squamous-cell carcinomas, with the smoking and education-adjusted risk among low-consumers reaching 1.6 compared to high-consumers. This effect was limited to smokers of long duration and current smokers, suggesting action on a late stage or promotional event. Opportunities to study nutritional hypotheses are also being pursued in other countries, particularly China, where several collaborative case-control and intervention studies are underway. In addition, E&B investigators have continued to develop and utilize national resources, including HANES I, the first Health and Nutrition Examination Study of the United States, in efforts to relate dietary habits with the subsequent risk of cancer.

Studies of Infectious Agents

Increasing attention was devoted to investigating the role of a type C retrovirus associated with a specific type of aggressive leukemia/lymphoma of T-cell origin. In collaboration with the NCI Laboratory of Tumor Cell Biology, a series of studies have clarified the relation of HTLV-I to the T-cell malignancy. The T-cell leukemias were found to occur primarily in areas where HTLV-I infection is endemic in the general population, such as southern Japan, the Caribbean basin, northern parts of South America, Central America, and certain parts of Africa and the Middle East. In the United States the cases have developed mainly in the black population of the southeastern United States and in migrant groups from high-risk areas. In these widely separated parts of the world, a high percentage of cases with T-cell leukemia have shown antibodies against HTLV-I. Within these endemic areas there appears to be marked variation in antibody prevalence, and the relatives of infected individuals have a much higher prevalence of antibodies than the surrounding population. Current emphasis is on the epidemiologic pattern of infection with this virus, including the identification of reservoirs, modes of transmission, and susceptibility states.

The Program has been heavily committed to investigating epidemic outbreaks of AIDS, which predisposes to Kaposi's sarcoma and opportunistic infections. Since HTLV-III is now implicated as a likely cause of AIDS, assays for this virus and T-cell subsets have been used in a series of epidemiologic studies of AIDS in the United States, Haiti, Denmark and Africa. Among male homosexuals and patients with hemophilia, the high risk of AIDS and AIDS-related complex has occurred almost exclusively among persons with this virus or its antibody. The finding of HTLV-III also clarified the previous known risk factors among male homosexuals; that is, multiple sexual partners, frequent anal receptive intercourse, and contact with individuals from high-risk areas. In the longitudinal study of hemophilia patients, the development of seropositivity with HTLV-III was primarily among patients given factor VIII concentrate, which is derived from a large number of blood donors. No evidence was found that HTLV-III was directly involved in the tumors associated with AIDS, notably Kaposi's sarcoma and lymphoma, but its immunosuppressive effect may trigger other mechanisms. Although the frequency of AIDS and HTLV-III infection is high in certain parts of Central Africa, no relation was found between the virus and the classical form of Kaposi's sarcoma, which is also endemic in this region. Also continued this year were studies to clarify the role of the Epstein-Barr virus in Burkitt's lymphoma and nasopharyngeal cancer, human papillomaviruses and herpes virus type II in cervical cancer, and hepatitis-B infection in primary liver cancer.

Tobacco-Related Cancer

Cigarette smoking is known to account for a substantial proportion of human cancer, and its effects on lung cancer were evaluated by a largescale case-control study in several European cities. The size of this study makes it possible to clarify the modification of risk due to changing smoking habits. It was found that cessation of smoking lowers lung cancer risk, but the reduction was proportionally greater for short-term vs. long-term smokers. Little excess risk was seen among the group of persons who smoked for less than 20 years and quit for 10 or more years. Hence, the effect of smoking on lung cancer risk appears largely reversible if the habit is stopped soon enough. In addition, the risk for lifelong filter smokers was about one-half that seen for lifelong non-filter smokers. However, the risk for those who quit smoking for 10 or more years was much lower, being 20 to 30 percent of the risk seen among those who continued smoking. Although the dangers of filter cigarettes are less than non-filters, there was a substantial increase in the risk of lung cancer proportional to the duration of smoking filters. The European study was of sufficient size to dismiss the claims of some investigators that smoking affects only squamous and small-cell lung cancers. Among 938 patients with lung adenocarcinoma, risk increased in proportion to the duration and amount of smoking. Excess risks of lung cancer were also associated with cigar and pipe smoking, with dose-response relationships independent of cigarette smoking.

Tobacco habits and lung cancer risks vary geographically in the United States. A case-control study of lung cancer in a high-risk area of southern Louisiana implicated the heavy use by Cajuns of local brands and hand-rolled cigarettes containing high-tar levels. Because of an earlier study linking smokeless tobacco to high rates of oral cancer in the southern United States, the Program has become involved in additional projects to clarify the cancer risks associated with snuff dipping and chewing tobacco.

Several studies are evaluating the possible association of lung cancer with passive smoking. Preliminary data from a case-control study of lung cancer among non-smoking women suggest an increased risk, in proportion to the number of cigarettes their husbands smoked, and indicate the importance of further studies with more quantitative exposure data on various sources of environmental tobacco smoke. Finally, the impact of tobacco on cancer incidence appears to be increasing with newer understanding about the causes of particular tumors. Recently completed case-control studies of renal cell cancer, renal pelvis cancer, nasal cancer, and cervical cancer indicate etiologic relationships to cigarette smoking, so that the list of cancers associated with tobacco products continues to grow and the need for preventive action is more urgent than ever.

Radiation Studies

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

A new survey of breast cancer among atomic bomb survivors revealed, for the first time, a dose-related excess risk among women exposed under age 10, indicating that the immature breast is susceptible to the carcinogenic effects of radiation. A study of childhood cancer in twins indicated a 2fold excess risk associated with prenatal x-ray, suggesting that the association is due to radiation rather than the indications for pelvimetry. A case-control interview study found that 9% of all thyroid cancers could be attributed to prior childhood head and neck irradiation, and that pregnancy subsequent to radiation exposure appeared to enhance risk. A further follow-up of children irradiated for ringworm of the scalp in Israel revealed an excess of thyroid cancer and nodules following doses on the order of 9 rads, as well as elevated risks of brain tumor and leukemia.

In an international survey of cervical cancer, radiotherapy was found to be associated with a small, but significant, increased risk of leukemia. This may be related to the low doses of radiation absorbed by bone marrow outside the pelvis, since pelvic marrow was probably destroyed or rendered inactive by the large therapeutic exposures. Ovarian damage by radiation may have contributed to a low breast cancer risk, which was evident even among postmenopausal women. The expression period for radiation-induced solid tumors appeared to continue to the end of life. Chromosomal aberrations following partial-body irradiation were found to persist in circulating lymphocytes for over 30 years.

In an international study of over 9000 children treated for cancer, the risk of second cancers of the bone was strongly associated with high-dose radiation therapy. Radiotherapy was not associated with increased rates of second leukemias, which could be attributed almost entirely to alkylating agent treatment. A joint monograph on multiple primary cancers, focusing on long-term survivors, was prepared in collaboration with the Connecticut Tumor Registry and the Danish Cancer Registry. Cancer patients in Connecticut were found to have a 31% increased risk of developing a second primary cancer, which rose to 49% among those surviving more than 30 years. Some constellations of cancers appeared to be due to smoking and alcohol (e.g., lung, larynx, esophagus, buccal cavity, and pharynx), whereas others seemed to be related to hormonal or dietary factors (e.g., colon, uterine

corpus, breast, and ovary). In some instances, second cancers appeared to be caused by radiotherapy (e.g., rectal cancer following cancers of the female genital tract, and leukemia following uterine corpus cancer) or by chemotherapy (e.g., acute non-lymphocytic leukemia following multiple myeloma, Hodgkin's disease, and cancers of the breast and ovary). The non-neoplastic effects of radiotherapy were examined in a registry of longterm survivors of childhood cancer at the Dana-Farber Cancer Center. Among Wilms' tumor survivors, an excess of low birth weight was found among the offspring of females, but not males, suggesting a consequence of radiogenic fibrosis of the developing uterus.

Occupational Studies

As a time-tested means of identifying physical and chemical carcinogens, occupational studies were pursued to assess hazards suspected on the basis of experimental, clinical, and field observations. During the past year a case-control study of bladder cancer in New England uncovered an elevated risk among truck drivers that rose to nearly 2.5-fold among those employed for five or more years. The risk was greatest among men who began driving in the 1930s and 1940s and remained unchanged after controlling for smoking and coffee drinking. The high rate of bladder cancer in both sexes in this area was found to be related, in part, to exposures in the leather and textile industries. A survey of professional artists revealed excess mortality from leukemia and cancers of the bladder and kidney among painters, and cancer of the prostate among sculptors. The excess of bladder cancer among painters was confirmed in a reanalysis of data from the National Bladder Cancer Survey. This Survey also revealed excess risks among truck drivers (especially those using diesel engines), railroad workers, metal machinists, metal workers, construction workers, lumbermen and woodworkers, hair dressers, drycleaners and cutting operators.

A screening program for colorectal cancer and polyps by flexible sigmoidoscopy among pattern makers uncovered a 2.6-fold excess of colon cancer, but the excess could not be linked to any specific characteristic of pattern making. Compared to asymptomatic populations, the proportion of pattern makers with polyps did not appear excessive. A systematic survey of the mortality experience of 293,958 U.S. veterans by occupation/industry and smoking habits provided new clues regarding workrelated factors that require further evaluation. Elevated risks for stomach cancer among carpenters, machinists, and steelworkers may reflect exposure to dusts and abrasives. Surveys of anatomists and morticians uncovered consistently elevated mortality from leukemia and brain cancer, and further projects to clarify the possible influence of formaldehyde in these and other formaldehyde-exposed groups are underway.

A case-control study of leukemia using death certificates uncovered associations between leukemias and farming-related activities. Case-control interview studies of leukemia, lymphoma, and soft-tissue sarcomas are underway to evaluate the role of herbicides, insecticides, and other agricultural factors in the origin of these tumors. A case-control study of nasal cancer in Virginia and North Carolina implicated occupational exposure to wood dust and textiles; the latter association may explain the high rates of nasal cancer among women in this area. A case-control study of renal pelvis cancer suggested the influence of occupational exposure to aromatic amines, which resembles the associations reported for bladder cancer.

Environmental Studies

Epidemiologic studies have utilized relevant environmental measurements to evaluate the effects of pollutants in the general environment. To test the hypothesis that arsenical air pollution is related to lung cancer, a case-control interview study was carried out in the vicinity of a large zinc smelter in Pennsylvania. An elevated risk was found among people living near the smelter and in areas with high soil levels of arsenic, even after controlling for the effects of smoking and occupational exposure (excess risks were seen among long-term workers in the smelter and steel plant). The risk of indoor air pollution is being investigated through case-control studies of lung cancer in New Jersey and Sweden, where radon daughter products will be measured, and in China, where coal-burning stoves generate high levels of polycyclic hydrocarbons.

The risk of water pollution from halogenated hydrocarbons was evaluated by using the national case-control study of bladder cancer. Despite previous findings of an association based on geographic correlation studies, no overall relationship was found between drinking water quality and the risk of bladder cancer after making appropriate adjustments. However, in certain western areas (Iowa, New Mexico, and Utah), a positive association was detected between bladder cancer risk and duration of exposure to surface (chlorinated) water, suggesting the possible role of agricultural chemicals. Further case-control studies are planned to clarify the effect of water pollutants on the risk of bladder, colon, and other cancers, with particular attention to agricultural areas.

Studies on Therapeutic Agents

Studies were continued to evaluate the carcinogenic effects of cytotoxic drugs, hormones, and other compounds. A survey of patients given methyl-CCNU, a nitrosourea used in cancer chemotherapy, provided the first quantitative evidence that the risk of developing a leukemic disorder was directly related to the total dose per surface area administered. Alkylating agents to treat childhood cancer were associated with an increased risk of leukemia and bone cancer--the latter provides evidence that solid tumors may result from chemotherapy. An analysis of cancer registry data suggested that women with breast cancer who received chemotherapy are prone to leukemia. In addition, women with breast cancer who received estrogen therapy were at an increased risk of endometrial cancer. Recent concerns about the possible tumor-promoting effects of thyroid supplements on breast cancer risk were not substantiated. Data from the Breast Cancer Detection Demonstration Project revealed an association between use of menopausal estrogens and the risk of benign breast disease of all histological types, with an increased risk associated with duration of use. Case-control studies revealed an increased risk of in situ and invasive cervical cancer associated with oral contraceptives, particularly with long-term use. This risk persisted after adjusting for sexual activity and smoking, which were independent risk factors. The use of phenacetin-containing analgesics was implicated in a case-control study of renal pelvis cancer, which also suggested the possible influence of acetaminophen; this association deserves further study because of the increasing use of this drug.

Family Studies

Enhanced by collaborative ties with laboratory investigators, epidemiologic and clinical observations have resulted in the delineation of familial cancer syndromes

and several leads to mechanisms of host susceptibility. The discovery of the dysplastic nevus syndrome has provided a marker of susceptibility to melanoma, enabling early detection and treatment of this potentially lethal cancer. In 14 families studied intensively over a 7 year period, 42 new primary melanomas have been detected in 22 patients, and all but one was surgically curable. An analysis of the segregation of melanoma and dysplastic nevi in high-risk families indicates an autosomal dominant pattern of inheritance. It is noteworthy that the Rh gene is located on the short arm of chromosome 1, which is the most frequently abnormal genomic segment in human melanoma tumor cells. Nontumor fibroblast lines from patients with melanoma and dysplastic nevi have shown increased cell killing after exposure to ultraviolet light and to a UV-mimetic chemical.

Studies of a familial syndrome featuring soft-tissue sarcomas, breast cancer, and other neoplasms have led to the discovery of in vitro cellular radio-resistance in this disorder. Preliminary cytogenetic studies of sarcomas indicate deletions or rearrangements in the region of 3p21, near the suspected gene locus for small-cell carcinoma of the lung. Study of a family with 10 cases of renal cell carcinoma has revealed a 3:8 translocation in the normal cells of all family members with renal carcinoma. The breakpoints seen on chromosomes 3 and 8 have prompted experimental studies into the role of chromosomal rearrangements and oncogenes. Preliminary findings suggest that the c-myc oncogene has been translocated without duplication in this family, and that sporadic renal cell cancers have non-random rearrangements in the short arm of chromosome 3. The repository of cancer-prone families in the Program has become of increasing interest to experimentalists involved in the identification of human oncogenes, and tissue specimens are made available upon request to the extramural community. The NIH Inter-Institute Medical Genetics Clinic, directed by two staff members, provides a multidisciplinary setting for studying families and individuals prone to cancer.

Activities in the Office of the Director

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

Activities in the area of environmental carcinogenesis are located in the Office of the Director. A number of cooperative projects and collaborations with other Federal agencies are carried out under interagency agreements with the U.S. Environmental Protection Agency (EPA), National Institute for Occupational Safety and Health (NIOSH), and National Oceanic and Atmospheric Administration (NOAA). In addition to managing and serving as Project Officers on these interagency agreements, staff from the Office of the Director interface with state agencies, industrial 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 Carinogenesis (ICGEC), organized within the Office of the Director 12 years ago, also serves as a vehicle for information exchange. The ICGEC was originally constituted to provide a mechanism for interagency contacts to secure access to data bases; it has provided, indirectly, a stimulus for development of projects in the area of environmental and occupational carcinogenesis. It consists of representatives from 28 agencies or subagencies, and meets every 2-3 months. By October 1985 there will have been 80 meetings. Topics of meetings held so far this year are as follows: (1) Approaches to Evaluation of Short-Term Test Data, (2) Cancer Prevention in the Workplace, (3) Oncogenes and the Environmental Toxicologist, and (4) Toxicity of Ethylene Dibromide.

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

The office 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 prior decisions on chemicals to be submitted to the CSWG.

Another information dissemination activity involves preparation, under contract, of the "Survey of Compounds Which Have Been Tested for Carcinogenic Activity." Previous contracts provided for the preparation of volumes for 1974-75, 1976-77, 1979-80, which have been distributed. The present contract provides for the preparation of volumes for 1981-86. These reports, as well as the IARC (International Agency for Research on Cancer) Monographs are distributed through this office. An interagency agreement with the Centers for Disease Control (CDC) facilitates collaboration with and funding of the Michigan State Department of Public Health (MDPH), and a liaison officer from this office is active in that project relating to population studies on polybrominated biphenyls (PBBs).

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

The Division supports a Cooperative Agreement with IARC which is managed by staff of the Office of the Director. IARC is located in Lyon, France and the title of the project is "Evaluation of the Carcinogenic Risk of Chemicals to Humans." IARC established this program in 1970 and monographs have been published in volumes so entitled for a large number of chemicals. Thus far 34 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, is evaluated on the basis of results in experimental animals, studies in in vitro systems and epidemiologic studies. The monographs also contain background information on the chemicals under consideration such as chemical and physical properties, analysis, occurrence, production, use and estimated human exposures from all sources. This information is provided to IARC by NCI through a resource contract currently held by Tracor-Jitco. The IARC monographs have become a highly respected and authoritative reference source for countries around the world. Another IARC activity supported under this agreement is the compilation of a listing of laboratories around the world into a compendium entitled "Survey of Chemicals Being Tested for Carcinogenicity." The IARC initiated this survey in 1973 on a worldwide basis; thus far 11 surveys have been published and the twelfth survey is in preparation. These surveys are made available so that laboratories involved in carcinogenesis research can coordinate their testing and research, thus avoiding unnecessary duplication.

Registry of Tumors in Lower Animals

The Division continues to support the Registry of Tumors in Lower Animals (RTLA) which is located at the Smithsonian Institution in Washington, D.C. The RTLA is the focal point through which information on neoplasms in lower animals is channelled and maintained. Neoplasms and tumor-bearing animals of invertebrate or cold-blooded vertebrate species are collected, studied, classified and preserved at the Registry, which maintains the largest collection on 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: Air, Water, Food, Drugs and Cosmetics

Aquatic Toxicology

A number of efforts, some in collaboration with other government agencies, continue in the area of aquatic toxicology. For example, an interagency agreement with the Northwest and Alaska Fisheries Service, NOAA, U.S. Department of Commerce, to investigate the "Etiology of Tumors in BottomDwelling Marine Fish" in Puget Sound, Seattle, Washington, is in its second year. Several species of fish have evidenced an abnormally high incidence of liver lesions, preneoplastic and neoplastic, in urban areas of the Sound with a high level of industrialization. Efforts are being made to identify causative carcinogens in the sediment of those areas and in the muscle tissue, liver, bile, etc., of affected fish captured at those points. In addition to analytical chemistry activities, suspect compounds fractionated from the sediment and model compound mixtures are being assayed in in vitro and in vivo tests. The project focuses on the English sole, a commercially significant fish that demonstrates neoplasia at incidences up to 16 or 17%--approximately 3 times the level of the next most afflicted species. The in vivo assay utilizes the English sole also, as this laboratory has demonstrated the capability to utilize this specie in long-term studies.

Another example is a project entitled "Biochemical, Pharmacological and Tumorigenic Studies on a Composite of Drinking Water - Carcinogens and Mutagens Utilizing Aquatic Animals as a Bioassay Model." Significant progress made on this project by a contractor laboratory has revealed that small aquarium-size fish rapidly develop tumors comparable to those in rodents when exposed to classical carcinogens such as MAM-acetate. From a spectrum of seven species studied, the Japanese medaka and the guppy showed the most sensitivity and will be the two species used throughout this study. This study, focusing on aquarium-size fish, has been extended to a carcinogenic evaluation or bioassay of major contaminants in the nation's water supply using these small fish as test models. This work was reported on at the Fifth Water Chlorination Conference in Williamsburg, Virginia (June 1984) and at the NCI/EPA/NIOSH Workshop on Environmental and Occupational Carcinogenesis held in Bethesda in March 1984. Several publications have already resulted from this effort, including a report of the first CNS (ocular) tumor in fish readily produced by carcinogens.

Another project in the area of aquatic toxicology is being carried out in collaboration with the U.S. Army. The Army has made significant funding contributions to DCE's development of fish bioassay models, including an RFA for various aspects of aquatic toxicology, and sponsorship (along with EPA and the American Petroleum Institute) of an atlas on fish oncology and related lesions.

Chemical Carcinogenesis in Nonhuman Primates

Staff of the Office of the Director direct a large project on chemical carcinogenesis in nonhuman primates. Twenty-eight substances, including antitumor agents, contaminants of human food-stuffs, rodent carcinogens, pesticides, and artificial sweeteners have been or are being evaluated in four species of nonhuman primates for their potential carcinogenicity and other long-term toxic effects. Seventeen of these substances have not as yet demonstrated carcinogenic activity, although some have been on test for less than 4 years. Eleven of the compounds are carcinogenic in nonhuman primates, producing tumors in 5-100% of the treated

animals. 1-Methyl-1-nitrosourea (MNU) induced squamous cell carcinomas of the oropharynx and esophagus, with the esophageal tumors possessing clinical and morphologic similarities to human esophageal carcinoma. Long-term treatment with procarbazine resulted in an increased incidence of malignancies, one-half of which were acute nonlymphocytic leukemia. The effects of six of the compounds (DENA, DPNA, 1-nitrosopiperidine, aflatoxin B_1 MAM-acetate and urethane) were manifested primarily as hepatocarcinogenicity. Single cases of malignant tumors have been diagnosed in animals treated with adriamycin (acute myeloblastic leukemia), butter yellow (bronchioalveolar carcinoma), sterigmatocystin (hepatocellular carcinoma) and cyclophosphamide (transitional cell carcinoma of the urinary bladder).

In Vitro Evaluation of Chemical Candidates for In Vivo Testing

Approximately 150 compounds have been tested in two in vitro mutagenicity assays (a mouse lymphoma assay and the Salmonella typhimurium assay), and results utilized by intramural scientists and the chemical selection process. Data have been exchanged with other Federal agencies, i.e., U.S. Army Medical Bioengineering Research and Development Laboratory and the FDA, for mutual benefit.

Expert Panel Review of Monographs on Drugs and Cosmetic Ingredients

The Federation of American Societies for Experimental Biology (FASEB) convened a panel of outside experts who completed their evaluation of the content of two reports prepared previously under a master agreement (Data Bank on Environmental Agents) by outside contractors. These reports contain monographs on selected drugs (109) and cosmetic ingredients (64) that summarize data on chemical/physical properties, production, occurrence and use, pharmacokinetics, in vivo and in vitro carcinogenesis, general toxicology and epidemiology. A final report was received in April 1985.

Data Bases on Carcinogens, Mutagens, Promoters and Inhibitors

Data bases developed by a contractor have been extended during the year with addition of new information under CCRIS (Chemical Carcinogenesis Research Information System). All data developed from studies on water and air pollutants, cosmetics, dye class studies, other use and structural class studies, IARC monograph chemicals, chemicals from the NTP Bioassay Summary Reports (about 230), GeneTox data from literature searches, food contaminants, inhibitors, comparative metabolism on carcinogens and drugs and metal-containing drugs are entered into this data base. As of August 1985, this resource data can be accessed online through the National Library of Medicine's TOXNET.

OFFICE OF THE DIRECTOR

CONTRACTS AND COOPERATIVE AGREEMENTS ACTIVE DURING FY 1985

Institution/Principal Investigator/ Contract Number	Title
Centers for Disease Control (CDC) Matthew Zack Y01-CP-60215	Studies on the Human Health Consequences of Polybrominated Biphenyls (PBBs) 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
Federation of American Societies for Experimental Biology (FASEB) Kenneth Fisher N01-CP-31014	Expert Panel to Review Monographs on Drugs and Cosmetics
Gulf Coast Research Laboratory Robin Overstreet N01-CP-26008	Biochemical, Pharmacological and Tumorigenic Studies on a Composite of Drinking Water Carcinogens and Mutagens Utilizing Aquatic Animals as a Bioassay Model
Hazleton Laboratories America, Inc. Dan W. Dalgard N01-CP-25601	Induction, Biological Markers and Therapy of Tumors in Primates
International Agency for Research on Cancer (IARC) Harri Vainio U01-33193	IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans
Microbiological Associates Andrea Back N01-CP-41004	In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Mouse Lymphoma Assay
Microbiological Associates Steve Haworth N01-CP-41030	In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Salmonella Typhimurium Assay
National Institute for Occupational Safety and Health (NIOSH) Roy M. Fleming Y01-CP-60505	Conduct of Research on Occupational Carcinogenesis
National Oceanic and Atmospheric Administration (NOAA) Donald C. Malins Y01-CP-40507	Etiology of Tumors in Bottom-Dwelling Marine Fish

Contractor	Project
Smithsonian Institution John Harshbarger N01-CP-26000	Operation of a Registry of Tumors in Lower Animals
SRI International C. Tucker Helmes N01-CP-95607	Resource to Support the Chemical, Economic, and Biological Needs of DCE and to Provide Support to the International Agency for Research on Cancer (IARC) (Contract expired February 1985)
Syracuse Research Corporation Joseph Santodonato N01-CP-26002-03	Occupational Carcinogens
Technical Resources, Inc. Anthony Lee N01-CP-15761	Survey of Compounds Which Have Been Tested for Carcinogenic Activity
Tracor Jitco, Inc. Stephen S. Olion N01-CP-41003	Resource to Support the Chemical, Economic, and Biological Information Needs of the Division of Cancer Etiology (DCE) and to Provide Chemical Process, Production and Economic Information as Support to the IARC (International Agency for Research on Cancer)
Tracor Jitco, Inc. Harold E. Seifried N01-CP-31046	Support of Evaluation of Monographs on Drugs and Cosmetic Ingredients

INTERNATIONAL AGREEMENTS AND INFORMATION EXCHANGE ACTIVITIES

(Fiscal Year 1985)

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

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

SCIENTIST EXCHANGES

U.S. to China:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. D. Mann Dr. C. Harris National Cancer Inst. (Bethesda, MD)	Dr. S. Tsung Cancer Institute (Beijing)	1 month	Investigating Microbial Agents and Chemicals in Human Carcinogenesis

China to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. Q. Xig Dr. Z. Sun Cancer Institute (Beijing)	Dr. C. Harris National Cancer Inst. (Bethesda, MD)	2 months	Risk Factors for liver and esophageal cancers

U.S.-Federal Republic of Germany. Dialogue between the U.S. coordinator and leading German scientists concerning the proposed cooperation has continued on an informal basis during both national and international meetings. Initially, the German committee had presented a broad proposal in cancer research and research training that represented the coordinated national program effort by the Deutsche Forschungsgemeinschaft and the Federal Ministry for Research and Technology. Subsequent discussions have served to refine this proposal in terms of specific research areas of current and mutual interest for collaborative exchanges. Plans are underway to organize a bilateral workshop on "Mechanisms of Initiation" in order to stimulate further action under the cooperative agreement. During the past year there have been numerous discussions between Dr. Brieskorn and the coordinator of the U.S. side and as a result the U.S. coordinator has assisted in evaluating Americans for fellowships in Germany and in making recommendations as to where information on carcinogenesis can be obtained.

U.S.-Italy Agreement. Research pertinent to this Division is included in the Cancer Prevention area of the U.S.-Italy Cancer Program. The program involves a variety of activities related to cancer etiology, with recent emphasis on epidemiology. This year a workshop reviewing cooperative epidemiology programs in childhood cancer, occupational cancer and stomach cancer was held in Santa Margherita Ligure, Italy. A protocol for a cooperative study of hepatitis-B virus associated with childhood leukemia was developed for implementation in Italy and the U.S. In addition, final plans were drawn for a collaborative case-control study of gastric cancer, the leading cause of cancer death in parts of northern and central Italy. The multi-center stomach cancer investigation involving high and low risk areas of Italy will be funded in part by a contract from the Division of Cancer Etiology with the Center for Study and Prevention of Cancer in Florence.

SCIENTIST EXCHANGES

U.S. to Italy:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. D. Palli Center for Study and Prevention of Cancer (Florence)	Dr. J. Fraumeni National Cancer Inst. (Bethesda, MD)	2 months	Assessment of Cancer Screening and Gastric Cancer Epidemiology

U.S.-Japan Agreement. This year marks the beginning of the third 5-year program of this agreement, which consists of four broad program areas: Etiology, Cancer Biology and Diagnosis, Cancer Treatment, and Interdisciplinary Research. The cooperation between U.S. and Japanese scientists still remains one of the most active of all the bilateral agreements and is especially well suited to the study of malignancies that differ markedly in their occurrences within the two nations. Within the Etiology Area of the U.S.-Japan Agreement, seminars were held on "Analysis of Tumorassociated Antigens of Digestive Organs with Monoclonal Antibodies and Their Clinical Application," and "Oncogenes and Experimental Carcinogenesis." Eight scientists from the U.S. and Japan participated in exchange

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

SCIENTIST EXCHANGES

U.S. to Japan:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. William Haseltine Dana-Farber Cancer Institute (Boston, Massachusetts)	Dr. Yohei Ito Univ. of Kyoto Medical School (Kyoto)	4 weeks	Human T-Cell Leukemia Virus
Dr. Thomas Benjamin Harvard University (Boston, Massachusetts)	Dr. Hiroto Shimojo National Institute of Health (Tokyo)	3 weeks	Identification and Analysis of Multiple Functions of SV40 Large T Antigen and the Use of MMTV-LTR to Regulate Expression of E-1a and E-1b Proteins of Human Adenoviruses
Dr. Stephen Leadon Stanford University (Stanford, California)	Dr. Motohisa Kaneko Natl. Cancer Ctr. Res. Inst. (Tokyo)	3 weeks	Free Radicals as Intermediates in Carcinogenesis

Japan to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. Kunitada Shimotohno Natl. Cancer Cntr. Res. Inst (Tokyo)	Dr. David W. Golde Univ. of California (Los Angeles, California) Dr. M. Yamada Cold Spring Harbor Lab. (Cold Spring Harbor, New York)	3 weeks	Gene Structure and Expression of Human T-Cell Leukemia Virus
Dr. Yoichi Taya Natl. Cancer Ctr. Res. Inst. (Toyko)	Dr. Fuyuhiko Tamanoi Cold Spring Harbor Lab. (Cold Spring Harbor, New York)	1 month	Studies on *ras* Gene Products

Dr. Yoshinari Ohnishi Univ. of Tokushima (Tokushima)	Dr. Frederick A. Beland National Center for Toxicological Research (Jefferson, Arkansas)	4 weeks	Studies on the Metabolism of Nitroarenes
Dr. Ikuo Abe Tohoku University (Sendai)	Dr. Gary M. Williams Naylor Dana Inst. of Disease Prevention (Valhalla, NY) Dr. Bruce Chin Univ. of Michigan (Ann Arbor, MI) Drs. Gary A. Boorman & Joseph K. Haseman National Institute of Environmental Health Sciences (Research Triangle Park, North Carolina)	3 weeks	Methodology and Assessment of Carcinogenicity Screening In Vivo
Dr. Kazuo Yanaghi Univ. of Tsukuba (Tsukuba)	Dr. Bernard Roizman Univ. of Chicago (Chicago, Illinois)	9 weeks	Mechanism of Morphological Transformation by Herpes Simplex Virus

<u>U.S.-France (NCI-Institut National de la Sante et de la Recherche Medicale-INSERM) Agreement.</u> The NCI-INSERM Committee on Basic Cancer Research has the responsibility to support high quality nonclinical research relating to understanding the process of carcinogenesis. The exchange program is open to all qualified American and French scientists who are presently engaged in basic research in carcinogenesis. Proposals submitted by candidates from each country are reviewed for scientific merit by both American and French committees. Information about this cooperation has been given wide circulation.

SCIENTIST EXCHANGES

<u>U.S. to France:</u>

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. Dennis W. Ross	Dr. Marcel Bessis Institut de Pathologie Cellulaire, Hopital de Bicetre (Bicetre)	6 mo	Quantitative Analysis of Maturation in Leukemic Cells
Dr. Merwin Moskowitz	Dr. Yves Courtois Unite de Recherches Gerontologigues INSERM (Paris	9 mo	Extraction and Study of Growth Inhibitory Factors from Human Plasma

Dr. W. H. Newman	Dr. J. Seylaz Laboratoire du Physiologie Cerebro-vasculaire (Paris)	8 mo	Study of Application of Ultrasonics and Hyperthermia
	and		
	Dr. A. Dittmar Claude Bernard Universit (Lyon)		Study of Application of Ultrasonics and Hyperthermia
Dr. R. W. Makuch	Prof. Flamant Institut Gustave Roussy (Villejuif)	1 mo	Design, Conduct and Analysis of Clinical Trials and Large Scale Epidemiologic Studies
	and		
	Dr. John Kaldor IARC (Lyon)		Design, Conduct and Analysis of Clinical Trials and Large Scale Epidemiologic Studies

France to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. C. Lacombe	Dr. Eugene Goldwasser University of Chicago (Chicago, Ill.)	12 mo	Regulation of Erythroprotein in Erythro-leukemia
Dr. C. Riva	Dr. Y. Rustum Roswell Park Memorial Inst. (Buffalo, N.Y.)	3 mo	Development of Assays for Predicting Response of Leukemia cells to Ara-C
Dr. C. Guenet	Dr. Maxine Singer National Cancer Inst. (Bethesda, Md.)	4 mo	Development of DNA Sequencing Techniques
Dr. J. Barbet	Dr. J. Weinstein National Cancer Inst. (Bethesda, Md.)	11 mo	Monoclonal Antibody Detection of Lymph Node Metastases

Dr. A. Rhodes-Feuillette	Dr. M. Siniscalco Cancer Center (New York, N.Y.)	1 mo	Study of In Situ Techniques with Iodinated Probes
Dr. P. A. Boulanger	Dr. A. Berk University of California (Los Angeles, Calif.)	6 mo	Purification and Analysis of Class III Stimulating Activity
Dr. J. Feunteun	Dr. T. Benjamin Harvard Medical School (Boston, Mass.)	3 mo	Analysis of Hamster Papova Virus Polypeptides
Dr. J. Boyer	Dr. J. Maller University of Colorado (Denver, Colo.)	8 mo	Study of Phosphorylation of Proteins in Oocytes Injected with Tyrosine Protein Kinase
Dr. P. Lointier	Dr. B. Levin M.D. Anderson Hospital (Houston, Tex.)	12 mo	Expression of Growth Promoting Substances
Ms. P. Baldacci	Dr. W. Haseltine Dana-Farber Cancer Inst. (Boston, Mass.)	6 mo	Transactivation of Virus Transcription in HTLV

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

in areas of carcinogenesis; and (3) the availability of Soviet principal scientists in carcinogenesis for in-depth discussion of research interests and results for consideration of carcinogenesis problem areas amenable to joint investigation. Without having this information, we cannot, in any meaningful way, plan collaboration and/or selection of American scientists unique and appropriate to the research areas in which American and Soviet specialists will interact. We have not as yet obtained from the Soviets a satisfactory response to our requests.

As before, the major contribution to the U.S. in this program, is the access to the large monkey colony in Sukhumi. Drs. Gallo and Saxinger (Division of Cancer Treatment, NCI) have found some of these primates to be seropositive for human T-cell leukemia/lymphoma virus antibody and further collaborative research in this area is planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CP03509-22 OD

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Carcinogenesis, Chemotherapy and Biological Markers in Nonhuman Primates

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

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

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

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.5	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.)

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

PROJECT DESCRIPTION

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

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

Objectives:

To obtain data on which to base a comparison of the response of nonhuman primates and rodents to materials known or suspected to be carcinogenic to man, and thereby to evaluate the relative merits of rodents and nonhuman primates in predicting carcinogenic risk for man.

To evaluate the carcinogenic potential and long-term adverse effects of clinically useful antineoplastic and immunosuppressive agents.

To determine whether the nonhuman primate resembles the rodent in that it is more susceptible to chemical carcinogenesis as a neonate than as an adult.

To use normal and tumor-bearing nonhuman primates for studying the pharmacological, toxicological, and chemotherapeutic properties of various anticancer, antiviral, and antimicrobial agents.

To carry out comparative biochemical and immunological studies using normal animals, animals with preneoplastic lesions, and animals with malignant neoplasms.

To develop methods for detecting preneoplastic changes and accomplishing the early diagnosis of tumors.

Methods Employed:

Compounds Under Investigation

Twenty-eight substances are currently under investigation or have been investigated, including antineoplastic and immunosuppressive agents (procarbazine, adriamycin, methylnitrosourea, melphalan, azathioprine and cyclophosphamide), food additives and environmental contaminants (aflatoxin B_1, methylazoxymethanol acetate, sterigmatocystin, cyclamate, saccharin, butter yellow, 3-methyl-DAB, DDT, arsenic and cigarette smoke condensate), "model" rodent carcinogens (urethane, 3-MC, 2-AAF, 2,7-AAF, copper chelate of N-OH-AAF, dibenzpyrene and dibenzanthracene), and various nitroso- compounds (DMNA, DENA, DPNA, 1-nitrosopiperidine and MNNG).

The compounds are administered subcutaneously, intravenously, intraperitoneally, or orally. For oral administration to newborn monkeys, the compound is added to the Similac formula at the time of feeding; when the monkeys are 6 months old, carcinogens given orally are incorporated into a vitamin mixture which is given to monkeys as a vitamin sandwich on a half slice of bread. An alternate

way of giving doses orally is to incorporate the compound into baited foods or to administer it by intubation. The dose level chosen is dependent on the chemical under evaluation. Antineoplastic and immunosuppressive agents are administered at doses likely to be encountered in a clinical situation; other substances, such as environmental contaminants, are given at levels 10-40 fold higher than the estimated human exposure level. The remainder of the chemicals tested are administered at maximally tolerated doses which, on the basis of weight gain, blood chemistry and hematology findings, and clinical observations, appear to be devoid of acute toxicity.

Animals

The present colony, consisting of 532 animals, is comprised of four species: Macaca mulatta (rhesus), Macaca fascicularis (cynomolgus), Cercopithecus aethiops (African green) and Galago crassicaudatus (bushbabies). Seventy-nine of these monkeys are adult breeders which supply juvenile animals for experimental studies. The majority of the animals are housed in an isolated facility which contains only animals committed to this study, and with the exception of the breeding colony, most animals are housed in individual cages. The administration of test compounds is continued until a tumor is diagnosed or until a predetermined exposure period is completed. A minimum of 30 animals is usually allotted to each treatment group, since in a sample of this size it is possible to detect a tumor incidence of 10% within 95% confidence limits.

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

Major Findings:

Carcinogenic Potential of Antineoplastic and Immunosuppressive Agents

Procarbazine

There is evidence that Hodgkin's disease patients receiving treatment with the MOPP regimen, one component of which is procarbazine, are at increased risk of developing acute myelogenous leukemia (AML). Although a causal relationship between cyotoxic drug therapy and the appearance of AML in these patients has not yet been established, there is ample evidence that procarbazine is a potent carcinogen in mice and rats. The carcinogenic potential of procarbazine in three species of nonhuman primates has been under study for 19 years. A total of 48 monkeys have survived 6 months or longer after the first dose of drug. Fifteen of the 42 monkeys (35.7%) necropsied thus far have had malignant neoplasms, eight of which were acute leukemia. The leukemias, all non-lymphocytic, arose in monkeys after latent periods ranging between 16 and 166 months (average 86.5 months). They developed in monkeys that had ingested an average cumulative procarbazine dose of 61.2 gm (range 2.7 to 170.4). Solid tumors were diagnosed in seven monkeys on the probarbazine study. Three monkeys developed osteogenic sarcomas, two monkeys developed hemangiosarcomas, and

single cases of lymphocytic lymphoma and astrocytoma were found. These tumors arose after latent periods of 68-192 months (average 111 months), and after an average cumulative procarbazine dose of 64.6 gm (range 23.8 to 154.4 gm). The six surviving monkeys in this study may represent a population at high risk for developing acute non-lymphocytic leukemia. For this reason, they are being utilized in hematologic and cytogenetic studies in order to determine whether a "pre-leukemic" phase is detectable prior to the development of frank drug-induced leukemia. For this purpose, sequential bone marrow samples from all of the monkeys receiving procarbazine are being examined for alterations in cellular morphology and for chromosomal aberrations. Since initiation of these studies, no specific chromosomal aberrations have been detected in bone marrow cells from the monkeys and no additional cases of acute leukemia have developed. However, a number of the toxic effects of procarbazine seen clinically are also noted in the monkeys, including vomiting and myelosuppression. Its most striking toxic effect, however, is on the reproductive system of the males. The majority of the adult males necropsied to date have had testicular atrophy with complete aplasia of the germinal epithelium.

Adriamycin

The potential carcinogenicity of adriamycin was also evaluated. A group of 10 monkeys received an IV dose of drug (12 mg/m^2) once each month for 23-27 months. At the end of the dosing period it was intended to hold the animals under observation for the remainder of their lives. However, approximately 2 months after the last dose of adriamycin 8 of 10 animals developed congestive heart failure. Histopathologic examination of cardiac muscle taken at necropsy revealed lesions characteristic of adriamycin-induced cardiomyopathy in humans, and in some cases these findings were confirmed by electron microscopy. In man, a cumulative adriamycin dose of 550 mg/m^2 has been associated with cardiac toxicity; nonhuman primates appear to be more sensitive to adriamycin-induced cardiomyopathy, as the monkeys in the present study had received an average cumulative dose of 310 mg/m^2 (range 276-336 mg/m^2). One of the 10 monkeys developed acute myeloblastic leukemia after receiving 324 mg/m^2 of adriamycin divided into 27 monthly doses. The 10th monkey in this series recently developed clinical signs of congestive heart failure and was sacrificed. Although histopathologic examination of cardiac tissue from this animal has not been completed, the gross appearance of the heart resembled that of the eight animals with histologically-confirmed adriamycin-induced cardiomyopathy. This animal received 25 injections of adriamycin totaling 300 mg/m^2, and a total of 84.5 months had elapsed since the animal was last exposed to adriamycin. This study is being repeated, using two groups of 10 monkeys each; the monkeys are receiving monthly IV injections of adriamycin at 2.4 and 4.8 mg/m^2; dosing will be terminated when a cumulative dose of 280 mg/m^2 is attained. That dose was attained in the 0.4 mg/kg group during the last year and these animals are no longer being treated. Thus far, none of the monkeys have as yet developed signs of congestive heart failure or other indications of ill health.

N-methylnitrosourea (MNU)

This compound administered by IV injection has been reported by clinicians in the Soviet Union to be effective in Hodgkin's disease and undifferentiated

carcinoma of the lung, and the nitrosoureas BCNU, CCNU and methyl-CCNU have been used in this country to treat a variety of human tumors. The latter three agents have been implicated in the development of second tumors in patients receiving treatment for central nervous system (CNS) neoplasms. We have evaluated the carcinogenic potential of MNU in three species of nonhuman primates. A total of 44 monkeys have received oral doses of MNU for periods up to 207 months. Thirteen of the 28 monkeys necropsied thus far have had squamous cell carcinoma (SCA) of the mouth, pharynx and/or esophagus; upper digestive tract lesions such as atrophy or dyskeratosis of the esophageal mucosa and esophagitis have been a consistent finding among all the monkeys necropsied to date. The tumors developed in monkeys ingesting cumulative MNU doses ranging from 53.2-246.4 gm over a range of 57-178 months. A minimum of approximately 50 gm of MNU appears necessary for tumor induction by the oral route. Many parallels were noted between the esophageal SCA observed in the present series of monkeys and human esophageal carcinoma, including the clinical manifestations of the tumor, its complications, its radiographic appearance and its morphology. The MNU-induced lesions of the oropharynx and esophagus of primates may therefore be a valuable model for the study of human esophageal carcinoma.

Melphalan

There is some evidence that patients receiving long-term treatment with melphalan for multiple myeloma or ovarian cancer may also be at increased risk of developing AML. Although a causal relationship between melphalan treatment and the development of AML in these patients has not yet been established, the carcinogenic activity of this agent has been demonstrated in mice and rats. The carcinogenic potential of melphalan in nonhuman primates is therefore under study. Twenty monkeys are currently being treated by the oral route with melphalan (0.1 mg/kg) daily, 5 days a week. The average cumulative dose administered to these animals is 1.113 gm and the dosing interval averages 106 months. Women receiving prophylactic melphalan therapy for ovarian carcinoma would receive, in the prescribed 18-month dosing period, a total melphalan dose of 660 mg/m^2, a dose lower than that already ingested by these monkeys. None of the monkeys on this study have died and all appear to be healthy.

Azathioprine

Kidney transplant recipients and other patients under chronic immunosuppressive therapy with azathioprine appear to be at risk of developing malignancies, primarily lymphomas. Whether this increased risk is related to a direct oncogenic effect of azathioprine or is secondary to a prolonged immunosuppressed state is at present unclear. The carcinogenic potential of azathioprine is being evaluated in two groups of monkeys receiving the drug daily, 5 days every week at doses of 2 and 5 mg/kg, respectively. The 2 mg/kg group is comprised of 14 animals that have thus far received an average cumulative azathioprine dose of 16.71 gm over the course of approximately 85 months. Fourteen animals are receiving azathioprine at 5 mg/kg, and have ingested an average cumulative dose of 28.67 gm over an average dosing interval of 65 months. None of the monkeys on this study have developed a malignancy and all appear to be healthy.

Cyclophosphamide

Cyclophosphamide is widely used as a single agent and in combination regimens for treating human cancer; it is also being used with increasing frequency for treating various non-malignant disorders such as rheumatoid arthritis. The association of transitional cell carcinomas of the urinary bladder and acute myelogenous leukemia with prolonged cyclophosphamide treatment of both malignant and non-malignant disease represents the basis for our study on the carcinogenic potential of cyclophosphamide in nonhuman primates. Cyclophosphamide is being administered to a group of 20 monkeys orally, 5 days every week, beginning at 6-7 months of age. The initial dose (3 mg/kg) is increased to 6 mg/kg after 6 months. This study has been underway for an average of 44 months; during this period an average cumulative cyclophosphamide dose of 15.66 gm has been administered. Three of the monkeys died during an outbreak of measles in the colony four years ago, but no evidence of malignancy was found upon histopathologic examination of their tissue. During the past year a fourth monkey died. Histopathologic examination of a lesion noted at autopsy in the urinary bladder revealed transitional cell carcinoma. This animal had received a cumulative cyclophosphamide dose of 19.02 gm for a total of 41 months.

Carcinogenic Potential of Food Additives and Environmental Contaminants

Aflatoxin B_1 (AFB_1)

AFB_1, a product of a mold (Aspergillus flavus) known to contaminate some human foodstuffs, is carcinogenic in a variety of experimental animals. The carcinogenicity of AFB_1 has been under evaluation in nonhuman primates for the past 18 years. A total of 39 Old World monkeys, chiefly rhesus and cynomolgus, has received AFB_1 by IP (0.125-0.25 mg/kg) and/or oral (0.1-0.8 mg/kg) routes for 6 months or longer, and all but one have been necropsied. Twenty-two of the 38 monkeys necropsied to date developed a total of 36 malignant neoplasms, yielding an overall tumor incidence of 56.4%. Five of the 22 tumor-bearing monkeys developed hemangioendothelial sarcomas of the liver, six developed bile duct or gallbladder adenocarcinomas, and two cases of hepatocellular carcinoma were diagnosed. Two monkeys developed osteosarcomas and six were found at necropsy to have multiple primary tumors. The tumors diagnosed in the 22 monkeys developed after latent periods ranging from 49-210 months, and after cumulative AFB_1 doses of 99-1650 mg. Eleven of the 16 (69%) necropsied monkeys without tumor showed histologic evidence of liver damage, including toxic hepatitis, cirrhosis and hyperplastic liver nodules. Our results indicate that AFB_1 is a potent hepatotoxin and carcinogen in nonhuman primates and further support the hypothesis that humans exposed to this substance may be at risk of developing liver cancer.

Cycads (Cycad Meal, Cycasin and MAM-Acetate)

Cycasin, the active principle in the cycad nut, induces liver and kidney tumors in rats and may be a human carcinogen as well. The carcinogenic potential of cycasin and its aglycone, MAM-acetate, is under investigation in nonhuman primates. Old World monkeys (rhesus, cynomolgus, and African greens) received cycasin and/or MAM-acetate by PO or IP routes for periods up to 14 years. Seventeen monkeys survived > 6 mo after initiation of treatment with cycasin

(50-75 mg/kg) or MAM-acetate (1.5-3.0 mg/kg) given PO daily 5 days/week, and 12 of the animals have been necropsied. Histopathologic examination of tissue from one of these monkeys revealed hepatocellular carcinoma. A second monkey was noted to have multiple tumors, including hepatocellular carcinoma, intrahepatic bile duct adenocarcinoma, renal carcinoma and adenomas, and adenomatous polyps of the colon. An adenocarcinoma of the pancreas was diagnosed in a third monkey. Although liver tumors were not observed in the other monkeys, all but one had hepatic lesions such as toxic hepatitis and cirrhosis. A group of 10 monkeys received MAM-acetate by weekly IP injections (3-10 mg/kg). Six of these animals developed tumors after receiving an average of 6.14 gm (range 3.58-9.66 gm) of MAM-acetate for an average of 75 mo (range 50-89 mo). Four of the monkeys developed hepatocellular carcinomas and two had multiple primary tumors including hepatocellular carcinomas, renal carcinomas, squamous cell carcinomas of the esophagus and adenocarcinomas of the small intestine. Our results show that MAM-acetate is a carcinogen in monkeys and add to the evidence that cycasin and its aglycone may be carcinogenic in man.

Sterigmatocystin

Sterigmatocystin has been under test for approximately 9 years. It is being administered PO, 1 day/week at 1 mg/kg (15 monkeys) and 2 mg/kg (15 monkeys). Three animals receiving the 1.0 mg/kg dose have been necropsied, and one was found to have developed a primary hepatocellular carcinoma. This animal had received a total of 1.13 gm of sterigmatocystin during the course of 74 months. Three animals at the 2.0 mg/kg dose have been necropsied, and histopathologic examination of the tissues revealed primary hepatocellular carcinoma in one of the animals. Severe toxic hepatitis with hyperplastic nodules was found in one of the other two animals. Laparoscopic examinations and biopsies of the livers of the remaining 25 monkeys have indicated that several have developed toxic hepatitis, hyperplastic liver nodules and atypical bile duct proliferation.

Butter Yellow

A total of seven animals survived 6 months or longer after receiving the initial oral dose of butter yellow, and all animals have been sacrificed at this time. One case with a bronchioalveolar carcinoma was found among the treated animals. Whether this tumor arose as a consequence of treatment with butter yellow or is a spontaneous tumor developing in an aged (20 year) animal is uncertain.

Cyclamate

Cyclamate has been under test for the past 13 1/2 years. Two groups of monkeys have received this compound orally, 5 days every week, at 100 and 500 mg/kg, respectively. The 100 mg/kg dose in monkeys corresponds, on an equivalent surface area basis, to a daily intake of 2.3 gm/day/70 kg man, and is equivalent to drinking about six diet drinks per day. The 500 mg/kg dose in monkeys corresponds, on an equivalent surface area basis, to a daily intake of 11.6 gm/day/70 kg man, and is equivalent to drinking about 30 diet drinks per day. Two of 12 monkeys at the low dose, and two of 11 monkeys at the high dose have been necropsied, but no evidence of a malignant neoplasm was found. Studies on the potential reproductive toxicity of cyclamate in males have been completed.

These studies have included evaluation of sperm motility and morphology, testicular biopsies, measurement of testicular size and assays of serum gonadotrophins. The results of these studies indicate that prolonged exposure of male monkeys to cyclamate is not associated with adverse effects on the reproductive system. During the past year all of the cyclamate-treated male monkeys were tested for relative levels of cyclamate and cyclohexylamine excreted in 24-hour urine. The results indicate that the 24-hour urinary excretion of cyclohexylamine is highly variable, ranging from zero mg to 15.74 mg.

Saccharin

Two groups of 10 monkeys each have been receiving oral doses of saccharin (25 mg/kg), 5 days every week. This dose corresponds, on an equivalent surface area basis, to a daily intake of five cans of diet soda by a 70 kg man. One group of monkeys has been receiving saccharin for an average of 162 months (range 161-164 months), and the second group of 10 monkeys began saccharin treatment approximately 7 years ago. Since the inception of the study, only one of the monkeys has died, and histopathologic examination of tissue from this animal gave no indication of tumor development.

DDT

Similarly, long-term administration of DDT has not resulted in the development of tumors in our nonhuman primates. A total of 25 animals has received DDT by the oral route (20 mg/kg) daily, 5 days every week, in a study that has been underway for the past 176 months. Administration of DDT is discontinued after a dosing interval of 130 months is completed. Although eight of the monkeys have died thus far, none were found to have developed tumor. The apparent cause of death in five of these animals was DDT-induced CNS toxicity, as they experienced severe tremors and convulsions immediately prior to death. The 17 surviving monkeys appear to be in good health.

Arsenic

The carcinogenic potential of arsenic has been under evaluation for approximately eight years. A total of 20 monkeys has received sodium arsenate PO (0.1 mg/kg) 5 days/week, and three monkeys in the group have died. The cause of death in the monkeys was unrelated to arsenic treatment, and the surviving monkeys are well and without signs of toxicity.

Cigarette Tobacco Smoke Condensate

Ten monkeys have received lung implants containing tobacco smoke condensate in a beeswax matrix. Three animals have been necropsied; no neoplastic lesions were found in these animals. The remaining seven monkeys are well and without evidence of toxicity approximately 11 years after implantation of the material.

Carcinogenic Potential of "Model" Rodent Carcinogens

With the exception of urethane, none of the compounds in this category have demonstrated carcinogenic activity in nonhuman primates.

Urethane

Rhesus and cynomolgus monkeys received urethane (250 mg/kg) orally, 5 days every week beginning within one month of birth. They received continuous urethane treatment for 5 years, during which time some monkeys also received 3-10 weekly courses of whole body irradiation (WBI) at 50 rads per course. Urethane administration was discontinued 15-18 years ago and since that time all animals have been held under close observation for development of tumor or other adverse effects of treatment. Thirty of a total of 44 monkeys survived 6 months or longer after the first dose of urethane, and 24 of these animals have been necropsied. A total of nine malignant tumors were found in six (16.7%) of the 30 treated monkeys; in comparison, seven of a total of 219 (3.2%) control monkeys have developed tumors during this period. One or more primary liver tumors (three cases of hemangiosarcomas, one case of adenocarcinoma of intrahepatic bile ducts, one case of hepatocellular carcinoma) were present in three monkeys; one monkey was found to have an ependymoblastoma, one monkey developed a pulmonary adenocarcinoma and one case with pancreatic adenocarcinoma was found. The animals with tumor had received an average cumulative urethane dose of 260 gm (range 230-339 gm); the latent period for tumor induction averaged 171 months (range 142-229 months). Two of the six monkeys developing tumors had received nine and ten courses of WBI, respectively. These results indicate that urethane, with or without WBI, is carcinogenic in monkeys; however, the latent period for tumor induction (> 14 years) is long, requiring approximately 50% of the usual lifespan of Old World monkeys in captivity.

Carcinogenic Potential of Nitroso- Compounds

Diethylnitrosamine (DENA)

DENA is highly predictable as a carcinogen in Old World monkeys, inducing hepatocellular carcinomas when given either orally or by IP injection. In one study, we are using DENA as a model hepatocarcinogen in Old World monkeys to examine the relationship between chronic (milligrams per kilogram) dose, cumulative dose, and latent period for tumor induction. To this end, groups of monkeys are being given bimonthly IP injections of DENA at doses of 0.1, 1, 5, 10, 20, and 40 mg/kg and are observed for the appearance of tumor. In the five groups of monkeys in which tumors have developed, we have found that the latent period increases as the milligram-per-kilogram dose decreases. Thus, the latent period at the 40 mg/kg dose averages 17 months; for the 20 mg/kg dose it is 23 months and at 10 mg/kg it is 38 months. A semilog plot of these three mg/kg doses of DENA against the latent periods for tumor induction yields a straight line which intercepts the y-axis at approximately 110 months. This point on the ordinate corresponds to a DENA dose of 0.1 mg/kg. Thus animals in the 0.1 mg/kg group should develop tumors after a latent period of 110 months if the relationship between the milligram-per-kilogram dose and latent period is strictly linear; however, the animals at this dose have only been on study approximately 40 months. Extrapolation from the curve to the ordinate for the 1 mg/kg group yields a latent period of 60 months, although 90% of the animals in this group remain tumor-free after an average of 111 months of observation. The tumors developing in the nine animals receiving the 5 mg/kg dose required a latent period averaging 70 months, a figure which shows a marked deviation from the value (42 months) expected if the relationship between dose and latent

period were linear. Our results thus far indicate that this relationship departs from linearity at lower chronic doses of DENA.

During the past year we have initiated a new study involving DENA. The objective of the study is to evaluate in monkeys the activity of DDT as a promoter of DENA-initiated hepatocellular carcinomas. As noted above, DENA induces primary hepatocellular carcinomas in Old World monkeys at high yield after a latent period which is directly related, at relatively high (10, 20, and 40 mg/kg) chronic doses, to the mg/kg dose administered. In protocols employing IP injections of DENA at bimonthly intervals, the cumulative DENA dose required for tumor development was found to lie between 1.720 gm and 2.232 gm; the earliest tumor to have appeared in the study developed 6 months after the first DENA exposure of an African green monkey. This tumor was induced by a cumulative DENA dose of 0.394 gm. In contrast to DENA, DDT has not provided any evidence of carcinogenicity in nonhuman primates. However, DDT produces tumors in rodents and is suspected of being a tumor promoter in rodents and other species. In our study, monkeys are receiving DENA only, DDT only or an initiating dose of DENA administered IP followed by daily oral doses of DDT for 120 months.

Dimethylnitrosamine (DMNA)

A total of 11 monkeys were treated with DMNA and seven survived longer than 6 months after first exposure. All seven aniamls have been necropsied, and histopathologic examination revealed severe liver damage in all cases although no liver tumors were present.

Dipropylnitrosamine (DPNA)

DPNA induced primary hepatocellular carcinomas in all six rhesus and cynomolgus monkeys given bimonthly IP doses of 40 mg/kg. The average total dose of DPNA was 7.0 gm; the average latent period for tumor development was 28.5 months.

1-Nitrosopiperidine (PIP)

This compound is also a hepatocarcinogen in macaques. Hepatic cell carcinomas developed in 11 of 12 monkeys receiving this compound by the oral route and in five out of ten monkeys treated by the IP route. The average cumulative dose necessary for tumor induction by PIP given orally (1742.5 gm) was higher than for oral DENA (18.0-55.1 gm); similarly, the average cumulative dose of PIP given by the IP route (39.4 gm) exceeded that required for tumor induction by IP DENA (1.7 gm) or IP DPNA (7.0 gm).

N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG)

MNNG is being administered by the oral route (1.0 mg/kg daily, 5 days every week). A group of 20 monkeys has received this compound for periods of up to 13 years; thus far, two animals have died of causes unrelated to MNNG toxicity. The remaining 18 animals appear to be in good health and without signs of toxicity. However, three additional monkeys were given MNNG as a colon implant; two of these monkeys have been necropsied and one monkey was diagnosed with a well-differentiated adenocarcinoma at the rectosigmoid junction. The latter

monkey had received a total MNNG dose of 8.65 gm; it was administered in gelatin cubes containing 5.3-42.7 mg MNNG which were inserted into the colon twice every week.

Significance to Biomedical Research and the Program of the Institute:

The present colony has been in continued existence for 23 years; it supports the largest study of chemical carcinogenesis in nonhuman primates undertaken in this country, and as such it represents a national resource. In addition to providing data on the carcinogenicity of a variety of chemicals, including antitumor and immunosuppressive agents in clinical use, it has also made it possible for us to acquire information in other important areas of primatology. Such information includes the spontaneous tumor incidence in various species of nonhuman primates, their lifespan in captivity, their reproductive characteristics, the organization and management required to hand-rear 40-50 neonates per year in a nursery, and parameters of growth and development in simian primates. Normal animals of all ages as well as tumor-bearing animals are used in a variety of pharmacologic studies. The availability of monkeys receiving chronic treatment with chemicals has made it possible to identify some consequences of long-term chemical exposures (other than tumor development) not previously recognized. Tumor-bearing monkeys have been employed in attempts to develop biological markers, such as α-fetoprotein, and other diagnostic tests for detecting premalignant lesions and early tumors. More recently, tumor-bearing monkeys have been used to develop new contrast media for computerized tomography of liver and spleen, and to compare the distribution of free and liposome-entrapped ara-C in tumor tissue and in adjacent normal tissue. The animals are also being used in a metabolic phenotyping study, the objective of which is to determine the correlation between drug metabolizing phenotype(s) and susceptibility to chemically-induced cancer. This project is of great significance to biomedical research in general and continues to serve well the objectives of the Program.

Proposed Course:

The studies described will be continued. A new study designed to evaluate the metabolism and carcinogenicity of IQ (2-amino-3-methylimidazo-quinoline) in monkeys has recently been initiated.

Publications

None

CONTRACT IN SUPPORT OF THIS PROJECT

HAZLETON LABORATORIES AMERICA, INC. (N01-CP-25601)

Title: Induction, Biological Markers and Therapy of Tumors in Primates

Current Annual Level: $561,288

Man Years: 9.2

Objectives: To maintain a breeding colony of various species of primates so that offspring are readily available for use in experimental studies. To make available normal and tumor-bearing animals for pharmacologic, toxicologic, biochemical and immunologic studies. To provide the facilities for maintaining and treating a large colony of nonhuman primates for studies of chemical carcinogenesis.

Major Contributions: This contract continues to satisfy the objectives of the project. Animals are bred, housed and dosed with test chemicals by the contractor according to protocols specified by the Project Officer.

Proposed Course: Ongoing studies on chemical carcinogenesis in nonhuman primates will be continued and several new studies will be initiated during the coming year.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04548-13 OD

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

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

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

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

COOPERATING UNITS (if any)

LAB/BRANCH

Office of the Director

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	2.5	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The objectives of the Registry of Experimental Cancers are the storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents) and the use of such information for research and educational purposes. The Registry has acquired a total of 2,869 (473 since the 1984 report) single or group accessions from investigators outside the NCI and approximately 61,475 records have been coded. Forty investigators have come to the Registry for study and consultation on single or multiple visits.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Harold L. Stewart	Scientist Emeritus	DCE	NCI
Bernard Sass	Veterinary Medical Officer	DCE	NCI
Margaret K. Deringer	Guest Researcher	DCE	NCI
Cornelia Hoch-Ligeti	Guest Researcher	DCE	NCI
Carel F. Hollander	Guest Researcher	DCE	NCI
Annabel G. Liebelt	I.P.A. Fellow	DCE	NCI

Objectives:

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

Methods Employed:

The methods employed in the work of the Registry involve the selection of protocols, pathologic material, including histologic slides, paraffin blocks, and illustrations in the form of lantern slides, gross photographs, and photomicrographs in black and white and in color. The work of the Registry also includes the collection of records of experiments, reprints and references on this material. The Registry of Experimental Cancers possesses a large collection of spontaneous and induced cancers and other lesions. The pertinent information on the collection is indexed and many of the data have been entered into the computer. The Registry accesses material from investigators at NCI, other institutes of NIH, other governmental agencies, industrial laboratories, and universities here and abroad. A total of 2,869 (473 since the 1984 report) single or group accessions from investigators outside of NIH have been processed. The Registry prepares Study Sets of slides, with explanatory notes, relating to particular cancers of rodents.

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

Investigators come to the Registry for study and consultation. There have been single or multiple consultations with 40 individuals since the 1984 report. Eight investigators have sent material to the Registry for diagnosis.

Major Findings:

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

Significance to Biomedical Research and the Program of the Institute:

The availability of the wealth of material possessed by the Registry advances the knowledge of spontaneous and induced disease processes in animals. It is a national and international resource.

The existence of the Registry will contribute to the standarization of nomenclature of cancers and other lesions in laboratory rodents. Slides and protocols from the Registry are used to illustrate and describe lesions discussed at weekly slide conferences.

The members of the Registry serve as consultants in the monitoring of pathology from laboratories of NCI, of other institutes of NIH and elsewhere.

During the period from January 1980 until April 30, 1985, the Registry has received 8,634 requests for reprints of Histologic Typing of Liver Tumors of the Rat (J. Nat'l. Cancer Inst. 64: 177-206, 1980). This histologic classification and typing of rat liver tumors is calculated to promote uniformity of diagnoses from one laboratory to another in this country.

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

Proposed Course of the Project:

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

Publications

Hoch-Ligeti, C. and Stewart, H. L.: Cardiac tumors in laboratory rodent-comparative pathology. In Kaiser, H. E. (Ed.): Progressive Stages of Malignant Neoplastic Growth. Oxford, Bergaman (In Press)

Hoch-Ligeti, C, and Stewart, H. L.: Cardiac tumors of mice. J. Natl. Cancer Inst. 72: 1449-1456, 1984

Hoch-Ligeti, C., Wagner, B. P., Deringer, M. K., and Stewart, H. L.: Tumor induction in Praomys (Mastomys) natalensis by N,N'-2,7-Fluorenylenebisacetamide. J. Natl. Cancer Inst. 74: 909-915, 1985.

Sass, B., and Liebelt, A. G. Metastatic tumors, lung, mouse. In Jones, T. C. Mohr, U. and Hunt, R. D. (Eds.): Respiratory System. New York, Springer-Verlag, 1985, pp. 138-159.

Stewart, H. L., Sass, B., Deringer, M. K., Dunn, T. B., Liotta, L. A. and Togo, S.: Pure yolk sac carcinoma of the uterus of the mouse: Report of 8 Cases. J. Natl. Cancer Inst. 73: 115-122, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP06134-10 OD

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Role of Lymphatic System in the Absorption and Distribution of Antitumor Agents

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

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

COOPERATING UNITS (if any)

None

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	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.)

The role of the lymphatic system in the absorption and biodistribution of antitumor agents and monoclonal antibodies in normal and tumor-bearing rodents is under investigation. Monoclonal antibodies given by subcutaneous injection are delivered via lymphatic channels to regional lymph nodes with greater efficiency than when administered intravenously. Typically, 30 to 50% of the injected antibody binds specifically to cells in these nodes, a finding now confirmed for a variety of antibodies with specificity for lymphoid cells. Pharmacokinetic studies and modelling indicate that optimal conditions for antibody uptake in target tissue will be achieved by a careful choice of dose, osmolarity, volume, and site of injection. Comparing the biodistribution of intact antibody with that of its F(ab')2 and Fab fragments suggest that the latter may be more appropriate for clinical use than intact antibody. Fragments were cleared from the bloodstream and excreted more rapidly than intact antibody which may be important in reducing toxicity due to circulating antibody and lowering background radioactivity when radiolabelled antibodies are used for diagnostic imaging. Information obtained from studies in animals is currently being applied to clinical studies for detection of lymph node metastases in patients with malignant melanoma, carcinoma of the breast and T-cell lymphoma. Studies in patients with T-cell lymphoma have demonstrated the most efficient imaging of tumor cells yet achieved in humans by any technique. In an attempt to utilize conjugates of monoclonal antibodies with cytotoxic agents for the treatment of lymph node metastases, we have synthesized conjugates of antibody covalently coupled to the plant toxin, ricin A-chain, and antibody labelled with the alpha-emitting radiometal, bismuth-212. Both conjugates were found to bind specifically to target cells and were highly cytotoxic in vitro. Studies to demonstrate cytotoxicity in vivo using the guinea pig hepatocarcinoma model for lymph node metastasis are currently underway.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

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

Objectives:

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

Major Findings:

We have conducted extensive studies in mice on the biodistribution and metabolism of radiolabelled monoclonal antibodies and their Fab and $F(ab')_2$ fragments. Animals received both specific (anti-murine B-cell antibody) and nonspecific (MOPC-21) antibodies in order to distinguish the effects of specific binding from those of non-specific binding and to establish baseline values for unreactive antibodies such as MOPC-21 which has no known binding specificity in the mouse. Major findings are as follows: (1) Intact antibodies are cleared more slowly from the circulation than are antibody fragments irrespective of their specificity or route of administration; (2) lymph node uptake of Fab fragments of specific antibody following subcutaneous injection is lower than for corresponding $F(ab')_2$ fragments or intact antibody; (3) specific antibodies (intact or fragments) given by subcutaneous injection show a dose dependent uptake in target tissues which is not observed with non-specific antibody; (4) binding of specific antibody in target tissues is associated with a significant increase in catabolism of the antibody and loss of radiolabel which was not observed with non-specific antibody.

Antibody-ricin A-chain conjugates were synthesized during antibodies of different specificities. Conjugates were shown to bind to target cells in vitro and inhibit protein synthesis at the ribosomal level. While the guinea pig hepatocarcinoma cell line (L10) showed only weak cyto-toxicity with anti-L10 monoclonal antibody coupled to ricin A-chain,

the same toxin coupled with monoclonal antibody against mouse the histo-comparibility antigen H2-K^k and anti-murine B-cell antibody proved highly cytotoxic against mouse lymphoid cells. While previous attempts at therapy using antibodies labelled with the gamma emitting radioisotope I-131 were unsuccessful, we were able to demonstrate specific cytotoxicity in lymphoid cells of mice receiving subcutaneous injections of anti-murine B-cell antibody labelled with the α-emitting radiometal, bismuth-212. Studies are currently in progress to optimize specific uptake and reduce non-specific toxicity.

Proposed Course:

(1) To continue to pursue goals listed under "Objectives" above; (2) to further develop systems for in vivo delivery of monoclonal antibodies against B and T cell sub-populations for optimizing regimes utilizing antibodies for diagnosis and therapy of lymph node metastases and as a means for modulating immune function; (3) to further optimize delivery of antibody to lymph nodes by modulating lymph flow using pharmacological agents; (4) continue development of antibody-cytotoxic agent conjugates with emphasis on plant toxins and α-emitting radioisotopes and refine their delivery to target tissue in order to minimize non-specific toxicity.

Significance to Biomedical Research and the Program of the Institute:

Monoclonal antibodies are potentially useful as carriers for cytotoxic agents and as diagnostic imaging agents when labelled with radioisotopes such as iodine-131 and indium-111. This project represents an attempt to improve the selectivity of cytotoxic agents by targeting them, through monoclonal antibodies to specific sites and to develop diagnostic imaging agents for efficient detection of tumor in lymph node. Tumor metastasis is a major clinical problem in treating cancer and presents a diagnostic challenge as well. Lymphatic channels are frequently the route by which tumors metastasize, with micrometastases lodging regional and even distant lymph nodes. It would therfore be of potential therapeutic and diagnostic advantage in early metastases to selectively concentrate agents in lymph channels and lymph nodes. The results of our studies suggest that targeting with monoclonal antibodies are feasible methods for directing antitumor agents to these sites.

Publications :

Weinstein, J. N., Black, C. D. V., Keenan, A. M., Holton, O. D., Larson, S. M., Sieber, S. M., Covell, D. G., Carrasquillo, J., Barbet, J. and Parker, R. J. Use of monoclonal antibodies for detection of lymph node metastases. In International Workshop on Monoclonal Antibodies and Breast Cancer. Symposium Volume, November 1984 (In Press)

Weinstein, J. N., Keenan, A. M., Holton, O. D., Covell, D. G., Sieber, S. M., Black, C. D. V., Barbet, J. and Parker, R. J. Use of monoclonal antibodies to detect metastases of solid tumors in lymph nodes. In Monoclonal Antibodies in Medicine. ICN-UCLA Symposium, 1985 (In Press)

Weinstein, J. N., Parker, R. J., Holton, O. D., Keenan, A. M., Covell, D. G. and Sieber, S. M.: Lymphatic delivery of monoclonal antibodies: Potential for detection and treatment of lymph node metastases. Cancer Invest. 3: 85-95, 1985.

ANNUAL REPORT OF

THE LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY

BIOLOGICAL CARCINOGENESIS PROGRAM

NATIONAL CANCER INSTITUTE

October 1, 1984 to Setpember 30, 1985

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

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

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

During the past year, our accomplishments have included assignment of the first normal cellular function for a proto-oncogene, and the demonstration that the normal coding sequence for a human growth factor possesses transforming properties when expressed in a cell susceptible to its growth promoting activities. Acute transforming retroviruses have arisen in nature by substitution of viral genes necessary for replication with discrete segments of host genetic information. When incorporated within the retroviral genome, such transduced cellular sequences, termed onc genes, acquire the ability to induce neoplastic transformation. Some of these same cellular genes or proto-oncogenes have also been implicated as important targets for genetic alterations that may lead normal cells to become malignant independent of virus involvement.

Despite advances in identifying cellular genes with transforming potential, little is known about proto-oncogene function or how the altered counterparts

of these genes disrupt normal growth regulation. Recently, however, studies of the onc gene of simian sarcoma virus (SSV), a primate transforming retrovirus, combined with investigations of platelet-derived growth factor (PDGF), a potent mitogen for connective tissue cells, have led to the discovery that the SSV transforming gene product and PDGF arise from the same or very closely related cellular genes. Thus the mechanism by which the SSV onc gene, v-sis, transforms cells may involve the constitutive expression of functions similar to those of PDGF.

Expression of the normal human sis/PDGF-2 coding sequence was demonstrated to induce cellular transformation. The human sis proto-oncogene was found to contain the coding sequence for one of two polypeptide chains present in preparations of biologically active PDGF. A human clone, c-sis clone 8, which contains all of the v-sis-related sequences present in human DNA, was transcriptionally inactive when transfected into NIH/3T3 cells. When placed under the control of a retrovirus LTR, the clone was transcribed at levels comparable to that observed in cells transformed by simian sarcoma virus (SSV) DNA. However, c-sis clone 8 DNA did not express detectable sis/PDGF-2 proteins and lacked biologic activity. A putative upstream exon was identified by its ability to detect the 4.2-kb sis-related transcript in certain human cells. When this sequence was inserted in the proper orientation between the LTR and c-sis clone 8, the chimeric molecule acquired high titered transforming activity, comparable to that of SSV DNA. Transformants containing this construct expressed human sis/PDGF-2 translational products. Thus the normal coding sequence for a human growth factor has transforming activity when expressed in an appropriate assay cell.

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

The v-sis transforming gene encodes the woolly monkey homologue of human platelet-derived growth factor polypeptide 2. After its synthesis on membrane bound polyribosomes, the glycosylated precursor dimerizes in the endoplasmic reticulum and travels through the Golgi apparatus. At the cell periphery, the precursor is processed to yield a dimer structurally analogous to biologically active PDGF. Small amounts of two incompletely processed forms are detectable in tissue culture fluids of SSV transformants. However, the vast majority remains cell associated. Thus this growth factor related transforming gene product is not a classical secretory protein. These findings define possible cellular locations where the transforming activity of the sis/PDGF-2 protein may be exerted.

During the past year, much new information was developed on characterization of human ras genes and the frequency with which members of this family are activated as oncogenes. Three human ras family proto-oncogenes, c-Ki-ras-1 and c-Ki-ras-2, and N-ras have been mapped to chromosome bands 6p11-12,

12p11.1-12.1, and 1p11-13, respectively, by *in situ* molecular hybridization. Certain human cancers display consistent and specific alterations involving chromosomes 1, 6 and 12. The ability to map the precise chromosomal localization of *ras* genes should permit evaluation of the possible effect of these chromosome changes on the structure and activities of *ras* proto-oncogenes in human neoplasia.

An N-*ras*-related transforming gene was detected in the human lung carcinoma cell line SW-1271 and molecularly cloned. The lesion responsible for its acquisition of transforming activity was localized to a single nucleotide transition from A to G in codon 61 of the predicted protein. This lesion in the second exon results in the substitution of arginine for glutamine at this position. These findings, together with previous studies, indicate that the activation of *ras* oncogenes in human tumors is most commonly due to point mutations at one of two major "hot spots" in the *ras* coding sequence.

Analysis of human mammary tumors for transforming genes detectable by transfection analysis revealed the presence of an H-*ras* oncogene in the HS578T mammary carcinosarcoma line. The oncogene was cloned in biologically active form and the activating lesion identified as a single nucleotide substitution of adenine for guanine within the 12th codon. This results in substitution of aspartic acid for glycine at this position of the p21 coding sequence. Knowledge that this alteration creates a restriction site polymorphism for Msp I/Hpa II within the H-*ras* proto-oncogene made it possible to survey for the presence of the activated H-*ras* allele in normal cells as well as in clonally derived tumor cell lines of the same patient. We demonstrated the presence of unaltered H-*ras* alleles in normal HS578T cells. In contrast, every clonally derived HS578T tumor cell line analyzed contained an H-*ras* oncogene possessing the genetic alteration at position 12. These findings establish that activation of this oncogene was the result of a somatic event, powerfully selected within all HS578T tumor cells. As such, the evidence strongly favors the concept that this oncogene played an important role in the development of the HS578T mammary carcinosarcoma.

A large series of urinary tract tumors were surveyed for *ras* oncogenes by DNA transfection and by molecular genetic analysis to determine their frequency in urothelial cells. H-*ras* oncogenes were detected in 2 of 38 tumors by transfection, molecularly cloned in biologically active form and shown to contain single base changes at codon 61 leading to substitutions of arginine and leucine, respectively, for glutamine at this position. One additional H-*ras* oncogene was identified in a bladder carcinoma by restriction polymorphisms at codon 12. In one of 21 tumors, a 40-fold amplification of the K-*ras* gene was observed. No amplification of other *ras* genes was detected in any of the tumors analyzed. These findings strengthen the conclusion that codons 12 and 61 are the major "hot spots" of *ras* oncogene activation and suggest that quantitative alterations in expression due to gene amplification may provide an alternative mechanism for *ras* gene activation in primary human tumors.

The nucleotide sequence of the v-H-*ras*-related oncogene of BALB/c murine sarcoma virus was determined. This oncogene contains an open reading frame of 189 amino acids that initiates and terminates entirely within the mouse cell-derived *ras* sequence. The protein encoded by this open reading frame matches the sequence predicted for the T24 human bladder carcinoma oncogene product, p21,

in all but two positions. The presence of a lysine residue in position 12 of BALB/c murine sarcoma virus p21 likely accounts for its oncogenic properties.

As noted, ras oncogenes are frequently activated in human tumors by mutations at position 12 or 61 in their coding sequences. To investigate how these subtle alterations exert such profound effects on the biologic activities of these genes, we studied structural and conformational properties of human ras oncogene-encoded p21 proteins. Striking differences were observed in the electrophoretic mobilities of the proteins under reducing and nonreducing conditions. These findings imply that intramolecular disulfide bonds affect native p21 conformation. The two activating lesions were shown to induce distinctly different alterations in p21 electrophoretic mobility unmasked only under reducing conditions. These results suggest that regions of the molecule containing such alterations are either not exposed or are under conformational constraints in the native p21 molecule. We confirmed the opposing effects on protein mobility induced by the two activating lesions using a recombinant gene containing both lesions. Its high titered transforming activity further established that the two lesions do not negatively complement one another with respect to transforming gene function. Our findings of distinct alterations in electrophoretic mobilities of position 12- and 61-altered p21 molecules should be applicable to the rapid immunologic diagnosis of ras oncogenes in human malignancies.

It remains to be determined what normal functions are served by ras proto-oncogenes, as well as how point mutations at position 12 or 61 can so markedly affect their biologic functions. The use of monoclonal or peptide antibodies may make it possible to develop immunologic reagents capable of specifically recognizing the altered gene products. Alternatively, oligonucleotide hybridization techniques may provide a method of detecting the changed alleles or transcripts. Such approaches could be used to identify cells with these genetic changes and thus determine whether such alterations are predictive of a particular clinical course.

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

Studies of the transcriptional status of the fgr proto-oncogene in human tumors has revealed in general that certain lymphomas but not sarcomas or carcinomas expressed fgr-related mRNA. Further examination of lymphoid tumors demonstrated that expression of c-fgr was highly correlated with Epstein-Barr virus (EBV) infection. This correlation was extended to include cord and peripheral blood lymphocytes established in culture by infection with EBV. Moreover, when EBV-negative Burkitt's cells were deliberately infected with the virus,

fgr proto-oncogene transcripts were elevated 50-fold. These findings demonstrated that EBV infection was responsible for enhanced expression of the c-fgr gene, a member of the tyrosine-specific protein kinase gene family.

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

Studies demonstrating the hematopoietic targets of ras-containing viruses within the myeloid lineage extended the spectrum of targets whose growth is altered by ras-containing retroviruses to cells at several stages of differentiation within each of the major hematopoietic lineages. Diffuse colonies were induced by BALB or Harvey murine sarcoma virus infection of murine bone marrow cells. Generally these colonies were made up of relatively mature macrophages which exhibited increased self-renewal capacity but eventually underwent terminal differentiation in culture. Cells from one BALB murine sarcoma virus-induced colony displayed phenotypic markers of more immature myelomonocytic cells. This colony, designated BAMC1, was readily established as a continuous cell line and was highly malignant in vivo. Exposure of these cells to 12-O-tetradecanoylphorbol-13-acetate led to the induction of a more mature myeloid phenotype, which was associated with decreased growth potential in vitro and in vivo. The effects of the inducing agent were not mediated by an alteration in the level of expression of the ras-coded p21 transforming protein.

Normal mast cells can be propagated in culture when medium is supplemented with interleukin-3 (IL-3). We demonstrated that Abelson-MuLV (Ab-MuLV) infection of mast cells eliminates their IL-3 dependence for growth. By contrast, Harvey-, BALB-, and Moloney-MSV, which also productively infect mast cells, are unable to supplant IL-3 dependence. Ab-MuLV-induced IL-3-independent lines express the v-abl-specific transforming protein and have phenotypic characteristics of mast cells. Moreover, these cells possess high cloning efficiencies in soft agarose and are tumorigenic in nude mice. In addition, Ab-MuLV induces transplantable mastocytomas in pristane-primed adult mice resistant to lymphoid transformation, defining a new hematopoietic target for malignant transformation by this virus. None of the Ab-MuLV-derived transformants express or secrete detectable levels of IL-3 nor is their growth inhibited by anti-IL-3 serum. These results argue that Ab-MuLV abrogation of the IL-3 requirement is not due to an autocrine mechanism.

Recent investigations have begun to dissect the number and nature of genetic alterations associated with cancer cells. We were able to demonstrate that primary human epidermal keratinocytes acquired indefinite lifespan in culture but did not undergo malignant conversion in response to infection with a hybrid of adenovirus 12 and simian virus 40. The addition of Kirsten murine sarcoma virus, which contains a K-ras oncogene, to these cells induced morphological

alterations associated with the acquisition of neoplastic properties. These findings demonstrate the malignant transformation of human primary epithelial cells in culture and support a multiple-step process for neoplastic conversion.

The role of LTR sequences in the efficient expression of Moloney murine sarcoma virus (MSV-124) transforming gene function was investigated. Recombinant plasmids containing a single LTR positioned 3' of v-mos were subjected to sequential deletions, and the relative transforming efficiency of these recombinants was analyzed in the NIH/3T3 transfection assay. Recombinants lacking CAAT, TATA, and poly(A) signals within the LTR were able to transform with an efficiency comparable to that of the wild type MSV-124 genome. Deletion of one of the two 74-bp tandem repeat units within the LTR did not abolish v-mos gene function, whereas removal of both 74-bp repeat units completely eliminated transforming activity. The addition of a fragment containing only a single 74-bp unit and 29-bp downstream sequences derived from the LTR to a position 3' of v-mos led to efficient activation of v-mos transforming function. Residual potentiating activity for v-mos expression was retained even when the distance between v-mos and the 3' LTR was increased by several kbp. All these findings are consistent with the concept that the potentiating action of the LTR in its 3' position is due to activator/enhancer sequences localized to one of its 74-bp repeats. A permuted MSV-124 molecule, whose single LTR was localized 5' of v-mos, was very inefficient at transformation. However, its transforming activity could be increased by approximately 1000-fold by tandemization of the molecule. These results suggest that the transcript for the MSV-124 transforming gene is not normally initiated within the 5' LTR, but instead utilizes promoter signals in close proximity to v-mos and enhancer elements localized in the 3' LTR.

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

Oncoviruses have been shown to be etiologically involved in naturally occurring tumors of a wide variety of vertebrate species. They appear to exert their oncogenic potential under conditions in which there is poor host cell control of virus replication. This is the case for horizontally transmitted oncoviruses of birds, rodents, artiodactyls, cats and subhuman primates. It has become evident that oncoviruses can interact with their hosts in a manner which appears to be unique among viruses of vertebrates. In many species, oncoviruses are transmitted from one generation to the next in an unexpressed form within the host cell genome. Under such conditions, these endogenous viruses appear to be subject to regulatory processes analogous to those affecting cellular genes. Over the past several years, our laboratory has isolated a number of new endogenous oncoviruses. Moreover, we have investigated the relationships of such viruses to known oncoviruses, as well as the distribution of related viral sequences within vertebrate cellular DNAs by molecular hybridization techniques.

In collaboration with scientists in the Laboratory of Tumor Immunology and Biology, NCI, we investigated a new class of human endogenous oncoviral genomes. This class is characterized by extensive sequence homology with type A, B, D and avian type C oncoviral _pol_ genes, weak homology with the type A oncoviral _env_ gene and type D oncoviral LTR sequences, and no detectable homology with mammalian type C oncoviral genomes. This unusual pattern of sequence homology suggests that genetic interactions between different oncoviral genera contributed significantly to the evolution of the progenitor of this class of human oncoviral genomes. The relationship of MMTV _env_ and LTR-related sequences in human cellular DNA to this family of human oncoviral genomes remains unresolved. One would suspect that human proviral genomes containing these sequences represent a subclass of this family. The role which these proviral genomes play in the etiology of human neoplasia is unknown. However, the reagents are now available to unambiguously approach this question.

Immunoglobulin synthesis and gene rearrangements in lymphoid cells transformed by replication-competent Rauscher murine leukemia virus (R-MuLV) were described. Lymphoid cells transformed by R-MuLV belonged to the B cell lineages. One group of cells exhibited Fc receptors but completely lacked immunoglobulin μ heavy and κ light chains. The majority of the cells resemble pre-B type. They displayed μ chains but κ chains were completely absent. Very rarely certain cells synthesized both μ and κ chains. Based on the presence of Fc receptors and IgM synthesis, the cells transformed by R-MuLV belonged to three B cell developmental stages. These cells were tested for immunoglobulin gene rearrangements using J_H and C_K probes. DNA from cell lines without any detectable levels of IgM μ exhibited embryonic as well as rearranged JJ_H genes, whereas cells expressing IgM possess, in addition, productive and nonproductive light chain gene rearrangements. The most terminally differentiated cell possesses J_H and C_K rearrangement associated with the synthesis of μ and κ chains. Presumably the cells with rearranged J_H and C_K genes without immunoglobulin synthesis represent a developmental transition. We conclude that cells transformed by R-MuLV belonged to five step-wise compartments of B cell development. Our findings implicate definite sequential events of immunoglobulin gene rearrangement and expression during B cell development.

Molecular cloning of integrated caprine arthritis encephalitis virus (CAEV) was accomplished. A full length DNA clone of the exogenous retrovirus, CAEV, was isolated from high molecular weight DNA of CAEV-infected Himalayan tahr ovary cells. Although other restriction maps of CAEV have been published, this is the first time that the proviral DNA has been cloned. The restriction enzyme map of the clone was determined and found to be identical to that of unintegrated linear CAEV DNA. The cloned CAEV genome was shown to contain terminal repeats of approximately 450 base pairs in length, and its restriction enzyme map was oriented with respect to the direction of viral RNA transcription. When the cloned CAEV DNA was used as a molecular probe, it failed to detect related proviral sequences in the genomes of a variety of vertebrate species, including the goat, sheep, horse, mouse and man. When CAEV DNA was hybridized under relaxed conditions to a variety of cloned DNAs, representing different oncoviral genera, homology to mouse mammary tumor virus (MMTV) was observed, while no homology to avian type C or mammalian type A, C and D retroviruses was detected. This homology was localized to a region in MMTV corresponding to the 3' end of the _gag_ gene and the 5' end of the _pol_ gene.

LCMB efforts to elucidate mechanisms of carcinogenesis are complemented by the program of the In Vitro Carcinogenesis Section which is directed toward elucidating through cell culture studies the mechanism(s) of neoplastic transformation in human cells. Major new findings are as follows: Thirteen cell lines derived from human tumors of diverse tissue origin and histopathology were compared with 12 lines of normal skin fibroblasts with respect to chromatid damage induced by 25, 50, or 100 R of x-irradiation during the G_2 period of the cell cycle. Only cells in metaphase were examined, and these had been irradiated 1.5 hr before fixation. When irradiated under identical conditions, the tumor cells showed significantly more chromatid breaks and gaps than did the normal cells at all doses tested. The data suggest that the increased G_2 chromosomal radiosensitivity of the tumor cells is associated with deficient DNA repair during the G_2-prophase period of the cell cycle.

The combined impact of visible light and oxygen on photosensitizing components in culture medium and/or cells has been shown to generate photoproducts, predominantly H_2O_2 and the derivative ·OH. These photoproducts have been shown to have important and diverse effects on human cells in culture, influencing their survival, proliferation and lifespan. They can produce membrane peroxidation, DNA strand breakage, DNA-protein crosslinks, sister chromatid exchanges and chromatid damage. Furthermore, the quantitative assessment of light-induced chromatid damage has revealed fundamental differences in the responses of normal as compared with cancer-prone and neoplastic cells. The results suggest that a deficiency in repair of DNA damage during G_2 phase is associated with both genetic susceptibility to cancer and the neoplastic state.

The generation of hydrogen peroxide (H_2O_2) and the derivative free hydroxyl radical (·OH) in cultures of mouse cells grown in the presence of visible light and ambient oxygen was shown previously to be implicated in chromatid damage. Furthermore, chromosome alterations apppear to be associated with the spontaneous neoplastic transformation of mouse cells in culture. An attempt was made in this study to reduce the incidence of chromosomal aberrations and delay or prevent the onset of spontaneous neoplastic transformation of freshly isolated mouse cells, both fibroblasts and epidermal keratinocytes, by adding catalase to the culture medium, shielding the cultures from wavelengths <500 nm and providing a gas phase of 0-1% O_2. These conditions significantly decreased the incidence of chromosomal aberrations in both cell types, prevented their tumorigenicity in non-irradiated syngeneic mice, and increased latent periods for tumor development in x-irradiated mice. The epidermal keratinocytes were particularly resistant to spontaneous neoplastic transformation under all conditions tested. These observations on the protective effect of extracellular catalase suggest that H_2O_2, a normal metabolite, and/or the derivative ·OH can directly or indirectly produce genetic damage and neoplastic transformation in mouse fibroblasts.

It had been previously reported that human cells after neoplastic transformation in culture had acquired an increased susceptibility to chromatid damage induced by x-irradiation during G_2 phase of the cell cycle. Evidence suggested that this results from deficient DNA repair during G_2 phase. Cells derived from human tumors also showed enhanced G_2 chromosomal radiosensitivity. Furthermore, skin fibroblasts from individuals with genetic diseases predisposing to a high risk of cancer, including ataxia-telangiectasia, Bloom syndrome, Fanconi anemia, and xeroderma pigmentosum, exhibited enhanced G_2

chromosomal radiosensitivity. The present study shows that apparently normal skin fibroblasts from individuals with familial cancer, i.e., from families with a history of neoplastic disease, also exhibit enhanced G_2 chromosomal radiosensitivity. This radiosensitivity appears, therefore, to be associated with both a genetic predisposition to cancer as well as malignant neoplastic state. Further, enhanced G_2 chromosomal radiosensitivity may provide the basis for an assay to detect genetic susceptibility to cancer.

G_2 chromosomal radiosensitivity of ataxia-telangiectasia heterozygotes was reported. Five lines of skin fibroblasts from individuals heterozygous for ataxia-telangiectasia (A-T), compared with six cell lines from age-matched normal controls, show much higher frequency of chromatid breaks and gaps following x-irradiation during the G_2 phase of the cell cycle. The magnitude of this difference suggests that G_2 chromatid radiosensitivity could provide the basis for an assay to detect A-T heterozygotes. Though clinically normal, A-T heterozygotes share a high risk of cancer with A-T homozygotes and constitute approximately 1% of the human population. Further, G_2 chromosomal radiosensitivity, which appears to result from a DNA repair deficiency, may be associated with a genetic predisposition to cancer.

Skin fibroblasts from Gardner syndrome (GS) compared with those from normal donors showed a significantly higher incidence of chromatid gaps and breaks following exposure to low intensity, cool white fluorescent light during G_2 phase of the cell cycle. Considerable evidence supports the concept that chromatid gaps and breaks seen directly after exposure to DNA-damaging agents represent unrepaired DNA single- and double-strand breaks, respectively. The changes in incidence of chromatid aberrations with time after light exposure are consistent with the sequence of events known to follow DNA damage and repair. Initially, the incidence of light-induced chromatid gaps was equivalent in GS and normal fibroblasts. In the normal cells, the chromatid gaps disappeared by one hour post-exposure, presumably as a result of efficient repair of DNA single-strand breaks. In contrast, the incidence of gaps increased in GS cells by 0.5 hour followed by a decrease at one hour and concomitant increase in chromatid breaks. It appears from these findings that the increased incidence of chromatid damage in GS fibroblasts results from deficient repair of DNA single-strand breaks which arise from incomplete nucleotide excision of DNA damage during G_2 phase.

Enhanced chromatid radiosensitivity during late G_2 phase, within 1.5 hours of mitosis, is associated with genetic susceptibility to cancer. It is inherent in cells from individuals genetically predisposed to cancer and it can be induced in normal cells undergoing neoplastic transformation spontaneously, by chemical carcinogens or tumor viruses in culture. It characterizes all neoplastic cells examined whether of in vitro or in vivo origin; thus its acquisition appears to be an early necessary, possibly initiating step, in carcinogenesis. In somatic cell hybrids it behaves as a recessive trait segregating with the neoplastic tumorigenic phenotype. Furthermore, the increased susceptibility of cancer-prone and neoplastic cells to chromatid damage compared to normal cells or cells from normal individuals results from deficient DNA repair mechanisms operative during late G_2 phase.

To study the mechanisms of formation and repair of DNA-protein cross-links in mammalian cells, the best general method to assay these lesions is the Kohn

membrane alkaline elution procedure. Use of this sensitive technique requires the introduction of random strand breaks in the DNA by x-irradiation to reduce the very high molecular weight so that it elutes off the filter at an appropriate rate. This report describes an alternative method for fragmenting the DNA in the absence of x-irradiation equipment. Convenient reproducible elution rates of DNA from various mouse and human cells in culture without x-irradiation result from elution through polyvinyl chloride filters with 75 mM sodium hydroxide (0.33 ml per minute) instead of the standard 20 mM EDTA-tetrapropylammonium hydroxide, pH 12.2 (0.03 to 0.04 ml per minute). Dose-dependent retardation of the DNA elution was observed over the range 0 to 30 {M trans-platinum(II)-diamminedichloride, and proteinase K treatment during cell lysis restored the elution rate to that of the untreated control cell DNA. In the absence of x-irradiation, this elution method measures DNA-protein cross-links with higher sensitivity and equivalent reproducibility as the standard procedure.

Two studies on DNA primate herpesviruses yielded the following results. In surveys of sera from 11 patients with acquired immunodeficiency disease (AIDS), transforming Epstein-Barr virus (EBV) was isolated from four. The latter expressed HTLV-III as well as significantly elevated levels of EBV antibodies. Uncultured peripheral mononuclear cells from these individuals contained 8-15% EBV-membrane antigen (MA)-positive cells. Transforming virus was isolated at various passage levels after culture. In comparison, healthy donors not at risk of developing AIDS did not contain MA-positive cells in the peripheral blood and no EBV could be isolated from plasma or cultured cells. None of the EBV-positive AIDS sera contained neutralizing antibody to EBV which were present in sera from healthy donors.

In response to a request from NASA, squirrel monkeys which were to be used on space lab flights 3 and 4 were tested for Herpesvirus saimiri (HVS) which is endemic in this species. NASA officials were concerned about the possibility of crew members being infected by HVS shed by the monkeys within the close confines of the spacecraft. Sera of 150 individuals occupationally exposed to squirrel monkeys were tested for the presence of HVS, and 11 (7.3%) were found positive for antibodies to HVS late antigen (LA) in the immunofluorescence test. No other HVS antibodies were evident. Eight of the 11 sera also immunoprecipitated HVS proteins. These results suggest that replication of HVS either does not occur in humans, or is too minimal to elicit immune responses. Although no demographic patterns in the HVS-LA positives were noted, the majority (7/11) had worked at primate centers around the country for periods ranging from less than one year to 25 years. This was the first report of the detection of HVS antibodies in humans.

In addition to their intramural research efforts, investigators within the LCMB serve on the editorial boards of major journals in their fields, serve as members of various review bodies, and participate in a large number of collaborative efforts with scientists in laboratories within and outside of the United States. The ultimate goal of these multidisciplinary studies of virus/carcinogen-induced and spontaneously occurring cancers is to apply the basic information derived to its most important application, the prevention of cancer in man.

CONTRACTS IN SUPPORT OF ALL LABORATORY PROJECTS:

HAZLETON LABORATORIES AMERICA, INC. (N01-CP-01017)

Title: Support Services for the Laboratory of Cellular and Molecular Biology

Current Annual Level: $22,200 (October 1, 1984-November 15, 1984)

Man Years: 0.9

Objectives: The purpose of the contract is to provide support services for research conducted by the LCMB.

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: LCMB elements previously residing on the contract site were relocated to the NIH Bethesda campus in December 1983. As a result, the contract was terminated February 15, 1985, with no funds beyond its November 15, 1984, expiration date.

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

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

Current Annual Level: $140,000

Man years: 3.05

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

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

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04930-14 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Biology of Natural and Induced Neoplasia

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

PI:	P. Arnstein	Veterinary Director	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. S. Rhim	Research Microbiologist	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NCI
	J. Pierce	Sr. Staff Fellow	LCMB	NCI
	A. Eva	Visiting Associate	LCMB	NCI

COOPERATING UNITS (if any)

California Dept. Health Services (J. Riggs, R. Emmons); Peralta Cancer Inst. (A. Hackett); University of California (M. Gardner, J. Levy, H. Rubin and M. Stampfer); Children's Hospital Medical Center (K. Walen)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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.)

In vivo studies of the sis transforming gene, either from the simian sarcoma virus (v-sis) or from human cells (c-sis), continue to yield data on the unique biologic effects of this important oncogene. When introduced to newborn mice in an infectious viral form, it produces sarcomas after a very long incubation period in either euthymic or athymic animals. Cells grown from the primary sarcomas are transplantable to athymic mice only, where they produce slow growing local sarcomas as well as spleen metastases.

Studies on malignancy of transformed cultures in vivo, using human cells transformed by oncogenic viruses or rodent cells transfected with human oncogenes, are producing valuable data on the nature of neoplastic progression.

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. S. Rhim	Research Microbiologist	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI
J. Pierce	Sr. Staff Fellow	LCMB	NCI
A. Eva	Visiting Associate	LCMB	NCI

Objectives:

1. Conduct experiments to determine the in vivo biology of available cloned oncogenes and selected genetically mapped tumor viruses. The goal is to correlate specific viral genomic sequences with a particular effect on the host (e.g., in vivo replication, viremia, target cell preference, tumor induction and tumor type induced). The viruses and cloned oncogenes for these studies are selected or especially engineered by molecular biology techniques in vitro.

2. Characterize neoplastic transformation in primate cells and selected non-primate cultures. Correlate morphological transformation with transplantability and tumor production in athymic nude mice.

3. Test spontaneous human tumors as well as selected animal specimens for relative xenograft malignancy in athymic nude mice; seek correlations of grafting results with cellular genomes and their translation products, including viruses.

Methods Employed:

1. In vitro portions of the viral and cellular oncogene experiments are collaborative studies in Dr. Aaronson's and Dr. Robbins' projects (Z01CP04940-18 and Z01CP05167-05 LCMB). Candidate inocula are furnished in coded vials as deep-frozen alilquots or cultures of transformed and control cells. Newborn mice are inoculated at approximately 24 hours of age with the cloned retroviruses by appropriate routes (customarily these are intraperitoneal, subcutaneous, intramuscular or intracerebral). Adult mice are inoculated, usually at two months of age, by similar routes. All inoculated mice are subsequently monitored by periodic physical examination and appropriate periodic blood sampling until onset of disease or, in case of nonpathogenic clones, for the designated holding periods up to the normal lifespan of the breed of mouse. Tumored mice are exsanguinated, all tissues examined for neoplasia, and appropriate specimens processed for virus isolation, cell culture, microscopic characterization, analysis of tumor DNA and RNA (for onc sequences) and proteins (for tumor-specific translation products).

Studies of an important oncogene, v-sis, derived from simian sarcoma virus (SSV) are continuing. It is of special interest because its translation product is closely related or identical to the human blood platelet-derived growth factor (PDGF). Using molecular biology techniques, new, fully mapped

viruses have been constructed, incorporating either the simian v-sis or its cellular counterpart, the human c-sis oncogene in type C virus "packages" which are infectious for newborn mice.

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

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

Major Findings:

1. V-sis oncogenesis. Genetically engineered viruses containing the v-sis oncogene continue to produce delayed onset sarcomas in athymic nude and in euthymic mice inoculated at birth. Incidence approaches 20% of mice at risk, with median onsets seven months after subcutaneous inoculation. Long-standing sarcomas allowed to grow to large size tend to metastasize to the spleen. Cell lines established from the primary tumors have an aberrant growth pattern, but morphologically do not resemble highly malignant cultures. When grafted on athymic mice, representative lines have produced sarcomas with as few as 10^3 cells. Grafted tumors are also relatively slow growing, but have a high incidence of metastases to the spleen (25-60%). Interestingly, tumor lines derived from primary neoplasms of either euthymic or athymic mice are transplantable to athymic mice only; high immunogenicity of a neoantigen in those lines may be responsible and is under study.

2. Transformation studies. In collaboration with Dr. Rhim, new lines of transformed human keratinocytes have been tested for tumor production in adult and suckling nude mice. Early results have been confirmed: cells "immortalized" by infection with the hybrid virus adenovirus 12-simian virus 40 (Ad12-SV40) exhibit some properties of neoplasia (high saturation density in vitro, rapid growth, low serum requirement), but do not produce tumors in nude mice. When infected with a second virus, Kirsten sarcoma baboon pseudotype, the cells undergo additional changes, lose contact inhibition, become heterogeneous and tumorigenic, causing rapidly progressive squamous cell carcinomas in suckling athymic nude mice; in adult nudes slowly progressive carcinomas are seen. This system is now being studied for its applicability in chemical and transfection oncogenesis studies.

A similar "two-hit" transformation in human amniocytes is under study in collaboration with the amniocentesis team at Children's Hospital Medical Center, Oakland, California.

A collaborative study on spontaneous transformation of murine 3T3 cells with Dr. Harry Rubin, University of California, Department of Molecular Biology, continues to yield data on the heterogeneity and variability of this system in assaying neoplasia potential.

In a continuing series of experiments, 3T3 cultures transfected with putative human onc sequences by Drs. Aaronson and Eva are being characterized for malignancy in athymic nude mice and their euthymic cohorts. Depending on which gene is integrated, the cells exhibit great variations in malignancy, particularly as to the minimum number of cells (threshold) needed to produce a malignant graft. In addition, some will graft to athymic mice only, suggesting high immunogenicity, whereas others are transplantable also to euthymics, possibly because they lack immunogenicity.

3. Human tumor studies. Xenografts of human tumor cultures in athymic nude mice revealed consistent differences in tumor propagation and malignancy among the tumor lines studied. In particular, using suckling and adult nudes as graft recipients, it was found that several malignant lines produce progressive neoplasia only in sucklings, but fail to grow in adults. This phenomenon may be due to the action of murine natural killer (NK) lymhocytes which are active in adults but absent in sucklings.

Significance to Biomedical Research and the Program of the Institute:

In vivo testing of genetically defined retroviruses and oncogenes in mice furnishes important information on molecular aspects of tumor induction using an economical, uniformly predictable experimental host system. Testing murine and simian oncogenes and their cloned human counterparts is furnishing valuable data on gene expression.

The ability (or inabililty) of cultures to form malignant growth by grafting to appropriate (euthymic or athymic nude) mice is still one of the best measures of malignancy in cell cultures. This test can be used to prove successful transformation by viruses or chemicals, or establishment of cancer cultures of naturally occurring neoplasms from any specimens. It is reproducible and relatively economical.

Proposed Course:

Continue collaborative studies described.

Publications:

Rhim, J. S., Jay, G., Arnstein, P., Price, F. M., Sanford, K. K. and Aaronson, S. A.: Neoplastic transformation of human epidermal keratinocytes by Ad12-SV40 and Kirsten sarcoma viruses. Science 227: 1250-1252, 1985.

Rhim, J. S., Sanford, K. K., Arnstein, P., Fujita, J., Jay, G. and Aaronson, S. A.: Human epithelial cell carcinogenesis: combined action of DNA and RNA tumor viruses produces malignant transformation of primary human epidermal keratinocytes. In Barrett, J. C. (Ed.): Carcinogenesis, a Comprehensive Survey. New York, Raven Press. (In Press)

Rubin, H., Arnstein, P. and Chu, B. M.: High-frequency variation and population drift in a newly transformed clone of BALB/3T3 cells. Cancer Res. 44: 5242-5247, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP04940-18 LCMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Viruses and Transforming Genes in Experimental Oncogenesis and Human Cancer

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

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NIC
	J. Pierce	Sr. Staff Fellow	LCMB	NCI
	A. Eva	Visiting Associate	LCMB	NCI

COOPERATING UNITS (if any)
U. CA, San Francisco (L. Williams); U. MA Med. Ctr., Worcester (J. Greenberger); U. SC, Charleston (G. Grotendorst); Weizmann Inst. Sci., Rehovot, Israel (D. Givol); Tel Aviv U., Israel (A. Yaniv); NIEHS, Res. Triangle Park, (M. Anderson)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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.)

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

During the past year, our investigations have provided important new insights regarding _sis_ and _ras_ genes, as well as identification of a new human oncogene, designated _dbl_, from a diffuse B cell lymphoma. Highlights of _sis_ include: demonstration that expression of the normal human _sis_/PDGF-2 coding sequence induces cellular transformation; the polypeptide sequence of the v-_sis_ transforming gene product of simian sarcoma virus (SSV) is divisible into four regions which are likely to represent structural domains of the protein; the v-_sis_ transforming gene encodes the woolly monkey homologue of human PDGF polypeptide 2; and definition of possible cellular locations where the transforming activity of the _sis_/PDGF-2 protein may be exerted. Regarding _ras_: three human _ras_ family proto-oncogenes were chromosomally mapped; _ras_ oncogenes were detected, cloned and at least partially characterized in lung and mammary carcinoma cell lines, as well as tumors of the urinary tract; activation of _ras_ oncogenes in human tumors appears to be most commonly due to point mutations at one of two major "hot spots" (codons 12 and 61) in the _ras_ coding sequence.

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
K. C. Robbins	Expert	LCMB	NCI
J. Pierce	Sr. Staff Fellow	LCMB	NCI
A. Eva	Visiting Associate	LCMB	NCI
J. Falco	Medical Staff Fellow	LCMB	NCI
J. Fujita	Visiting Fellow	LCMB	NCI
A. Gazit	Visiting Fellow	LCMB	NCI
C. R. King	Staff Fellow	LCMB	NCI
M. Kraus	Visiting Fellow	LCMB	NCI
S. K. Srivastava	Visiting Fellow	LCMB	NCI
R. Callahan	Chief, Oncogenetics Section	LTIB	NCI

Objectives:

1. To study the mechanisms of action of RNA tumor viruses and transforming genes; and

2. To apply knowledge gained from experimental systems to the search for etiologic agents and mechanisms involved in neoplastic transformation of human cells.

Methods Employed:

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

Major Findings:

Expression of the normal human sis/PDGF-2 coding sequence was demonstrated to induce cellular transformation. The human sis proto-oncogene was found to contain the coding sequence for one of two polypeptide chains present in preparations of biologically active PDGF. A human clone, c-sis clone 8, which contains all of the v-sis-related sequences present in human DNA, was transcriptionally inactive when transfected into NIH/3T3 cells. When placed under the control of a retrovirus LTR, the clone was transcribed at levels comparable to that observed in cells transformed by simian sarcoma virus (SSV) DNA. However, c-sis clone 8 DNA did not express detectable sis/PDGF-2 proteins and lacked biologic activity. A putative upstream exon was identified by its ability to detect the 4.2-kb sis-related transcript in certain human cells. When this sequence was inserted in the proper orientation between the LTR and c-sis clone 8, the chimeric molecule acquired high titered transforming activity, comparable to that of SSV DNA. Transformants containing this construct expressed human sis/PDGF-2 translational products. Thus the normal coding sequence for a human growth factor has transforming activity when expressed in an appropriate assay cell.

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

The v-sis transforming gene encodes the woolly monkey homologue of human platelet-derived growth factor polypeptide 2. After its synthesis on membrane bound polyribosomes, the glycosylated precursor dimerizes in the endoplasmic reticulum and travels through the Golgi apparatus. At the cell periphery, the precursor is processed to yield a dimer structurally analogous to biologically active PDGF. Small amounts of two incompletely processed forms are detectable in tissue culture fluids of SSV transformants. However, the vast majority remains cell associated. Thus this growth factor-related transforming gene product is not a classical secretory protein. These findings define possible cellular locations where the transforming activity of the sis/PDGF-2 protein may be exerted.

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

An N-ras-related transforming gene was detected in the human lung carcinoma cell line SW-1271 and molecularly cloned. The lesion responsible for its acquisition of transforming activity was localized to a single nucleotide transition from A to G in codon 61 of the predicted protein. This lesion in the second exon results in the substitution of arginine for glutamine at this position. These findings, together with previous studies, indicate that the activation of ras oncogenes in human tumors is most commonly due to point mutations at one of two major "hot spots" in the ras coding sequence.

Analysis of human mammary tumors for transforming genes detectable by transfection analysis revealed the presence of an H-ras oncogene in the HS578T mammary carcinosarcoma line. The oncogene was cloned in biologically active form and the activating lesion identified as a single nucleotide substitution of adenine for guanine within the 12th codon. This results in substitution of aspartic acid for glycine at this position of the p21 coding sequence. Knowledge that this alteration creates a restriction site polymorphism for Msp I/Hpa II within the H-ras proto-oncogene made it possible to survey for the presence of the activated H-ras allele in normal cells as well as in clonally derived tumor cell lines of the same patient. We demonstrated the presence of unaltered H-ras alleles in normal HS578T cells. In contrast, every clonally derived HS578T tumor cell line analyzed contained an H-ras oncogene possessing the genetic alteration at position 12. These findings establish that activation of this oncogene was the result of a somatic event, powerfully selected

within all HS578T tumor cells. As such, the evidence strongly favors the concept that this oncogene played an important role in the development of the HS578T mammary carcinosarcoma.

As noted, ras oncogenes are frequently activated in human tumors by mutations at position 12 or 61 in their coding sequences. To investigate how these subtle alterations exert such profound effects on the biologic activities of these genes, we studied structural and conformational properties of human ras oncogene-encoded p21 proteins. Striking differences were observed in the electrophoretic mobilities of the proteins under reducing and nonreducing conditions. These findings imply that intramolecular disulfide bonds affect native p21 conformation. The two activating lesions were shown to induce distinctly different alterations in p21 electrophoretic mobility unmasked only under reducing conditions. These results suggest that regions of the molecule containing such alterations are either not exposed or are under conformational constraints in the native p21 molecule. We confirmed the opposing effects on protein mobility induced by the two activating lesions using a recombinant gene containing both lesions. Its high titered transforming activity further established that the two lesions do not negatively complement one another with respect to transforming gene function. Our findings of distinct alterations in electrophoretic mobilities of position 12- and 61-altered p21 molecules should be applicable to the rapid immunologic diagnosis of ras oncogenes in human malignancies.

A large series of urinary tract tumors were surveyed for ras oncogenes by DNA transfection and by molecular genetic analysis to determine their frequency in urothelial cells. H-ras oncogenes were detected in 2 of 38 tumors by transfection, molecularly cloned in biologically active form and shown to contain single base changes at codon 61 leading to substitutions of arginine and leucine, respectively, for glutamine at this position. One additional H-ras oncogene was identified in a bladder carcinoma by restriction polymorphisms at codon 12. In one of 21 tumors, a 40-fold amplification of the K-ras gene was observed. No amplification of other ras genes was detected in any of the tumors analyzed. These findings strengthen the conclusion that codons 12 and 61 are the major "hot spots" of ras oncogene activation and suggest that quantitative alterations in expression due to gene amplification may provide an alternative mechanism for ras gene activation in primary human tumors.

The nucleotide sequence of the v-H-ras-related oncogene of BALB/c murine sarcoma virus was determined. This oncogene contains an open reading frame of 189 amino acids that initiates and terminates entirely within the mouse cell-derived ras sequence. The protein encoded by this open reading frame matches the sequence predicted for the T24 human bladder carcinoma oncogene product, p21, in all but two positions. The presence of a lysine residue in position 12 of BALB/c murine sarcoma virus p21 likely accounts for its oncogenic properties.

A new human oncogene, designated dbl, was isolated from a diffuse B cell lymphoma. The transforming gene was serially transmissible, conferred the neoplastic phenotype to NIH/3T3 cells, and appeared to be larger than 20 kbp by analysis of transfectants for conserved human DNA sequences. From a cosmid recombinant DNA library of a third-cycle transfectant, overlapping clones spanning 80 kbp of cellular DNA were isolated. One clone, which contained a

45-kbp insert comprised entirely of human sequences, was shown to be biologically active, with a specific transforming activity of 650 focus-forming units/pmole. This transforming gene was unrelated to any previously reported oncogene by restriction mapping and hybridization analysis.

Studies demonstrating the hematopoietic targets of ras-containing viruses within the myeloid lineage extended the spectrum of targets whose growth is altered by ras-containing retroviruses to cells at several stages of differentiation within each of the major hematopoietic lineages. Diffuse colonies were induced by BALB or Harvey murine sarcoma virus infection of murine bone marrow cells. Generally these colonies were made up of relatively mature macrophages which exhibited increased self-renewal capacity but eventually underwent terminal differentiation in culture. Cells from one BALB murine sarcoma virus-induced colony displayed phenotypic markers of more immature myelomonocytic cells. This colony, designated BAMC1, was readily established as a continuous cell line and was highly malignant in vivo. Exposure of these cells to 12-O-tetradecanoylphorbol-13-acetate led to the induction of a more mature myeloid phenotype, which was associated with decreased growth potential in vitro and in vivo. The effects of the inducing agent were not mediated by an alteration in the level of expression of the ras-coded p21 transforming protein.

Normal mast cells can be propagated in culture when medium is supplemented with interleukin-3 (IL-3). We demonstrated that Abelson-MuLV (Ab-MuLV) infection of mast cells eliminates their IL-3 dependence for growth. By contrast, Harvey-, BALB-, and Moloney-MSV, which also productively infect mast cells, are unable to supplant IL-3 dependence. Ab-MuLV-induced IL-3-independent lines express the v-abl-specific transforming protein and have phenotypic characteristics of mast cells. Moreover, these cells possess high cloning efficiencies in soft agarose and are tumorigenic in nude mice. In addition, Ab-MuLV induces transplantable mastocytomas in pristane-primed adult mice resistant to lymphoid transformation, defining a new hematopoietic target for malignant transformation by this virus. None of the Ab-MuLV-derived transformants express or secrete detectable levels of IL-3 nor is their growth inhibited by anti-IL-3 serum. These results argue that Ab-MuLV abrogation of the IL-3 requirement is not due to an autocrine mechanism.

Recent investigations have begun to dissect the number and nature of genetic alterations associated with cancer cells. We were able to demonstrate that primary human epidermal keratinocytes acquired indefinite lifespan in culture but did not undergo malignant conversion in response to infection with a hybrid of adenovirus 12 and simian virus 40. The addition of Kirsten murine sarcoma virus, which contains a K-ras oncogene, to these cells induced morphological alterations associated with the acquisition of neoplastic properties. These findings demonstrate the malignant transformation of human primary epithelial cells in culture and support a multiple-step process for neoplastic conversion.

The role of LTR sequences in the efficient expression of Moloney murine sarcoma virus (MSV-124) transforming gene function was investigated. Recombinant plasmids containing a single LTR positioned 3' of v-mos were subjected to sequential deletions, and the relative transforming efficiency of these recombinants was analyzed in the NIH/3T3 transfection assay. Recombinants lacking

CAAT, TATA, and poly(A) signals within the LTR were able to transform with an efficiency comparable to that of the wild-type MSV-124 genome. Deletion of one of the two 74-bp tandem repeat units within the LTR did not abolish v-mos gene function, whereas removal of both 74-bp repeat units completely eliminated transforming activity. The addition of a fragment containing only a single 74-bp unit and 29-bp downstream sequences derived from the LTR to a position 3' of v-mos led to efficient activation of v-mos transforming function. Residual potentiating activity for v-mos expression was retained even when the distance between v-mos and the 3' LTR was increased by several kbp. All these findings are consistent with the concept that the potentiating action of the LTR in its 3' position is due to activator/enhancer sequences localized to one of its 74-bp repeats. A permuted MSV-124 molecule, whose single LTR was localized 5' of v-mos, was very inefficient at transformation. However, its transforming activity could be increased by approximately 1000-fold by tandemization of the molecule. These results suggest that the transcript for the MSV-124 transforming gene is not normally initiated within the 5' LTR, but instead utilizes promoter signals in close proximity to v-mos and enhancer elements localized in the 3' LTR.

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

In collaboration with scientists in the Laboratory of Tumor Immunology and Biology, NCI, we investigated a new class of human endogenous oncoviral genomes. This class is characterized by extensive sequence homology with type A, B, D and avian type C oncoviral pol genes, weak homology with the type A oncoviral env gene and type D oncoviral LTR sequences, and no detectable homology with mammalian type C oncoviral genomes. This unusual pattern of sequence homology suggests that genetic interactions between different oncoviral genera contributed significantly to the evolution of the progenitor of this class of human oncoviral genomes. The relationship of MMTV env and LTR-related sequences in human cellular DNA to this family of human oncoviral genomes remains unresolved. One would suspect that human proviral genomes containing these sequences represent a subclass of this family. The role which these proviral genomes play in the etiology of human neoplasia is unknown. However, the reagents are now available to unambiguously approach this question.

Immunoglobulin synthesis and gene rearrangements in lymphoid cells transformed by replication-competent Rauscher murine leukemia virus (R-MuLV) were described. Lymphoid cells transformed by R-MuLV belonged to the B cell lineages. One group of cells exhibited Fc receptors but completely lacked immunoglobulin μ heavy and κ light chains. The majority of the cells resemble pre-B type. They displayed μ chains but κ chains were completely absent. Very rarely certain cells synthesized both μ and κ chains. Based on the presence of Fc receptors and IgM synthesis, the cells transformed by R-MuLV belonged to three

B cell developmental stages. These cells were tested for immunoglobulin gene rearrangements using J_H and C_K probes. DNA from cell lines without any detectable levels of IgM μ exhibited embryonic as well as rearranged J_H genes, whereas cells expressing IgM possess, in addition, productive and nonproductive light chain gene rearrangements. The most terminally differentiated cell possesses J_H and C_K rearrangement associated with the synthesis of μ and κ chains. Presumably the cells with rearranged J_H and C_K genes without immunoglobulin synthesis represent a developmental transition. We conclude that cells transformed by R-MuLV belonged to five step-wise compartments of B cell development. Our findings implicate definite sequential events of immunoglobulin gene rearrangement and expression during B cell development.

Molecular cloning of integrated caprine arthritis encephalitis virus (CAEV) was accomplished. A full length DNA clone of the exogenous retrovirus, CAEV, was isolated from high molecular weight DNA of CAEV-infected Himalayan tahr ovary cells. Although other restriction maps of CAEV have been published, this is the first time that the proviral DNA has been cloned. The restriction enzyme map of the clone was determined and found to be identical to that of unintegrated linear CAEV DNA. The cloned CAEV genome was shown to contain terminal repeats of approximately 450 base pairs in length, and its restriction enzyme map was oriented with respect to the direction of viral RNA transcription. When the cloned CAEV DNA was used as a molecular probe, it failed to detect related proviral sequences in the genomes of a variety of vertebrate species, including the goat, sheep, horse, mouse and man. When CAEV DNA was hybridized under relaxed conditions to a variety of cloned DNAs, representing different oncoviral genera, homology to mouse mammary tumor virus (MMTV) was observed, while no homology to avian type C or mammalian type A, C and D retroviruses was detected. This homology was localized to a region in MMTV corresponding to the 3' end of the *gag* gene and the 5' end of the *pol* gene.

Significance to Biomedical Research and the Program of the Institute:

The systems that are being intensively investigated have provided a much better understanding of the biology and biochemistry of malignant transformation. It is felt that a clear understanding of these phenomena will significantly speed progress in the search for causes of human cancer as well as mechanisms involved in neoplastic transformation of human cells.

Proposed Course:

To continue research already in progress in the following major areas: (1) mechanisms of action of mammalian sarcoma and leukemia viruses; (2) regulation and functions of endogenous retroviral sequences in mammalian cells; (3) determination of the role of human homologues of retroviral transforming genes in human neoplasia; and (4) application of basic research advances to the investigation of mechanisms involved in malignant transformation of human cells.

Publications:

Aaronson, S. A.: Transforming genes of retroviruses and human cancer cells. In Fortner, J. G. and Rhoads, J. E. (Eds.): *Accomplishments in Cancer Research, 1983*. Philadelphia, J. B. Lippincott Company, 1984, pp. 139-150.

Aaronson, S. A., Frati, L., and Verna, R. (Eds.): Genetic and Phenotypic Markers of Tumors. New York, Plenum Press, 1984, 379 pp.

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Yaniv, A., Dahlberg, J. E., Tronick, S. R., Chiu, I.-M. and Aaronson, S. A.: Molecular cloning of integrated caprine arthritis encephalitis virus. Virology (In Press)

Yuasa, Y., Eva, A., Kraus, M. H., Srivastava, S. K., Needleman, S. W., Pierce, J. H., Rhim, J. S., Gol, R., Reddy, E. P., Tronick, S. R. and Aaronson, S. A: Ras-related oncogenes of human tumors. In Vande Woude, G. F., Levine, A. J., Topp, W. C. and Watson, J. D. (Eds.): Cancer Cell 2/ Oncogenes and Viral Genes. New York, Cold Spring Harbor Laboratory, 1984, pp. 433-439.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04941-13 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Biochemical Characterization of Retroviruses and onc Genes

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

PI:	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NCI
	A. Gazit	Visiting Fellow	LCMB	NCI
	G. Vecchio	Expert	LCMB	NCI
	A. Eva	Visiting Associate	LCMB	NCI

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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.)

Molecularly cloned human sequences related to the tyrosine-specific protein kinase domain of the transforming gene of Gardner-Rasheed feline sarcoma virus (GR-FeSV) were shown to represent the c-fgr (human) proto-oncogene. Restriction endonuclease analysis of human DNA revealed that c-fgr (human) was distinct from sequences homologous to the oncogenes v-yes and v-src which encode a product closely related in predicted amino acid sequence to that of v-fgr. The c-fgr (human) proto-oncogene was localized to human chromosome 1 bands p36.1-36.3. The c-fgr gene was shown to be expressed in certain normal human tissues. In order to study the molecular pathogenesis of lentivirus diseases of mammals, the genome of caprine arthritis and encephalitis (CAEV) virus was molecularly cloned and characterized. Southern analyses delineated the genetic relationship of CAEV to other retroviruses and also indicated that a variety of mammalian species do not harbor sequences homologous to CAEV within their genomes. The in situ chromosome hybridization technique was used to map the sites of residence of H-ras, K-ras, and N-ras within the human genome in greater detail. A survey of human urinary tract tumors provided information regarding the frequency of the occurrence of activated ras oncogenes and their associated molecular lesions. Human DNA sequences corresponding to a new human oncogene isolated by A. Eva were recovered from a library of normal fetal liver DNA. These sequences are being characterized structurally in order to determine the mechanism of activation of this gene during the neoplastic process.

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
K. C. Robbins	Expert	LCMB	NCI
A. Gazit	Visiting Fellow	LCMB	NCI
A. Eva	Visiting Associate	LCMB	NCI
G. Vecchio	Expert	LCMB	NCI
J. E. Dahlberg	Research Microbiologist	LCMB	NCI
S.-C. Cheah	Medical Staff Fellow	LCMB	NCI
R. Callahan	Chief, Oncogenetics Section	LTIB	NCI
J. Schlom	Chief	LTIB	NCI
N. C. Popescu	Research Microbiologist	LB	NCI

Objectives:

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

Methods Employed:

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

Major Findings:

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

A full length DNA clone of the exogenous retrovirus, caprine arthritis encephalitis virus (CAEV), was isolated from high molecular weight DNA of CAEV-infected Himalayan tahr ovary cells. Although other restriction maps of CAEV have been published, this is the first time that the proviral DNA has been cloned. The restriction enzyme map of the clone was determined and found to be identical to that of unintegrated linear CAEV DNA. The cloned CAEV genome was shown to contain terminal repeats of approximately 450 base pairs in length, and its restriction enzyme map was oriented with respect to the direction of viral RNA transcription. When the cloned CAEV DNA was used as a molecular probe, it failed to detect related proviral sequences in the genomes of a variety of vertebrate species, including the goat, sheep, horse, mouse and man. When CAEV DNA was hybridized under relaxed conditions to a variety of cloned DNAs, representing different oncoviral genera, homology to mouse mammary tumor virus (MMTV) was observed, while no homology to avian type C or mammalian type A, C and D retroviruses was detected. This homology was localized to a region in MMTV corresponding to the 3' end of the gag gene and the 5' end of the pol gene.

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

A large series of urinary tract tumors were surveyed for ras oncogenes by DNA transfection and by molecular genetic analysis to determine their frequency in urothelial cells. H-ras oncogenes were detected in 2 of 38 tumors by transfection, molecularly cloned in biologically active form and shown to contain single base changes at codon 61 leading to substitutions of arginine and leucine, respectively, for glutamine at this position. One additional H-ras oncogene was identified in a bladder carcinoma by restriction polymorphisms at codon 12. In one of 21 tumors, a 40-fold amplification of the K-ras gene was observed. No amplification of other ras genes was detected in any of the tumors analyzed. These findings strengthen the conclusion that codons 12 and 61 are the major "hot spots" of ras oncogene activation and suggest that quantitative alterations in expression due to gene amplification may provide an alternative mechanism for ras gene activation in primary human tumors.

We determined the nucleotide sequence of the v-H-ras-related oncogene of BALB/c murine sarcoma virus. This oncogene contains an open reading frame of 189 amino acids that initiates and terminates entirely within the mouse cell-derived ras sequence. The protein encoded by this open reading frame matches the sequence predicted for the T24 human bladder carcinoma oncogene product, p21, in all but two positions. The presence of a lysine residue in position 12 of BALB/c murine sarcoma virus p21 likely accounts for its oncogenic properties.

Significance to Biomedical Research and the Program of the Institute:

Highly sensitive and specific biochemical probes for retroviral gene products and cellular onc genes generated in our studies have been extremely valuable in order to demonstrate the possible etiologic involvement of these viral and cellular genes in human cancers and to study the role that retroviral gene products and cellular onc genes may play in normal cellular functions. The availability of molecularly cloned human gene fragments related to viral onc genes has greatly facilitated studies on the role these sequences play in the causation of human cancer.

Proposed Course:

A detailed biochemical and biological analysis of molecularly cloned human DNA analogues of retroviral onc genes will continue.

Publications:

Aaronson, S. A., Robbins, K. C. and Tronick, S. R.: Human proto-oncogenes, growth factors, and cancer. In Ford, R. J. and Maizel, A. L. (Eds.): Mediators in Cell Growth and Differentiation. New York, Raven Press, 1985, pp. 241-255.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04951-09 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Molecular Characterization of Retroviruses

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

PI:	J. S. Dahlberg	Research Microbiologist	LCMB	NCI
Others:	D. V. Ablashi	Research Microbiologist	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

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Tel Aviv University (A. Yaniv); Hebrew University, Rehovot, Israel (K. Perk); U. California School of Veterinary Medicine, Davis, CA (N. East, G. Thielen); Dept. Pathology, Colorado State U., Fort Collins, CO (J. DeMartini).

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NCI, NIH, Bethesda, Maryland 20205

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

Lentiviruses, a subfamily of retroviruses that cause slow viral diseases in domestic animals, are being intensively analyzed by immunological techniques and by molecular cloning technology. We have recently obtained molecular clones of equine infectious anemia virus (EIAV) and demonstrated that this virus is relatively closely related to the prototype caprine lentivirus, CAEV, previously cloned in this laboratory. Nucleotide sequence analysis of the 5' end of the pol genes of both EIAV and CAEV revealed that this highly conserved region is closely related to the corresponding region of HTLV-III, demonstrating that the causative agent of acquired immunodeficiency syndrome (AIDS) and these lentiviruses are evolved from a common progenitor. Currently, immunoassays and candidate vaccines are being developed to facilitate early diagnosis of infected animals and to determine if these important diseases (and models of AIDS) can be controlled.

Analysis of the sera of individuals who handle squirrel monkeys who harbor the oncogenic Herpesvirus saimiri (HVS) revealed the important finding that about 7% of this group were positive for antibodies to the virus. Analysis of these sera, and additional experiments which examined the capacity of HVS to replicate in human cells cultured in vitro, suggest that either HVS does not replicate in vivo or does so by a mechanism inconsistent with what is seen in vitro. Nevertheless, it seems prudent to suggest that the guidelines recommended for class II oncogenic viruses continue to be followed.

PROJECT DESCRIPTION

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

J. E. Dahlberg	Research Microbiologist	LCMB	NCI
D. V. Ablashi	Research Microbiologist	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

1. To understand the molecular biology of lentiviruses and other retroviruses associated with slow diseases of domestic animals and man; the use of molecular techniques to analyze mechanism of pathogenesis and to develop vaccines capable of controlling disease.

2. To use molecular techniques, particularly monoclonal antibodies, to analyze the replication of transforming viruses and cellular and viral *onc* gene products.

Methods Employed:

The methodology required to develop monoclonal antibody-producing hybridoma cell lines includes maintaining mycoplasma-free cell lines, cell fusion techniques, preparation of thymocyte conditioned medium, single cell cloning procedures, and a variety of immunological assays to screen for antibody-producing cells. These include solid phase immunoassays, ELISA tests, and immunoprecipitation followed by analysis on polyacrylamide gels.

The lentiviruses are grown in 2-8 liter batches and purified by a combination of ultrafiltration and isopycnic gradient centrifugation. Purified virus is used both as a source of protein for purification of virion proteins in order to develop intraspecies and interspecies radioimmunoassays and as a source of genomic RNA to allow preparation of cDNA probes to permit cloning of proviral DNA from infected cells.

Major Findings:

1. The lentiviruses are a subgroup of the retroviruses which are principally detected in domesticated ungulates. They are exogenous viruses which cause a significant amount of morbidity and mortality in herds of sheep, goats and horses on a worldwide basis. Recently, a retrovirus isolated by French scientists from humans with acquired immunodeficiency disease syndrome (AIDS) has been shown to be immunologically related to a lentivirus, equine infectious anemia virus (EIAV). We have been interested in molecular characterization of EIAV and a goat virus, caprine arthritis-encephalitis virus (CAEV), and comparing these viruses to the prototype visna virus of sheep. Visna and the closely related progressive pneumonia virus (PPV), also of sheep, are excellent models of slow virus disease development, and we are interested in developing reagents and techniques which will aid in understanding how these diseases develop.

Within the past year, a full length molecular clone of CAEV has been further characterized and compared to unintegrated linear DNA obtained from acutely infected cells. Although we have been able to demonstrate biological activity, the restriction map of one CAEV molecular clone is identical to that of the integrated linear viral DNA, except that the clone contains cellular flanking sequences, suggesting that any defect present in the clone must be relatively minor. The presence of flanking sequences in this clone constitutes the first direct proof that lentiviruses are able to integrate into the host cellular genome. More recently, several molecular clones of EIAV have been obtained and they are currently being characterized. When the CAEV and EIAV cloned DNAs are hybridized to each other and to other restriction enzyme digested retroviral genomes under stringent and relaxed conditions, we observed that significant homology exists between the gag and pol genes of these two viruses. This verifies the immunological cross reactivity observed in this laboratory. A limited homology was detected between a portion of the murine mammary tumor virus (MMTV) genome encompassing the 3' end of the gag gene and the 5' end of the pol gene, and the comparable regions of both CAEV and EIAV. These hybridizing regions of CAEV and EIAV were sequenced and the derived amino acid sequences, representing the 5' end of the reverse transcriptase gene, compared to those already published of the major groups of retroviruses. This analysis revealed that EIAV and the causative agent of AIDS, HTLV-III, are relatively closely related to each other and to CAEV, the prototype caprine lentivirus. Furthermore, this group of retroviruses is more distantly related to other groups of retroviruses, indicating that HTLV-III and the lentiviruses have evolved from a common progenitor. This observation is supported by our recent sequence data which indicates that the LTR of CAEV shares a number of structural features with that of HTLV-III but not other retroviruses, and by Western blot analysis of the structural proteins of HTLV-III and lentiviruses, which revealed that antisera to CAEV and EIAV recognize HTLV-III gag proteins.

2. During the past year, this laboratory has been collaborating with officials from NASA involving an assessment of risk to astronauts flying with squirrel monkeys in space. Squirrel monkeys harbor a virus, *Herpesvirus saimiri*, which, while nononcogenic in its natural host, is capable of inducing T cell lymphomas in heterologous hosts such as marmosets, owl and spider monkeys, and rabbits. Since it has been classified by NCI as a class II oncogenic virus, and has been shown to replicate to a limited extent in human cells in vitro, a survey of the sera of 150 long-term handlers of squirrel monkeys was initiated. Although a substantial body of epidemiological data fails to indicate that people exposed to squirrel monkeys are at risk for cancer or any other disease, it was considered essential to determine if humans could seroconvert as a result of exposure to squirrel monkeys.

   Our results, using both immunofluorescence and immunoprecipitation techniques, demonstrate for the first time that a low but significant fraction (7.3%) of individuals who handle squirrel monkeys on a daily basis have antibodies to the major structural proteins of HVS. By comparison of the reactivities of the human sera with the specificity of various

monoclonal antibodies previously isolated by us, it was possible to identify the antigens recognized by human sera as being the three major glycoproteins of the virus and the major capsid antigen. The human sera were negative for antibodies to early antigens (EA), and their reactivities to HVS could be blocked by preincubation with purified virus. This contrasts with the results seen with other heterologous hosts, such as the owl monkey, where the sera are EA-positive and contain reactivities to nonstructural virion proteins which are not blocked by purified virus, as well as reactivities that are blocked.

To further understanding of these results, some experiments were undertaken to analyze the molecular events occurring when human cells are infected with HVS. We were able to show by immunofluorescence that both EA and late antigens (LA) are made in acutely infected lymphoid and nonlymphoid human cells. When the level of EA and LA antigens in human fibroblasts and owl monkey kidney cells at comparable stages of infection (cytopathic effect) were compared by immunoprecipitation, it was shown that the level and type of EA antigens were very similar. Analysis of the LA antigens, on the other hand, revealed that while the level of some of the LA antigens was equivalent in the human and monkey cells, the human cell extracts contained no detectable glycoprotein or capsid protein. The absence in the human cells of the very proteins which induce the major reactivities seen in the human sera was unexpected. A possible explanation could be that HVS does not replicate effectively in vivo and that the observed reactivities in human sera result from an immunization occurring from chronic exposure to virus. Alternatively, our in vitro results may not accurately reflect what can occur in vivo, particularly if the virus replicates more efficiently in other cell types. We are currently investigating the replication of HVS in a wider range of human lymphoid and nonlymphoid cell types.

Significance to Biomedical Research and the Program of the Institute:

1. Lentiviruses are retroviruses which cause widespread disease and may represent a reservoir from which human viruses such as HTLV-III may have originated. Our work with these agents will lead to increased understanding of how slow viral diseases originate and progress, and may represent important models for developing and testing retroviral vaccines relevant to the control of human disease.

2. Earlier work with a large group of monoclonal antibodies to the structural proteins of the oncogenic HVS has unexpectedly facilitated our analysis of the semipermissive replication of HVS in human cells and adds to our ability to interpret the newly described presence of antibodies to HVS in human sera. This observation emphasizes the need to further study, at the molecular level, a primate herpesvirus which causes malignant lymphoma in every other heterologous host studied so far in which the virus has been shown to replicate.

Proposed Course:

1. The major objectives with regard to lentivirus research are to develop sensitive diagnostic assays, to facilitate assessment of the status of animals both for the purpose of disease control and for monitoring biological and vaccine experiments. We are currently developing ELISA

tests to supplement our radioimmunoassays, since the ELISA is more rapid and can be more readily carried out by collaborators elsewhere. Nucleotide sequence analysis of our cloned lentiviruses is a prerequisite to the development of mammalian expression vectors, such as a recombinant vaccine, which can be tested for their ability to protect animals. The inability of CAEV to normally induce neutralizing antibody makes it an ideal model for testing adjuvants for efficiency. This work has great relevance to HTLV-III research, since the AIDS virus also fails to induce neutralizing antibody.

2. Continued analysis of the events occurring during infection of human cells by HVS will continue. It is important to determine the degree of permissiveness that a variety of human cells have for virus replication in order to more fully understand the mechanism by which a significant fraction of individuals having frequent contact with squirrel monkeys manufacture antibodies to the major structural proteins of HVS. The panel of monoclonal antibodies previously developed by this laboratory will greatly facilitate this analysis.

Publications:

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

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Dahlberg, J. E., Zintz, E. and Ablashi, D. V.: Analysis of Herpesvirus saimiri structural proteins with monoclonal antibodies. J. Virol. 53: 279-286, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04976-08 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Carcinogenesis of Mammalian Cells in Culture

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

PI:	K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
Others:	R. Gantt	Research Chemist	LCMB	NCI
	R. E. Tarone	Mathematical Statistician	BB	NCI
	J. S. Rhim	Research Microbiologist	LCMB	NCI
	M. H. Greene	Epidemiologist	EEB	NCI
	M. Potter	Chief	LG	NCI
	J. H. Robbins	Dermatologist	D	NCI

COOPERATING UNITS (if any)

Howard U. College of Medicine (R. Parshad); College of Medicine, U. California (E. Stanbridge); Johns Hopkins U. School of Medicine (J. K. Frost)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.25	1.25	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.)

Cultures of skin fibroblasts from normal and cancer-prone individuals, as well as neoplastic cells transformed in culture or in vivo, are utilized in evaluating the relationship between radiation-induced chromosomal DNA damage, deficient DNA repair, cancer susceptibility and malignant neoplastic transformation. An increased incidence of chromatid damage after x-irradiation during GS phase of the cell cycle is associated with both a predisposition to cancer and malignant transformation and may provide the basis for a test for cancer susceptibility. A genetic basis for this radiosensitivity is indicated from studies with somatic cell hybrids between normal and malignant cells. The chromosomal radiosensitivity appears to result from deficient DNA repair during G2. Another aspect of this project is to develop a reproducible transformation system with human epidermal keratinocytes as an in vitro model for following the progression of biologic and biochemical changes leading to neoplastic transformation. An associated problem is to identify cytomorphologic changes diagnostic of neoplastic transformation to facilitate transfection and transformation studies. Efforts are in progress to develop a biochemical correlate of enhanced G2 chromosomal radiosensitivity to provide a more rapid assay for cancer susceptibility and to understand the DNA repair deficiencies of cancer-prone individuals.

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
R. Gantt	Research Chemist	LCMB	NCI
R. E. Tarone	Mathematical Statistician	BB	NCI
J. S. Rhim	Research Microbiologist	LCMB	NCI
M. H. Greene	Epidemiologist	EEB	NCI
M. Potter	Chief	LG	NCI
J. H. Robbins	Dermatologist	D	NCI

Objectives:

The objective of this project is to elucidate, through cell culture studies, mechanisms of neoplastic transformation in human cells. Current emphasis is on the relationship between radiation-induced chromosomal DNA damage, deficient DNA repair, cancer susceptibility and malignant transformation. Efforts are also directed toward developing a transformation system with human epidermal keratinocytes as an in vitro model of human cell carcinogenesis for characterizing changes in DNA repair capacity during neoplastic transformation. Additionally, efforts are in progress (1) to identify cytomorphologic markers of neoplastic transformation for facilitating transfection and transformation studies, and (2) to develop a biochemical correlate of enhanced G_2 chromosomal radiosensitivity to provide a more rapid assay for cancer susceptibility and to understand the DNA repair deficiencies of cancer-prone individuals.

Methods Employed:

Chromatid breaks, gaps, and interchanges following low level x-irradiation (25, 50, 100 R) or 2- to 5-hour exposure to low intensity fluorescent light (effective wavelength 405 nm in visible range) are quantified in cells grown on coverslips in sealed Leighton tubes and processed in situ following experimental treatment. DNA repair inhibitors, caffeine and cytosine arabinoside, are used following irradiation to analyze mechanisms. Since repair of lesions is influenced by the stage of the cell cycle and since the chromatid damage is scored in metaphase cells only, the stage of the cell cycle at the time of irradiation can be experimentally manipulated by varying the interval from treatment to fixation of cells.

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

Efforts to devise methods for comparison of DNA repair capacities during G_2 phase in normal and transformed epidermal keratinocytes by biochemical methods will be actively pursued.

Major Findings:

Major new findings reported in this year's publications follow:

Apparently normal skin fibroblasts from individuals with several genetic disorders predisposing to a high risk of cancer, ataxia telangiectasia, Gardner syndrome, Fanconi anemia, Bloom syndrome, xeroderma pigmentosum variant and groups C and E, all showed a significantly higher incidence of light-induced chromatid breaks than skin fibroblasts from normal donors if exposed to cool-white fluorescent light during late S-G_2 phase. However, no consistent difference was observed if exposed during G_1 phase. Xeroderma pigmentosum group A cells did not show the late S-G_2 enhanced chromosomal radiosensitivity. These cells are deficient in repair of DNA damage requiring endonucleolytic incision. This observation implicates endonuclease incision of DNA as a requirement for chromatid break formation after late S-G_2 light exposure. The responses of these mutant human cells to visible light mimic those observed with low level x-irradiation described previously. This similarity is not surprising since both agents generate intracellular ·OH, ionizing radiation by radiolysis of H_2O and visible light through reaction of the photoproduct H_2O_2 with reduced transition metals in cells or medium.

The generation of H_2O_2 and the derivative free hydroxyl radical (·OH) in cultures of mouse cells grown in the presence of visible light and ambient oxygen was shown previously to be implicated in chromatid damage. Furthermore, chromosome alterations appear to be associated with the spontaneous neoplastic transformation of mouse cells in culture. An attempt was made to reduce the incidence of chromosomal aberrations and delay or prevent the onset of spontaneous neoplastic transformation of freshly isolated mouse cells, both fibroblasts and epidermal keratinocytes, by adding catalase to the culture medium, shielding the cultures from wavelengths <500 nm, and providing a gas phase of 0-1% O_2. These conditions significantly decreased the incidence of chromosomal aberrations in both cell types, prevented their tumorigenicity in non-irradiated syngeneic mice, and increased latent periods for tumor development in x-irradiated mice. The epidermal keratinocytes were particularly resistant to spontaneous neoplastic transformation under all conditions tested. These observations on the protective effect of extracellular catalase suggest that H_2O_2, a normal metabolite, and/or the derivative ·OH can directly or indirectly produce genetic damage and neoplastic transformation in mouse fibroblasts.

Skin fibroblasts from Gardner syndrome (GS) compared with those from normal donors showed a significantly higher incidence of chromatid gaps and breaks following exposure to low-intensity, cool-white fluorescent light during G_2 phase of the cell cycle. Considerable evidence supports the concept that chromatid gaps and breaks seen directly after exposure to DNA-damaging agents represent unrepaired DNA single- and double-strand breaks, respectively. The changes in incidence of chromatid aberrations with time after light exposure are consistent with the sequence of events known to follow DNA damage and repair. Initially, the incidence of light-induced chromatid gaps was equivalent in GS and normal fibroblasts. In the normal cells, the chromatid gaps disappeared by one hour post-exposure, presumably as a result of efficient repair of DNA single-strand breaks. In contrast, the incidence of gaps increased in GS cells by 0.5 hour, followed by a decrease at one hour and concomitant increase in chromatid breaks. It appears from these findings

that the increased incidence of chromatid damage in GS fibroblasts results from deficient repair of DNA single-strand breaks which arise from incomplete nucleotide excision of DNA damage during G_2 phase.

Epidermal keratinocytes acquired indefinite lifespan in culture but did not undergo malignant conversion in response to infection with a hybrid of adenovirus 12 and simian virus 40. Addition of Kirsten murine sarcoma virus, which contains a K-ras oncogene, to these cells induced morphological alterations associated with the acquisition of neoplastic properties. These findings demonstrate the malignant transformation of human primary epithelial cells in culture and support a multiple-step process for neoplastic conversion.

Cell lines derived from human tumors of diverse tissue origin and histopathology were compared with lines of normal skin fibroblasts with respect to chromatid damage induced by x-irradiation during G_2 period of the cell cycle. Only cells in metaphase were examined, and these had been irradiated 1.5 hours before fixation. When irradiated under identical conditions, the tumor cells showed significantly more chromatid breaks and gaps than did the normal cells at all doses tested. The data suggest that the increased G_2 chromosomal radiosensitivity of the tumor cells is associated with deficient DNA repair during the G_2 period of the cell cycle.

Lines of skin fibroblasts from individuals heterozygous for ataxia telangiectasia, compared with six lines from age-matched normal controls, show a much higher frequency of chromatid breaks and gaps following x-irradiation during the G_2 phase of the cell cycle. The magnitude of this difference suggests that G_2 chromatid radiosensitivity could provide the basis for an assay to detect ataxia telangiectasia heterozygotes. Though clinically normal, ataxia telangiectasia heterozygotes share a high risk of cancer with ataxia telangiectasia homozygotes, constitute approximately 1% of the human population, and contribute to about 5% of all cancers. Further, we propose that G_2 chromosomal radiosensitivity, which appears to result from a DNA repair deficiency, may be associated with a genetic predisposition to cancer.

Apparently normal skin fibroblasts from individuals with familial cancer, i.e., from families with a history of neoplastic disease, exhibit enhanced G_2 chromosomal radiosensitivity. This appears to be the first report of a common defect in individuals with diverse forms of neoplastic disease. Since the cell lines were mostly from relatively young members of families having a high incidence of cancer, they were probably all individuals genetically susceptible and at high cancer risk. Thus, G_2 chromatid radiosensitivity appears to be associated with both a genetic predisposition to cancer as well as malignant neoplastic state. Furthermore, enhanced G_2 chromosomal radiosensitivity may provide the basis for an assay to detect genetic susceptibility to cancer.

Significance to Biomedical Research and the Program of the Institute:

Enhanced G_2 chromosomal radiosensitivity characterizes cells transformed in culture, cells derived from human tumors of diverse tissue origin and histopathology, skin fibroblasts from cancer-prone individuals and skin fibroblasts of individuals from high cancer families. This enhanced radiosensitivity appears, therefore, to be associated with both a genetic predisposition to cancer and neoplastic transformation. Certain observations suggest that it

results from deficient DNA repair during G_2 and that it has a genetic basis. This discovery may have a significant impact on clinical aspects of cancer as well as on our understanding of fundamental mechanism(s) in the etiology of cancer. The study promises to aid efforts to elucidate the mechanism of cancer susceptibility in members of high-risk cancer families. The results clearly provide the basis for a test for genetic susceptibility to cancer. Currently we are adapting this assay procedure to samples of one to five ml of blood. Such a test would be of value in epidemiology studies, provide guidelines for cancer therapy with mutagenic agents such as radiation or chemotherapy that could further increase the risk of cancer, and provide means for cancer prevention through genetic counseling. It is possible, for instance, to distinguish carriers of genes for ataxia telangiectasia and hereditary cutaneous melanoma who are clinically normal individuals but at high cancer risk. The ataxia telangiectasia carriers constitute about 1% of the human population and contribute to about 5% of all cancers. Conceivably, many more genes for cancer susceptibility than those presently recognized will be identified in screening human populations with this assay because there is a strong possibility that a much larger fraction of the population is genetically susceptible to cancer than hitherto estimated.

In the realm of basic research on the mechanism(s) of etiology, this discovery appears to have the necessary properties ascribed to the "initiation step" in carcinogenesis. If further work continues to support this concept, the finding will be of extraordinary fundamental importance, since the molecular basis of "initiation" has been an enigma for years. Enhanced chromosomal radiosensitivity associated with deficient DNA repair during G_2 can also explain the heterogeneity and mutability of tumors that make therapy so difficult.

Rapid progress is greatly hampered by the technical skill and tedium involved in quantifying chromatid breaks and gaps in mitotic cells. Therefore, current efforts are directed toward developing a rapid biochemical assay. Such an assay would further promote analysis of the DNA repair deficiency(ies) and encourage other laboratories to develop and extend these studies to identify the relevant lesions in DNA repair pathways during G_2 phase and their mutant genes.

Proposed Course:

The following studies in progress will be continued:

An attempt to determine whether G_2 chromosomal radiosensitivity is inherited as a Mendelian characteristic. Through the use of cells from inbred and congenic mouse strains, it may be possible to identify the chromosomes or genes responsible for susceptibility or resistance.

Biochemical studies on G_2 phase cell populations to correlate these cytogenetic observations with measures of deficient DNA repair.

Attempts to extend our observations to peripheral lymphocytes to develop a practical and rapid assay for cancer susceptibility.

Study of a kindred, some members of which have hereditary cutaneous malignant melanoma or its precursor, dysplastic nevus syndrome, to correlate G_2 chromosomal radiosensitivity with predisposition to this cancer, which may not appear until late in life.

Studies of cells from patients with other genetic disorders predisposing to cancer or degenerative neurologic disorders.

Cytogenetic and biochemical studies to understand the molecular mechanisms underlying radiation-induced chromatid gaps and breaks.

Studies of cells, fibroblasts or lymphocytes, from cancer patients of families known to be relatively free of cancer to determine whether G_2 chromosomal radiosensitivity is limited to individuals from cancer-prone families.

In view of the successful transformation of human epidermal keratinocytes with adenovirus 12-simian virus 40 hybrid virus and superinfection with Kirsten murine sarcoma virus, we will continue efforts to cotransfect these cells with pSV3 neo (containing the origin of simian virus 40 and information for large and small T antigens) and the cloned transforming region from Kirsten murine sarcoma virus.

Because of the reported role of oxygen-derived free radicals and anions in DNA damage and carcinogenesis, an O_2^- (superoxide radical) generating system (xanthine, xanthine oxidase) will be evaluated for administering chronic damage to proliferating human keratinocytes in an effort to transform these cells. Dosage will be determined in short-term studies by measuring DNA and chromosome damage.

Efforts to devise methods for comparison of DNA repair capacities during G_2 phase in normal and transformed epidermal keratinocytes by biochemical methods will be actively pursued.

Publications:

Evans, V. J. and Sanford, K. K.: Tissue culture 1. The Tissue Culture Section, NCI. In Stetten, Jr. and Carrigan, W. T. (Eds.): An Account of Research in its Laboratories and Clinics. New York, Academic Press, Inc., 1984, pp. 88-98.

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Parshad, R., Gantt, R., Sanford, K. K. and Jones, G. M.: Chromosomal radiosensitivity of human tumor cells during the G_2 cell cycle period. Cancer Res. 44: 5577-5582, 1984.

Parshad, R., Sanford, K. K. and Jones, G. M.: Chromatid damage induced by fluorescent light during G_2 phases in normal and Gardner syndrome fibroblasts. Interpretation in terms of deficient DNA repair. Mutat. Res. (In Press)

Parshad, R., Sanford, K. K. and Jones, G. M.: Chromosomal radiosensitivity during the G_2 cell cycle period of skin fibroblasts from individuals with familial cancer. Proc. Natl. Acad. Sci. USA (In Press)

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Sanford, K. K., Parshad, R. and Gantt, R.: Enhanced G_2 chromosomal radiosensitivity, susceptibility to cancer and neoplastic transformation. In Skamene, E. (Ed.): Proceedings of the Second International Symposium on Genetic Control of Host Resistance to Infection and Malignancy. New York, Alan R. Liss, Inc. (In Press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04977-08 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

DNA Damage, Repair, and Neoplastic Conversion in Cultured Mouse and Human Cells

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

PI:	R. R. Gantt	Research Chemist	LCMB	NCI
Others:	K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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.)

Two aspects of DNA damage and repair associated with malignant transformation are being studied in mouse cells and human fibroblasts, including cells from xeroderma pigmentosum (XP) patients and normal individuals. First, the biochemical mechanism responsible for the enhanced G_2 radiosensitivity which characterizes the cultured fibroblasts of cancer-prone individuals and the malignant state is being investigated. To date, experiments measuring the kinetics of DNA strand-break repair of newly synthesized DNA from cells enriched in the G_2 phase indicate that G_2 radiosensitive cells either repair x-ray-induced DNA strand breaks more slowly than normal cells, or they acquire DNA strand breaks more rapidly than normal cells. These results were found comparing normal KD fibroblasts with the transformed derivative, Hut 24, and with glioblastoma cells, Wilm's tumor cells, and nontumorigenic fibroblasts from a retinoblastoma patient versus normal human skin fibroblasts. The second aspect of DNA damage and repair concerns the mechanisms involved in repair of DNA-protein cross-links induced with <u>trans</u>-platinum(II) diaminedichloride. Numerous and varied carcinogenic agents induce DNA-protein cross-links, and, since they have been reported to lead to transformation of 3T3 and 10T-1/2 mouse cells, their mechanisms of repair and possible deficiencies are important. We previously showed that these lesions were repaired by a pathway in addition to the nucleotide excision mechanism and that cell cycling is necessary for activation of this pathway. We have now shown that not only is DNA replication, per se, not required for repair but that "loosening up" of the chromatin, one consequence of DNA replication events, is not associated with repair in either of two XP group A cell lines (XP12BE, XP20S) or in mouse L1210 cells.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

R. R. Gantt	Research Chemist	LCMB	NCI
K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI

Objectives:

To identify primary changes in cellular and viral nucleic acids during chemical, viral, and radiation carcinogenesis and to develop and apply techniques for assaying the repair responses of the cells. The use of human epithelial cells is emphasized where technically feasible. The study of the formation and mechanism of repair of DNA strand breaks in human cells which are more susceptible to x-irradiation during the G_2 phase of the cell cycle than normal cells and the study of the induction, consequences, and repair of DNA-protein cross-links is currently stressed.

Methods Employed:

The standard laboratory techniques for measuring single- and double-strand DNA breaks, repair synthesis, base damage, base adducts, and cross-links are used with the usual adaptations and innovations. The conditions chosen for these studies minimize cell toxicity to increase the relevance of the data to conditions of chronic exposure and to allow the cells as large a range of response as possible (e.g., the ability to proliferate).

Major Findings:

Repair deficiency in G_2 phase of human cells susceptible to cancer. Our earlier demonstration that fibroblasts from patients susceptible to cancer manifest an increase in chromatid aberrations from x-irradiation in the late S-G_2 phase of the cell cycle strongly suggests a DNA repair deficiency in the susceptible cells. We have now discovered in at least five cases that, under experimental conditions which enrich G_2 phase cells, there appears to be a significant difference in repair of DNA strand breaks between G_2 susceptible and normal cells. These comparisons include KD cells versus the transformed derivative, Hut 14, Wilm's tumor cells versus a normal human foreskin fibroblast, a glioblastoma cell line and non-tumorigenic fibroblasts from a retinoblastoma patient versus a second normal human foreskin fibroblast and finally a human foreskin line that is susceptible to x-irradiation in the G_2 phase versus a third normal foreskin.

Repair of DNA-protein cross-links. The only known way to repair bulky DNA adducts is by the nucleotide excision pathway. It has been reported, as expected, that excision-deficient XP12BE, a xeroderma pigmentosum (XP), group A, cell line is deficient in repair of DNA-protein cross-links. However, we previously have found that eventually XP12BE, as well as other XP groups including C, D, E, and the XP variant, are able to repair completely the DNA-protein cross-links induced by 20 μM trans-platinum. Since the excision capacity of the group A cells has been estimated at less than 2% of normal cells, the eventual repair of DNA-protein cross-links suggests that another mechanism is

involved. Many laboratories have reported enhanced repair of DNA damage in cells which are synthesizing DNA. This finding implicates a coupling of repair to DNA replication and/or the necessity for cell cycling, but doesn't differentiate between a simple enhancement of nucleotide excision repair or the presence of a second pathway. A widely held view regarding the lesion for XP-A cells is that all the repair enzymes are present in the nucleotide excision pathway but that the XP cells are unable to "loosen" the chromatin to allow access of the repair enzymes to the lesion. Since XP-A cells are essentially devoid of nucleotide excision repair, and since DNA-protein cross-links are repaired by this mechanism, we tested the hypothesis that repair in XP-A cells takes place in association with DNA replication to the extent that preparation for replication events "loosened" the chromatin to allow access of the repair enzymes. Careful kinetic studies of DNA-protein cross-link repair and DNA replication provide strong evidence that replication of DNA per se is not the mechanism by which XP-A cells "loosen up" the chromatin or allow access to the lesions for repair. This is further evidence that an alternative pathway is utilized by nucleotide excision deficient cells for repair of these bulky adducts.

Significance to Biomedical Research and the Program of the Institute:

The finding that there apparently is a DNA repair deficiency associated with G_2 susceptibility to x-irradiation indicates that there may be a much larger number of people suceptible to cancer because of a DNA repair deficiency than previously thought. The characteristics of this lesion suggest it is an initiation step in the series of events leading to malignant transformation and may provide the means by which both tumor promotion and progression are manifested. (More information on these points: See Project Z01CP04976-08.) Another significant aspect of this biochemical difference is that it may lead to a simple reliable assay for detecting G_2 susceptibility instead of the arduous task of scoring chromatid gaps and breaks.

Reports of others show that DNA-protein cross-links (trans-platinum induced) increase sister chromatid exchanges and transform 3T3 and 10T-1/2 mouse cells, observations which indicate important perturbations of DNA. These observations, taken together with our finding that repair of the cross-links is by two pathways, one of which is cell-cycle dependent, have two important aspects. First, they suggest that DNA-protein cross-links (which are induced by a wide variety of carcinogens, including x-rays, light, and many chemicals such as benzopyrene, methylmethane sulfonate, AAF, etc.) may play a role in epigenetic events leading to malignant transformation. Second, DNA-protein cross-links may accumulate with time in noncycling cells of animals if repair is dependent solely on cycling. In animals, this accumulation would be expected to impair the function of organ systems containing significant numbers of nondividing cells, particularly at the level of mRNA production; a general decline of organ response would ensue.

Proposed Course:

1. Extend the present results to more paired cell lines to generalize the DNA strand repair deficiency.

2. Examine the possibility that deficient ADP-ribosylation is involved, as suggested by the role of 3-acetylaminobenzamide.

3. Determine whether the DNA strand-break repair involves single-strand breaks, double-strand breaks, or both.

4. Systematize and simplify the procedure so that it may be useful as an assay for people susceptible to cancer.

5. With regard to the DNA-protein cross-links:

   a. Attempt to establish unambiguously the existence of this proposed pathway for DNA-protein cross-link repair. Possible approaches:

      i. Develop an alternate assay, e.g., an antibody assay, so that synchronized cells can be studied despite the problem of DNA fragmentation.

      ii. Develop a cell-free system using an antibody assay.

   b. Isolate and characterize the cross-linked protein. Possible approaches:

      i. CsCl banding followed by nuclease digestion and gel electrophoresis analysis.

      ii. Use of antibody techniques.

   c. Determine whether other bulky adducts, such as AAF, can be repaired by a pathway other than nucleotide excision repair.

   d. Compare repair of DNA-protein cross-links from other agents with those induced by _trans_-platinum.

   e. Look for the accumulation of DNA-protein cross-links in animals as a function of age of "deterioration."

Publications:

Gantt, R., Stephens, E. V. and Davis, S. R.: Measurement of DNA-protein cross-links in mammalian cells without x-irradiation. _Ann. Biochem._ (In Press)

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Jones, G. M., Sanford, K. K., Parshad, R., Gantt, R., Price, F. M. and Tarone, R. E.: Influence of added catalase on chromosome stability and neoplastic transformation of mouse cells in culture. _Brit. J. Cancer_ (In Press)

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Sanford, K. K., Parshad, R. and Gantt, R.: Enhanced G_2 chromosomal radiosensitivity, susceptibility to cancer and neoplastic transformation. In Skamene, E. (Ed.): Proceedings of the Second International Symposium on Genetic Control of Host Resistance to Infection and Malignancy. New York, Alan R. Liss, Inc. (In Press)

Sanford, K. K., Parshad, R. and Gantt, R.: Responses of human cells in culture to hydrogen peroxide and related free radicals generated by visible light: relationship to cancer susceptibility. In Johnson, J. E., Jr. (Ed.): Free Radicals, Aging and Degenerative Diseases. New York, Alan R. Liss, Inc. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05060-07 LCMB

PERIOD COVERED

October 1, 1984 to Septembeer 30, 1985

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

Studies of Oncogenic Expression in Animal and Human Cancers

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

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

COOPERATING UNITS *(if any)*

Columbia University, College of Physicians & Surgeons, New York, NY (Borek, C.)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	1.5

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

Major findings were: (1) Malignant transformation of human primary epithelial cells in culture by the combined action of a DNA hybrid virus (adenovirus 12-SV40 [Ad12-SV40]) and a retrovirus (Kirsten murine sarcoma virus [Ki-MSV]), supporting a multistep process for neoplastic conversion. (2) The addition of thyroid hormone optimized transformation by Ki-MSV and was found to exert its effects during the early phase of Ki-MSV-induced transformation. (3) Hydrocortisone was found to enhance expression of Epstein Barr virus (EBV) genomes in human cells and led to increased levels of EBV antigen expression and virus production.

PROJECT DESCRIPTION

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

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

Objectives:

1. To develop sensitive in vitro transformation assays to identify carcinogenic agents and humans at high risk for cancer.

2. To develop and study human cell transformation systems, particularly epithelial cells, and the factors regulating cell transformation to elucidate mechanisms of cell transformation by carcinogenic agents and viruses.

3. To search for human and primate oncogenes from human and primate cancers.

4. To develop measures to prevent and/or control cell transformation and cancer in animals and ultimately in humans.

Methods Employed:

Biological methods include cell and virus cloning, transformation focus, cell aggregation, soft agar, genome rescue and transfection assays. Biochemical methods include reverse transcriptase assay, radioimmunoprecipitation, and I^{125} protein A assay.

Major Findings:

Development of an in vitro multistep model for human epithelial cell carcinogenesis. In studies to develop models in tissue culture for malignant transformation of cells by oncogenes, several investigators have reported that primary rodent fibroblasts can undergo neoplastic conversion in response to the combined actions of two transforming genes from viral or cellular oncogenes or from the transforming genes of DNA tumor viruses, but that one transforming gene by itself was not sufficient. However, there have been only a few investigations of the minimum number and nature of genes that could induce malignant transformation of human cells, particularly those of epithelial origin, in culture.

In this study, we used newly developed tissue culture methods to ascertain whether prototype RNA or DNA tumor viruses containing well-defined transforming genes could confer the malignant phenotype to human epithelial cells.

Primary human epidermal keratinocytes acquired indefinite lifespan in culture but did not undergo malignant conversion in response to infection with a hybrid of adenovirus 12 and simian virus 40 (Ad12-SV40). The Ad12-SV40-altered cells contained both SV40 large and small tumor antigens, but did not contain adenovirus early region (E1A and E1B) messages. Subsequent addition of Kirsten murine sarcoma virus (Ki-MSV), which contains a K-ras oncogene, to these cells induced striking morphological alterations associated with the acquisition of neoplastic properties. These findings demonstrate the malignant transformation of human primary epithelial cells in culture by the combined action of Ad12-SV40 and Ki-MSV and support a multiple-step process for neoplastic conversion.

Thyroid hormone modulation of transformation induced by Ki-MSV. The role of hormones at a cellular level during virus-induced neoplastic transformation is relatively unknown. Cell culture systems offer powerful tools to evaluate such questions in vitro. It has been reported that thyroid hormones are essentially permissive factors for the induction of transformation by x-rays and by chemical carcinogens in hamster embryo cells and the C3H/10T 1/2 cell line.

We have investigated the effect of triiodothyronine (T_3) on the transformation of normal rat kidney (NRK) cells by Ki-MSV. When NRK cells were grown and infected with Ki-MSV in medium lacking T_3, the yield of transformed foci was about one-half that observed in the cultures supplemented with T_3. Individual foci appeared somewhat later in cells grown out in medium devoid of T_3. The yield of Ki-MSV released from transformed NRK cells was lower when these cells were maintained in T_3-depleted medium. The results cannot be attributed to cell growth modification by T_3. Normal and Ki-MSV-transformed NRK cells grew equally well in monolayer culture in medium containing or lacking T_3. Selective maintenance and removal of T_3 during various phases of the transformation process indicated that T_3 exerted its maximum effect on transformation rates when added to the medium 24 hours prior to virus infection. T_3 was less effective in modulating transformation when added simultaneously with virus infection and was ineffective if added 24 hours after virus infection. The results indicate that thyroid hormone is a required factor for optimal transformation by Ki-MSV and that the hormone exerts its effects during the early phase of Ki-MSV-induced transformation.

Hydrocortisone enhancement of both Epstein Barr virus (EBV) replication and transformation of human cord lymphocytes. Certain hormones are known to influence carcinogenesis in vitro as well as in experimental animals. It has been reported that in mouse cells glucocorticoids (hydrocortisone and dexamethasone) enhanced the production of certain DNA and RNA tumor viruses, but did not promote the production of murine sarcoma virus (MSV). In contrast to the reported lack of effect of steroid hormones on MSV-induced transformation in mouse cells, we have found that glucocorticoids enhanced transformation of NRK and human cells induced by Ki-MSV. Successful cultivation of normal human nasopharyngeal carcinoma (NPC) epithelial cells up to 146 days has also been achieved when the culture medium contained hydrocortisone. These cells also could be subcultured up to 50 passages, thus providing a method for culturing NPC cells in quantities suitable for extensive experimental work with EBV.

We investigated the interaction of hydrocortisone and EBV. The treatment of P3HR-1 cells (propagated at 34°C and 37°C) with various concentrations of hydrocortisone for 7 and 21 days resulted in enhanced levels of antigen-positive cells with a maximum increase at 21 days. Virus harvested from hydrocortisone-treated P3HR-1 cells grown at 34°C had a 1- to 2-log higher titer in Raji cells when compared to control virus. Treatment of AG876 EBV-producer cells grown at 34°C with 5 and 10 µg/ml of hydrocortisone exhibited a 3- to 5-fold increase in virus capsid antigen-positive cells. When human cord blood mononuclear cells were infected with AG876 EBV and maintained in hydrocortisone, earlier transformation was observed.

These data suggest that hydrocortisone is able to enhance the expression of the EBV genomes present in human cells and leads to increased levels of antigen expression and virus production. The mechanism by which this glucocorticoid hormone modulates EBV expression remains to be determined.

Frequency of molecular alterations affecting ras proto-oncogenes in human urinary tract tumors. Cellular transforming genes have been identified in various human malignancies by DNA-mediated gene transfer using mouse NIH/3T3 cells as recipients. Molecular characterization of these transforming genes has revealed most to be members of the *ras* gene family. Three human *ras* oncogenes, H-*ras*, K-*ras* and N-*ras*, have been described, all of which encode closely related proteins of 189 amino acid residues, generically designated p21. Several *ras* oncogenes have been molecularly cloned in active form from human as well as animal tumors. DNA sequence analysis has indicated that these genes commonly acquire transforming activity by single point mutations affecting either the 12th or 61st amino acid residue of their p21 proteins.

Sporadic evidence indicates that about 10-20% of human tumors register as positive for *ras* oncogenes in the NIH/3T3 transfection assay. However, the actual incidence of *ras* oncogene activation in specific tumors remains to be defined. We have recently surveyed 23 primary human tumors of the bladder, ureter and renal pelvis by DNA transfection and found activated H-*ras* oncogenes in two cases. To determine further the frequency at which such genes might be involved in the neoplastic process affecting a specific target tissue, urothelial cells, we surveyed a large series of urinary tract tumors for *ras* oncogenes by DNA transfection and by molecular genetic analysis. H-*ras* oncogenes were detected in 2 of 38 tumors by transfection, molecularly cloned in biologically active form, and shown to contain single base changes at codon 61 leading to substitutions of arginine and leucine, respectively, for glutamine at this position. One additional H-*ras* oncogene was identified in a bladder carcinoma by restriction polymorphisms at codon 12. In one of 21 tumors, we observed a 40-fold amplification of the K-*ras* gene. No amplification of other *ras* genes was detected in any of the tumors analyzed. Our findings strengthen the conclusion that codons 12 and 61 are the major "hot spots" of *ras* oncogene activation and suggest that quantitative alterations in expression due to gene amplification may provide an alternative mechanism for *ras* gene activation in primary human tumors.

Significance to Biomedical Research and the Program of the Institute:

1. The development of testing systems for identification of carcinogenic agents and humans at high risk for cancer could prove very useful in cancer diagnosis and prevention.

2. Definition of factors associated with cell transformation should provide important insights into the mechanisms of carcinogenesis induced by chemicals, oncogenic viruses, hormones and other environmental influences.

3. Development of primate and human cell lines for rescue and identification of primate and human cancer genes will provide the tools for eventual development of protective vaccines against cancer.

4. Development of a new model for the study of human epithelial cell carcinogenesis is important in understanding the process of neoplastic conversion in human epithelial cells.

Proposed Course:

1. Continuation of studies to elucidate the various factors (with emphasis on the effect of hormones) regulating cell transformation induced by oncogenic viruses and chemical carcinogens.

2. Further attempts to develop a system for transforming human epithelial cells by viruses and chemicals in order to elucidate human epithelial cell carcinogenesis.

3. Continued characterization of Ad12-SV40 transformed human epithelial cells and their clonal lines in order to establish permanent lines for further genetic studies and for understanding the mechanisms involved in cellular gene expression and tumorigenicity.

4. Continuation of oncogene isolation studies from human tumors, with emphasis on those derived from carcinomas, using the DNA transfection 3T3 cell assay.

5. Search for DNA transfection-susceptible cell lines other than the NIH/3T3 mouse fibroblast line.

Publications:

Ablashi, D. V., Whitman, J., Dahlberg, J., Armstrong, G. and Rhim, J. S.: Hydrocortisone-enhancement of both EBV replication and transformation of human cord lymphocytes. In Levine, P. H., Ablashi, D. V., Pearson, G. R., and Kottaridis, S. D. (Eds.): Developments in Medical Virology, Vol. 1, EBV and Associated Diseases. Hingham, Massachusetts, Martinus Nijhoff Publishing Co. (In Press)

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

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

Rhim, J. S.: Viruses, oncogenes and cancer. In Yun, T. K. (Ed.): International Cancer Symposium. Seoul, Korea Cancer Center Hospital. (In Press)

Rhim, J. S., Jay, G., Arnstein, P., Price, F. M., Sanford, K. K. and Aaronson, S. A.: Neoplastic transformation of human epidermal keratinocytes by Ad12-SV40 and Kirsten sarcoma viruses. Science 227: 1250-1252, 1985.

Rhim, J. S., Sanford, K. K., Arnstein, P., Fujita, J., Jay, G. and Aaronson, S. A.: Human epithelial cell carcinogenesis: combined action of DNA and RNA tumor viruses produces malignant transformation of primary human epidermal keratinocytes. In Barrett, J. C. (Ed.): Carcinogenesis, a Comprehensive Survey. New York, Raven Press. (In Press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05062-07 LCMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Transforming Genes of Naturally Occurring and Chemically Induced Tumors

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

PI:	A. Eva	Visiting Associate	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	G. C. Vecchio	Expert	LCMB	NCI
	S. K. Srivistava	Visiting Fellow	LCMB	NCI
	L. Varesio	Visiting Scientist	LMI	NCI
	J. Ward	Chief, TPPS	LCC	NCI
	W. McBride	Chief, CRS	LB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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

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

In a small series of methylcholanthrene (MCA)-induced fibrosarcoma-derived cell lines, we earlier had shown the specific activation of K-ras in 50% of the tumor cells analyzed. In more extensive studies, we analyzed by transfection assay, DNAs of MCA-induced fibrosarcomas in BALB/c and NIH Swiss mice for their ability to transform NIH/3T3 cells. An activated K-ras gene was detected in 50% of the tumor DNA analyzed. We noticed a relationship between the frequency of activated K-ras in MCA-induced tumors and the latency period as well as the growth capability of the tumors themselves. The K-ras-positive cells also demonstrated strikingly faster growth of tumor cells in vivo as compared with the K-ras-negative cells. We also analyzed, by transfection experiments, DNAs of thymic lymphomas induced in RFJ mice by MCA and DNAs of liver tumors induced in mice by diethylnitrosomine (DEN). We found that K-ras was specifically activated in the MCA-induced lymphomas, while H-ras was the transforming gene found in about 40% of DEN-induced liver tumors.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

A. Eva	Visiting Associate	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
G. C. Vecchio	Expert	LCMB	NCI
S. K. Srivistava	Visiting Fellow	LCMB	NCI
L. Varesio	Visiting Scientist	LMI	NCI
J. Ward	Chief, TPPS	LCC	NCI
W. McBride	Chief, CRS	LB	NCI

Objectives:

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

Methods Employed:

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

Major Findings:

We have identified a transforming gene associated with a human diffuse B cell lymphoma. This newly identified oncogene, termed dbl, conferred the transformed phenotype to NIH/3T3 cells, inducing an unusual focus comprised of small and spindle shaped refractile cells as well as large multinucleated giant cells. Such transfectants acquired other phenotypic properties of transformed cells, including neoplastic potential in athymic mice. In efforts to isolate this apparently large transforming gene, we constructed a cosmid genomic library with a partially digested third-cycle transfectant DNA. From a library of 300,000 recombinants, we isolated four overlapping clones whose 40-45-kbp inserts contained human repetitive sequences. One of these recombinant cosmid clones demonstrated transforming activity in NIH/3T3 cells in several separate transfection experiments. Hybridization experiments of third-cycle transfectant DNA with cloned viral and cellular oncogenes, as well as reciprocal hybridization studies between these probes and the cloned oncogene, indicated lack of homology between this transforming gene and previously reported oncogenes.

We have detected the presence of an activated K-ras gene in 50% of MCA-induced fibrosarcoma DNAs analyzed by NIH/3T3 transfection assay. We also detected a p21 protein with altered migration properties in cell lines established from MCA-induced fibrosarcomas and in their respective NIH/3T3 transfectants containing an activated K-ras gene. We also noticed a relationship between the frequency of an activated K-ras in MCA-induced tumors and the latency period of the tumors themselves. We found that about 90% of the tumors harvested three months after MCA treatment of the mice showed an activated K-ras gene,

while the percent of tumor DNAs capable of transforming NIH/3T3 seemed to substantially decrease when the tumors were harvested three weeks later, and was even lower when tumors were harvested 18 weeks after MCA treatment. Finally, the presence of an activated K-ras gene seems to be associated with a strikingly faster growth of the tumor cells in vivo, as compared with the growth of MCA tumor cells that do not contain an activated ras gene.

Significance to Biomedical Research and the Program of the Institute:

We have used DNA transfection analysis with the continuous NIH/3T3 cell line as assay cells in efforts to detect transforming genes of human hematopoietic malignancies. By this technique, the most frequently detected transforming genes of human tumors have been members of the ras family, activated by point mutation as oncogenes in a wide variety of malignancies.

Our newly detected transforming gene is one of the very few non-ras oncogenes isolated to date and, moreover, represents the first transforming gene identified associated with a human diffuse B cell lymphoma. The distinct phenotype of transformants induced by this oncogene suggests also that the NIH/3T3 transfection assay may reveal other oncogenes which induce alterations that differ from those caused by ras oncogenes. Evidence implicating transforming genes detected by transfection with the human neoplastic process is as yet indirect. Nonetheless, in the case of ras oncogenes, this evidence appears to be compelling. Ras oncogenes are somatically activated and powerfully selected within the tumor. The mechanism of activation of this newly isolated transforming gene and its potential role in the etiology of B cell lymphomas, as well as other tumors, remains to be determined. The cloning of this gene in a biologically active form, though, provides an opportunity to elucidate potentially new and important pathways in the human malignant process.

While there is substantial evidence implicating the activation of ras oncogenes in the development of 10-20% of human cancers, it has been difficult to establish when in the course of tumor development ras gene activation occurs and how it may contribute to the neoplastic phenotype. The availability of animal model systems to investigate oncogenes that may be activated in chemical carcinogen-induced tumors can provide some insights into these questions. Our work in chemically-induced tumor systems was undertaken to more precisely define the frequency of ras oncogene activation as well as the specific ras gene implicated.

We showed that there is an extraordinary specificity for the activation of one of the three members of the ras family in any given chemical carcinogenesis animal model we analyzed. We demonstrated that K-ras is specifically and reproducibly detectable in 50% of MCA-induced fibrosarcomas. We also showed that DEN-induced live tumors carry an activated H-ras gene. Moreover, K-ras is also specifically activated in MCA-induced lymphomas at an even higher frequency. These results suggest that, at least for MCA-induced tumors, specificity in the activation of a particular ras gene is due to the chemical used and not to the tissue affected, the animal strain used or the way the drug was administered. Moreover, the apparent association of an activated K-ras in MCA-induced fibrosarcomas with the growth capability of the tumors themselves seems to indicate that the activation of a K-ras gene is not associated with MCA-induced fibrosarcomas as a necessary factor for tumor development, while

an activated K-*ras* gene is associated with a strikingly faster growth of the tumor cells *in vivo* as compared with the growth of MCA tumor cells that do not contain an activated *ras* gene.

Proposed Course:

Alu-free fragments from the cloned gene, *dbl*, are being prepared in order to identify the mRNA coded by this transforming gene. The isolation of these probes will allow us to attempt cDNA cloning of the transforming gene as well as of the mouse allele in order to determine the structure and mechanism of activation of this gene. Efforts will also be made to determine the chromosomal mapping of the gene. Finally, identification of the gene product is being attempted by immunoprecipitation techniques using polyclonal antibodies produced in mice by immunizing them with transfectant cell lines.

Cell lines have been established from several MCA-induced fibrosarcomas. Analysis of their growth capability *in vivo* in correlation with the frequency of an activated K-*ras* allele will give further and more definite information about the association of a transforming *ras* gene with a particular time course of tumor development and tumor phenotype.

Publications:

Aaronson, S. A., Yuasa, Y., Robbins, K. C., Eva, A., Gol, R. and Tronick, S. R.: In Bolis, C. G., Frati, L. and Verma, R. (Eds.): *Genetic and Phenotypic Markers of Tumors*. New York, Plenum Press, 1984, pp. 261-278.

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Eva, A. and Aaronson, S. A.: Isolation of a new human oncogene from a diffuse B cell lymphoma. *Nature* (In Press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05063-07 LCMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies on Epstein-Barr Virus and Herpesvirus Saimiri

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

PI:	D. V. Ablashi	Research Microbiologist	LCMB	NCI
Others:	J. E. Dahlberg	Research Microbiologist	LCMB	NCI
	J. S. Rhim	Research Microbiologist	LCMB	NCI
	S. Z. Salahuddin	Expert	LTB	NCI
	P. H. Levine	Senior Investigator	CEB	NCI

COOPERATING UNITS (if any)
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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.)

In studies of sera from 11 patients with acquired immunodeficiency disease (AIDS), transforming Epstein-Barr virus (EBV) was isolated from the plasma of four. The latter expressed HTLV-III antibody as well as significantly elevated levels of EBV antibodies. Uncultured peripheral mononuclear cells from these individuals contained 8-15% EBV-membrane antigen (MA)-positive cells. Transforming virus was isolated at various passage levels after culture. In comparison, healthy donors not at risk of developing AIDS did not contain MA-positive cells in the peripheral blood and no EBV could be isolated from plasma or cultured cells. None of the EBV-positive AIDS sera contained neutralizing antibody to EBV which were present in sera from healthy donors. This is the first report of the isolation of EBV from the plasma of AIDS patients.

In response to a request from NASA, squirrel monkeys which were to be used on space lab flights 3 and 4 were tested for Herpesvirus saimiri (HVS) which is endemic in this species. NASA officials were concerned about the possibility of crew members being infected by HVS being shed by the monkeys within the close confines of the spacecraft. Sera of 150 individuals occupationally exposed to squirrel monkeys were tested for the presence of HVS, and 11 (7.3%) were found positive for antibodies to HVS late antigen (LA) in the immunofluorescence test. No other HVS antibodies were evident. Eight of the 11 sera also immunoprecipitated HVS proteins. These results suggest that replication of HVS either does not occur in humans, or is too minimal to elicit immune responses. Although no demographic patterns in the HVS-LA positives were noted, the majority (7/11) had worked at primate centers around the country for periods ranging from less than one year to 25 years. This was the first report of the detection of HVS antibodies in humans.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

D. V. Ablashi	Research Microbiologist	LCMB	NCI
J. E. Dahlberg	Research Microbiologist	LCMB	NCI
J. S. Rhim	Reseaarch Microbiologist	LCMB	NCI
S. Z. Salahuddin	Expert	LTB	NCI
P. Levine	Senior Investigator	CEB	NCI

Objectives:

EBV has been shown to be implicated in the etiology of Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). The incidence of BL in AIDS patients has significantly increased in the United States since 1973; the majority of these casgs contain EBV-DNA in the tumor. This suggested that besides HTLV-III, EBV may also be responsible for polyclonal B cell activation and might thereby be partially responsible for immunosuppression due to antigen load. Based on this, we (a) studied the EBV antibody profile in AIDS patients who had demonstrable HTLV-III antibodies; (b) attempted to isolate EBV from fresh blood peripheral lymphocytes and plasma, which could suggest excessive EBV production; (c) compared results in the (a) and (b) populations with results obtained in EBV seropositive healthy individuals not at risk for AIDS; and (d) sought to demonstate neutralizing antibody to EBV in sera from AIDS patients from whom EBV had been isolated.

2. To detect EBV nuclear antigen (EBNA) in cells from the fossa Rosenmüller, thought to be the site of initiation of the malignant process leading to NPC.

3. Herpesvirus saimiri (HVS) has been shown to replicate in human fibroblasts and T lymphoblastoid cells, suggesting that individuals working closely with squirrel monkeys, which are naturally infected with HVS, might be susceptible to infection with HVS. This study, initiated by NASA, consisted of a survey of individuals occupationally exposed to squirrel monkeys for evidence of HVS seroconversion. This was of interest to NASA because they wished to include squirrel monkeys for biomedical experiments in space labs 3 and 4. Since HVS is shed from the oropharynx, NASA was concerned it could pose a hazard for flight crew members. Thus the objectives were: (a) to collect sera from individuals working with squirrel monkeys; (b) clinically define the test population as to type and degree of exposure to the monkeys, age and sex of the individuals, and any demographic patterns which might emerge; (c) develop a profile of the sera for HVS late, early and membrane antigens (LA, EA and MA), and neutralizing antibody; (d) attempt to isolate HVS from peripheral blood lymphocytes and oral washings; and (e) study the cross reactivities of HVS antibody-positive sera with other human herpesviruses and define the specificity of positive HVS sera by radioimmunoprecipitation.

Methods Employed:

Primary cell cultures and continuous cell lines of human and animal origins were used for virus isolation, biological and biochemical assays. Standard biochemical and immunological procedures were applied. Human sera were obtained

from AIDS patients, healthy donors, individuals working with oncogenic herpesviruses in the laboratory, and individuals occupationally exposed to squirrel monkeys. Monoclonal antibodies to HVS and EBV, as well as sera from nonhuman primates and humans with or without HVS-induced tumors or EBV infection were used. Three strains of HVS (prototype-S29SC, OMI, and 11), and both transforming (AG876) and nontransforming strains (P3HR1) of EBV were employed.

Major Findings:

1. Fresh plasma and peripheral blood mononuclear leukocytes were obtained from 11 AIDS patients. Their sera had been shown to contain HTLV-III antibodies as detected by ELISA. All of the patients had elevated levels of IgG antibodies to EBV virus capsid antigen (VCA) and EA. However, 4/11 patients had EBV-VCA/EA antibody levels comparable to those found in BL and NPC patients. One patient also demonstrated VCA IgA, specific to NPC patients. Transforming EBV was isolated from the plasma of these four patients. No neutralizing antibody was found in the plasma and cultured peripheral blood of the four AIDS patients from whom transforming EBV had been isolated. The freshly processed uncultured lymphocytes from these patients were also positive (8-15%) for EBV-MA, as detected by EBV monoclonal antibody, and EBV could be isolated from in vitro cultured lymphocytes. In comparison, no EBV was isolated from EBV seropositive healthy donors not at risk for AIDS, nor did their lymphocytes exhibit EBV-MA. However, their sera did neutralize EBV.

These data, for the first time, demonstrate the isolation of EBV from the plasma of EBV-associated immunodeficient diseases or EBV-associated cancers (BL and NPC). This finding suggests that in AIDS patients there is not only general immunosuppression but also an abnormality of B cell functions, such as massive polyclonal B cell activation due to ineffective T cell regulation. It may also be that the HTLV-III may activate the unusual EBV production. The absence of EBV neutralizing antibodies may reflect (a) a tie up of this antibody in immune complexes commonly observed in AIDS patients, or (b) due to overproduction of EBV, there is not enough antibody to neutralize the virus. Thus, it is evident that overproduction of EBV in AIDS patients may be a harmful complicating factor by amplifying serious abnormalities in cellular immune functions.

2. Tumor cells from the fossa Rosenmüller from 21 individuals with clinical syndromes that might be related to NPC were tested for EBNA. Histologic and cytologic findings did not show any microscopic lesions. The sera from these patients showed elevated EBV-VCA (IgG) and EA (IgG) antibodies (>250 titer for VCA and >20 EA); VCA-IgA antibodies (>10) were detected in six patients. EBNA-positive cells were found prior to any detectable malignant process. Thus, these patients were harboring EBV which might have been involved in the malignant process. Approximately 11 months later, the second biopsy from the fossa Rosenmüller of 2/3 patients were not only EBNA positive, but were histologically confirmed as undifferentiated NPC. These findings reveal that the presence of EBNA in cells from the fossa Rosenmüller suggest premalignant activity that may lead to NPC. Secondly, they further strengthen the role of EBV in NPC.

3. Eleven sera out of 150 (7.3%) from individuals occupationally exposed to squirrel monkeys for periods ranging from >1 year to 25 years were found

to possess HVS-LA antibody (IgG). The reactivity of the antibody by immunofluoresence was predominantly restricted to the nucleus of the cell. The HVS-LA antibody titer ranged from 1:10 - 1:80. These sera were reactive to all three strains of HVS-infected cells. No HVS-EA or neutralizing antibodies were detected. Eight of 11 sera immunoprecipitated HVS structural proteins as shown by polyacrylamide gel electrophoresis. No cross reactivities to known human herpesviruses (EBV, cytomegalovirus, Herpes simplex virus, varicella zoster virus) were detected by immunofluorescence, suggesting specificity of HVS-LA antibody in human sera. No demographic patterns were observed in HVS antibody-positive sera. A larger proportion of the antibody-positive individuals were females (55%), and the majority of them (64%) were from regional primate centers. Seven of the 11 individuals containing HVS antibody (64%) had been bitten by squirrel monkeys. The median hours of exposure in the antibody-positive group ranged from one to 40 hours per week, which was not different from the antibody-negative group. Follow-up serum samples (3-7 months) from eight HVS antibody-positive individuals had maintained HVS-LA titers.

Peripheral blood and oral washings of 9/11 HVS-LA antibody-positive individuals were negative for HVS or HVS antigens detectable in immunofluorescence. Most of these individuals were interviewed regarding symptoms. Except for a few allergy problems and Herpes simplex type 1 infections, no clinical manifestations were evident.

This is the first report of HVS-specific antibodies in humans. These data suggest, but do not prove, that replication of HVS either does not occur in humans, or occurs in such a transient way as to not elicit an immune response against nonstructural virally encoded proteins. Secondly, the possibility of cross reactivity of an as yet unidentified human herpesvirus cannot be ruled out.

Significance to Biomedical Research and the Program of the Institute:

1. Our finding of transforming EBV from AIDS patients' plasma raises many interesting points. Immunosuppression is a commonly observed phenomenon in EBV-associated disorders and malignancies. It is evident that specific suppressor cells may be partly responsible for immunosuppression due to overload of antigen, particularly during persistently active EBV infection, as is the case in the AIDS patients studied. Since T cell function in AIDS is abnormal, the natural killer cells may be far too few to counteract EBV-activated B cells, resulting in further immunosuppression. The control of EBV infection in AIDS patients by antiviral therapy or by use of immunization of MA proteins that produce neutralizing antibody may improve their immunologic condition and their response to other forms of therapy. Secondly, studying the interaction between EBV and HTLV-III may provide insight into the increased incidence of EBV-related B cell lymphomas in AIDS patients.

2. The ability to detect EBNA-positive cells in the fossa Rosenmüller of populations at risk for NPC prior to any clinical or microscopic lesions supports the etiological role of EBV in the malignant process and suggests that the EBV virus may have been present for a long period in these cells. Initiation of the malignant process might also be due to other factors that stimulate EBV

to transform cells. This study may be important not only in understanding the malignant process in NPC but may provide the means of detecting precursors to NPC. It thus has prognostic implications as well in that treatment and control measures can be instituted earlier.

3. Detection of HVS antibodies in human subjects is an important contribution because it raises possibilities of the role and fate of HVS in human cells. Since we have demonstrated that HVS replication in human cells is semi-permissive and that late viral proteins, particularly the major structural proteins, are either not made or are made in too small amounts to be detectable, the virus may be defective. Whether defective virus particles are capable of infecting and/or transforming cells has not been fully investigated. Secondly, whether this virus is capable of producing an in vivo immune response is also unclear. The chances of cell transformation by nonlytic viruses are much greater than by a lytic virus that kills the cell. The lack of expression of the major structural proteins of HVS in human infected cells raises the possibility of the immortalization of cells. It is important to follow HVS antibody-positive individuals for changes in antibody titer, detectable virus, and any clinical abnormalities. Since antibody reflects viral infection, this study shows conclusively that exposure to HVS-infected monkeys does occur. Precautions should therefore be taken to protect against such exposures with appropriate biohazard safeguards. NASA's decision to use only HVS-free squirrel monkeys in its space flights, based on the above findings, was well taken, since there the possibility of virus shedding is probably greater under zero gravity conditions. In addition, immunosuppression has been observed in human subjects in prior space flights, further increasing their susceptibility.

Proposed Course:

Studies on the role of EBV in AIDS patients will be continued. The mechanism of B cell infection with HTLV-III may provide further evidence of interaction between EBV and HTLV-III. Secondly, EBV isolated from HTLV-III-positive AIDS patients will be characterized biologically and biochemically and compared with the known EBV strains.

The role of EBV in the fossa Rosenmüller will be continued to determine the relationship of EBV to NPC.

Studies relating to HVS will be followed by testing the sera from individuals never exposed to monkeys. This will help elucidate whether there are unidentified human herpesviruses which cross-react with HVS. Secondly, HVS antibody-positive individuals will be followed for HVS antibodies and any clinical abnormalities that may develop due to HVS infection.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05164-05 LCMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Interaction of Hematopoietic Cells and Mammalian Retroviruses

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

PI:	J. H. Pierce	Sr. Staff Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	P. Di Fiore	Visiting Fellow	LCMB	NCI
	A. Gazit	Visiting Fellow	LCMB	NCI
	M. Potter	Chief	LG	NCI

COOPERATING UNITS (if any)
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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.)

(1) Abelson murine leukemia virus (Ab-MuLV) was shown to induce mast cell transformation by a nonautocrine mechanism in vitro when interleukin-3 (IL-3) was initially utilized to enhance the proliferation of the mast cell phenotype. Ab-MuLV was the only retrovirus analyzed that conferred factor-independence and leukemogenicity to the mast cell cultures. Ab-MuLV was also capable of inducing mastocytomas in vivo under specific conditions. These results define a new target for transformation by Ab-MuLV. (2) Human bone marrow cells were infected with ras-containing retroviruses pseudotyped with amphotroph (Am)-MuLV. No direct transformation of hematopoietic cells infected with these viruses was observed in long-term cultures. However, there was a significant increase in the emergence of Epstein-Barr virus (EBV)-positive pre-B cell lines from the virus-infected cultures which released virus and expressed the p21 transforming protein. (3) A recombinant murine retrovirus containing the erb B gene of avian erythroblastosis virus was generated and shown to induce transformation of NIH/3T3 and murine hematopoietic cells in vitro. Characterization of these transformants is currently underway. (4) Several retrovirus-transformed hematopoietic cell lines derived in our laboratory by transformation with various acute transforming retroviruses were analyzed to determine their phenotype and stage of differentiation by fluorescence activated cell sorter analysis of lineage-specific cell surface antigens and examination of their immunoglobulin and T cell receptor gene rearrangement patterns. The majority of the lines analyzed expressed characteristics of very immature B cells. However, certain lines appeared to be less differentiated. Preliminary evidence has indicated they may represent transformed counterparts of lymphoid or lymphoid/myeloid progenitor stem cells.

PROJECT DESCRIPTION

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

J. H. Pierce	Sr. Staff Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
P. Di Fiore	Visiting Fellow	LCMB	NCI
A. Gazit	Visiting Fellow	LCMB	NCI
M. Potter	Chief	LG	NCI

Objectives:

To determine whether hematopoietic growth factors that allow certain types of normal cells to proliferate in culture will cause the transformation of previously undefined target cells by certain replication-defective retroviruses.

To investigate whether human hematopoietic cells can be transformed in long-term bone marrow culture systems utilizing amphotroph murine leukemia virus (Am-MuLV) pseudotypes of replication-defective retroviruses. In addition, to determine whether Am-MuLV can be utilized as a vector for inserting normal genes into human hematopoietic cells by recombinant techniques.

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

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

Methods Employed:

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

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

Identification of retrovirus-specific transforming proteins in hematopoietic transformants was performed by radioimmunoprecipitation and immunofluorescence techniques.

Major Findings:

Although pre-B cells are preferential hematopoietic targets for transformation by Abelson-murine leukemia virus (Ab-MuLV) in vitro and in vivo, there have been reports that Ab-MuLV induces rare mastocytomas in vivo. Normal basophil/mast cells can be grown in long-term culture when medium is supplemented with interleukin-3 (IL-3). IL-3-dependent fetal liver cultures were generated in order to determine effects of Ab-MuLV infection on an enriched population of normal mast cells in vitro. Ab-MuLV infection led to the conversion of mast cells from dependence on IL-3 for growth to factor-independence. Although other acute transforming retroviruses, including Harvey-, BALB-, and Moloney-murine sarcoma virus (MSV) were capable of productively infecting mast cell cultures, they were unable to release them from dependence on IL-3. Ab-MuLV-induced mast cell lines expressed abl-specific transforming proteins, were tumorigenic in nude mice, and had high cloning efficiencies in soft agar containing no exogenous factors. Ab-MuLV-induced mastocytomas were also derived in vivo by treatment of adult mice known to be resistant to pre-B cell transformation by Ab-MuLV. Cell lines were established in culture from four of several transplantable mast cell tumors isolated in vivo. The Ab-MuLV-derived lines did not autonomously produce IL-3 nor was their growth inhibited by anti-IL-3 serum. None of the Ab-MuLV-derived transformants expressed IL-3-related RNA message as determined by Northern blot hybridization analysis. These results argue that Ab-MuLV abrogation of the IL-3 requirement was not the result of an autocrine mechanism involving direct or indirect induction of an IL-3-like growth factor. Moreover, release of normal mast cells from dependence on IL-3 for growth by Ab-MuLV infection corresponded with the conversion of these cells to a transformed phenotype, defining a new hematopoietic target cell for transformation by Ab-MuLV.

Human continuous bone marrow cultures were established from intraoperative marrow specimens and infected with Am-MuLV pseudotypes of Kirsten- or Harvey-MSV, and the biologic effects were compared with mouse continuous bone marrow cultures. Cultures were tested for production of total nonadherent granulocytes and granulocyte-macrophage progenitor cells (GM-CFUc); virus replication by supernatant reverse transcriptase activity; percentage of adherent and nonadherent cells and GM-CFUc that released virus by infectious center assay; and for synthesis of Harvey ras p21 protein. High efficiency, stable infection of over 90% of human marrow culture nonadherent and adherent cells was detected by infectious center assay. Synthesis of Harvey ras p21 was detected in the adherent and nonadherent cell populations of human as well as mouse continuous marrow cultures infected with Kirsten or Harvey pseudotype virus. In contrast to data with mouse cultures, cumulative production of GM-CFUc and differentiated granulocytes in human cultures was not detectably altered by Harvey or Kirsten virus infection, and all cultures ceased to produce hematopoietic cells by 20 weeks. Of 54 virus-infected cultures in 10 separate experiments, 13 produced a second peak of nonadherent cells ($>10^5$ per flask) after 20 weeks. Significantly, this occurred more frequently than in control uninfected cultures (one of 32). When subcultured, these harvests produced permanent Epstein-Barr virus (EBV)-transformed pre-B cell lines that released the original inoculating pseudotype virus. Thus, Am-MuLV is a potentially valuable vector for inserting genetic sequences by recombinant techniques into human hematopoietic and stromal cells in culture. However, activation of EBV may be a significant complication.

The major oncogene of avian erythroblastosis virus, erb B, corresponds to a truncated portion of the human EGF receptor and possesses tyrosine-specific protein kinase activity. Since v-erb B is the first oncogene shown to share extensive homology to a growth factor receptor and this relationship to cellular transformation is not well understood, a murine recombinant retrovirus construct that contains the erb B gene was generated in our laboratory. This construct was capable of inducing foci after DNA transfection of NIH/3T3 cells, and transforming virus could be rescued from these foci by infection with murine helper viruses. NIH/3T3 cells transformed by the erb B recombinant virus expressed glycoproteins that were antigenically related to avian v-erb B proteins. These cells had a high cloning efficiency in soft agar and were tumorigenic in nude mice. Since avian erythroblastosis virus is capable of inducing erythroleukemia in chickens and transforming avian erythroblasts in culutre, we examined the abililty of the erb B recombinant virus to alter the growth properties of mouse hematopoietic cells in culture. The erb B recombinant virus induced compact colonies from bone marrow or fetal liver which follow single-hit kinetics and required mercaptoethanol in the soft agar medium. Cells from the transformed colonies could be established as continuous cell lines which demonstrated unrestricted self-renewal and were tumorigenic. All hematopoietic transformants expressed high levels of v-erb B proteins. Further analysis of the hematopoietic transformants, other murine cellular targets for transformation by this virus, and its in vivo properties are in progress.

Several clonal murine hematopoietic cell lines derived in our laboratory by infection with various acute transforming retroviruses other than Ab-MuLV were characterized by fluorescence-activated cell sorter analysis for a range of cell surface antigens specific to different hematopoietic lineages and stages of differentiation. These transformed lines were also analyzed for immunoglobulin and T cell receptor gene rearrangement patterns. The majority of lines tested appeared to express markers of very immature B cells, including some rearrangements of the immunoglobulin heavy chains. However, one Harvey-MSV-transformed fetal liver line possessed a germline immunoglobulin gene pattern and preliminary analysis has indicated that certain subclones of this line spontaneously rearrange either the immunoglobulin heavy chain genes or the T cell receptor genes. These results indicate that this line may be representative of a lymphoid progenitor cell. Another Harvey-MSV-transformed line was shown to display markers of both pre-B and early myeloid phenotypes. Subclones of this clonal transformant could be shown to express increased levels and/or new markers specific to either the myeloid or lymphoid lineages. These preliminary results indicate that this line may be a hematopoietic stem cell capable of differentiation along either the lymphoid or myeloid pathways.

Significance to Biomedical Research and the Program of the Institute:

Investigation of the diversity of target cells for neoplastic transformation by a particular transforming retrovirus might be suspected to provide insights into pathways of transformation and, in particular, the relationship of the differentiation state of the cell to its susceptibility to onc gene action. The isolation of a variety of transformed hematopoietic cell lines frozen at various stages in hematopoietic cell differentiation should provide a useful approach for analysis of normal development of differentiation within a particular lineage and may help to explain the possible functions of certain markers, such as the enzyme terminal deoxynucleotidyl transferase in hematopoietic cell

differentiation. The ability of recombinant retroviruses which contain certain genes analogous to human growth factors or receptors may help to define the role of these genes in the stimulation of cell proliferation and their relationship to neoplastic transformation. The ability of particular retroviruses to induce factor independence to previously factor-dependent hematopoietic lineages may help to define relationships of hematopoietic growth factors, or growth factor receptors, to specific retrovirus transformation-specific proteins.

Investigation of the effects of mammalian retroviruses on the growth and differentiation of human hematopoietic cells may be useful in determining the etiology of different human leukemias. This project should also help to define the feasibility of using retroviruses as vectors for introducing non-transforming genes into human hematopoietic cells.

Proposed Course:

Efforts are underway to determine whether several different hematopoietic growth factors that allow certain types of normal cells to proliferate in culture will allow for the transformation of previously undefined target cells by certain replication-defective retroviruses. The ability of particular retroviruses to induce factor independence to previously dependent hematopoietic lineages may help to define relationships of hematopoietic growth factors or their receptors to specific retrovirus transforming proteins.

Recombinant retroviruses which contain certain human oncogenes have been or presently are being generated in our laboratory. We are currently analyzing the ability of these viruses to induce murine hematopoietic cell transformation in the bone marrow assay system. We are also extending the analysis of the ability of mammalian retroviruses to transform human bone marrow cultures utilizing amphotrophic virus pseudotypes of replication-defective retroviruses.

We plan to extensively characterize the effects of the *erb* B recombinant retrovirus in the murine system. Hematopoietic cells transformed by this virus are currently being analyzed for phenotypic marker and response to growth and differentiation factors. Studies of the effect of this virus on normal epithelial cell lines, which have high levels of EGF receptors, are also underway. The transforming effects of *erb* B in cooperation with other oncogene-containing viruses is also being explored. Finally, the mechanism by which a retrovirus that expresses a protein which is analogous to a normal growth factor receptor will be investigated.

We plan to continue our efforts to define hematopoietic transformants which have the ability to differentiate along different developmental lineages through the use of temperature-sensitive mutant viruses or exposure to agents which promote differentiation in other systems.

Publications:

Eva, A., Pierce, J. and Aaronson, S. A.: Interactions of oncogenes with hematopoietic cells. *UCLA Symposia on Leukemia, 1985*. (In Press)

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Pierce, J. H., Di Fiore, P. P., Aaronson, S. A., Potter, M., Pumphrey, J., Scott, A. and Ihle, J. N.: Neoplastic transformation of mast cells by Abelson-MuLV: abrogation of IL-3 dependence by a nonautocrine mechanism. Cell (In Press)

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Yuasa, Y., Eva, A., Kraus, M. H., Srivastava, S. K., Needleman, S. W., Pierce, J. H., Rhim, J. S., Gol, R., Reddy, E. P., Tronick, S. R. and Aaronson, S. A: Ras-related oncogenes of human tumors. In Vande Woude, G. F., Levine, A. J., Topp, W. C. and Watson, J. D. (Eds.): Cancer Cell 2/ Oncogenes and Viral Genes. New York, Cold Spring Harbor Laboratory, 1984, pp. 433-439.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05167-05 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Mechanisms of Transformation Induced by Retrovirus onc Genes

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

PI:	K. C. Robbins	Expert	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	H. Igarashi	Visiting Fellow	LCMB	NCI
	F. Leal	Guest Researcher	LCMB	NCI
	M. Cheah	Medical Staff Fellow	LCMB	NCI
	T. Kawakami	Visiting Fellow	LCMB	NCI
	R. King	Staff 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 20205

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.)

The v-sis gene arose from an evolutionarily conserved, unique sequence cellular gene, designated c-sis. A structural analysis of human c-sis DNA clones has shown that regions corresponding to the v-sis coding sequence are arranged in five exons which stretch over approximately 12 kbp of the human genome. The nucleotide sequence of these exons has revealed that the human sis proto-oncogene is the structural gene for one of the two major polypeptides of PDGF. Using probes derived from sequences flanking the v-sis-related regions of human c-sis, it has been possible to identify an upstream exon of the c-sis transcriptional unit. This exon is not related to v-sis but is transcribed in certain human tumor cells. In attempts to assess the transforming potential of the normal c-sis human locus, it has been shown that transcriptional activation of a construct containing all of PDGF-2 coding exons as well as the upstream c-sis exon leads to the acquisition of high titered transforming activity.

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

PROJECT DESCRIPTION

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

K. C. Robbins	Expert	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
H. Igarashi	Visiting Fellow	LCMB	NCI
F. Leal	Guest Researcher	LCMB	NCI
M. Cheah	Medical Staff Fellow	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI
C. R. King	Staff Fellow	LCMB	NCI
N. Giese	Guest Researcher	LCMB	NCI

Objectives:

1. To define the interaction of the simian sarcoma virus (SSV)-transforming protein ($p28^{sis}$) with the platelet-derived growth factor (PDGF) receptor.

2. To assess the role of sis-related genes in human neoplasia.

3. To determine the mechanism of SSV-induced oncogenesis and to apply this knowledge to understanding the etiology of cancers in humans.

4. To determine the mechanism of Gardner-Rasheed feline sarcoma virus (GR-FeSV)-induced oncogenesis.

Methods Employed:

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

Major Findings:

Expression of the normal human sis/PDGF-2 coding sequence was demonstrated to induce cellular transformation. The human sis proto-oncogene was found to contain the coding sequence for one of two polypeptide chains present in preparations of biologically active PDGF. A human clone, c-sis clone 8, which contains all of the v-sis-related sequences present in human DNA, was transcriptionally inactive when transfected into NIH/3T3 cells. When placed under the control of a retrovirus LTR, the clone was transcribed at levels comparable to that observed in cells transformed by simian sarcoma virus (SSV) DNA. However, c-sis clone 8 DNA did not express detectable sis/PDGF-2 proteins and lacked

biologic activity. A putative upstream exon was identified by its ability to detect the 4.2-kb sis-related transcript in certain human cells. When this sequence was inserted in the proper orientation between the LTR and c-sis clone 8, the chimeric molecule acquired high titered transforming activity, comparable to that of SSV DNA. Transformants containing this construct expressed human sis/PDGF-2 translational products. Thus the normal coding sequence for a human growth factor has transforming activity when expressed in an appropriate assay cell.

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

The v-sis transforming gene encodes the woolly monkey homologue of human platelet-derived growth factor (PDGF) polypeptide 2. After its synthesis on membrane bound polyribosomes, the glycosylated precursor dimerizes in the endoplasmic reticulum and travels through the Golgi apparatus. At the cell periphery, the precursor is processed to yield a dimer structurally analogous to biologically active PDGF. Small amounts of two incompletely processed forms are detectable in tissue culture fluids of SSV transformants. However, the vast majority remains cell associated. Thus this growth factor related transforming gene product is not a classical secretory protein. These findings define possible cellular locations where the transforming activity of the sis/PDGF-2 protein may be exerted.

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

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

single transcript, 3 kb in length, was detected in approximately 50% of the Burkitt's lymphomas examined.

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

Significance to Biomedical Research and the Program of the Institute:

Our work on SSV in combination with independent investigation on the structure of PDGF has provided a very important link between retrovirus transforming genes and growth factors. Subsequent studies have provided evidence that the mechanism by which SSV induces morphologic transformation involves pathways also associated with the growth promoting activities of PDGF. These findings may also have etiologic relevance to some naturally occurring human cancers. Our findings provide strong evidence that expression of human c-sis in cells responsive to PDGF (fibroblasts, glial cells and smooth muscle cells) is an important step in the malignant process leading to tumors of these cell types.

Several acute transforming retroviruses encode tyrosine-specific protein kinases which possess structural and functional relationships to cell surface receptors for certain growth factors. One such tyrosine kinase is encoded by the onc gene, v-fgr, of GR-FeSV. We have described the isolation and characterization of the human gene corresponding to v-fgr and have determined that the fgr proto-oncogene is a distinct sequence located on the short arm of chromosome 1. We have shown a correlation between fgr proto-oncogene expression and EBV infection of transformed lymphocytes. Activation by EBV infection was shown to occur in tumor cells which had previously been established in culture and possessed no prior evidence of EBV infection, suggesting that c-fgr expression did not nonspecifically result from the transformation process. Moreover, each of the normal cord and peripheral blood lymphocyte cell lines established by EBV infection expressed the 3-kb c-fgr transcript. Thus, transcriptional activation of the fgr proto-oncogene occurs in response to EBV infection of normal or transformed lymphocytes. These studies establish a direct interaction between a DNA tumor virus and a human proto-oncogene.

Proposed Course:

1. Examine the interaction between the v-sis coded growth factor and the PDGF receptor utilizing a v-sis mutagenesis approach.

2. Define the cellular events occurring in response to synthesis of the v-sis coded growth factor in susceptible cells.

3. Continue to assess the involvement of the human sis proto-oncogene in naturally occurring human malignancies.

4. Determine the significance of our finding that an acute transforming retrovirus contains a domain of the cytoskeletal protein, actin.

5. Define the role of the fgr proto-oncogene in EBV-induced cell immortalization.

Publications:

Aaronson, S. A. and Robbins, K. C.: Activation of a gene coding for a normal human growth factor to one with transforming properties. In Furmanski, P., Hager, J. C. and Rich, M. A. (Eds.): RNA Tumor Viruses, Oncogenes, Human Cancer and AIDS: On the Frontiers of Understanding. Boston, Martinus Nijhoff Publishing, 1985, pp. 54-66.

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Gazit, A., Igarashi, H., Chiu, I.-M., Srinivasan, A., Yaniv, A., Tronick, S. R., Robbins, K. C. and Aaronson, S.A.: Expression of the normal human sis/PDGF-2 coding sequence induces cellular transformation. Cell 39: 89-97, 1984.

Igarashi, H., Gazit, A., Chiu, I.-M., Srinivasan, A., Yaniv, A., Tronick, S. R., Robbins, K. C. and Aaronson, S. A.: Expression of a normal growth factor gene causes morphologic transformation. In Giraldo, E., Beth, E., Castello, G., Giordono, G. G., and Zarrili, D. (Eds.): From Oncogenes to Tumor Antigens. Amsterdam, Elsevier, 1985, pp. 3-15.

Igarashi, H., Gazit, A., Chiu, I.-M., Srinivasan, A., Yaniv, A., Tronick, S. R., Robbins, K. C. and Aaronson, S. A.: Normal human sis/PDGF-2 gene expression induces cellular transformation. Conferences on Cell Proliferation and Cancer. Cold Spring Harbor series Vol. 12 (In Press)

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

McBride, O. W., Swan, D. S., Robbins, K. C., Prakash, K. and Aaronson, S.A.: Chromosomal mapping of tumor virus transforming gene analogues in human cells. In Pearson, M. L. and Sternberg, N. L. (Eds.). Gene Transfer and Cancer. New York, Raven Press, 1984, pp. 197-205.

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Robbins, K. C., Antoniades, H. N., Devare, S. G., Hunkapiller, M. W. and Aaronson, S. A.: Close similarities between the transforming gene product of simian sarcoma virus and human platelet-derived growth factor. In Arnold, J. L., Vande Woude, G. F., Topp, W. C., and Watson, J. D. (Eds.): Cancer Cells 1/The Transformed Phenotype. New York, Cold Spring Harbor Laboratory, 1984, pp. 35-42.

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

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05234-04 LCMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular Cloning and Characterization of Transforming Genes

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

PI:	V. Notario	Visiting Associate	LCMB	NCI
Others:	K. C. Robbins	Expert	LCMB	NCI
	T. Kawakami	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

Laboratory Biochemical Pharmacology, NIADDK (Dr. Reed B. Wickner)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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

1. The detection and molecular cloning of yeast DNA sequences related to the v-sis oncogene were previously reported. A 1.5-kbp fragment (Xba I-Bam HI) encompassed the hybridizing region. Nucleotide sequence analysis showed that the homology was localized in a stretch of 16 bp, with one mismatch. The cloned gene, therefore, does not belong to the sis family. However, it was found to be a single copy gene in yeasts with coding capacity for a protein of about 20 kd. It was mapped to chromosome X of Saccharomyces cerevisiae, at about 7.5 cM from the centromere, linked to the ilv-3 gene. Gene disruption experiments proved that it is a nonessential gene, and may be a useful tool for the integration of heterologous genes in the yeast genome.

2. By means of standard recombinant DNA techniques, a number of yeast expression vectors have been constructed. Yeast promoters such as ADC-1, PGK (constitutive), and GAL-1, GAL-10 and MF-α (inducible) are currently used to express sis-related proteins in yeasts.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

V. Notario	Visiting Associate	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI

Objectives:

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

Methods Employed:

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

Major Findings:

A 9-kbp Eco RI fragment hybridizing to v-sis probes was cloned from the yeast Saccharomyces cerevisiae under relaxed hybridization conditions. An internal 1.5-kbp Xba I-Bam HI fragment enclosing the v-sis-related sequences was subcloned into pBR322, and its nucleotide sequence determined. Computer analysis of the sequence revealed a 28% and 33% homology at the amino acid level between the 5' and 3' ends of the yeast fragment, and the first and fifth exons of the human c-sis/PDGF-2 gene. However, the best detectable homology within the main hybridizing portion of the fragment corresponded to a stretch of only 30 bp with four mismatches, even if a 1-bp gap was introduced in the yeast sequence. It was not a real sis gene. This agrees with the fact that neither total yeast extracts nor yeast culture fluids showed PDGF-like mitogenic activity in assays where mammalian sis proteins scored as positive.

On the other hand, the cloned yeast gene has been found to be a new gene which is transcribed during active yeast growth. It is a single copy gene in the yeast genome and, by means of meiotic mapping techniques, has been localized to the short arm of chromosome X, closely linked to the centromere and tightly linked to ilv-3. Gene disruption experiments, in which the ura 3 gene was introduced into the coding region of the new gene, showed that it is nonessential, since no observable difference became evident between disrupted and undisrupted haploid strains.

By means of Southern blot analysis of the total genomic DNA, sis-related sequences were undetectable in the genomic DNA of nematode Caenorhbditis elegans and the fruit fly Drosophila.

Significance to Biomedical Research and the Program of the Institute:

The obvious conclusion is that *Saccharomyces cerevisiae* does not have an endogenous *sis* gene, and that *sis* genes are not present in invertebrates, yet they are very well conserved in vertebrate evolution. Contrary to the case of the *ras* gene family in which its members have been found to be extremely well conserved in eukaryotic evolution, the *sis*/PDGF gene must have arisen much later. This argues in favor of an essential function for *ras* genes in eukaryotic cells, while *sis* genes may carry out rather specialized functions in certain types of tissues in higher organisms.

The lack of endogenous *sis* genes in yeasts, and the similarity between protein processing systems in yeasts and higher eukaryotes, make yeasts an excellent system for the regulated expression of *sis*-related proteins. Overproduction of v-*sis* oncogene products may be a powerful way to gain some knowledge of their mechanism of oncogenic transformation.

Proposed Course:

Current work is focused on the characterization of the *sis* proto-oncogene in lower vertebrates, and on the expression of *sis* proteins in *Saccharomyces cerevisiae*.

Publications:

Kang, M. S., Szaniszlo, P. J., Notario, V. and Cabib, E.: The effect of papulacaudin B on $\beta(1\rightarrow3)$ glucan synthetases. A possible relationship between inhibition and enzyme conformation. *Carbohydr. Res.* (In Press)

Lacal, J. C., Santos, E., Notario, V., Barbacid, M., Yamazaki, S., Kung, H., Seamans, C., McAndrew, S. and Crowl, R.: Expression of normal and transforming H-*ras* genes in *Escherichia coli* and purification of their encoded p21 proteins. *Proc. Natl. Acad. Sci. USA* 81: 5305-5309, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05306-03 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Properties of Human ras Oncogene-encoded Transforming Protein

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

PI:	S. K. Srivastava	Visiting Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. C. Lacal	Visiting Fellow	LCMB	NCI
	J. Fujita	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 20205

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.)

Project 1: Extension of previous studies on structure and function of ras oncogene-encoded p21 proteins required quantities of purified protein extremely difficult to obtain from eukaryotic cells. To circumvent this problem, column chromatographic procedures were developed to purify normal and transforming p21 proteins from bacterial expression systems. From 1.5 L culture, about 2 mg of apparently homogeneous protein could be obtained. The purified proteins were biochemically active for guanine nucleotide-binding function, autophosphorylation (where applicable) and GTPase activities. Knowledge gained by the studies on GTP-binding function of eukaryotic protein has been extended to purified p21 proteins. p21 proteins with threonine at position 59 show significant GTP binding as compared to the variants with alanine at position 59 without any detectable activity under nonreducing conditions. However, under reducing conditions, p21 proteins with alanine 59 also show enhanced nucleotide binding, although only one-fourth of their threonine counterpart. These results suggest that residue 59 affects the binding of nucleotide to protein.

Project 2: Using synthetic antipeptide antibodies corresponding to residues 161-176 in H-ras p21 protein, a structurally and functionally important region in the p21 molecule has been identified which affects the binding of guanine nucleotide to p21 protein. Results with purified p21 proteins and carboxy terminal antibody strongly suggest that the amino and carboxy terminal regions in the p21 molecule are proximal to each other in the native conformation of p21 protein.

Project 3: Studies on the characterization of Hs242, a lung carcinoma-derived cell line, have been extended. This cell line, although exhibiting normal phenotype, was found to harbor activated H-ras gene mutated at position 61. Further studies show that H-ras-specific RNA is expressed in the Hs242 cell line. However, analysis of proteins either by immunoprecipitation or by Western blots failed to detect any mutated p21.

PROJECT DESCRIPTION

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

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

Objectives:

1. Purification of normal and transforming p21 proteins from bacterial expression systems. Characterization of guanine nucleotide binding activities of variant p21 proteins.

2. Dissection of functionally important domains of p21 protein using oligopeptide antibody.

3. Characterization of Hs242 cell line with regard to the presence of activated H-*ras* gene.

Methods Employed:

1. Column chromatographic procedures involved in purification of proteins.

2. Immunoprecipitation of p21 proteins using monoclonal and antioligopeptide antibodies and their analysis on SDS-polyacrylamide gel electrophoresis and Western blot analysis of p21 proteins.

3. In vitro assays for GTP binding autophosphorylation and GTPase activities of p21.

4. Analysis of the transforming gene in Hs242 cells. RNA expression analysis by oligonucleotide probes.

5. In vitro deletion mutagenesis using synthetic oligonucleotides.

Major Findings:

Studies on eukaryotic as well as purified bacterially-expressed p21 proteins strongly suggest that residue 59 affects the binding of guanine nucleotide to p21 protein.

Analysis of effects of antioligopeptide antibody on various activities of purified p21 protein demonstrates that amino- and carboxy- terminal regions of p21 proteins are proximal to each other or part of nucleotide binding site originates from carboxy terminal domain in the native structure of p21.

Significance to Biomedical Research and the Program of the Institute:

Activated ras genes have been found in a variety of neoplasms. Analysis of the ras gene and its translational product would help in understanding the mechanism of cellular transformation by this family of transforming genes.

Proposed Course:

1. A functionally important region in the p21 molecule has been mapped by use of truncated proteins and monoclonal antibody Y13-259 (J. C. Lacal). Defined deletion mutagenesis will be carried out in this region of the molecule and various biochemical and biological functions will be assessed.

2. Studies on the Hs242 cell line will be continued.

3. Studies towards analysis of putative substrate(s) with which p21 may interact will be continued.

Publications:

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Tronick, S. R., Eva, A., Srivastava, S. K., Kraus, M., Yuasa, Y. and Aaronson, S. A.: The role of human ras proto-oncogenes in cancer. In Castellani, A. (Ed.): Epidemiology and Quantitation of Environmental Risk in Humans from Radiation and Other Agents. New York, Plenum Press, 1985 (In Press)

Yuasa, Y., Eva, A., Kraus, M. H., Srivastava, S. K., Needleman, S. W., Pierce, J. H., Rhim, J. S., Gol, R., Reddy, E. P., Tronick, S. R. and Aaronson, S. A.: Ras-related oncogenes of human tumors. In Vande Woude, G. F., Levine, A. J., Topp, W. C. and Watson, J. D. (Eds.): Cancer Cell 2/Oncogenes and Viral Genes. New York, Cold Spring Harbor Laboratory, 1984, pp. 433-439.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05307-03 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Expression of Normal Human sis/PDGF-2 Coding Sequence Induces Transformation

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

PI:	A. Gazit	Visiting Fellow	LCMB NCI
Others:	S. Aaronson	Chief	LCMB NCI
	H. Igarashi	Visiting Fellow	LCMB NCI
	S. Tronick	Chief, Gene Structure Section	LCMB NCI
	K. Robbins	Expert	LCMB NCI

COOPERATING UNITS *(if any)*

Meloy Laboratories, Springfield, Virginia (I.-M. Chiu);
Sackler School of Medicine, Tel Aviv, Israel (A. Yaniv)

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

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

The human sis proto-oncogene contains the coding sequence for one of two polypeptide chains present in preparations of biologically active human platelet-derived growth factor (PDGF). We sought to determine whether this normal coding sequence could be activated as a transforming gene by appropriate in vitro manipulations. A human clone, c-sis clone 8, which contains all of the v-sis-related sequences present in human DNA, was shown to be transcriptionally inactive when transfected into NIH/3T3 cells. When placed under the control of a retrovirus LTR, the clone was transcribed at levels comparable to that observed in cells transformed by SSV DNA. In spite of its transcriptional activation, c-sis clone 8 DNA did not express detectable sis/PDGF-2 proteins and lacked biologic activity.

A putative upstream exon was identified by its ability to detect the 4.2-kb sis-related transcript in certain human cells. When this putative exon was inserted in the proper orientation between the LTR and c-sis clone 8, the chimeric molecule acquired high titered transforming activity, comparable to that of SSV DNA. Moreover, transformants containing this construct were shown to express human sis/PDGF-2 translational products. These findings establish that the normal coding sequence for a human growth factor has transforming activity when expressed in an appropriate assay cell.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

A. Gazit	Visiting Fellow	LCMB	NCI
S. Aaronson	Chief	LCMB	NCI
H. Igarashi	Visiting Fellow	LCMB	NCI
S. Tronick	Chief, Gene Structure Section	LCMB	NCI
K. Robbins	Expert	LCMB	NCI

Objectives:

To determine whether the normal human coding sequence for PDGF-2 could be activated as a transforming gene by providing it with necessary regulatory signals for its expression.

Methods Employed:

To construct recombinant plasmids, the conventional methods of recombinant DNA technology are employed, including preparation of plasmid DNAs, purification of DNA fragments, ligation and bacterial transformation. To check the biological activity of the cloned DNA fragments, DNA transfection, as well as cotransfection assays with simian virus-2 (SV-2), are carried out on NIH/3T3 cells. Presence and quantitation of the transfected DNA within cell DNA is checked by procedures including isolation of high molecular weight DNA, restriction enzyme digestion, electrophoresis and Southern filter DNA blot hybridization. RNA expression is assayed by the RNA blot technique, gel electrophoresis and the Northern blotting procedures.

Major Findings:

The v-sis transforming gene contains coding sequences for a protein closely related to PDGF-2, a major polypeptide chain of human PDGF. The primary v-sis gene product, $p28^{sis}$, has been shown to be the precursor of a smaller molecule corresponding in size as well as amino acid sequence to a PDGF-2 monomer. Moreover, $p28^{sis}$ undergoes dimer formation and subsquent processing to a form analogous in structure to that of biologically active human PDGF. Thus, it seems likely that the transforming activity of the v-sis gene product is mediated by this processed PDGF-2-like dimer.

There is as yet no information concerning the sequence of the normal woolly monkey homologue of v-sis or of its gene product. Thus, it is not known whether v-sis contains alterations in its coding sequence which contribute to the transforming properties of this PDGF-2-related gene. The normal human homologue of v-sis has been shown to be the structural gene for PDGF-2.

These findings provided the opportunity for us to investigate whether expression of the normal PDGF-2 coding sequence would be sufficient to induce cellular transformation. Our efforts to activate human c-sis/PDGF-2 took into account the fact that v-sis derives both transcription and translation initiation signals from its helper viral sequences and that human c-sis cl 8, which contained all of the coding information for PDGF-2, lacked transcription and translation initiation signals as well.

We were able to activate this human sequence as a transforming gene by two in vitro manipulations, neither of which was sufficient alone. Placement of human c-sis cl 8 under the control of a retroviral LTR led to its transcription but not to the synthesis of detectable sis-related translational products. We identified a putative upstream exon in a c-sis flanking genomic DNA clone isolated from a normal human library. Insertion of this region upstream of c-sis cl 8 was ineffective in activating transforming properties. However, addition of an LTR to this construct led to acquisition of high titered transforming activity, comparable to that of SSV DNA. Moreover, the foci induced were indistinguishable in their appearance from those induced by SSV. The extraordinary high efficiency at which the LTR human sis chimera induced transformed foci excludes the possibility of a minority population of altered DNA molecules accounting for this activity.

Nucleotide sequence analysis of the upstream region revealed several open reading frames which could serve to initiate translation of a PDGF-2 precursor. A plasmid containing a 2.7-kbp human c-sis cDNA clone from a T-cell lymphoma line infected with HTLV, as well as the SV40 enhancer and early promoter regions, was able to induce transformation of NIH/3T3 cells. This cDNA clone contains a region of 180 bp directly upstream from its v-sis-related sequence. An identical sequence is present at position 581 to 761 in the activating region of our normal human fetal liver genomic DNA, confirming that this region contains an upstream exon of the human sis/PDGF-2 proto-oncogene. Moreover, our findings that PDGF-2 gene products were expressed in pLTR-c-sis RH-9/8 transformants convincingly demonstrates that this upstream region provides signals necessary for translation of these proteins.

Transformants induced by the activated human sis/PDGF-2 coding sequence contained two major sis/PDGF-2-related protein species of 52,000 and 35,000 daltons, which were detected using anti-sis peptide serum and nonreducing assay conditions. In contrast, a 26,000 dalton species, presumed to be the primary translational product, was observed under reducing conditions. These findings imply that the putative upstream exon sequences served to initiate translation of a PDGF-2 precursor molecule, which underwent dimer formation and subsequent processing. In light of our knowledge that the human sis gene codes for PDGF-2 and our findings of highly efficient transforming activity by the construct, we conclude that normal PDGF-2 expressed in NIH/3T3 cells is sufficient to induce transformation.

Significance to Biomedical Research and the Program of the Institute:

The present studies have potentially important implications concerning the role of normal genes coding for growth regulatory molecules in the neoplastic process. A number of growth promoting molecules have been shown to be released by a variety of tumor cells. In some cases, such molecules have been postulated to play a role in the neoplastic state of these cells. However, the alternative possibility exists that the expression of such factors is a secondary result of the genetic instability and dedifferentiated state known to exist in human cells.

Our present findings establish that derepression of the coding sequence for a normal human growth factor can cause it to acquire transforming properties in an appropriate target cell. Moreover, when incorporated by a retrovirus, the v-*sis*/PDGF-2 transforming gene has been shown to induce fibrosarcomas and glioblastomas. Many human glioblastomas and fibrosarcomas express *sis*/PDGF-2 transcripts, whereas normal fibroblasts and glial cells so far analyzed do not. Thus, transcriptional activation of this gene could be involved in the induction of naturally occurring tumors of connective tissue origin. If so, it will be important to gain more detailed knowledge of the regulation of the human *sis* proto-oncogene in order to determine what mechanisms may lead to its transcriptional activation.

Proposed Course:

The project will be terminated this year.

Publications:

Gazit, A., Igarashi, H., Chiu, I.-M., Srinivasan, A., Yaniv, A., Tronick, S. R., Robbins, K. C. and Aaronson, S. A.: Expression of the normal human *sis*/PDGF-2 coding sequence induces cellular transformation. *Cell*: 89-97, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05308-03 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Role of the sis Proto-oncogene in Human Malignancies

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

PI:	H. Igarashi	Visiting Fellow	LCMB	NCI
Others:	K. C. Robbins	Expert	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	F. Leal	Guest Researcher	LCMB	NCI
	N. A. Giese	Guest Researcher	LCMB	NCI

COOPERATING UNITS (*if any*)

None

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Molecular Biology Section

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NCI, NIH, Bethesda, Maryland 20205

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.*)

Expression of the PDGF-2 gene has been detected in a variety of human cells, both normal and transformed. Never in our survey were we able to detect expression of PDGF-2 in normal cells susceptible to the mitogenic effect of PDGF. As a means of further assessing the meaning of PDGF-2 expression in susceptible cells, molecular constructs containing all of the coding sequences of the normal PDGF-2 gene were prepared and introduced into NIH/3T3 fibroblasts. When provided with a retrovirus LTR promoter, the normal PDGF-2 gene was converted to an oncogene. These findings provide strong evidence for the concept that transcriptional activation of the PDGF-2 gene is involved in the process leading to tumors of connective tissue origin.

PROJECT DESCRIPTION

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

H. Igarashi	Visiting Fellow	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
F. Leal	Guest Researcher	LCMB	NCI
N. A. Giese	Guest Researcher	LCMB	NCI

Objectives:

1. To determine the role of c-sis in naturally occurring human tumors.

2. To assess the role of noncoding c-sis sequences in the transcriptional activation of the PDGF-2 gene.

Methods Employed:

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

Major Findings:

To investigate the possible role of retrovirus onc-related genes in human neoplasia, we have analyzed human cell lines and tissues for evidence of expression of sis-related genes. Our demonstration of sis-related transcripts in a high fraction of human fibrosarcoma and glioblastoma cell lines, whose normal counterparts are highly sensitive to the growth promoting activity of PDGF, suggests that the expression of this onc gene is associated with the continuing proliferation of these cells.

The human sis proto-oncogene contains the coding sequence for one of two polypeptide chains present in preparations of biologically active human PDGF. We sought to determine whether this normal coding sequence could be activated as a transforming gene by appropriate in vitro manipulations. A human clone, c-sis clone 8, which contains all of the v-sis-related sequences present in human DNA, was shown to be transcriptionally inactive when transfected into NIH/3T3 cells. When placed under the control of a retrovirus long terminal repeat (LTR), the clone was transcribed at levels comparable to that observed in cells transformed by SSV DNA. In spite of its transcriptional activation, c-sis clone 8 DNA did not demonstrate focus-forming activity.

A putative upstream exon was identified by its ability to detect the 4.2-kb sis-related transcript in certain human cells. Nucleotide sequence analysis revealed that this exon contained potential translation initiation signals which were not present in the first v-sis related exon of human c-sis. When this putative exon was inserted into the proper orientation between the LTR and c-sis clone 8, the chimeric molecule acquired high titered transforming activity, comparable to that of SSV DNA. These findings establish that the normal coding sequence for a human PDGF polypeptide has transforming activity when provided necessary signals for transcription and initiation of translation.

We utilized $p28^{v-sis}$ peptide and PDGF antibodies in an attempt to identify the sis/PDGF-2 polypeptide in human tumor cells. We were able to detect PDGF-2 only in human tumor cell lines expressing v-sis related transcripts. Although the human PDGF-2 precursor was distinct in electrophoretic mobility from $p28^{v-sis}$, more mature forms of the human protein were indistinguishable from processed forms of $p28^{v-sis}$. The PDGF-2 protein localizes to cell membrane components but is not a secretory protein.

Significance to Biomedical Resarch and the Program of the Institute:

Our studies have defined and characterized the SSV transforming gene and its product. In addition, this work has provided the basis for the finding that the sis gene encodes a growth factor-like molecule, suggesting that the pathway by which PDGF causes cellular proliferation may also be involved in the process leading normal cells toward malignancy. Studies of the human c-sis gene have revealed its transforming capacity. This finding, in combination with the pattern of our expression studies, strongly suggests the involvement of human c-sis in the etiology of tumors of mesenchymal origin.

Proposed Course:

1. Isolate the entire c-sis (human) proto-oncogene.

2. Determine what mechanisms lead to c-sis transcriptional activation.

3. To compare the physical and biological properties of the human c-sis gene product with those of PDGF.

Publications:

Gazit, A., Igarashi, H., Chiu, I.-M., Srinivasan, A., Yaniv, A., Tronick, S. R., Robbins, K. C. and Aaronson, S. A.: Expression of the normal human sis/PDGF-2 coding sequence induces cellular transformation. Cell 39: 89-97, 1984.

Igarashi, H., Gazit, A., Chiu, I.-M., Srinivasan, A., Yaniv, A., Tronick, S. R., Robbins, K. C. and Aaronson, S. A.: Expression of a normal growth factor gene causes morphologic transformation. In Giraldo, E., Beth, E., Castello, G., Giordano, G. G. and Zarrili, D. (Eds.): From Oncogenes to Tumor Antigens. Amsterdam, Elsevier (In Press)

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

Robbins, K. C., Igarashi, H., Gazit, A., Chiu, I.-M., Tronick, S. R., and Aaronson, S. A.: Establishing the link between human proto-oncogenes and growth factors. In Castellani, A. (Ed.): Epidemiology and Quantitation of Environmental Risk in Humans from Radiation and Other Agents: Potentials and Limitations (NATO). New York, Plenum Publishing Corp. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05362-02 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Studies of Oncogene Expression During Cellular Growth and Differentiation

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

PI:	J. Falco	Medical Staff Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	P. P. DiFiore	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (*if any*)

None

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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

☐ (a1) Minors

☐ (a2) Interviews

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

Proto-oncogene expression was studied in the BALB-MK epithelial cell line following mitogenic stimulation with epidermal growth factor (EGF). The following results were obtained:

C-fos expression: C-fos is undetectable in total cellular RNA of quiescent cells prior to addition of EGF. After addition of EGF, c-fos expression rises sharply to a sharp peak, one hour post-stimulation. It then drops off rapidly to low but detectable levels within the next few hours.

C-myc expression: These cells express constitutively high levels of c-myc when quiescent. When EGF is added, c-myc expression rises four- to eight-fold to a plateau beginning at two hours and ending at eight hours post-stimulation. It then steadily declines to steady-state levels higher than prior to addition of EGF.

Effects of TPA: The tumor promoter TPA was found to mimic the mitogenic effects of EGF and, like EGF, to stimulate a sharp one-hour peak of c-fos when given to BALB-MK cells in the absence of EGF.

Ca++ and c-fos expression: Ca++, which is a signal for terminal differentiation in BALB-MK cells, was found to cause a decline in c-fos expression.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

J. Falco	Medical Staff Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
P. DiFiore	Visiting Fellow	LCMB	NCI

Objectives:

To determine the possible role of proto-oncogenes as intracellular transducers of mitogenic and differentiating signals.

Methods Employed:

Cells: BALB-MK cells were selected for study because they provide a single system in which both signals for mitogenesis (EGF) and differentiation (Ca++) can be studied. These cells require supplemental EGF for sustained growth and must be maintained in subphysiologic levels of Ca++ (.05mM) for continuous passage. Increasing Ca++ levels to 1.5 mM causes terminal squamous differentiation of these cells.

EGF stimulation: Confluent plates of cells were rendered mitotically quiescent via 48 hours of EGF deprivation in the presence of 8% fetal calf serum (FCS). DNA synthesis was stimulated by giving the cells fresh media containing FCS and EGF (4 ng/ml). This treatment is known to cause the cells to move synchronously through S phase, with a peak of thymidine incorporation 100 times quiescent levels occurring 20 hours after EGF stimulation.

Studies with TPA: The possibility that the tumor promoter TPA could mimic the effects of EGF in this system was investigated. The same protocol as that for EGF was utilized but with varying concentrations of TPA substitutions for EGF.

Ca++ signaling: Growing cells were given fresh media contained FCS, EGF, and 1.5 mM Ca++. Thymidine incorporation and oncogene expression were checked at various time points following Ca++ addition.

RNA studies: Total cellular RNA was extracted from the study cells at various time points following EGF, TPA, or Ca++ signaling. Equal amounts (20 μg) of these RNA samples were run on formaldehyde gels, blotted (Northern blots) and hybridized to nick-translated probes of the various oncogenes to be studied.

Major Findings:

Oncogene expression following EGF stimulation: (1) c-fos expression: Pre-stimulation, c-fos was not detectable. The transcript appeared within 15 minutes of stimulation, peaked at one hour, and then fell rapidly to low but detectable steady-state levels over the next few hours. (2) c-myc expression: Quiescent BALB-MK cells express moderate levels of c-myc. EGF was able to stimulate a rise in expression of c-myc. This rise succeeded the c-fos peak and was much broader and less intense that the c-fos peak. Highest c-myc levels were four to eight times as high as pre-stimulation levels and occurred two to eight hours after the stimulus. Thereafter, levels dropped slowly to slightly higher than pre-EGF. (3) Other oncogenes: The following oncogenes were found to be constitutively expressed in BALB-MK cells, with no change of expression following EGF stimulation: C-Ha-ras, c-Ki-ras, c-abl. C-mos is not expressed in these cells.

Oncogene expression following shift to high Ca++: levels of c-fos were found to decline steadily during the first three days after shift to 1.5 mM Ca++.

Studies with TPA: The tumor promoter TPA was found to be nearly as strong a mitogen as EGF, with overall timing of post-stimulation DNA synthesis similar to that with EGF. The optimal dose of TPA (50 ng/ml), when given with fresh serum, stimulated a peak of thymidine incorporation 75% as high as that stimulated by EGF. In addition, it was found that TPA also stimulated a one-hour peak of c-fos approximately equal in intensity to that stimulated by EGF. These results suggest that EGF, in stimulating DNA synthesis, is acting through protein kinase C, the target enzyme bound and stimulated by TPA. Expression of the c-fos proto-oncogene then occurs distal to this step, and may or may not be a later step in the pathway ultimately leading to DNA synthesis and cell division.

Significance to Biomedical Research and the Program of the Institute:

Central to the understanding of the phenomenon of oncogenesis is the question of how cancerous cells escape the normal constraints on cell growth, and what normally regulates cell growth. Important in answering this question have been the discoveries of various growth factors. Recently it has been found that these growth factors have roles in moving quiescent cells (G-0) into the G-1 stage of the cell cycle (competency), and that other growth factors can allow the progression of cells through subsequent control points in G-1, leading to DNA synthesis and cell division. Recent discoveries that some oncogenes are homologues of growth factors or their receptors has led to the hypothesis that growth factor action may be mediated within the cell via oncogenes. Myc and fos are oncogenes whose protein products localize in the nucleus, suggesting a role in modulating gene expression or DNA replication. Recent studies in fibroblasts and lymphocytes have shown that mitogens can stimulate early expression of fos and/or myc. Our study is an important addition to these studies in that it shows analagous results in an epithelial cell line, lending credence to the view that surges in fos and perhaps myc mediate the mitogenic signal within the cell, ultimately leading to DNA synthesis and cell division.

Our TPA results further suggest a step through which EGF is acting--the phosphatidyl inositol pathway leading to stimulation of protein kinase C--and suggests that _fos_ expression is a subsequent step in this pathway.

Proposed Course:

The present study will be complemented by experiments distinguishing the roles of EGF vs. other serum factors in specifically stimulating _fos_, _myc_, or other oncogenes, and their various roles in competency and progression in this cell system.

Along these lines, we will attempt to set up a serum-free, defined-media system for these cells. Using this system, we will continue a study already underway, in which oncogenes of interest are being transfected into BALB-MK cells to study any effect vis-a-vis the abrogation of growth factor control of cell growth. Of particular interest would be transfection of the _myc_, _fos_, and _erb_ B oncogenes.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05363-02 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Analysis of Oncogenes Encoding a Growth Factor and Putative Receptor

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

PI:	C. R. King	Staff Fellow	LCMB	NCI
Others:	N. A. Giese	Guest Researcher	LCMB	NCI
	M. H. Kraus	Visiting Fellow	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*

None

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

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

Two areas are under investigation: (1) The structure of the v-sis oncogene is being assayed with respect to its function by means of in vitro mutagenesis. The resulting variants were tested by their ability to transform NIH/3T3 cells in culture using DNA-mediated gene transfer. The structure of the variant protein products was investigated. (2) Using the cloned v-erb B DNA as a probe, we have detected, cloned, and partially characterized a gene similar to but distinct from the EGF receptor gene. This gene is likely to encode a tyrosine kinase protein. Its gene has been found to be amplified in a human mammary carcinoma and thus may have a role in the phenotypic alterations of some human cancers.

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

C. R. King	Staff Fellow	LCMB	NCI
N. A. Giese	Guest Researcher	LCMB	NCI
M. H. Kraus	Visiting Fellow	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

In vitro mutagenesis has revealed that the PDGF-2 homologous region of v-sis is required for transforming activity. Continued mutagenesis will be used to identify more specifically the molecular residues required for activity.

The identification of a v-erb B-related cellular proto-oncogene distinct from the EGF receptor gene makes possible further characterization of this gene and its encoded protein product. The possible role of this gene in human cancer will be investigated.

Methods Employed:

We have used the directed mutagenesis approach of Wallace and Itakura to introduce single base changes into the v-sis coding region. The transforming function of v-sis is easily assayed by the ability of cloned proviral DNA to transform NIH/3T3 cells in culture following introduction using calcium phosphate precipitates. Since we expect that some variants will lose the ability to transform cells, we linked the simian sarcoma virus (SSV) provirus to the plasmid pSV2gpt. The pSV2gpt gene confers to eukaryotic cells resistance to media containing mycophenolic acid, hypoxanthine, aminopterin, and thymidine (killer HAT). Cells which have taken up the pSV2 simian sarcoma virus (SSV) plasmid can thus be identified and studied with respect to whether or not they have been transformed by the linked SSV provirus. This selection makes it possible to analyze v-sis protein expresssion in cells transfected with non-transforming variants. Other methods which generate mutations in a nonspecific manner will also be employed.

Using a cloned probe for the v-erb B oncogene, we screened a variety of DNAs isolated from mammary carcinoma tissue using Southern blot hybridization. We detected in one tumor sample the amplification of a cellular DNA fragment which hybridized to v-erb B but was distinct from the cellular EGF receptor gene. DNA from this tumor, MAC117, was cleaved with Eco RI and inserted into bacteriophage λ gtWES·λ B. Using a cloned library generated in this way, we used hybridization to v-erb B as a means of isolating homologous gene fragments. One phage, λ MAC117, contained the cellular gene fragment amplified in the tumor DNA of mammary carcinoma 117.

To further establish the relationship of this putative gene to the v-erb B oncogene, we determined the nucleotide sequence of a related region using the chemical degradation method of Maxam and Gilbert. The fragment homologous to the v-erb oncogene was subcloned into a plasmid and can be used as a hybridization probe to analyze this novel gene. Studies of the RNA product of this gene

are underway using Northern blot hybridization and the screening of cDNA libraries.

Major Findings:

Studies at the protein level have indicated that $p28^{sis}$ undergoes several post-translational processing events. These include formation of dimeric structures and cleavage to remove N and C terminal residues. Analysis of mutations suggests that the v-sis-encoded protein can be divided into four domains. The N-terminal domain is encoded by a virally derived nucleotide sequence and contains a signal-like sequence for membrane-associated synthesis of $p28^{sis}$. The central v-sis-encoded domain bears homology to the mature PDGF-2 polypeptide sequence and is essential for transforming function. Two domains flank the PDGF-related domain on N and C termini. These regions are dispensable for transforming function or the formation of dimeric structures. Analysis of N and C terminal deletion mutations also conclusively establishes the homodimer structure of the v-sis-encoded transforming protein.

We have identified and partially characterized a novel gene partially homologous to the viral oncogene v-erb B. The nucleotide sequence of two exons of this gene shows 85% amino acid sequence homology with the predicted amino acid sequence of v-erb B and human EGF receptor. This information establishes the cloned gene as distinct from but related to the EGF receptor gene. Moreover, the homology falls within a region shared by all tyrosine kinase-encoding genes. This strongly suggests that the novel gene identified by us is likely to also encode tyrosine kinase. Our observation that this gene is amplified in a mammary carcinoma suggests that genetic alterations affecting it may play a role in human cancer.

Significance to Biomedical Research and the Program of the Institute:

The SSV can cause oncogenic transformation both in vivo and in vitro. In addition, a variety of human cell lines have been found to transcribe the cellular proto-oncogene, c-sis. We have developed a system for the structural analysis of the v-sis oncogene product based on site-specific mutagenesis.

Our findings provide functional evidence for the presence of domains within the v-sis oncogene protein product. We anticipate that this information may be used to further characterize functional sites within the protein.

The identification of a new member to the tyrosine kinase-encoding gene family allows the exploration of how its protein product is related to cellular control processes and to the initiation or progression of neoplasia.

Proposed Course:

Continued analysis of the variants already generated will be required. The rationale and methodology described here are directly applicable to the study of other aspects of this sis protein structure/function relationship. The dimerization of the protein will be approached by introducing mutations to

cysteine codons of v-*sis*. Conserved structural elements responsible for receptor binding will also be investigated.

Using the molecular probes already isolated, we will investigate the structure of the transcript of the v-*erb*-related gene. We propose to do this by examination of Northern blot hybridization experiments and cDNA cloning.

Publications:

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05365-02 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Studies of Oncogenes in Human Urinary Tract Tumors

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

PI:	J. Fujita	Visiting Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. S. Rhim	Research Microbiologist	LCMB	NCI
	S. K. Srivastava	Visiting Fellow	LCMB	NCI
	M. Kraus	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 20205

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.)

In order to assess the significance of activated oncogenes in the pathogenesis of human urological malignancies, fresh urothelial tumors of the bladder and kidney were screened by both DNA transfection and restriction size polymorphism analysis. The frequency of Ha-*ras* activation by point mutations at the 61st or 12th codons was estimated to be about 10%. No rearrangement of *ras* genes was detected. In one of 21 tumors, a 40-fold amplification of the Ki-*ras* gene was detected.

Similar findings were made with fresh human renal cell carcinomas, suggesting the importance of quantitative as well as qualitative alterations of *ras* oncogenes in urological tumors. In nitrosamine-induced rat bladder tumors, activated Ha-*ras* was found in one of nine tumors by DNA transfection. The feasibility of this system as a model of human bladder carcinogenesis is being studied. Modification of the effect of activated *ras* oncogenes by anti-sense RNA or chemical mutagens is also being tried.

PROJECT DESCRIPTION

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

J. Fujita	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. S. Rhim	Research Microbiologist	LCMB	NCI
S. K. Srivastava	Visiting Fellow	LCMB	NCI
M. Kraus	Visiting Fellow	LCMB	NCI

Objectives:

To study the relevance of cellular oncogenes in pathogenesis and clinical findings of human urinary tract tumors. Studies are directed toward identification of the oncogenes involved and the specific molecular events leading to their activation. Specific methods to detect an activated oncogene are being sought and mechanisms to prevent or reverse transformation are being studied.

Methods Employed:

This laboratory has developed and utilized DNA-mediated gene transfer (DNA transfection) using NIH/3T3 cells as recipients to detect activated cellular transforming genes in malignant tumors. DNA is extracted from human urinary tract tumors and used for assay. Morphology is followed by light microscopy after DNA transfection and transformed foci are picked up and propagated for further analysis. A number of biochemical and molecular biological techniques, including gel electrophoresis, Southern blotting, immunoprecipitation and restriction endonuclease analysis, are used to characterize the activated oncogene. Gene cloning into phage and plasmid is used for characterization of the oncogene.

Major Findings:

Forty-seven samples of human urothelial tumors (bladder, ureter and renal pelvis) were analyzed by DNA transfection and restriction size polymorphism analysis: Ha-*ras* was found to be activated in ~10%. The Ki-*ras* gene was found to be amplified in one out of 21.

Of 16 human renal cell carcinomas (fresh frozen tissue), two were positive in the DNA transfection assay. Both had activated Ha-*ras* (one at 12th and the other at 61st codon mutation).

Of nine rat bladder carcinomas (fresh frozen tissue) induced by N-butyl-(N-4 hydroxybutyl) nitrosamine, one produced foci in DNA transfection. Activated Ha-*ras*, possibly at the 61st codon, is being explored by oligonucleotide hybridization.

Significance to Biomedical Research and the Program of the Institute:

Urothelial tumors are considered to be gross manifestations of a generalized neoplastic change of the urothelium and several chemicals have been identified

as bladder carcinogens in humans. The knowledge of which oncogenes are involved in human urothelial tumors and the factors influencing their activation will help elucidate the pathogenesis of the tumors and possible approaches to their prevention. The demonstration that the Ha-*ras* proto-oncogene is activated in 10% of human urothelial tumors by point mutation confirms the importance of oncogenes in clinical pathology and encourages the development of new diagnostic and therapeutic modalities based on studies of oncogenes and their products.

Proposed Course:

Further studies will build on present findings. Point mutation is not the only mechanism of oncogene activation, and gene amplification, gene rearrangement and the level of mRNA and proteins encoded by oncogenes is continuing to be investigated. A major effort will address itself to the effect of specific inhibition of Ha-*ras* gene expression on the transformed phenotype, using anti-sense Ha-*ras* RNA.

Publications:

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

Rhim, J. S., Sanford, K. K., Arnstein, P., Fujita, J., Jay, G. and Aaronson, S. A.: Human epithelial cell carcinogenesis: combined action of DNA and RNA tumor viruses produces malignant transformation of primary human epidermal keratinocytes. In Barrett, J. C. (Ed.): *Carcinogenesis, a Comprehensive Survey*. New York, Raven Press (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05366-02 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Studies on the Mechanisms of Oncogene Activation in Human Tumors

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

PI:	M. H. Kraus	Visiting Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	C. R. King	Staff Fellow	LCMB	NCI
	J. Fujita	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

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Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

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

The mechanism of oncogene activation in two urinary tract tumor tissues positive in the NIH/3T3 transfection assay was determined. Nucleotide sequence analysis of the first and second exon of H-*ras* oncogenes molecularly cloned from first-cycle transfectants identified single base changes at codon 61 in comparison to the human H-*ras* proto-oncogene. In the renal pelvic tumor JPT26, guanine was substituted for adenine, resulting in a change of glutamine to arginine; and in the bladder tumor JBT44, the substitution of thymidine for adenine at the same nucleotide position resulted in an amino acid change of glutamine to leucine.

Southern blot analysis of human mammary tumor DNAs with v-*erb* B as a probe revealed two patterns of aberrations: the mammary tumor cell line BT20 exhibited an approximately eight-fold gene amplification of the majority of Eco RI restriction fragments, whereas tissue from a primary mammary adenocarcinoma (MAC 117) showed distinct amplification of a ~6-kb restriction fragment. Southern blot analysis using cDNA from the human EGF receptor gene as a probe confirmed, under stringent hybridization conditions, an amplification of the EGF receptor gene in BT20 and established the amplification of a v-*erb* B-related gene distinct from the EGF receptor in MAC 117. Molecular cloning of this fragment and nucleotide sequence analysis defined two putative exons with closer homology to the v-*erb* B/EGF receptor than to other known tyrosine kinases.

Gene product analysis of EGF receptor mRNA and protein in BT20 demonstrated that amplification of the EGF receptor gene resulted in an elevated receptor level. Furthermore, comparative EGF receptor gene/gene product analyses of human tumor cell lines with increased EGF-binding capacity demonstrated that elevated EGF receptor levels can be found in the presence and absence of gene amplification or rearrangement of the EGF receptor gene.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

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

Objectives:

1. To determine the activation mechanism of transforming DNA sequences associated with human tumors.

2. To screen human tumor DNAs for structural gene aberrations, such as gene amplification or gene rearrangement leading to overexpression of proto-oncogenes.

3. To search for unknown transforming genes amplified in human tumors and related to known oncogenes by relaxed stringency hybridization.

4. To characterize molecular pathways leading to overexpression of the EGF receptor on human tumor cells.

Methods Employed:

1. DNA transfection onto NIH/3T3 cells by the calcium phosphate coprecipitation assay.

2. Southern blotting.

3. Molecular cloning, using λ phage and bacterial plasmid systems.

4. Nucleotide sequence analysis according to Maxam and Gilbert.

5. Immunoprecipitation, phosphorylation assay, and SDS-PAGE analysis of proteins.

Major Findings:

1. Nucleotide substitution of guanine for adenine resulting in an amino acid change of glutamine to arginine activates the H-*ras* oncogene in a renal pelvic tumor, JPT 26. The H-*ras* oncogene of a bladder tumor, JBT 44, became activated at the same position by a point mutation from adenine to thymidine, substituting leucine for glutamine in the amino-acid sequence of the p21 gene product.

2. The EGF receptor gene was found to be amplified eight-fold in the human mammary tumor cell line BT20, leading to an overexpression of the EGF receptor protein.

3. Increased EGF receptor protein levels without gene amplification or rearrangement were observed in the human glioblastoma cell line HA 1781, indicating a dysregulation on transcriptional and/or translational level.

4. A novel v-*erb* B-related gene, distinct from the EGF receptor and amplified in a human mammary carcinoma, was identified and partially isolated. Nucleotide sequence analysis identified its closer relationship to v-*erb* B and EGF receptor than to other known protein kinase genes.

Significance to Biomedical Research and the Program of the Institute:

The identification of the activating site of two human urinary tract tumors at codon 61 of H-*ras* supports the evidence that *ras* gene activation associated in varying frequencies with human malignancies occurs predominantly at amino acid positions 12 and 61 of the predicted p21 gene product. As activated *ras* oncogenes have been found in 10-30% of carcinomas, 10% of sarcomas, and 10-50% of hematopoietic malignancies, we searched for activating mechanisms of proto-oncogenes other than point mutations associated with human tumors. The detection of an amplified EGF receptor gene leading to overexpression of the EGF receptor in a mammary carcinoma cell line, BT20, a tumor category in which we had previously reported a comparatively low incidence of activated *ras* oncogenes (less than 5% of the cases analyzed), indicates that amplification of a growth factor receptor with homology to the v-*erb* oncogene may be involved in human tumorigenesis. By overexpression of its gene product, it could have given growth advantage to the tumor cell at a certain stage of tumor development. Furthermore, we demonstrated that this growth factor receptor could be overexpressed due to gene amplification (BT20) or in the absence of gene amplification and gross gene rearrangements (HA 1781), indicating abnormalities on the genomic, transcriptional and/or translational levels which are responsible for the elevated expression of an oncogene/growth factor receptor product.

The identification and isolation of a novel v-*erb* B-related gene provides an opportunity to investigate its potential role in human malignancy. The amplification of this gene in a human mammary carcinoma suggests that increased expression of this gene may have provided a selective growth advantage to this tumor.

Proposed Course:

The relevance of the novel v-*erb* B-related gene for human tumorigenesis is suggested by sequence homology with an oncogene (v-*erb* B) and a growth factor receptor gene (EGF receptor), as well as its amplification in a mammary carcinoma. Studies are underway to obtain complementary DNA clones for a more precise molecular characterization of this novel gene member of the tyrosine kinase family. Moreover, clones containing the entire coding sequence facilitates the analysis of its normal function and of potential activation mechanisms in tumors. Antibodies raised against synthetic peptides can be used to analyze protein expression and structure coded for by this gene. Furthermore, Southern and Northern blot analysis of a variety of human tumors will address the question whether this gene is commonly activated in human tumors or its activation is tissue- or stage-specific.

Publications:

Fujita, J., Srivastava, S. K., Kraus, M., Rhim, J. S., Tronick, S. R. and Aaronson, S. A.: Frequency of molecular alterations affecting ras proto-oncogenes in human urinary tract tumors. Proc. Natl. Acad. Sci. USA (In Press)

Tronick, S. R., Eva, A., Srivastava, S. K., Kraus, M., Yuasa, Y. and Aaronson, S. A.: The role of human ras proto-oncogenes in cancer. In Castellani, A. (Ed.): Epidemiology and Quantitation of Environmental Risk in Humans from Radiation and Other Agents. New York, Plenum Press (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05456-01 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Transformation Induced by Viral and Cellular fgr Oncogenes

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

PI:	S. C. Cheah	Medical Staff Fellow	LCMB	NCI
Others:	K. C. Robbins	Expert	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	T. Kawakami	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

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

The GR-FeSV onc gene, v-fgr, appears to have arisen as a result of a recombinational event involving genes coding for actin and a tyrosine-specific protein kinase. In an effort to understand the role of the actin domain in transformation, a series of mutant GR-FeSVs with successive deletions in their gag and/or actin domains were constructed and tested for their ability to transform NIH/3T3 cells in a transfection assay. Preliminary data suggest that the actin sequence has little effect on transforming activity in vitro.

The human fgr proto-oncogene has been isolated and characterized and shown to be a distinct sequence located on the short arm of chromosome 1. Furthermore, it has been shown that the fgr proto-oncogene is evolutionarily conserved and is distinct from other related proto-oncogenes which encode proteins that are as much as 80% related in amino acid sequence. These findings strongly imply evolutionary pressure to conserve similar structure and kinase functions at different human loci.

A survey of human tumor cell lines and normal tissues was conducted for evidence of fgr proto-oncogene expression. Wide distribution of a single fgr-related mRNA species of 3 kb was found in lymphoproliferative disorders and neurologic tumors but limited expression in carcinomas and sarcomas. Further scrutiny of the data revealed that expression of c-fgr was associated with Epstein-Barr virus (EBV) genome-positive Burkitt's lymphoma lines. Additional experiments have shown that the immortalization function of EBV may involve activation of c-fgr, a member of the tyrosine kinase gene family.

PROJECT DESCRIPTION

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

S. C. Cheah	Medical Staff Fellow	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
T. Kawakami	Visiting Fellow	LCMB	NCI

Objectives:

1. To elucidate the molecular mechanisms by which GR-FeSV transforms cells.

2. To determine the normal function of the fgr proto-oncogene.

Methods Employed:

Standard biochemical techniques for DNA and nuclei RNA isolation and analysis, restriction enzyme analysis, gel electrophoresis, molecular hybridization techniques to analyze genes, recombinant DNA techniques for purification and amplification of genes, standard gene cloning methods, radioimmunoassays for qualitative and quantitative characterization of gene products, peptide synthesis and antibody production, DNA transfection and standard recombinant techniques for construction of mutant viruses; standard cell culture techniques.

Major Findings:

1. To investigate the role of the actin sequences of v-fgr in the biological activity of the virus, a series of mutant viruses were constructed with successive deletions in their gag and/or actin domains. These deletion mutants were tested for their ability to transform NIH/3T3 cells in a transfection assay. Preliminary data suggest that the actin sequences have little effect on transforming activity in vitro.

2. Studies of the transcriptional status of the fgr proto-oncogene in human tumors has revealed that in general certain lymphomas and neurologic tumors, but not sarcomas or carcinomas, express a 3-kb fgr-related mRNA. Further examination of lymphoid tumors demonstrated that expression of c-fgr was highly correlated with Epstein-Barr virus (EBV)-infected but not EBV-negative Burkitt's lymphoma cells. When EBV-negative Burkitt's cells were deliberately infected with EBV, the fgr proto-oncogene was transcriptionally activated. These findings demonstrate that EBV regulates expression of c-fgr in lymphocytes, and suggest that the fgr proto-oncogene cooperates in the immortalization of normal lymphocytes by EBV.

3. We have described the isolation and characterization of the human fgr proto-oncogene as a distinct sequence located on the short arm of chromosome 1. The fgr proto-oncogene is evolutionarily conserved and is distinct from other proto-oncogenes such as yes and src which encode proteins that are as much as 80% related in amino acid sequence.

Significance to Biomedical Research and the Program of the Institute:

Our studies have defined and characterized the GR-FeSV transforming gene as a novel oncogene which possesses tyrosine kinase activity. Studies of the fgr proto-oncogene have shown that it is distinct from other proto-oncogenes with which it shares a strong homology. These findings strongly imply evolutionary pressure to conserve oncogenes with closely similar structure and kinase functions at different human loci. Furthermore, our expression studies have demonstrated that EBV infection can be responsible for enhanced expression of c-fgr, and suggest that the cooperation of genetic changes affecting more than one proto-oncogene may be involved in the etiology of Burkitt's lymphoma.

Proposed Course:

1. Detection of variant proteins derived from mutant GR-FeSV transformants and/or co-transfectants, and analysis of transforming and nontransforming proteins by localization.

2. Rescue mutant GR-FeSVs with helper virus and assess their transformability in vivo.

3. To isolate c-fgr cDNA clones from human poly A+ mRNA templates to further characterize the human c-fgr locus and to determine the structure of the protein it encodes.

4. To determine the region of the EBV genome responsible for c-fgr activation and whether fgr proto-oncogene expression is involved in EBV-induced lymphocyte immortalization.

5. To determine whether fgr proto-oncogene expression is affected by other transforming DNA viruses.

Publications:

Tronick, S. R., Popescu, N. C., Cheah, S. C., Swan, D. C., Amsbaugh, S. C., Lengel, C. R., DiPaolo, J. A. and Robbins, K. C.: Isolation and chromosomal localization of the human fgr proto-oncogene, a distinct member of the tyrosine kinase gene family. Proc. Natl. Acad. Sci. USA (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05457-01 LCMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular Cloning of the Subunits of Platelet-Derived Growth Factor

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

PI:	P. Di Fiore	Visiting Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NCI
	J. H. Pierce	Sr. Staff Fellow	LCMB	NCI

COOPERATING UNITS (if any)

Litton-Bionetics, Frederick, Maryland (J. N. Ihle)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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 molecular cloning of subunit 1 of the platelet-derived growth factor (PDGF) is being attempted. The strategies used so far have been: (a) screening of human genomic libraries with digonucleotides made on the presumptive nucleotide sequence reconstructed from the partial amino acid sequence available for PDGF-1; (b) immunological detection of PDGF-1 and identification of the PDGF 1 transcript to identify a candidate cell line for construction of a cDNA library.

Normal mast cells, which display an absolute dependence for growth on interleukin (IL)-3, have been infected with several retroviruses in vitro. Infection with Harvey-, BALB- and Moloney-MSV is unable to relieve IL-3 dependence, whereas infection and transformation with Abelson murine leukemia virus (Ab-MuLV) induces IL-3-independent cell growth. The abrogation of IL-3 dependence is not due to an autocrine mechanism.

PROJECT DESCSRIPTION

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

P. Di Fiore	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI
J. H. Pierce	Sr. Staff Fellow	LCMB	NCI

Objectives:

To clone subunit 1 of the PDGF molecule. This will lead to a better understanding of the structure and biological function of the PDGF molecule, whose subunit 2 is a transforming protein encoded by the human proto-oncogene c-*sis*.

To identify new hematopoietic targets for retroviruses and to study the relationships between transformation and growth factor requirement in hematopoietic systems.

Methods Employed:

1. Oligonucleotides have been generated on the basis of the published amino acid sequence of PDGF-1. Two different kinds of probes have been used: (a) long oligonucleotides (40 bp) representing the most likely sequence encoding for a given peptide. In these probes the third base of each codon is guessed according to the codon preference in high eukaryotes and humans; (b) short oligonucleotides (14-17 bp) consisting of a mixture of probes having all the sequence possibilities encoding for a given peptide.

   A human genomic library (fetal liver) has been screened several times with these probes.

   Hybridizing phages have been purified and the regions of interest (hybridizing to the oligonucleotides) subcloned. Nucleotide sequence according to Maxam-Gilbert is being performed for comparison to the PDGF-1 sequence.

2. Normal IL-3-dependent mast cells from primary mouse fetal liver cultures have been infected with Abelson-murine leukemia virus (Ab-MuLV) or BALB-, Harvey- and Moloney-murine sarcoma virus (MSV). Productive infection by the viruses was assayed by the infectious center assay. Transformation was assayed by the soft agar assay and tumorigenicity in nude mice. The target of transformation for Ab-MuLV in vitro was established by cytochemical and biochemical markers (like metachromasic, histamine or IgE receptors). Induction of IL-3 dependence was assayed by comparing the colony-forming efficiency of the virally infected cell lines in the presence or absence of IL-3.

   Production of IL-3 by the infected cell lines has been assayed either at the protein level by means of a biological assay or at the mRNA level by Northern blot hybridization.

Major Findings:

1. Screening of a human genomic library with oligonucleotide probes has yielded a number of phages hybridizing with at least one of the oligonucleotides. The major problem encountered in this approach is the high number of false positive phages yielded by screening on a high complexity genomic library with oligonucleotides. Thus, we are now developing approaches to identify suitable probes to screen less complex libraries, such as cDNA libraries. To this end, antibodies have been raised against a synthetic peptide move on the base of the known amino terminal sequence of PDGF-1. This antibody should be a more specific reagent to study the expression of PDGF-1 molecules in various tissues as a preliminary approach to cloning.

2. Ab-MuLV is able to transform in vitro normal mast cells abrogating their dependence on IL-3 for growth. This finding is paralleled by the ability of Ab-MuLV to induce in vivo mastocytomas in pristane-primed adult mice resistant to lymphoid transformation (the major target for Ab-MuLV transformation). These two findings combined clearly establish a new hematopoietic target for transformation by Ab-MuLV.

   The release from IL-3 dependence brought about by Ab-MuLV infection is not due to an autocrine mechanism (i.e., IL-3 secretion by the infected cells). In fact, Ab-MuLV-infected mast cells do not product IL-3-like factors active in a biological assay, do not synthesize IL-3-specific mRNA, and are not inhibited in growth by antibodies against IL-3.

Significance to Biomedical Research and the Program of the Institute:

1. The homology between subunit 2 of PDGF and the proto-oncogene c-*sis* is the first finding relating oncogenes to defined cellular functions. In addition, a certain number of human tumors (mainly fibrosarcomas and glioblastomas) have been shown to express high levels of c-*sis* transcript. Cloning of subunit 1 of PDGF will shed light on the structure of the PDGF molecule, the relationship in terms of reciprocal regulation between the two subunits and possibly the role of PDGF in human neoplasms and/or growth control.

2. A precise relationship between oncogenes and the mechanisms regulating cell growth in normal and transformed cells is becoming clearer. To study the interaction of oncogenes with the growth factor requirement in an in vitro model system can contribute to the understanding of how oncogenes can interfere with the normal control of cell growth, thus leading to malignant proliferation.

Proposed Course:

1. To screen with antibodies against PDGF-1 various tissues known to produce PDGF or PDGF-like activities (like endothelial cells); to make expression cDNA libraries from the positive tissues identifed as outlined above; and to screen those libraries with the anti-PDGF-1 antibody and/or available oligonucleotides.

2. To continue the study on the interaction of retroviruses with the hematopoietic system using a newly constructed murine recombinant of the v-*erb* B oncogene on mouse bone marrow and fetal liver explants.

Publications:

Pierce, J. H., Di Fiore, P. P., Aaronson, S. A., Potter, M., Pumphrey, J., Scott, A. and Ihle, J.: Neoplastic transformation of mast cells by Abelson MuLV: abrogation of IL-3 dependence by a nonautocrine mechanism. *Cell* (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05458-01 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Gene Organization of Human c-fgr and Its Related Sequences

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

PI:	T. Kawakami	Visiting Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	S. C. Cheah	Medical Staff Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

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NCI, NIH, Bethesda, Maryland 20205

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 gene organization of a human proto-oncogene, c-*fgr*, is under investigation as a basis for understanding its normal functions and role in tumorigenesis. The cDNA library of human endothelial cells was screened by molecular hybridization using as a probe the tyrosine kinase portion (v-*fgr*) of the Gardner Rasheed feline sarcoma virus (GR-FeSV). Four unique clones of different sizes were isolated and the sequence of the longest one (1.5 kb) determined. The sequence showed a high degree of homology with v-*fgr*, v-*yes* and v-*src* oncogenes. It also revealed that the genomic sequence corresponding to this cDNA has a stop codon 36 bp downstream to the common recombination site of v-*fgr* and v-*yes*.

The cDNA libraries from human cells which produce c-*fgr* mRNA in large quantities are under construction.

PROJECT DESCRIPTION

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

T. Kawakami	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI
S. C. Cheah	Medical Staff Fellow	LCMB	NCI

Objectives:

To study the gene structures of human c-*fgr* and its related sequences. Studies are directed to give insight into the normal function of a c-*fgr* proto-oncogene product and its role for regulation of cell growth. Efforts are also directed to study its potential functions in human malignancies.

Methods Employed:

General biochemical methods for nucleic acid extraction and purification. Construction of a human cDNA library, screening of cDNA library by molecular hybridization using v-*fgr* as a probe, DNA sequencing methods (dideoxy chain termination method and Maxam-Gilbert chemical degradation method), S1 nuclease protection mapping, etc.

Major Findings:

1. C-*fgr* mRNA is found in most lymphoid cells and some other hematopoietic cell lines.

2. The DNA sequence analysis of a truncated *fgr*-related cDNA clone showed a high degree of homology to v-*fgr*, v-*yes* and v-*src* oncogenes.

3. The truncated cDNA sequence has a stop codon 36 bp downstream of the recombination site of the v-*fgr*/helper sequence or v-*yes*/helper sequence.

Significance to Biomedical Research and the Program of the Institute:

The isolation and DNA sequencing of the *fgr*-related cDNA clones make it possible to characterize the genomic organization of the human c-*fgr* proto-oncogene. This will be the basis for elucidation of a possible role of this proto-oncogene in tumorigenicity.

Proposed Course:

1. To construct the human cDNA library which contains full length c-*fgr* transcripts.

2. To determine the sequences of the full length c-*fgr* mRNA.

3. To study the regulatory mechanism of c-fgr expression in hematopoietic cells.

4. To study the effects of v-fgr expression in transgenic mice.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05459-01 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Structural and Functional Characterization of ras p21 Proteins

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

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

COOPERATING UNITS (if any)

None

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Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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

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

☐ (a1) Minors

☐ (a2) Interviews

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

The structural and functional properties of bacterially expressed ras p21 proteins were investigated by means of in vitro and in vivo analysis. A series of ras proteins, including BALB-MSV, Harvey-MSV and Kirsten-MSV, were expressed in E. coli and the products purified to >95% purity by extraction of bacterial pellets with 7 M urea followed by a sephadex G-100 chromatography. The same procedure was utilized to obtain deleted mutants of Harvey-MSV protein and to generate BALB-, Harvey- and Kirsten-MSV chimeric proteins carrying the normal 12th codon. Small deletions were generated at both amino and carboxy termini. Furthermore, larger deletions spanning almost the whole coding sequence generated a series of p21 derivatives lacking from 30 to 115 amino acid residues from the carboxy terminus. In vitro analysis of GTP binding, autophosphorylation and GTPase activities of all the expressed proteins show that at least two regions are required to generate all the activities. Amino acid sequences between positions 6-23 and 153-165 are necessary but not sufficient. In addition, monoclonal antibodies were generated against native p21 ras-H and the epitopes localized by means of deleted mutants. Monoclonals directed against positions 1-69 and 130-152 showed a complete blockage of GTP binding and related activities. Both sets of experiments indicate that at least these two regions are required for the in vitro activities of the p21 ras proteins. In addition, microinjection of NIH/3T3 cells by deletion mutants that showed lack of GTP binding activity showed a clear correlation between GTP binding, GTPase and transforming activity of the protein. The deleted derivatives of p21 were utilized as well to characterize a new functional domain by means of the localization of the epitope which is recognized by the monoclonal antibody Y13-259. This antibody has been proved to be able to block the normal activity of p21 proteins and revert the transformed phenotype of ras-, fms-, fes- and raf-transformed cells.

PROJECT DESCRIPTION

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

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

Objectives:

To study conformational structural and functional domains in the in vitro and in vivo activities of the p21 ras proteins. Efforts to understand the mechanisms of malignant transformation induced by ras oncogenes are in progress.

Methods Employed:

The development of a series of expression vectors has been achieved in order to express in E. coli several members of the ras gene family. These vectors have proved to be useful for the expresion of v-Ha-ras, v-Ki-ras and v-bas genes and derivatives. Purification of the expressed proteins has been achieved utilizing traditional fractionation procedures. In vitro enzymatic analysis and in vivo studies by microinjection of purified proteins have been utilized for characterization of the expressed proteins.

Major Findings:

1. The GTP binding site of p21 ras proteins requires sequences located at the $-NH_2$ and -COOH termini of the molecule.

2. When the GTP binding activity is lost by means of small deletion mutants, p21 is no longer transforming, indicating that the normal function of the protein requires the GTP binding activity.

3. Chimeric proteins containing Gly in position 12 and Thr in position 59 show in vitro GTPase activity comparable to the normal protein. However, they show a high transforming activity similar to activated p21s detected by microinjection of NIH/3T3 cells.

4. In vitro GTPase activity is not a valuable tool to distinguish between normal and transforming p21 ras proteins.

5. Monoclonal antibody Y13-259, which has been shown to interfere with the normal function of p21 proteins, does not interfere with the known in vitro activities of the protein.

6. This finding implies that Y13-259 antibody recognizes a new functional domain of the protein.

7. This new domain has been localized between amino acid residues 69-89.

8. We have generated several monoclonal antibodies against p21 proteins and localized the epitopes that recognize the p21 sequence. Some of them block in vitro activities of the protein.

Significance to Biomedical Research and the Program of the Institute:

The progress made in the understanding of conformation and function of p21, as well as the localization of a new functional domain in the molecule, will facilitate the understanding of the mechanism by which activation of the oncogenic potential of ras p21 proteins occurs. This knowledge will lead to the design of strategies that would block specifically the function of activated proteins reverting the transforming phenotype.

Proposed Course:

1. Deleted proteins will be tested for their competition in the effects induced by wild-type v-Ha-ras p21.

2. Microinjection studies of NIH/3T3 cells will be carried out in chemically defined medium to try to understand the requirements, if any, of p21 activity.

3. Our studies will be extended to other cellular systems in order to understand regulation of proliferation and differentiation in a variety of cell lines.

4. Screening of monoclonal antibodies will be continued in order to localize antibodies that specifically recognize the normal or mutated products.

Publications:

Lacal, J. C., Santos, E., Notario, V., Barbacid, M., Yamazaki, S., Kung, H., Seamans, C., McAndrew, S. and Crowl, R.: Expression of normal and transforming H-ras genes in *Escherichia coli* and purification of their encoded p21 proteins. *Proc. Natl. Acad. Sci. USA* 81: 5305-5309, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05460-01 LCMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Characterization of c-sis/PDGF-2 Locus

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

PI:	C. D. Rao	Visiting Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI

COOPERATING UNITS *(if any)*

None

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

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

The simian sarcoma virus transforming gene, v-sis, encodes a protein closely related to PDGF-2, a major component of platelet-derived growth factor (PDGF). Nucleotide sequence analysis has revealed that the human c-sis proto-oncogene, the normal homolog of v-sis, is the structural gene for PDGF polypeptide chain-2. The transcriptional product of the PDGF-2 gene is a 4.2-kb mRNA detected in certain human tumor cell lines as well as in normal human placenta and endothelial cells.

To study the structure and function of this oncogene we constructed a cDNA library from normal human placental mRNA. We have isolated five cDNA clones representing the PDGF-2 transcript and the complete nucleotide sequence of one clone, pSD1, 2.6 kb in length, has been determined. Our data revealed a noncoding sequence of 1625 bp at the 3' end of the mRNA of which 1597 bp were contained within a single exon in genomic DNA. In spite of the presence of long 3' noncoding sequences, the c-sis transcript did not contain the highly conserved polyadenylation signal, AATAAA. Instead, this signal was observed 18 bp downstream of the site of polyadenylation in genomic DNA. The PDGF-2 coding sequence consisted of 723 bp located immediately upstream of the 3' noncoding stretch. We detected two upstream TGA codons, both of which were in phase with the initiator ATG codon. These and other findings establish that the 2.6-kb cDNA clones contain the entire coding sequence of the normal PDGF-2 precursor.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

C. D. Rao	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI

Objectives:

To molecularly characterize the c-sis/PDGF-2 locus and clone full length complementary DNA.

Methods Employed:

For constructing cDNA libraries from normal human placental mRNA, we used the Okayama and Berg vector system. Other methods used include standard gene cloning methods, restriction enzyme analysis, gel electrophoresis, DNA sequencing by the method of Maxam and Gilbert, and library screening.

Major Findings:

1. The 4.2-kb PDGF-2 transcript contains only 723 bp of coding sequence which is represented in the 2.6-kb cDNA clone.

2. The coding sequence is followed by 1625 bp of noncoding ssequence at the 3' end.

3. Two TGA terminator codons in phase with the ATG initiator codon were observed in the upstream noncoding sequences.

4. The PDGF-2 transcript lacks the highly conserved polyadenylation signal, AATAAA, as observed from the sequence analysis of the 3' ends of the five c-sis cDNA clones.

5. Our data reveal that 723 bp of the 4.2-kb c-sis mRNA codes for the polypeptide chain-2 of PDGF.

6. Enhancer-like TG element is observed downstream of poly(A) site.

Significance to Biomedical Research and the Program of the Institute:

The PDGF-2 gene is actively transcribed in certain human glioblastomas and sarcomas. PDGF is also a potent growth factor involved in the repair mechanisms of blood vessels. Characterization and studies on regulation of the PDGF-2 gene can help shed light on the role of PDGF in cancer and growth.

Proposed Course:

1. To characterize the 5' noncoding sequences of the PDGF-2 gene by primer extension and S1 mapping analyses.

2. To study the transcriptional regulation of PDGF-2 by characterization of upstream promoter sequences.

3. Role of 3' sequences in regulation of c-sis expression.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05461-01 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Regulation of c-sis Expression

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

PI:	D. Ron	Visiting Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NCI

COOPERATING UNITS (if any)

None

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☐ (a1) Minors

☐ (a2) Interviews

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

The regulating mechanism involved in c-*sis* expression is being studied utilizing the technique of somatic cell hybridization. The strategy employed is to fuse cells negative for c-*sis* expression with those actively transcribing this gene. We postulate the existence of a trans-acting factor that will turn on a previously silent c-*sis* gene. Specifically, fusions between human glioblastoma or carcinoma cell lines and a mouse 3T3 cell line were performed. The human-derived cell lines expressed c-*sis*, while the mouse cell line did not produce detectable amounts of the mouse c-*sis* transcript, as determined by Northern blotting and hybridization under relaxed conditions using v-*sis* and human c-*sis* probes.

Since the size of the mouse transcript was not known, it was necessary to find tissues or cell lines which expressed this gene. To do this, RNAs of several mouse cell lines, as well as embryos and placentas were screened. The transcript was detected in mouse placenta RNA and its size was found to be very similar to that of the human c-*sis* transcript. These results will make it difficult to distinguish between RNAs in the somatic cell hybrids. To overcome this problem, efforts are now underway to clone the mouse c-*sis* gene. Specific probes from this gene will be used to detect the mouse transcript in the cell hybrids using the the S1 nuclease technique. This should distinguish the mouse c-*sis* RNA from the human counterpart. If positive results are obtained, attempts will be made to isolate the gene coding for a putative regulating factor utilizing molecular cloning and an appropriate selection system.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

D. Ron	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI

Objectives:

To study the regulatory mechanism underlying c-sis expression. Experiments are directed to see if a trans-acting factor regulates c-sis expression. If identified, the role of such a factor under physiologic and neoplastic conditions will be studied. Efforts will also be made to clone the gene coding for mouse c-sis and to identify highly conserved sequences which might be important regulatory signals and thus essential for c-sis expression.

Methods Employed:

The cell fusion method was performed to make hybrids between human- and mouse-derived cell lines. Hybridization conditions were developed in order to detect the mouse c-sis transcript. Other methods used include RNA extraction from tissues and cell lines, Northern and Southern blotting, restriction enzyme analysis, gel electrophoresis, S1-nuclease analysis and preparation of high molecular weight DNA.

Major Findings:

1. Human cell lines expressing c-sis transcript at readily detectable levels were identified. Mouse cell lines which are negative for mouse c-sis expression were also identified. Among these, cell lines containing suitable markers for cell hybrid selection were chosen for the fusion.

2. Hybridization conditions were developed to detect the mouse c-sis transcript. The transcript was found in mouse placenta RNA in late but not early stages of gestation. This may indicate that mouse c-sis expression is developmentally regulated. The size of the mouse c-sis transcript was determined to be very similar to that of the human c-sis RNA.

Significance to Biomedical Research and the Program of the Institute:

The human c-sis is expressed in several types of tumor cells. Studies of its regulation should shed light on the role of this gene in human malignancies. In addition, if a trans-acting factor regulates c-sis expression, isolation and identification of such a factor in normal and neoplastic tissues could contribute to the understanding of the steps involved in the malignant process and cell differentiation.

Proposed Course:

1. To screen human-mouse hybrid cells for expression of the c-*sis* transcript, using the S1-nuclease technique with human- and mouse-specific probes.
2. If trans-activation of the mouse c-*sis* can be established, attempts will be made to isolate the gene coding for the human regulatory factor, utilizing cloning and an appropriate selection system. If successful, different tissues and tumors at different stages will be screened for expression of this factor in order to examine its role in malignant processes and in cell differentiation.

3. Cloning of the mouse c-*sis* gene and preparation of specific probes from this gene.

4. Comparison of the nucleotide sequences of the mouse c-*sis* to that of the human c-*sis* in order to identify highly conserved sequences which might be important as regulatory signals.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05462-01 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Molecular Genetic Analysis of *ras* Genes

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

PI:	M. Ruta	Staff Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	C. R. King	Staff Fellow	LCMB	NCI
	S. Srivistava	Visiting Fellow	LCMB	NCI
	J.-C. Lacal	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

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

Ras genes are found to be activated in a high proportion of human tumors. The p21 proteins from these tumors have amino acid alterations at critical positions. In order to characterize the structural and functional domains of the *ras* genes, we have used a variety of techniques to mutate these genes. Both site-directed mutagenesis using oligonucleotides and linker insertion techniques were employed. We found that certain mutants alter the transforming ability of *ras* genes. When mutated proteins were analyzed for their biochemical properties, certain mutants were no longer able to autophosphorylate in an in vitro assay. We are analyzing these mutants to characterize the altered biochemical properties of p21 proteins.

PROJECT DESCRIPTION

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

M. Ruta	Staff Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
C. R. King	Staff Fellow	LCMB	NCI
S. Srivistava	Visiting Fellow	LCMB	NCI
J.-C. Lacal	Visiting Fellow	LCMB	NCI

Objectives:

To identify the structural and functional domains of the ras p21 protein.

Methods Employed:

Oligonucleotide-mediated, site-directed mutagenesis was used to generate mutations at specific amino acids. Linker insertion mutagenesis was used to insert Xho I linkers into specific areas of the ras gene. Mutants were ligated to a viral LTR and then assayed for biological activity by NIH/3T3 transfection. The mutated proteins were assayed for biochemical properties (i.e., altered mobility, in vitro autophosphorylation, GTP binding) by standard procedures.

Major Findings:

1. Linker insertion mutants at amino acid 63 and 111 affect the biological properties of p21.

2. Specific linker insertion mutants affect the biochemical properties of p21. These mutants no longer are able to autophosphorylate in an in vitro assay.

3. Gross deletion mutants eliminate biological activity of p21.

4. Site-directed mutagenesis of cys 51, 80, 118, and 186 indicates that only alteration of cys 186 inactivates the protein. This supports the idea that cys 186 is involved in lipid addition. Protein analysis of the mutants with or without reducing agent suggests that cys 51 and 80 are disulfide linked.

5. Site-directed mutagenesis of amino acids 59 and 60 suggests that these amino acids affect GTP binding.

Significance to Biomedical Research and the Program of the Institute:

Ras genes have been implicated in 15% of human tumors. Knowledge of the structural and functional domains of the p21 protein is important in understanding the possible functions of p21.

Proposed Course:

To complete the biochemical analysis of the mutated p21 proteins that have been generated.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05463-01 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Cellular and Oncogene Products which Participate in Growth Regulation

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

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

COOPERATING UNITS (if any)

None

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1.0	1.0	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

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

The long-term objective of this program is to understand the mechanisms of cellular changes fundamental to neoplastic transformation. Mammalian cells in culture represent a model with which to study growth regulation. Tumor cells have been shown to produce growth factors with mitogenic activity. Moreover, recent studies have shown that the gene products of certain tumor viruses are virtually identical to the normal cellular homologs which participate in the regulation of cell proliferation. Since mammalian cells in culture usually require undefined serum for proliferation, an assessment of the biologic impact of retroviral gene products on growth control required development of serum-free culture systems. Presently, precoating of the growth surface, and "optimized" nutrient medium and a battery of hormones and growth factors have been identified as critical for cell growth in serum-free medium.

PROJECT DESCRIPTION

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

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

Objectives:

To determine if proto-oncogenes alter growth regulation in characterized cell lines (e.g., NIH/3T3, BALB/MK). To achieve this objective, it is necessary to: (a) define conditions in which nontransformed cells will respond to known concentrations of growth factors and/or hormones in the absence of serum; (b) determine if specific oncogenes complement growth factor requirements; and (c) test whether interaction of the *sis* gene product with the cell surface is required for its transforming activity.

Methods Employed:

Standard methods are adapted so that cells usually grown in serum-containing medium survive and proliferate in serum-free medium without adaptation. It is necessary to simulate a basement membrane by coating the growth surface with one or more proteins which augment adhesion and spreading, and to supplement the basal medium with known growth factors and hormones. Several nutrient media have been screened to determine which would best support proliferation of NIH/3T3 fibroblasts at low cell density, either with 0.1% v/v calf serum or in serum-free medium without selection. Once growth factor/hormone concentrations are optimized, nontransformed fibroblasts or epithelial cells are compared with viral transformants to determine the degree of complementation conferred by *onc* gene products resulting in aberrant growth regulation.

Major Findings:

First, precoating of the growth surface was found essential to fibroblast proliferation under serum-free conditions. Though useful for clonal growth of normal fibroblasts and primary cultures of epidermal keratinocytes, this requirement was not initially thought critical for established lines such as NIH/3T3. Second, several media were tested for growth promotion under serum-free conditions, a marked insulin requirement observed with NIH/3T3 in the absence of serum, and both medium and growth factor requirements "optimized." As this is a developmental project, efforts continue to: (a) simplify the basal nutrient medium, and (b) adjust hormone and growth factor concentrations to the lowest efficacious level in order to enhance sensitivity of the biologic assay. These studies have progressed sufficiently, however, to allow orientation studies on gene activation and assessment of the action of purified p21 protein under serum-free conditions. Finally, a similar approach is being used for BALB/MK epithelial cells; preliminary studies suggest these cells will proliferate under similar serum-free conditions.

Significance to Biomedical Research and the Program of the Institute:

Rapidly transforming retroviruses are thought to have arisen by recombination of a nontransforming virus genome and transduced, normal cellular gene sequences (proto-oncogenes). Proto-oncogenes presumably act, directly or indirectly, at different points in the mitogenic pathway of normal cells. Irrespective of the normal cellular homolog, constitutive expression of *onc* gene products allows viral participation in the regulation of cell proliferation. The frequent loss of growth control in transformed cells can be interpreted as an alteration of one or more controlling genes during proliferation. Biologic manifestations include: changes in extracellular growth factor requirement(s); changes in cell membrane receptors which transduce extracellular mitotic stimuli; or changes in intracellular sensing mechanisms associated with initiation of DNA synthesis.

Serum, usually essential for proliferation in culture, contains hormones and growth factors, carrier proteins, proteases and inhibitors, attachment and spreading factors, metals and low molecular weight nutrients. Sato *et al.* showed that in some cases the serum requirement can be met by supplying only those serum components critical for proliferation; Ham *et al.* showed that mammalian cells grow as well or better in serum-free medium than serum-containing medium if the nutrient mixture is optimized for the cell type under study. These complementary observations suggest that a more rigorous biologic analysis of the impact which proto-oncogenes have on regulation of proliferation is possible. The demonstrated homology between the v-*sis* gene product and PDGF-2 further illustrates the need to refine biologic systems in which to study related proto-oncogene products and normal cell products associated with metabolism and proliferation.

Proposed Course:

1. Continue refinement of serum-free culture systems for both fibroblasts and epithelial cells.

2. Application of serum-free culture systems to obtain a better understanding of growth regulation.

3. Determination by immunologic and pharmacologic means if the *sis* gene product is secreted and subsequently interacts with the cell surface to exert its transforming activity.

Publications:

Taylor, W. G.: Toxicity and hazards to successful culture: cellular responses to damage induced by light, oxygen or heavy metals. In Stevenson, R. E. (Ed.): *Uses and Standardization of Vertebrate Cell Culture.* Tissue Culture Association Monograph No. 5, 1984, pp. 58-70.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05464-01 LCMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Characterization and Subcloning of a New Human Oncogene

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

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

COOPERATING UNITS (if any)

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Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

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

☐ (a1) Minors

☐ (a2) Interviews

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

A new human oncogene derived from the DNA of the spleen of a patient with a poorly differentiated B lymphocytic lymphoma has recently been cloned in cosmids in our laboratory. We have begun characterization of two cosmid clones by restriction endonuclease mapping of the oncogene. The first clone, designated C-8-X-1-1, has been shown to contain both human and mouse (NIH/3T3)-derived sequences. The human sequences of the C-8-X-1-1 (which had previously been shown to be devoid of biological activity, i.e., transformation of NIH/3T3 cells), have been shown to overlap partially with the human sequences contained in the cosmid clone C-14-1-2, which was shown to possess transforming activity. This last clone has been partially characterized as far as the enzyme restriction map is concerned and several fragments of it have been purified and subcloned either in pBR322 or in pAT-153. Some of the fragments, which are almost completely free of repetitive human sequences (Alu-negative), have been used to screen a normal human genomic library in order to detect sequences homologous to the new oncogene in normal human DNA. Three phages containing sequences homologous to some of the isolated fragments have been isolated so far and their further characterization is currently being performed. The Alu-negative fragments isolated from the cloned oncogene, as well as some Alu-containing sequences, also isolated by us, are being used to detect the mRNA for the gene in transfected NIH/3T3 cells and will be used to screen fresh normal and tumor tissues for expression of the newly isolated oncogene.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

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

Objectives:

1. Full characterization of the biologically active clone by restriction endonuclease mapping.

2. Isolation of the normal allele of the oncogene from phage libraries of normal human DNA.

3. Identification of the mRNA for the oncogene and for the normal gene in transfected NIH/3T3 cells as well as in human normal and tumor tissues.

Methods Employed:

The methods used include library screening, restriction enzyme analysis, standard gene cloning methods, and gel electrophoresis analysis.

Major Findings:

1. Development of restriction endonuclease maps of two cosmid clones of the oncogene, one designated C-8-X-1-1, containing both mouse and human sequences; and the other, designated C-14-1-2, containing only human-derived sequences and possessing full biological activity.

2. Isolation and plasmid subcloning of several Alu-free and Alu-containing fragments from the cosmid C-14-1-2.

3. Isolation and further characterization of three human normal DNA-containing phage clones which cross hybridize with two Alu-free DNA fragments of the oncogene.

Significance to Biomedical Research and the Program of the Institute:

The complete characterization of the newly isolated oncogene as well as of the normal human DNA containing homologous sequences will allow a better understanding of the new oncogene structure and possibly the mechanism of its activation.

Proposed Course:

1. Analysis of the size of the mRNA transcribed by the oncogene and possible definition of the portion of the gene from which the mRNA derives.

2. Definition of the portion of the cloned sequences which are an integral part of the oncogene and possibly those sequences which represent flanking human DNA sequences.

3. Definition of the sequences of the oncogene which are necessary for the transforming activity.

4. Screening of several human B cell lymphomas as well as other types of malignancies related to malignant B cell lymphomas for the presence and expression of the newly isolated oncogene.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF MOLECULAR ONCOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1984 to September 30, 1985

The Laboratory of Molecular Oncology plans and conducts research defining the molecular and genetic elements responsible for the development and expression of the malignant phenotype in humans and animals. Towards this end, the Laboratory of Molecular Oncology (1) applies the skills of molecular biology, recombinant DNA technology and the methods of immunology to identify and isolate cellular transforming onc genes from malignant and normal cells and tissues, as well as to characterize the product(s) encoded by these genes; (2) identifies, isolates, characterizes and determines the function(s) of these onc genes as determined by the expression and functional analysis of their specified oncoproteins; (3) develops the molecular probes and methodologies required to identify the target(s) of the onc gene product(s), relative to the process of malignant transformation and the normal mode(s) of cellular action; (4) determines and evaluates the organizational structures and function(s) of the normal cellular homologs (proto-onc genes) of the acute transforming viral onc genes by expression in normal eukaryotic and prokaryotic cellular systems; and (5) examines the molecular controlling elements and mechanisms regulating prokaryotic and eukaryotic gene expression, aiming to adapt, modify and apply this understanding to the expression and control of the neoplastic processes. The Laboratory of Molecular Oncology conducts research on the molecular elements responsible for the development and expression of malignant phenotypes in humans and animals. The Laboratory applies skills in molecular biology, recombinant DNA technology and hybridoma-monoclonal antibody production in a comprehensive program to identify and isolate cellular transforming genes and to characterize products expressed by these genes. This mission is accomplished by bringing together expertise in the diverse disciplines of eukaryotic and prokaryotic virology, molecular biology and genetics. The Carcinogenesis Regulation Section studies the relationship between oncogenic gene expression and the conversion of cells from the normal to the transformed, malignant state. Specific regions of molecularly cloned, acute transforming retrovirus genomes are tested for transforming activity and to determine the molecular mechanisms by which their oncogene products act in concert with cellular factors to activate the neoplastic process. This will be accomplished by examining the organization and products of normal essential cellular proto-onc gene(s) and the mechanism(s) by which these genes are subverted into transforming genes, as well as by comparing them to the organization and expression of their corresponding malignant counterparts. The Cellular Transformation Section investigates the malignant transformation of cells by avian sarcoma viruses, including the function of the virus-coded protein(s) directly responsible for transformation, the primary physiological effects of the functioning protein(s), the sequence of metabolic changes resulting in the altered metabolic profile characteristic of malignant cells, and the metabolic changes necessary for the maintenance of the malignant state. The Microbiology Section investigates the mechanism of cell transformation using biological, biochemical and immunological techniques. The transforming potential in mammalian cells of specific viral and cellular

DNA sequences amplified by cloning in appropriate prokaryotic vectors is determined under selective conditions using characterized markers. Transformation studies are augmented using monoclonal antibodies prepared against various DNA sequences and amplified by cloning in appropriate prokaryotic vectors, and then determined under selective conditions using characterized markers. Transformation studies are also augmented using monoclonal antibodies prepared against various viral- and cell-coded proteins. Specifically, this section develops systems to assay and analyze the mechanism of malignant transformation in human primary and established lines of cells using sequences derived from viral and genomic cellular DNA that may have oncogenic potential. The Molecular Control and Genetics Section conducts studies to understand how gene expression is controlled in the prokaryote, E. coli, and its phage, lambda. Their focus is on the elucidation of gene regulation at the levels of transcription initiation, transcription termination, RNA translation and RNA processing. In addition, this section, applying its expertise developed with prokaryotic systems, studies the basic molecular mechanism by which genes are expressed and normally regulated during differentiation using a simple eukaryotic model system (the cellular slime mold, Dictyostelium discoideum). The Office of the Chief, in addition to coordinating the administrative responsibilities of the Laboratory and its sections, conducts research to investigate the molecular structure and function and biochemical properties of select oncogenes, including the ras family of oncogenes and the p21 ras onco-protein(s), in particular. Such studies are directed towards a well-defined molecular and biochemical description of malignant transformation by select oncogenes and their products.

The major portion of the present and future emphases of this Laboratory concerns the identification, isolation and analysis of oncogenic sequences by molecular cloning techniques, as well as their oncogene protein products, in order to evaluate their relationship to the malignant transformation process. We have pursued studies in several major areas. We are analyzing the structural and biological properties of retroviral onc genes present in both avian and mammalian acute transforming retroviruses. We are identifying, isolating and characterizing normal cellular homologs, the proto-onc genes, from their species of origin, as well as from the human genome. Through combined efforts of the sections within the Laboratory of Molecular Oncology, we have developed strategies and protocols to address the mechanisms by which cellular proto-oncogenes can become transduced, activated and expressed, and have attempted to delineate the pleiotropic molecular changes effected by these concerted neoplastic events. We have also capitalized on our research emanating from investigations performed on acute transforming viruses. In particular, the Carcinogenesis Regulation Section has found that the avian carcinoma virus, MH2, shares a transformation-specific sequence, myc, in common with other acute transforming retroviruses such as MC29, CMII, and OK10. We have molecularly cloned a 5.2 kb MH2 provirus DNA and determined its complete genomic structure. In addition to Δgag, Δenv, and a c genetic region, shared in common with other nondefective retroviruses, we have discovered a unique mht genetic element which appears to be a MH2 transformation-specific onc gene. Hybridizations with normal chicken cellular DNA, as well as with cloned, chicken c-myc DNA, reveal that mht sequences are probably derived from a normal cellular gene, quite distinct from the c-myc proto-onc gene. Significantly, this avian mht region is 94% homologous to the murine raf oncogene at the deduced amino acid sequence level, the closest such homology determined thus far. Thus, it appears that the genome of the acute transforming retrovirus encodes two distinct genes having possible oncogenic functions, unlike the other avian retroviruses, MC29, OK10, and CMII, each carrying a

single hybrid Δgag-myc oncogene. Despite this fundamental difference, MH2 and MC29 viruses have similar oncogenic properties. The 5.2 kb genome of the avian retrovirus, MH2, has been analyzed and the nucleotide sequence 3.5 kb from the 3' end of the Δgag region to the 3' end of the retroviral vector has been elucidated and compared to other mht- and myc-containing oncogenic viruses, as well as to the chicken proto-myc oncogene. These data reveal that MH2 contains two potential transforming genes: Δgag-mht and myc. Deletion and frameshift mutants of each of these two MH2 oncogenes were constructed and were transfected into primary chicken and quail cells using helper virus to rescue viable virus and to assay the transforming functions of these constructs. We have found that the myc gene transforms primary cells by itself without the second potential onc gene, mht. In fact, the Δgag-mht gene was without detectable transforming function in these primary cell assays. Further work in animals will be necessary to determine the role in oncogenesis for each of the mht and myc genes. But, the significance of these data, relating to MH2, is that out of 19 known different viral oncogenes, 5 have been observed in viruses of different taxonomic groups. This observation suggests, therefore, that the number of different cellular proto-onc genes most likely is limited, since unrelated taxonomic groups like avian MH2 and murine 3611 retroviruses have transduced the same onc gene-specific sequences from vastly different cellular species. Furthermore, these observations suggest that specific deletions and linkages of the same proto-onc sequences integrated into different retroviral vector elements also effects differences in their oncogenic potential and transformation capabilities. Using nucleic acid hybridization, heteroduplex and DNA sequence analyses have indicated that the v-mht sequence captured by the MH2 virus is dispersed over 25 kb of the chicken genome. The chicken proto-mht contains 11 exons homologous to the v-mht sequence. Thus, it appears that the v-mht onc gene transduced a truncated version of the proto-mht gene; it lacks introns and possibly 5' coding sequences. Because there is no sequence homology between the proto-mht and the retroviral helper sequences, the virus transduction probably occurred by legitimate recombination. This section has also developed a prokaryotic vector which allows the expression of high levels of onc genes in E. coli. This vector (pJL6) has provided a means for studying the chemistry of the protein and for preparing appropriate immunological reagents for studying the expression of the oncogene product in transformed cells and tumor cell lines. This efficient expression vector contains several unique restriction enzyme sites 12 codons beyond the lambda phage cII gene initiation codon. A number of variants of this vector have been constructed containing a number of restriction enzyme sites suitable for cloning in a variety of viral and cellular onc genes. Thus far, fusion proteins of v-myc, first and third exon onc genes, v-myb, Ha-MuSV ras, v-mht, and c-sis are among the products expressed so far with this vector, in addition to specific portions of the HTLV-I envelope gene. These viral gene fragments were expressed as a 16,000 dalton portion of the N terminus of the p21E transmembrane fusion protein, as well as a 13,000 dalton portion of the gp46c-terminal portion of the HTLV-I env gene product. In addition, a fusion protein representing amino acid sequences from the carboxy terminus of the p42 LOR protein using the vector with the HTLV-I pX gene. These HTLV-specific proteins have been isolated, characterized and purified to homogeneity. Since these proteins reacted with antibodies in patient sera, they have the potential for use in diagnostic assays. Additionally, these proteins can be used to map epitopes recognized by monoclonal antibodies directed against these bacterially-expressed proteins. Studies in the Carcinogenesis Regulation Section have also included work on the cellular ets gene to determine if a pattern of truncated normal genes in the transforming retrovirus can be extended to other onc genes.

Consistent with this pattern, the cellular ets transcript is considerably larger than that DNA transduced by the E26 virus. In addition, it has been determined that the mammalian homolog of v-ets consists of two distinct domains, ets-1 and ets-2, located on different, yet homologous, chromosomes in cats, mice, and humans. Using chromosome-specific probes it has been demonstrated that both loci, ets-1 and ets-2, are transcriptionally active and distinct from one another. Since the sequences homologous to ets-1 and ets-2 are colinear in chicken proto-ets, it is possible that they diverged and became functionally distinct prior to their evolutionary separation in mammalia. We have identified a number of human leukemias, such as the acute undifferentiated leukemias (AUL) and the acute myeloid leukemia with maturation (AML-M2), which show specific chromosomal alterations involving chromosomes 11 and 21 which are known to contain the ets-1 and ets-2 loci. Specifically, we have observed that there is a translocation of the ets-1 proto-onc gene locus from chromosome 11 to chromosome 4 in AUL patients presenting t(4;11)(q21;q23) translocations. Similarly, the c-ets-2 gene is entirely translocated from chromosome 21 to chromosome 8 in AML-M2 patients with the t(8;21)(q21;q22) translocation. This event seems to also affect the expression of the c-ets-2 gene in leukemic cells of AML-M2 patients. Finally, the proteins of the c-myc locus have been examined in various human cell lines. Polyclonal antibodies prepared against peptides representing putative amino acid sequences deduced from the DNA sequences from each of the three myc exons are being used to probe myc-specific proteins. Several variants of c-myc proteins have been identified and purified from nuclear and cytoplasmic extracts of human cells, with definite differences in size and anionic charges being noted for these various forms. There is preliminary evidence that the c-myc protein fractionating with the nuclear, but not the cytoplasmic, extracts co-purifies and associates with a major cellular structural protein.

The overall objectives of the Microbiology Section have been to identify and isolate human oncogenic sequences and understand how these sequences act to transform human cells. The development over the last several years of gene transfer techniques, particularly using transfection and transformation of the NIH3T3 line of mouse fibroblasts, has allowed the identification and isolation of sequences from transformed human cells and fresh primary human tumors which express a transformation potential. With few, but significant, exceptions, these active cellular oncogenes have represented the human homologues of viral oncogenes previously isolated and identified as the transforming genes in members of the family of acute transforming retroviruses. Comparisons of these activated cellular oncogenes with the normal cellular homologues have allowed the beginnings of a molecular understanding of what may prove to be critical steps in human oncogenic progression.

The members of the Microbiology Section are now identifying and isolating human oncogenes and studying how they are activated and how they transform cells. They are utilizing the retroviral oncogene, mos, and its human homolog as a model system, to determine how normal cellular genes are activated to express their transforming potential. Projects underway include the development of biological assays which allow the identification of new oncogenes with relevance to human cancer. They are also attempting to determine the ability of specific oncogenes, which have been isolated and identified using rodent cell culture systems, to transform human cells, both immortalized cell lines as well as diploid cells with finite life spans. Recently, they have identified specific oncogenes isolated from human primary tumor tissue as well as human cells

transformed in vitro by chemical and viral agents. The interrelationships between these individual projects within the Microbiology Section and the interactions among the various investigators, formally through Section meetings, and Laboratory and Building research seminars, as well as informally through day-to-day contact, help to advance the overall goals of the program.

A particular area of this section's interest has been its attempts to develop new methods to screen for and detect transforming genes present in transformed cells and tissues. They have developed an assay based on the ability of freshly transfected mouse fibroblasts to induce tumors in athymic nude mice. The rationale behind this assay is twofold: first, that tumorigenesis represents a more significant and unambiguous phenotype of transformation; second, that this will lead to the isolation of previously unknown, oncogenic sequences, since malignant transformation need not lead to an altered morphology. Previously, this section identified several DNAs isolated from transformed human cell lines which would induce tumors in nude mice. This assay has recently been refined and the sensitivity of the assay has been increased markedly by including a selective co-transfection plasmid carrying the bacterial neomycin gene (pSV2neo) and then utilizing the neomycin analogue, G418, to select cells which have taken up cellular DNA for injection into mice. As a result, an activated human homologue of the Kirsten MSV transforming sequence, ras^K, was detected and identified in the genomic DNA from the human pancreatic carcinoma cell line, PANC-1. The activated sequence contains a G→A transitional mutation within the 12th codon of the first coding exon resulting in aspartic acid, rather than glycine, at position 12. Also, an activated ras^N in the DNA has been isolated directly from a gastric adenocarcinoma. This gene has been cloned directly from the primary tumor DNA in a biologically active form. The activating lesion has been identified at the 61st codon located within the 2nd coding exon. An A→G transition results in the substitution of an arginine for lysine at this position.

Recently, this section has exploited a novel cell line, PA-1, originally isolated from a patient with a metastatic ovarian germ line tumor for transfection studies. Early passage PA-1 cells were found to grow slowly in culture and form tumors in athymic mice at low efficiency and with long latent periods; the late passage cells grew rapidly and induced tumors with short latencies. A dominant transforming gene was detected in late passage PA-1 cells and was identified as a new isolate of the human ras^N locus. It contains a single G→A point mutation in the 12th codon, which results in a replacement of glycine with aspartic acid. DNA from early passage PA-1 cells does not yield foci in transfection assays; however, NIH3T3 cells transfected with this DNA formed tumors in nude mice, although at a slower rate than cells transfected with late passage PA-1 DNA. Tumors induced by early passage PA-1 DNA transfection did not contain human ras^N DNA sequences, but these sequences were readily detectable in tumors induced by late passage PA-1 DNA. Introduction of active ras^N transforming sequences into early passage PA-1 cells results in PA-1 cells which now rapidly form tumors in nude mice. These results suggest that early passage PA-1 cells do not contain an activated ras^N sequence and that their inability to form tumors in nude mice is not due to a block in ras^N function.

Previous studies in this section have indicated that the human homologue of mos, c-mos^{Hu}, was inactive in DNA transfection assays, even when linked to the Moloney murine sarcoma virus (MSV) long terminal repeat (LTR), which contains both enhancer and promoter activities. However, certain recombinants

containing both c-mos^{Hu} and v-mos (MSV viral mos) coding sequences were able to transform NIH3T3 cells in DNA transfection assays, with the levels of activity varying from <0.2% to 20% of the v-mos parental constructs. Both active and inactive constructs have been shown to express mos gene products when inserted into a bacterial expression vector, indicating that the transforming block does not lie at the level of expression. NIH3T3 cells transformed by recombinants consisting of human-derived 3' mos sequences contain low copy numbers of transfected DNA and express high levels of a mos gene product of approximately 35,000 daltons. This protein was specifically detectable with antisera prepared against the predicted peptide sequence of the c-mos^{Hu} carboxy terminus. In contrast, cells transformed by reciprocal recombinants with 3' v-mos derived sequences contain 20-50 copies of transfected DNA but expressed barely detectable levels of mos product. Mos-specific RNA was detected at similar levels in both classes of transformants.

In an attempt to develop a transfection system utilizing human cells in order to measure the transforming ability of cloned oncogenes, the Microbiology Section has screened a number of non-tumorigenic human cell lines for their suitability as recipients for DNA transfection. An SV40-transformed human cell line (SV80) and a cell hybrid between HeLa and a normal diploid fibroblast has allowed the identification of cells which can be used and transfected for cloned, selected markers with high efficiency. Although introduction of viral mos or human ras did not induce morphologically-transformed foci, individual SV80 cells showed expression of the MSV-p30 containing polyprotein antigen and morphological alterations, and infectious virus could be rescued from the MSV-transfected cells. Tumors arose which contained functional mos, but the frequency was low, suggesting additional sequences; these SV80 cells have been analyzed in the non-tumorigenic and tumorigenic hybrids and compared with the parental cells, to determine if their phenotypes could be correlated with respect to the tumorigenic potential of the cells. It was found that the levels of ras and myc expression were constant among the various cells, but the levels of fos and myb expression show variations which correlate with the ability of cells to form tumors.

Additionally, the Microbiology Section is continuing a series of studies in collaboration with members of the Basic Research Program, Litton Bionetics, Inc., using the transfected HOS cell. The HOS cell line, originally derived from a human osteosarcoma, is non-tumorigenic but was transformed to anchorage-independent growth and tumorigenicity by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). It was previously shown that DNA from MNNG-HOS cells, but not HOS cells, would transform NIH3T3 cells in DNA transfection assays. The MNNG-HOS transforming gene, designated met, has been cloned in several overlapping lambda clones totalling 40 kb of human sequence by members of this section. This new oncogene shows no detectable homology with the known members of the ras oncogene family, nor with the oncogenes, mos, myc, myb, src, erb, sis, rel or B-lym. No rearrangement or amplification can be detected in the met gene in MNNG-HOS cells when compared with HOS DNA or the DNA from several normal and transformed human cells and cell lines. Cloned probes detect multiple species of polyadenylated RNA in HOS and MNNG-HOS cells, as well as in several human cell lines. A unique RNA species of 6.5 kb is present in NIH3T3 cells transformed by met, and a corresponding RNA species is present in MNNG-HOS cells, but not in the HOS parent cell line. In collaborative studies it has been found that the met onc gene maps to chromosome 7 between 7p11.4 to 7qter.

The Molecular Control and Genetics Section studies the regulation of gene expression in the bacterium, E. coli, and its phage, lambda, as well as differentiation in the lower eukaryote, Dictyostelium discoideum. The control of gene expression is being examined at several levels: transcription initiation, transcription termination and antitermination, and post-transcriptional RNA processing and mRNA decay.

Members of this section have identified a 50 bp sequence in phage lambda DNA that controls the expression of the gene(s) upstream (5') in mRNA containing this sequence. This controlling sequence contains two important elements which overlap: a) a transcription termination signal and (b) an endoribonuclease processing site. Transcripts that terminate at this site express their genes efficiently. Transcripts that do not terminate fail to express upstream genes because of an E. coli enzyme, RNaseIII, which cuts the read-through RNA initiating its rapid degradation. Mutations of both the site and the RNaseIII gene have been isolated and confirm these findings. The Molecular Control and Genetics Section has previously identified a 100 bp DNA sequence in phage lambda that contains sites required for Rho-dependent transcription termination activity (tR1) and lambda N-dependent antitermination activity (nut R). In addition, this region was found to contain another site(s) involved in transcription termination and antitermination activities (Box A and Box C) that are used by host proteins in E. coli (Nus proteins). A set of deletions in this region has been generated in vitro and its effects on each of these functions of termination and antitermination are being evaluated, as well as the effect of translation and relative spacing of the sites on each of these functions.

Integrative and excisive site-specific recombination in lambda is carried out by the phage Int protein. The Int protein has been found to have several reactive sites: (a) for binding different DNA sequences; (b) for a topoisomerase I activity; (c) to interact with the Xis protein, also required for excision; and (d) to interact with IHF, a host factor required for both reactions. Int mutants have been isolated by members of the Molecular Control and Genetics Section and analyzed genetically not only for their inability to carry out certain of these reactions but also for their ability to prevent a wild-type protein from carrying out the normal reaction during the mixed infection of mutant and wild-type.

A plasmid vector capable of expressing open reading frames (ORF's) has been constructed. This expression vector consists of the lambda p_L promoter, the first 13 codons of the lambda phage cII gene providing the translation start signal, and the major active portion of the β-galactosidase gene (lacZ) from E. coli. The cII-lacZ genes are fused out of reading frames with each other. Small DNA fragments from genomic DNA of eukaryotes are cloned into the vector plasmid, pWS50. The introduction of inserts with open reading frames restores complete translation of the lacZ gene. The resulting recombinants are detected by a simple colorimetric assay for β-galactosidase. High-level expression of the hybrid proteins is provided by transcription from the inducible p_L promoter. The hybrid proteins synthesized are readily distinguished from native E. coli proteins by their large size (>117,000 M_r), and which may then be easily purified. In addition, these proteins are suitable for antibody production directed against determinants specified by the inserts. With this system it may be possible to conveniently map open reading frames from total genomic DNA.

The cellular slime mold, Dictyostelium discoideum, is one of the simplest organisms that undergoes true multicellular differentiation. It is being used as a model system to study mechanisms which control developmental gene activation during normal differentiation. Dictyostelium discoideum (NC4) grows as a single-celled amoeba. After food is exhausted, the cells aggregate forming a multicellular organism in which two predominant cell types, prespore and prestalk cells, differentiate. Utilizing the appearance of specific antigens and enzyme activities, it was previously shown that preaggregating cells cannot form clumps and cannot differentiate into prespore cells under liquid shaking culture conditions in media containing glucose-albumin-EDTA and cyclic AMP (GAC). Post-aggregating cells, however, can form clumps and differentiate into prespore and prestalk cells in GAC. Differentiation is dependent on cAMP and cell contact. Members of the Molecular Control and Genetics Section have investigated expression of cell type-specific mRNAs under these variable conditions. Preaggregating cells transferred into GAC did not express prespore or prestalk mRNAs. By contrast, aggregating cells transferred to GAC expressed large amounts of both prespore and prestalk mRNAs. When clump formation was inhibited by rapid shaking, aggregating cells in GAC expressed only very low levels of prespore and prestalk mRNAs. Addition of ammonium sulfate to GAC could partially restore prespore mRNA expression and completely restore prestalk type 1 mRNA expression without clump formation. These results show that expression of both prestalk and prespore mRNAs requires a specific differentiation step which does not proceed in GAC media. Additionally, at least part of the effects of clump formation (cell contact) on prespore, and all of the effects on prestalk cell differentiation can be replaced by addition of $(NH_4)_2SO_4$.

Post-aggregation Dictyostelium cells contain 2000 to 3000 new mRNA species which are absent from growing and preaggregation stage cells. The transcription of these new mRNAs is initiated late in development and requires cellular interaction and cAMP. As a result of exploration of the structural organization of these late genes in chromatin, two major conclusions emerge: 1) In nuclei of growing cells the late genes are in a fully DNaseI-sensitive "active" configuration, even though they are not transcribed in these cells. The DNaseI sensitivity of active and inactive genes is the same. 2) Digestion with micrococcal nuclease, however, reveals a major structural difference in the organization of the constitutively expressed genes that are actively transcribed in the growing cells and the developmentally-controlled late genes. The inactive late genes are found in a regular nucleosomal ladder with a repeat of about 168 bases. The actively-transcribed genes, however, are cut by the enzyme, both between nucleosomes, and at additional sites whose spacing is consistent with either (a) cuts within the core particle or (b) the presence of close-packed core particles lacking a linker region. This gives rise to an irregular ladder of bands with a spacing of 70-130 nucleotides between bands. The irregular ladder is superimposed on a smear with the ratio of DNA in bands and between bands correlating with the level of expression of the particular gene.

The Cellular Transformation Section has been investigating the malignant transformation of cells by avian sarcoma viruses, including identification of virus-coded proteins directly responsible for transformation, determining the cellular location and direct function of these proteins, the primary physiological effects of the functioning protein, the sequence of metabolic changes resulting in the altered metabolism of malignant cells, and the metabolic changes necessary for the maintenance of the malignant state.

Currently, this section's investigations deal with an analysis of the location, function, and structure of the transforming protein of v-myc, a 110 Kd protein encoded by the avian MC29 virus. This protein is typically found in the nucleus in monomeric and dimeric forms, and has a short half-life typical of regulatory proteins.

The synthesis of transformation-associated proteins encoded by several avian oncogenic viruses has been analyzed with respect to their intracellular localization and stability, and the sizes of the native proteins were analyzed by sedimentation in sucrose gradients. The MC29 $p110^{gag-myc}$, and the avian myeloblastosis virus (AMV) p48, were found to be located in the nucleus, had a rapid turnover rate, and existed primarily as dimers. The normal cellular homologue of the viral myc protein, as well as myc-related proteins produced by MH2 and OK10 viruses, were also found as intranuclear dimers. The relevance of the location and structure of these proteins to malignant transformation is under investigation.

The avian MH2 virus contains two potential oncogenes, mht and myc, which have the potential to transform cells to malignant forms. A DNA clone containing both mht and myc has been constructed, and was capable of transfecting chick embryo fibroblasts (CEF). To examine the oncogenic potential of each gene, and determine the regions of each gene responsible or required for transformation, deletion mutants have been constructed from this clone. Such deletion constructs currently confirm observations that the myc gene is essential for the transformation of the CEF, while deletions in the mht region had no observable effect on cellular transforming ability by the reserved virus in these primary cell assays.

Several human and animal tumors have been examined for alterations in proto-oncogene sequences using Southern blot analysis and DNA-mediated transfection assays. Members of the Cellular Transformation Section have focused attention on the human neuroblastoma and myeloid leukemia cell lines, where the c-myc oncogene was found to be amplified, i.e., present in several additional copies per cell. Amplification at the DNA level is being studied in relation to the increased expression of any c-myc protein(s).

The Office of the Chief, LMO, contains an Oncogene Biochemistry Working Group which conducts research on the molecular biology and biochemistry of the ras oncogenes and the p21 ras oncogene proteins. In collaboration with other members of the LMO, members of this group have succeeded in expressing, at high levels, the p21 of Harvey murine sarcoma virus in E. coli. A procedure was developed to isolate the p21 from bacteria in bulk quantity to homogeneity. The bacterially-expressed enzyme has a functionally-active autokinase and guanine nucleotide-binding activities. This protein and the p21 purified from virus-transformed cells were used to explore new biochemical properties associated with the p21. Using the vector expressed, purified protein of the p21 autokinase site has been mapped by phosphopeptide sequencing and a reactive tryptic phosphopeptide isolated by HPLC. Further characterization by NEM titration and photoaffinity labeling has characterized the peptide region proximate to the guanine nucleoside binding site. Using site-directed oligonucleotide mutagenesis techniques, members of this group have modified specific amino acid residues of the $p21^{ras}$ which have resulted in marked alterations in substrate specificity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04876-13 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Oncogenic Virus Influence on the Biochemical Events of Host Cells

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

PI:	P. S. Ebert	Chemist	LMO	NCI
Others:	J. P. Bader	Research Microbiologist	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

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

A new ATPase assay was developed to determine enzymatic activity in _myc_ antigen immune complexes. ATPase activity was demonstrated using _gag_ and _myc_ antisera against _myc_ proteins solubilized from the nuclei and nuclear matrix of Q8 cells, a source of p110. The enzyme required a neutral pH, Mg, detergent, dithiothreitol, and KCl. The enzyme cleaved only the terminal phosphate from ATP. ATP hydrolysis could be competitively inhibited by ATP, ADP, dATP and dADP. The immune complex also expressed GTPase activity. Nuclear extracts from Q8, QMH2 and OK10 cells precipitated with _gag_ antiserum produced about the same specific ATPase activity. ATPase activity co-migrated with p110 from Q8 nuclei on a molecular sizing column.

PROJECT DESCRIPTION

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

P. S. Ebert	Chemist	LMO	NCI
J. P. Bader	Research Microbiologist	LMO	NCI

Objectives:

To determine the biological function and enzymatic activities of proteins expressed by the myc and other oncogenes.

Methods Employed:

A new adenosine triphosphatase (ATPase) assay was developed utilizing a staphylococcal protein A sepharose (PAS)-bound immune complex of a protein transcript of the myc oncogene. Following immune precipitation of the oncogenic protein transcript, the immune complex was adsorbed to PAS and washed extensively. The immune complex was mixed with an incubation buffer containing λ-^{32}P-labeled ATP and incubated at 37°C for 1 hr. The labeled phosphate released from ATP was measured by liquid scintillation counting. The oncogene-specific p110 produced by MC29 nonproducer quail cells (Q8) was determined by labeling cells with ^{35}S-methionine, extracting the nuclei with detergents, sonicating the nuclei, and subjecting the immune-precipitated products to polyacrylamide gel electrophoresis. Labeled protein bands were visualized by exposure of the dried gels to X-ray film. The Q8 nuclear matrix was prepared by treating pooled Q8 nuclei with DNase and RNase. This procedure produced a greater purification of the Q8-p110. The p110 concentration of Q8 cells could be enhanced by incubating the cells for 1 hr in medium containing an elevated concentration of serum.

Major Findings:

An ATPase assay was developed to measure enzyme activity in myc-antigen immune-precipitated protein complexes. ATPase activity was optimized using p27-gag-precipitated myc proteins from the nuclei and nuclear matrix of Q8 cells, a source of p110$^{gag\text{-}myc}$. The enzyme required a pH of about 7.0, Mg as the preferred divalent cation, detergent, dithiothreitol, and KCl. The enzyme cleaved only the terminal phosphate from ATP. Q8 nuclear extracts were precipitated by p27-gag antiserum, two antisera made from the amino-terminus of myc, and two antisera made from the carboxy-terminus of myc, and ATPase activity was demonstrated in the washed immune precipitates. ATP hydrolyzing activity could be competitively inhibited substantially by adding ATP, ADP, dATP, and dADP. The immune complex also expressed GTPase activity; but at lower efficiency. Nuclear extracts from Q8, QMH$_2$, and OK10 cells precipitated with p27-gag antiserum produced about the same specific ATPase activity. The lowest specific activity was found in chick embryo fibroblasts. Studies were run to determine if the p110 myc protein and the ATPase activity co-migrated during column chromatography.

When ^{35}S-labeled immune-precipitated myc proteins were subjected to a Sephacryl S-300 molecular sizing column, p110 was detected in fractions containing molecular weights of 158K and larger. The fractions also were associated with a peak of ATPase activity.

Significance to Biomedical Research and the Program of the Institute:

The myc oncogene has been shown to affect the control of proliferation and the competence for differentiation irreversibly and thus serves as an important model for the study of oncogenic transformation. The MC29 myc oncogene is translated as a fusion protein which is located in the nucleus and which can bind to DNA. The mechanism of action of transformation by the myc oncogene is presently unknown. The finding of an enzymatic activity associated with the myc protein will help elucidate the mechanism of action of transformation and suggest a possible site of attack for anticancer agents.

Proposed Course:

Efforts will be made to further purify the myc protein by cation and anion exchange and chromatofocusing column chromatography. When more purified p110 is available, enzymatic kinetics and inhibitor studies can be performed on the associated ATPase. Attempts to determine the function of the ATPase will be continued. Factors determining the enhancement, inducibility, and inhibition of the enzyme will be studied.

Publications:

Ebert, P. S., Hess, R. A. and Tschudy, D. P.: Augmentation of hematoporphyrin uptake and in vitro-growth inhibition of L1210 leukemia cells by succinylacetone. JNCI 74: 603-608, 1985.

Weinbach, E. C. and Ebert, P. S.: Effects of succinylacetone on growth and respiration of L1210 leukemia cells. Cancer Lett. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04899-13 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Transforming Genes of Avian RNA Tumor Viruses

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

PI:	T. S. Papas	Acting Chief	LMO	NCI
Others:	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
	D. K. Watson	Senior Staff Fellow	LMO	NCI
	N. C. Kan	Visiting Associate	LMO	NCI
	K. P. Samuel	Visiting Associate	LMO	NCI
	C. S. Flordellis	Visiting Fellow	LMO	NCI
	R. J. Fisher	Expert	LMO	NCI

COOPERATING UNITS (*if any*)

Department of Biology, Johns Hopkins University School of Medicine, Baltimore, MD (G. Scangos and E. Moudrianakis); Department of Biology, University of California, Berkeley, CA (P. Duesberg)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

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 are pursuing the study of the process by which viral oncogenes, as well as their cellular homologys, induce the activation of biochemical events that direct the cell towards malignant transformation and neoplasia. Utilizing retroviruses as tools to understand the structural and molecular nature of the cellular oncogenes transduced by these viruses has enabled the resolution of these details in both normal and malignant systems. Further, we have utilized these onc genes as probes revealing the genomic organization of myc and mht proto-oncogenes in a variety of cells, such as human, all the way down evolutionarily to fish and Drosophila. In addition to studying the genomic organization of the three retroviruses (MC29, MH2 and OK10), all part of the myc oncogene family, we have approached the organization of several dual oncogene systems in an attempt to resolve the oncogenic interaction of the genes mht and myc in MH2, and ets and myb in E26. Utilizing these probes has enabled us to expand our study to include the organization and expression of ets and myc in several human neoplasias which undergo chromosomal rearrangements. Additionally, we have constructed and utilized expression vectors to produce large amounts of specific oncogene products and then to purify and characterize them chemically and immunologically, producing valuable immunodiagnostic reagents in the process. Further, our knowledge of the molecular structure of specific oncogenes has enabled us to make unique synthetic oligopeptides and produce cognate antisera to probe these relatively scarce proteins in normal cellular systems, which has led to their intracellular localization and cellular association. These studies on normal and activated oncogenes will have important diagnostic, as well as functional, implications towards understanding their role in the process of malignancy.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

T. S. Papas	Acting Chief	LMO	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
D. K. Watson	Senior Staff Fellow	LMO	NCI
N. C. Kan	Visiting Associate	LMO	NCI
K. P. Samuel	Visiting Associate	LMO	NCI
C. S. Flordellis	Visiting Fellow	LMO	NCI
R. J. Fisher	Expert	LMO	NCI

Objectives:

The scope of this investigation is to delineate the relationship between virus gene expression and conversion of cells from the normal to the malignant state, to study the molecular anatomy of known tumor viruses, and to describe the mechanism by which subviral structures act in concert with cellular factors to regulate oncogenesis. To investigate the process by which viral oncogenes, as well as their cellular homologs, induce the activation of the metabolic processes and participate in malignant transformation. To delineate, at the molecular level, the mechanism by which oncogenes act in concert with cellular factors to induce oncogenesis. To isolate and characterize specific viral and cellular transforming genes and identify the products specified by these sequences, particularly to identify the target(s) and function(s) of these oncogene products and their functional mode of action. To introduce functionally-modified oncogenes to specific target cells in an effort to analyze and identify the function of their normal counterparts. The techniques of molecular cloning, DNA sequence analysis, and site mutagenesis will be used to identify specific nucleotides in the transformation process.

Methods Employed:

1. Plasmid Construction Techniques. Plasmid DNAs were cleaved by the appropriate restriction enzymes and subjected to electrophoresis on polyacrylamide gels. Fragments were eluted from the gels by the method of Maxam and Gilbert (Methods Enzymol. 65: 499-560, 1980). Vector DNA was prepared for ligation by cleavage with the appropriate restriction enzymes and treated with calf intestinal alkaline phosphatase. Ligation of each isolated fragment to vector DNA was performed using T4 DNA ligase (New England Biolabs). Calcium chloride-treated E. coli DC646 cells were transformed as described by Cohen et al. (Proc. Natl. Acad. Sci. USA 6: 2110-2114, 1972) and ampicillin-resistant colonies were screened for plasmids. To study gene expression, plasmid DNAs prepared from the transformed DC646 cells were introduced into N4830 or N4831 cells by the same procedure. Extensive use was made of published DNA sequences in the design of the recombinant plasmids.

2. Radiolabeling and Electrophoresis of Bacterial Proteins. E. coli cells were grown at 32°C in supplemented M56 minimal media and 50 μg/ml of ampicillin. When the OD_{590} of the cultures reached 0.2, the temperature was shifted to 41°C.

Aliquots (150 µl) of the cells were taken after the temperature shift. These cells were added to media containing [^{35}S]-cysteine and incubated for 1.5 min. After labeling, some cultures were chased by adding unlabeled cysteine. Cellular protein was precipitated with trichloroacetic acid, washed with acetone, and resuspended in SDS/0.1% β-mercaptoethanol. The proteins were then resolved by electrophoresis on SDS-polyacrylamide gels and visualized by autoradiography.

3. Preparation of Bacterial Extracts. Unfractionated extracts were prepared from cells grown at 32°C in supplemented M56 media to an OD_{590}=0.3. The cultures were then induced by shaking at 41°C. The cells were pelleted by centrifugation, resuspended in supplemented M56 media and heated for 5 min. at 95°C after being placed in SDS and β-mercaptoethanol.

4. Immunodetection of Proteins by Western Blotting. Proteins were resolved on SDS-polyacrylamide gels and transferred electrophoretically onto diazobenzyloxymethyl (DBM) paper. Nonspecific protein binding sites were blocked by bovine serum albumin and incubated overnight with antibody. After being washed briefly in a sonicating cleaner, the paper was incubated with ^{125}I-labeled protein A from *Staphylococcus aureus*. Since protein A binds antibody, the antibody-antigen complex was detected by autoradiography.

5. Construction of Deletion Mutants. (i) Digestion of recombinant plasmid DNA with restriction enzymes; (ii) preparation of DNA fragments with blunt termini by repairing the ends with *E. coli* DNA polymerase large fragments, or by removing the protruding nucleotides with S1 nuclease; (iii) preparation of randomly shortened DNA fragments by treatment with Bal-31 exonuclease; (iv) dephosphorylation of DNA termini by treatment with calf intestinal alkaline phosphatase to prevent self-ligation of DNA fragments; (v) ligation of DNA fragments, either with blunt ends or with sticky ends, using T4 DNA ligase; (vi) use of small preparations of recombinant DNA (mini-lysates) to identify the desired DNA clones; (vii) colony hybridization using nick-translated DNA probes to screen for desired recombinant DNA clones; (viii) gel electrophoresis analysis of recombinant DNA fragments on agarose or polyacrylamide gels; and (ix) DNA sequence analysis of deletion mutants by the method of Maxam and Gilbert to determine the precise nature of the deletions.

6. Insertion of viral oncogenes into the expression vector, pJL6 or pJLA16, using the methods described above.

7. Polyacrylamide gel electrophoresis analysis of radioisotope-labeled or unlabeled bacterial proteins containing oncogene sequences.

8. Immunoprecipitation of labeled cell lysates with *onc*-specific antisera and analysis by SDS-polyacrylamide gel electrophoresis. The specific *onc* gene-related polypeptide was purified for further analysis by electroelution.

9. S^{35}-methionine and -cysteine labeling of expressed protein in bacteria utilizing the plasmid, pJL6, (vector constructed by Dr. James Lautenberger).

10. Enzymatic and chemical peptide analyses of viral and cellular, onc-related polypeptides by two-dimensional isoelectric focus electrophoresis and HPLC and reverse phase chromatographic techniques.

11. Microsequence analysis of onc-related polypeptides to define the initiation of translation and the nature of the oncogene-specific proteins synthesized in transformed and non-transformed cells.

Major Findings:

1. The integrated proviral genome of the avian carcinoma virus, MH2, has been molecularly cloned and its nucleotide sequence has been determined. Our data indicate that MH2 contains two potential transforming genes: Δgag-mht and myc. In order to determine which role each potential onc gene of MH2 plays in oncogenesis, we have constructed deletion and frameshift mutants of each of the two MH2 genes, and have tested these mutants for their virus production and transforming function in cultures of primary chicken and quail cells. We have found that the myc gene transforms primary cells by itself without the second potential onc gene, and that the Δgag-mht gene is without detectable transforming function in the primary cell assay. However, further work is necessary to determine whether the Δgag-mht gene has a role in oncogenesis in the animal. In order to understand the molecular mechanisms involved in the transduction of cellular mht genes (c-mht) into the MH2 virus, we have molecularly cloned the chicken c-mht from a phage lambda library containing genomic chicken sequences. Nucleic acid hybridization, and heteroduplex and DNA sequence analyses indicate that the v-mht sequence captured by the MH2 virus is spread over 25 kb of chicken genomic DNA. The c-mht locus contains 11 exons which are homologous to the v-mht sequence. Thus, the v-mht onc gene is a subset of its normal cellular homolog in that it lacks introns, and possibly lacks 5' coding sequences. Because there is no sequence homology between c-mht and retroviral helper sequences, the viral transduction of the cellular mht gene occurred through recombination. Finally, we have used the temperature-inducible bacterial expression vector developed in our laboratory to express large amounts of the v-mht protein. Purification of antibodies against the bacterially-synthesized mht protein is in progress. The cellular homolog of v-mht in chicken (c-mht) is conserved throughout evolution, being present in species as divergent as chickens and humans, and is transcriptionally active. An RNA species of 4.0 kb has been detected in normal chicken embryo fibroblasts (CEF) and a 3.7 kb mht-related RNA has been found in the human Daudi and HL60 cell lines.

2. We have determined the complete nucleotide sequence of the chicken and human c-myc genes and compared them to two members of the myc family of transforming retroviruses: MC29 and OK10. Although a close relationship between the viral and cellular myc genes has been found, these genes are not isogenic. The myc-related genes of MC29, MH2, and OK10, and the myb-related genes of AMV and E26 are genetic hybrids with sequences derived from viral structural genes and parts of essential cellular proto-onc genes. The cellular genes contain additional 5' sequences. The substitution of viral genes for parts of the normal cellular

We have initiated studies of the cellular ets gene to determine whether this pattern of a truncated normal gene in the transforming retrovirus can be extended to other onc genes. The cellular gene transcript is considerably larger than the amount of DNA transduced by the virus. We have determined that the mammalian homologs of v-ets consist of two distinct domains located on different chromosomes. Using chromosome-specific probes, we have shown that both loci (ets-1 and ets-2) are transcriptionally active producing mRNA species. The sequences homologous to ets-1 and ets-2 are colinear in chicken proto-ets and have possibly become separate and functionally distinct since before the evolutionary divergence of mammalia.

When viral probes related to ets-1 and ets-2 are hybridized to a single chicken c-ets clone, we find that sequences related to both loci are present in this clone. Portions of the human ets-1 and ets-2 loci have been sequenced, demonstrating a strong conservation of amino acid (over 90%), suggesting that this gene performs an important function. Using a previously characterized panel of mouse X hamster hybrids (Kosak et al., J. Virol. 49: 297-299, 1984), we have been able to assign ets-1 and ets-2 to murine chromosomes 9 and 16. The domestic cat homologs of the ets proto-oncogenes were found to be D1 (ets-1) and C2 (ets-2). Both genetic loci are transcriptionally active in man yielding distinct products.

3. A rainbow trout genomic library has been screened using 5' and 3' v-myc-specific sequences and multiple, overlapping clones have been isolated. Restriction enzyme and Southern blot analyses using defined chicken c-myc probes have been carried out to characterize these clones. Sequence analysis has shown that the fish myc is almost 70% homologous to chicken c-myc at the amino acid level. Additional restriction enzyme and Southern blot studies have suggested that the c-myc gene is rearranged in the DNA of Northern Pike genome isolated from lymphosarcomas relative to those observed in Northern Pike cells obtained in tissue culture.

4. Recently, we have mapped the c-ets locus in humans onto two separate chromosomes, 11 and 21. For convenience we have named these proto-oncogene loci ets-1, located on chromosome 11, and ets-2, located on chromosome 21. Given the known correlation of many forms of human cancers to specific chromosomal aberrations, we are using the distinct probes to investigate the involvement of the ets proto-oncogenes in the pathogenesis of certain suspect leukemia malignancies. A number of these human leukemias, such as the acute undifferentiated leukemias (AUL) and the acute myeloid leukemia with maturation (AML-M2), show specific chromosomal aberrations involving the chromosomes known to contain the ets-1 and ets-2 proto-oncogene loci. We have identified in a number of human cells from these acute leukemia patients, chromosomal aberrations such as deletions and translocations involving the 11q23.3 and 21q22 bands, specifically. The expression of c-ets-2 is affected in leukemic cells of AML-M2 patients with the t(8;21)(q21;q22) translocation. Similarly the c-ets-1 probe revealed that there was a translocation of this gene from chromosome 11 to chromosome 4 in AUL patients presenting a t(4;11)(q21;q23) translocation. Expression studies are being pursued to assess qualitative, as well as quantitative, changes which may arise as a consequence of

5. An expression vector was constructed in which 13 codons of the phage λ cII protein were placed adjacent to the E. coli β-galactosidase gene (lacZ) such that the lacZ sequence is out of frame with respect to the lambda cII sequence. The fused gene was placed under the transcriptional control of the strong, well-regulated phage λ p_L promoter. A restriction enzyme recognition site (NruI) is located between cII and lacZ, allowing insertion of DNA fragments. Inserted open reading frames that are properly aligned with the cII and lacZ sequences can be easily identified, since plasmids containing such sequences confer a lac^+ phenotype on the host bacterium. This vector was used to express the envelope protein of human T-cell leukemia virus type-I (HTLV-I). The ends of a restriction fragment containing envelope sequences were randomly digested by BAL 31 nuclease and inserted into the NruI site of the vector. A blue colony was isolated that was found to be synthesizing a β-galactosidase with a molecular weight larger than that of the wild type enzyme. This protein was shown to contain HTLV-I envelope sequences since it reacted on Western blots with antibodies in patient sera. The junctions between the HTLV-I envelope DNA and the vector were precisely established by Maxam-Gilbert and dideoxynucleotide DNA sequencing. The protein produced by this plasmid has great promise in diagnostic assays. Since the region of the envelope that is expressed is completely defined by the DNA sequence of the insert, this protein may also be used to map the epitopes recognized by monoclonal antibodies directed against the HTLV-I envelope.

6. HTLV-I env gene-containing plasmids were introduced in Escherichia coli MZ1, a strain that contains a partial λ prophage bearing the mutant c1857 temperature-sensitive repressor. At 32°C the repressor is active, and the p_L promoter on the plasmid is repressed. At 42°C the repressor is inactive, and the p_L promoter is induced, allowing a high level of expression of genes under its transcriptional control. When lysogens carrying either of the two plasmids containing different portions of the HTLV-I envelope gene were grown at 32°C and induced by shifting the temperature to 42°C, prominent bands were revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that were not found in uninduced cells containing the pJL6 vector alone. These proteins were observed in gels of isotopically-labeled bacterial extracts and in gels stained for total proteins. On the basis of DNA sequence data of the envelope gene fragments, the calculated molecular sizes of the pKS300 and pKS400 proteins are 12.84 and 15.88 kilodaltons (Kd), respectively. These sizes include the 1.56 kD coding sequence contributed by the amino terminal codons of the λ cII gene. The molecular weights of both proteins determined by SDS-PAGE are consistent with those calculated for a 321-base pair 9pKS300 insert) and a 397-base pair (pKS400 insert) coding sequence of the gp46 c-terminal and p21E N-terminal regions of HLTV-I env protein. The HTLV-I env gene codes for a glycoprotein (gp61), molecular weight 61,000 (61 Kd), that is cleaved into the 46 Kd exterior glycoprotein (gp46) and the 21 kD transmembrane protein (gp21E). The precise site of proteolytic cleavage has been determined by locating isotopically-labeled valine residues with respect to the amino terminal end of gp21. Because the BamHI site separating the inserted fragments is close to the region coding for the proteolytic cleavage site that separates gp46 from p21E, the protein from pKS300 contains sequences corresponding to the carboxyl terminal portion of gp46, and the protein from pKS400 predominantly consists of sequences from p21E.

We have designed experiments to determine whether such antibodies can recognize a bacterially-synthesized envelope product. Prominent bands corresponding to reaction of antibody to the 15 Kd bacterial envelope product developed when the sera used was from patients with HTLV-I-associated ATL or from HTLV-I antigen positive individuals. No such reactions were observed with sera from healthy control individuals. This procedure was used to screen a group of 28 coded sera. Antibodies that recognized the bacterially-synthesized HTLV-I envelope protein sequences were found in all sera that had been shown to have antibodies to HTLV-I by an ELISA (enzyme-linked immunosorbent assay) with disrupted virions as the antigen. None of the normal control sera were found to have reacting antibodies. Antibodies from a patient (Mo) with a hairy cell leukemia, whose disease is associated with HTLV-II, strongly reacted to the protein coded for in pKS400. This indicates that there is a high degree of relatedness between the p21E regions of HTLV-I and HTLV-II.

Because the bacterially-synthesized HTLV-I env protein was recognized by antibodies in sera from an HTLV-II (positive) patient, it was of interest to see if this assay could be used to screen for HTLV-III, an even more distantly related subgroup. Therefore, we examined a number of serum samples from AIDS patients, some of whom were also sero-positive for HTLV-I. The sera positive for AIDS that reacted with HTLV-I in the ELISA contained antibodies that recognized the bacterially-synthesized HTLV-I env protein. None of the sera from AIDS patients that were HTLV-I negative contained antibodies that reacted with this protein. Because antibodies that react with HTLV-III proteins can be found in the sera of more than 90 percent of all AIDS patients, this result indicates that there is little or no cross-reaction between the carboxyl terminal portion of the envelope proteins of HTLV-I and HTLV-III.

7. A pX-IV fragment of the LOR region of HTLV-I was fused to our expression vector plasmid pJLA16 by blunt-end ligation to produce a recombinant plasmid pKL296C, expressing a fusion protein with an apparent molecular size of about 15 Kd in SDS-PAGE. Its mobility on SDS-PAGE is slower than that expected for a DNA fragment size of 296 bp. The pX region, bounded by the envelope gene and 3'-LTR sequence, is approximately 1.6 kbp long and contains four open reading frames (numbered pX-I to pX-IV in HTLV-I and pX-a to pX-c in HTLV-II). The predicted DNA sequence of the long open reading frame (LOR) of pX-IV in HTLV-I and pX-c of HTLV-II can encode a protein of 357 amino acids (p42) and 337 amino acids (p38), respectively. Specific anti-peptide antisera to these regions have immunoprecipitated proteins of those sizes in HTLV-I and HTLV-II immortalized cell lines and HTLV positive human sera as reported by other investigating laboratories. Western blot analyses of the cellular extract of the bacterially-produced pX-IV C-terminal LOR protein with two positive control sera, #1990 and 4993, are from patients with AIDS. The bacterially-produced pX protein was recognized by antibodies to the p42 protein in those HTLV-I positive sera. No serological reactivity was seen with the other sera. In a comparative Western blot experiment, four different human sera were used to test for serological reactivity to both the bacteriallyproduced p21E envelope and pX proteins of HTLV-I. The results showed that serum D32 reacted with envelope protein in pKS400, but not with the pX protein of pKL296C. This serum and serum MI were from ATL patients with relatively high (MI) and moderate (D32) titers to anti-p24.

In contrast, serum A195 reacted with neither protein (low anti-p24 titer). Only one-quarter of all ATLL-positive patients (HTLV-MA seropositive) and healthy seropositive carriers were reported to have detectable antibody to the p42-pX protein. Recent attempts to clone the env-LOR open reading frame of HTLV-III have been successfully able to produce a new bacterial expression protein of the predicted molecular weight size. Synthetic antigens also have the potential of being the basis of a subunit vaccine. Studies of the bacterially-synthesized HTLV proteins are of special importance since the hazards of employing a live attenuated virus or inactivated virus as a potential vaccine for the disease AIDS precludes this approach.

8. The first exon of Hu-c-myc is not thought to be expressed because it contained many translational stop signals in all of its reading frames (Staton, L. W., et al., Nature 305: 401-406, 1983). Thus, it was inferred that the myc translational initiation codon started at exon 2. However, Papas, T. S., et al., (Cancer Cell 2: 153-160, 1984), and Gazin, C., et al. (EMBO J. 3: 383-387, 1984) have shown that the first exon indeed contains an open reading frame which could potentially code for a protein of 20,000 daltons. Furthermore, Psallidopoulos and Papas (unpublished results) have cloned the human c-myc first exon in an E. coli expression vector and demonstrated that this first exon open reading frame does indeed code for a protein.

To resolve some of these issues, we have identified and isolated subcellular fractions containing the oncoprotein, Hu-c-myc. In order to purify analytical quantities of c-myc and to detect the proteins(s) synthesized by the myc exons, we have developed a series of site-specific antibodies (Lerner, R. A., Nature 299: 592-596, 1982) against putative amino acid sequences within each of the 3 exons. These site-specific antibodies have been used to probe the organization of the myc protein in the various myc overproducing and translocated cell lines such as colo 320 human cells and human foreskin fibroblasts. We have thus far isolated five variants of the cellular myc product which react specifically against our epitope-specific anti-myc probes. Thus far, by the immunologic cytoplasmic analyses, components are uniquely compartmentalized and distributed from those of the nuclear-perinuclear complex. Further, N-terminal sequence analysis will enable us to resolve the molecular nature of these differences. Nuclear and perinuclear c-myc moreover appears to copurify and coelectrophorese with an important cellular structural component which is not observable with the cytoplasmic c-myc product. Further elucidation of the nature of this interaction is needed to determine if a functional interrelationship for these components is biologically significant. Towards this end we have highly purified the 58 kilodalton c-myc protein by using a combination of reverse phase HPLC in conjunction with Fast Protein Liquid Chromatography, and have treated the polypeptide with cyanogen bromide (CnBr). Several unique CnBr fragments that have been identified are recognized by specific immunologic anti-myc probes we have purified and made using defined putative myc encoded oligopeptides.

9. The sequence of the putative pol gene of HTLV-III was analyzed for homology with other retroviruses. Considerable homology was found in two regions that are the cores of the polymerase and the endonuclease proteins. In almost all cases where most other retroviruses were concordant with a consensus sequence, the HTLV-III pol gene was also concordant. However, the HTLV-III sequence does

not have an extremely close homology with any of the sequenced pol genes. To study the relationship of the HTLV-III pol gene with other pol genes in a consistent manner, comparable segments of the polymerase and endonuclease regions were aligned with their counterparts in several other retroviruses. A matrix was created from the fraction of matching amino acid residues in each pair of sequences. This data was used with the method of Fitch and Margoliash to create a phylogenetic tree. This tree indicated that HTLV-III evolved in a lineage separate from other retroviruses for which pol sequences are available. The other retroviruses examined fell into three other lineages: They are (1) HTLV-I and BLV; (2) RSV, MMTV, and SMRV; and (3) Moloney MLV, AKV, REV, and endogenous retroviruses.

Significance to Biomedical Research and the Program of the Institute:

To elucidate the role of oncogenes in the process of oncogenesis, both induced by retroviruses and spontaneously occurring, it is important to define them molecularly and determine the mechanism by which these genes become expressed and deregulated. It is also invaluable to determine their functional role(s) in both normal and malignant cells at the level of their protein products. Towards this end these studies, utilizing the specific reagents we have developed in the Laboratory, we can identify and locate these unique and important cellular proteins which are normally found only at very low levels intracellularly. Moreover, we can utilize our expression vector systems to make enzymatically- or biologically-active oncogene-specific proteins in quantities which will facilitate purification and molecular characterizations. Such materials can also be employed in useful immunodiagnostic applications and ultimately should play a role in the preparation of antigens for vaccine purposes. Thus, by these multifaceted approaches we can project the ability to focus our efforts on understanding the basic mechanisms by which retroviral captured oncogenes, as well as normal cellular oncogenes, become activated and involved in the malignant process.

Proposed Course:

1. Development of improved expression vectors. Several alterations to the bacterial expression vector, pJL6, will be performed. A synthetic oligonucleotide will be cloned into the unique Nde I site such that the sequence up to the cII initiation codon will be preserved, but a unique BamHI site will be introduced adjacent to this codon. The cII translation initiation site will be unaltered, and proteins introduced into the BamHI site will not have any codons (other than the initiation codon) derived from phage sequences. In addition, the oligonucleotide used will contain terminators in all three frames such that translation will not be able to proceed past the inserted sequence. Another modification will place a segment of phage M13 mp18 into pJL6. This segment contains several restriction sites, including one for Sph I that produces 3' protruding ends. This allows the production of deletions by the procedure of Henikoff (Gene 28: 351, 1984) in which the DNA is removed only in one direction. This method relies on the property of E. coli exonuclease III in which it degrades recessed 3' ends, but not protruding 3' ends. A piece of DNA containing several genes can be inserted and the sequence upstream from each gene can be selectively removed. This should allow the rapid construction of plasmids suitable for the expression of several genes at once. In addition, this vector will contain appropriate restric-

being necessary to isolate restriction fragments. This will be done by a scheme similar to that of Rüther (Nucleic Acid Res. 10: 5765, 1982) and should allow the rapid expression and sequence determination of uncharacterized DNA segments.

2. Production of specific mutants in the MC29 v-myc gene. A comparison of the sequences of three transforming avian retroviruses that contain the transforming v-myc gene indicated that they differ from the chicken proto-myc oncogene only at one common amino acid residue. To determine if that residue is necessary for transformation activity, the MC29 v-myc sequence will be converted to the equivalent chicken proto-myc sequence at that position. This will be done by oligonucleotide based site-specific mutagenesis. To facilitate this procedure, a portion of the v-myc gene will be cloned into M13 in p8. After mutagenesis, this segment will be rejoined with the rest of the MC29 proviral DNA and transformation of chicken embryo fibroblasts will be determined by a helper dependent transfection protocol. Any DNAs found to have lost transformation ability will be reconverted to the v-myc sequence by site-specific mutagenesis. This will establish that the residue that was mutated has a specific role in transformation.

3. In the proposed course of doing this work we will answer the following questions: (i) What is the N-terminal amino acid sequence of Hu-c-myc? Is there a difference in the N-terminal amino acid sequence of the myc protein in cell lines where the myc gene has been deregulated by gene translocation or amplification? (ii) Does the internal amino acid sequence of Hu-c-myc correspond to the amino acid sequence predicted by the DNA sequences? (iii) Do site-specific antibodies immunoprecipitate the same protein(s) from the various cell lines with deregulated myc synthesis? (iv) Is there post-translational processing of Hu-c-myc? Does this processing have only physiological significance? (v) Where is the native c-myc located within the nucleus? (vi) What is the molecular weight of Hu-c-myc? Does the native protein have any subunit structure? (vii) Does this native Huc-myc demonstrate sequence-specific DNA binding?

4. We will focus on answering two questions in the following year: (i) Is mht an oncogene? (ii) What are the functions of mht-encoded protein products in normal and cancerous cells?

5. Portions of the genomic ets clones will be subcloned into expression vectors (pJL6 and/or pUC plasmid derivatives), and the expressed polypeptides will be used as antigens for the preparation of ets-specific antibodies. In addition, synthetic peptides derived from the predicted amino acid sequence of v-ets and human c-ets will also be used as antigens to elicit antibody production. Cellular transformation assays will also be conducted using v-ets DNA in order to define sequences essential for tumorigenesis. DNA from human cell lines and solid tumors will be analyzed for polymorphism and gene rearrangement of c-ets. Using RNA isolated from these sources, the overexpression of the ets gene will also be investigated. Also, we will attempt to isolate cellular ets clones from lower eukaryotes to facilitate our understanding of the functional role of ets in simple cellular systems.

6. Cellular transformation studies will also be conducted using v-ets DNA in order to define sequences essential for tumorigenesis. DNAs from human leukemic cell lines and solid tumors will be analyzed for polymorphism and

gene rearrangement of c-ets. Using RNA isolated from these sources, expression of the ets gene will be investigated.

7. Attempts will be made to search for chromosomal abnormalities in relation to the mht gene, such as DNA rearrangements or amplification of the mht gene.

8. Ongoing and proposed experiments will involve (i) expression of portions of the envelope gene of HTLV-III in bacteria, (ii) characterization of the regions of the pX sequences that code for the expressed LOR protein(s) in HTLV-I and -III, (iii) utilizing these abundantly produced proteins for raising specific antibodies in rabbits to both env and pX genes, (iv) use of these antibodies in the development of radioimmunoassays (RIAs) for the quick and efficient screening for HTLV-infected patients and for identifying those at risk in endemic regions.

9. The extent of homology among human, chicken and fish c-myc genes will be assessed by heteroduplex analyses of cloned DNA, and by further DNA sequencing analyses. These analyses will also be carried out on fish c-ets, c-mht and other c-onc genes. Fish c-myc will be used to probe genomic DNA from a variety of fish malignancies for chromosomal rearrangements.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04963-09 LMO

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Toward a Molecular Description of Malignant Transformation by p21 *ras* Oncogenes

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

PI:	T. Y. Shih	Research Chemist	LMO	NCI
Others:	L. S. Ulsh	Microbiologist	LMO	NCI
	S. Hattori	Visiting Fellow	LMO	NCI
	D. J. Clanton	Senior Staff Fellow	LMO	NCI
	Z. Q. Chen	Visiting Fellow	LMO	NCI
	C. C. Yuan	Guest Researcher	LMO	NCI
	J. M. Ward	Vet. Medical Officer	LCC	NCI

COOPERATING UNITS (if any) LMB, NIADDK, NIH (D. Davies); Lab. of Cell. & Devel. Biol., NIADDK, NIH (M. Lin & S. Beckner); Lab. of Mol. Virol. and Carcin., LBI, Frederick, MD (S. Oroszlan); NAPS, Program Res., Inc., Frederick, MD (M. Zweig & S. Showalter)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.5	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 major focus of this project is to investigate the molecular biology and biochemistry of the *ras* oncogenes and the p21 *ras* oncogene proteins. The long-range goal is to elucidate molecular mechanisms of cell transformation induced by these genes and their protein products. We have characterized in detail the biochemical properties of p21 overproduced in *E. coli*. The GTP/GDP binding, autokinase and GTPase activities of this fusion protein are very similar to p21 in mammmalian cells. Importantly, we have found that these p21 activities can be specifically inhibited by a monoclonal antibody (Y13-259), indicating that all these activities are conferred by a single active center within the p21 molecule. This observation, together with the finding by Stacey *et al*. that the same monoclonal antibody can specifically block p21 *in vivo* function, provides the first direct evidence to implicate the p21 GTP/GDP binding in its cellular function. The active center of p21 has also been probed by photoaffinity labeling and other chemical methods. Extensive studies are undertaken to delineate the structural requirement of p21 for its biological activity by construction of many p21 mutant proteins with specific amino acid substitutions. The p21 post-translational acylation site has been mapped by a synthetic tetrapeptide of the p21 C-terminus. p21 is palmitylated at cysteine-186, presumably through a thioester linkage. The fatty acid is identified as palmitic acid, not myristic acid as has been observed in N-terminal acylation of oncogene proteins such as p60 *src*. The functional relationship of p21 to G proteins of adenylate cyclase has been evaluated, and attempts are being made to identify p21 cellular targets by chemical cross-linking.

PROJECT DESCRIPTION

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

T. Y. Shih	Research Chemist	LMO	NCI
L. S. Ulsh	Microbiologist	LMO	NCI
S. Hattori	Visiting Fellow	LMO	NCI
D. J. Clanton	Senior Staff Fellow	LMO	NCI
Z. Q. Chen	Visiting Fellow	LMO	NCI
C. C. Yuan	Guest Researcher	LMO	NCI
J. M. Ward	Vet. Medical Officer	LMO	NCI

Objectives:

The major focus of this project is to investigate the molecular biology and biochemistry of the ras oncogenes and the p21 ras oncogene proteins. The long-range goal is to elucidate molecular mechanisms of cell transformation induced by these genes and their protein products. The ras oncogenes were first identified in Harvey and Kirsten murine sarcoma viruses (Ha-MuSV and Ki-MuSV). Earlier work of this project has contributed to the elucidation of the molecular structure of the viral genome (Shih et al., J. Virol. 25: 238-252, 1978), the first definitive identification of the p21 proteins coded for by the oncogenes of these sarcoma viruses (Shih et al., Virol. 96: 64-79, 1979), and the identification of GTP/GDP binding and autokinase activities of the p21 proteins (Shih et al., Nature 287: 686-691, 1980). Recently, many laboratories have discovered oncogenes from many human tumors. Most of these human oncogenes are the activated cellular homologues of the ras genes of Ki-MuSV and Ha-MuSV. These observations provided a strong impetus for intensive studies to elucidate the molecular mechanisms of human carcinogenesis. The scientific program of the Oncogene Biochemistry Working Group is directed toward the long-range objective of seeking a molecular description of malignant transformation induced by the p21 ras oncogenes. The major emphasis of current research involves application of genetic engineering and monoclonal antibody technology to investigate the biochemical properties and to delineate the structure-function relationship of the p21 ras proteins. From these studies, we wish to address two central questions: (1) through what molecular mechanisms do the p21 proteins transform the normal cells into the malignant cancer cells; and (2) how are proto-oncogenes activated when they have been transduced in the highly oncogenic tumor viruses, or when they are activated in human tumor cells? It is conceivable that understanding of these basic problems of cancer etiology will provide us with new insights for developing creative new approaches to cancer prevention, diagnosis and treatment.

Methods Employed:

1. Enzymology of p21 ras proteins. To characterize the biochemical activities of p21, the proteins were purified in their native states from cells transformed by Ha-MuSV, and recently, with the success of overproduction of this protein in E. coli, from bacterial cells carrying the plasmid with the inserted ras^{H} gene of Ha-MuSV (Lautenberger et al., Science 221: 858-860, 1983). Briefly, bacterial cells were broken by grinding with glass beads, and the p21 was recovered from the supernatants of high-speed centrifugation. After ammonium sulfate precipitation, the p21 was isolated to over 95% purity by chromatography on DEAE-

Sephacel and Sephadex G-150. The GTP/GDP binding was assayed by the retention of the binary complex after incubation of p21 with ^{3}H-GTP or -GDP on a nitrocellulose filter. The autokinase was assayed by transfer of the ^{32}P-labeled -phosphate of GTP to p21 and visualization of the phosphorylated p21 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The GTPase activity was assayed by hydrolysis of GTP by detecting the liberated inorganic phosphate labeled with ^{32}P or the GDP product by thin-layer chromatography.

2. Cell labeling and immunoprecipitation. Cells were labeled either with ^{35}S-methionine, ^{35}S-cysteine or ^{32}P-orthophosphate. The p21 proteins were identified by immunoprecipitation with antibodies directed against the p21. The immunoprecipitated proteins were analyzed by SDS-PAGE and visualized by autoradiography.

3. Monoclonal antibodies against p21. The p21 isolated from the bacteria was used to immunize Balb/c mice. Spleen cells of the immunized animals were fused to a mouse myeloma cell line. The hybrid cells secreting antibodies were screened and selected by a plate-binding assay using the purified antigens. Hybridoma cell lines were cloned from the positive cells. The antibodies produced by hybridoma cells were characterized with various cell lines transformed by different *ras* genes.

4. Photoaffinity labeling of p21. A photo-reactive analogue of GTP, P^{3}-(4-azidoanilido)-5' GTP was synthesized by condensation of p-azidoaniline to radioactively-labeled nucleotides with carbodiimide. The covalent affinity labeling of p21 was effected by UV photolysis of p21 nucleotide complexes. The labeled proteins were visualized by fluorography after SDS-PAGE.

5. Oligonucleotide-directed mutagenesis of ras genes. Mutant *ras* genes were constructed by point mutagenesis with oligonucleotides to change specific codons of *ras* oncogenes. The substrate was the H-1 clone of the Ha-MuSV genomic DNA. Mutagenesis was performed either with the gapped H-1 clone DNA or the single-stranded M13 subclone. Mutant p21 proteins were obtained by expression of the *ras* genes in the pJL bacterial expression vector using the convenient HindIII site of the *ras*^{H} genes. Transforming activity of the mutant DNA was evaluated by a transfection assay with an NIH3T3 cell line.

6. Peptide chemistry of p21. Peptides generated by protease digestion were analyzed by a two-dimensional, thin-layer chromatography method or high performance liquid chromatography (HPLC) with a reversed phase column. The purified peptide was analyzed by an automatic Edman degradation procedure for amino acid composition. The fatty acid involved in p21 post-translational processing was also analyzed by HPLC after cleavage from p21 with hydroxylamine.

7. Chemical cross-linking of p21. p21 from plasma membranes prepared from Ha-MuSV transformed cells labeled with ^{35}S-methionine was cross-linked with several bifunctional cross-linkers in order to identify proteins associated with p21 in cells. After cross-linking, p21 was precipitated with antibody directed against p21. The immunoprecipitated proteins were examined on SDS-PAGE.

8. Adenylate cyclase activity. Plasma membranes were incubated with ATP and the cyclase activity was measured by cAMP production.

Major Findings:

1. A monoclonal antibody to p21 identifies p21 activities responsible for its cellular function. Several biochemical activities of p21 have been identified to be the intrinsic properties of p21 proteins. These activities include GTP/GDP binding, autokinase and GTPase. Importantly, the latter activity is associated with oncogenic activation of p21 due to point mutations of the *ras* genes. However, so far there has been no evidence to show that these activities are responsible for p21 cellular function. In the present study, we have found a monoclonal antibody to p21 which specifically affects p21 *in vitro* activities and p21 cellular function upon microinjection of the same antibody into NIH3T3 cells. The direct link of p21 *in vitro* activities to its cellular function provided by the Y13-259 monoclonal antibody strongly suggests that guanine nucleotide binding is an essential activity for p21 *in vivo* function. The reduced GTPase activity associated with oncogenic activation of p21 proteins apparently affects conversion of the putative active form of a GTP-p21 complex to an inactive GDP-p21 complex. These conclusions were derived from our detailed biochemical characterization of a highly-purified p21 overproduced in *E. coli*. The purified protein possesses the full p21 activities of GTP/GDP binding, autokinase and GTPase with specific activities of the *E. coli* p21 very close to those observed in p21 obtained from Ha-MuSV transformed mammalian cells. To probe for the active center of p21, we employed a battery of six monoclonal antibodies against p21. We found that only one monoclonal antibody, Y13-259, was capable of inhibiting both GTP/GDP binding and autokinase to a maximum extent of 80% at an equivalent ratio of p21 to antibody. No other antibody has a similar effect, thus suggesting that Y13-259 affects the active center of p21 molecules. The GTP/GDP binding site can also be specifically labeled with a ^{3}H-GTP photoaffinity analogue, P^3-(4-azidoanilido)-5' GTP. The recent observation by D. Stacey's group (*Nature* 313: 241, 1985) that microinjection of the same monoclonal antibody into NIH3T3 cells specifically blocks p21 *in vivo* function strongly suggests that p21 *in vitro* activities are responsible for its cellular function.

2. Post-translational processing of p21 ras proteins involves palmitylation of the C-terminal tetrapeptide containing cysteine-186. Our previous studies indicate that post-translational processing of p21 appears to be a common pathway for the synthesis of all p21 *ras* proteins. Sefton *et al*. demonstrated acylation of p21 in the processed product. The importance of p21 acylation to its transforming function is most clearly shown by Willumsen *et al*. in a series of genetic mutants located near the p21 C-terminus. Studies of these mutants indicate that cys-186 is essential, although not sufficient, for p21 processing. However, the exact role of cys-186 in p21 processing is uncertain from the mutant studies. We demonstrate here direct chemical evidence to indicate that cys-186 is the palmitylation site. We suggest that the sequence surrounding this site may be required for recognition by cellular palmitylation enzymes. This is accomplished by comparative peptide mapping by HPLC between ^{35}S-cysteine labeled p21 from NRK cells transformed by Ha-MuSV and p21 overproduced in *E. coli*. The *E. coli* p21 is not processed by acylation. A synthetic tetrapeptide of the p21 C-terminus is used to identify the acylation site in eukaryotic p21 as cys-186. Acylation occurs, presumably, through a thioester linkage. The same peptide of bacterial p21 is not acylated. Although p21 of Ha-MuSV transformed NRK cells can be metabolically labeled either with ^{3}H-palmitate or ^{3}H-myristate, the lipid moiety of the hydrophobic peptide is identified as palmitic acid, not myristic acid. We suggest

that the enzymatic mechanism for p21 palmitylation may be different from N-terminal myristylation of many other membrane proteins.

3. Construction of mutants affecting properties of p21 ras proteins. A systematic study has been initiated to investigate the structure-activity relationship of p21 that is important for its function and mechanism of oncogenic activation. Approaches using oligonucleotide-directed mutagenesis were used to construct point mutations in v-ras oncogenes to effect specific amino acid substitutions of p21 proteins. Properties of mutant p21 proteins were evaluated by expression in the plasmid expression vector in E. coli. Biological activities of the mutant ras genes were also evaluated by transfection into NIH3T3 cells. The major objective is to evaluate the relationship between the known p21 activities, to identify the guanine nucleotide binding site and to correlate the p21 in vitro activities to its biological functions. The first series of mutants concern the specificity of the autokinase activity and its significance in p21 activation. Serine and alanine mutants have been constructed from the wild-type threonine at the 59 position. The serine mutant p21 has one-tenth of the wild-type p21 autokinase activity with a proportional increase in its GTPase activity. The effect on its transforming activities is being evaluated. Other classes of mutants have been constructed with sites which may be the nucleotide binding sites, as based on amino acid sequence comparison with other guanine nucleotide binding proteins. The properties of these mutants are also in the process of being characterized.

4. Functional relationship of p21 and the regulatory G proteins of adenylate cyclase. Biochemical properties of p21 strongly resemble several guanine nucleotide binding proteins which serve as signal transducers in membrane function, such as G proteins of adenylate cyclase and transducin of retina rod outer segments. Implication of ras genes in adenylate cyclase regulation has recently been highlighted by observations in yeast mutants involving ras genes. We have investigated the plasma membrane adenylate cyclase to evaluate the functional analogy of p21 to the G proteins of adenylate cyclase. One of the biochemical properties that appears to be common to these regulatory proteins is ADP-ribosylation by several bacterial toxins. We have examined the p21 overproduced and purified from E. coli or p21 in the membranes of Ha-MuSV transformed NRK cells for its ability to be ADP-ribosylated by cholera toxin and pertusis toxin. The result is completely negative, although all the control experiments show strong reactions with G proteins of adenylate cyclase. The other approach is reconstitution of purified p21 from E. coli or Ha-MuSV transformed cells to the plasma membrane of a mouse lymphoma mutant cell line (S49) defective in the stimulatory G protein of adenylate cyclase. No complementation effect can be observed in restoring the adenylate cyclase activity. Although it has long been known that in these virus-transformed cells, the adenylate cyclase activity is decreased (in yeast, increased), but not specifically induced by the ras genes, the present results suggest that these effects may be indirect consequences rather than direct functional replacement of G proteins for p21 proteins.

5. Attempts to identify the p21 cellular targets by biochemical approaches. If p21 functions by regulating the activities of its cellular effectors and mediating signal transduction from transmembrane receptors to the intracellular effectors, we reasoned that these interactions must be effected by direct physical contacts between these macromolecules. We have initiated a line of investigation by trying to cross-link these protein molecules under appropriate conditions

by bifunctional chemical cross-linkers. Proteins cross-linked to p21 were immunoprecipitated with anti-p21 antibodies and visualized by SDS-PAGE. Cross-linkers with various spacer lengths such as dithiobis (succinimidyl propionate), disuccinimidyl suberate and bis (2-(succinidimideoxycarbonyloxy)ethyl) sulfone are among others being employed.

Significance to Biomedical Research and the Program of the Institute:

The p21 ras oncogenes have been demonstrated to be associated with many human neoplasms, such as carcinomas of the lung, colon, bladder, pancreas, and some forms of malignancy of the hematopoietic system. The ras genes have also been implicated as possible targets in carcinogenesis induced by chemical carcinogens. Furthermore, the p21 ras genes are the viral oncogenes of the highly oncogenic Harvey and Kirsten murine sarcoma viruses, and v-ras oncogenes probably represent one of the best model systems to study the molecular mechanisms of malignant transformation. The current studies characterized in detail the biochemical properties of p21 ras oncogene proteins purified from genetically engineered E. coli overproducing this protein. For the first time, we have been able to obtain direct evidence implicating the guanine nucleotide binding properties of p21 to its cellular function. This evidence is provided by the current observation of a monoclonal antibody to p21 (Y13-259) which affects the GTP/GDP binding activity of p21, and upon microinjection of the same antibody into NIH3T3 cells. The p21 in vivo activity of entering the cell in its S phase in cell division is neutralized. The identification of the crucial p21 activity in its cellular function is important as a first step toward understanding p21 oncogenic activation and the possible future development of drugs for cancer treatment. The current study also established a fact that cysteine-186 of p21 is the p21 palmitylation site in its post-translational processing. Other genetic mutant studies have suggested the crucial importance of this processing event in the p21 trans forming function. The present study provides the experimental system to delineate the enzymatic mechanism in protein palmitylation. This post-translational modification may also present a possible focal point for future designing of chemotherapeutic agents for human cancer.

Proposed Course:

1. The guanine nucleotide binding site of p21 will be mapped by photoaffinity labeling with the ^{32}P- and ^{3}H-labeled GTP analogue, P^3-(4-azidoanilido)-5' GTP or by modification of cysteine residues involved in GTP/GDP binding with ^{3}H-N-ethylmaleimide. The labeled peptide will be isolated and identified.

2. p21 enzymology will be continued with emphasis on exploring the structure of the active center.

3. Site-directed mutagenesis will be expanded to construct mutants which affect GTP/GDP binding in order to evaluate further the biological significance of these activities.

4. In collaboration with David Davies of the NIADDK, NIH, the purified p21 isolated from E. coli overproducing this protein will be exploited to determine its structure by X-ray crystallography. The mutant p21 constructed in this lab will be valuable for detailed structural comparison.

5. An Effort will be made to search for the p21 cellular targets with an emphasis on biochemical means using the p21 as a handle.

6. The enzyme systems for p21 palmitylation will be explored by using the pro-p21 substrate of the p21 protein expressed in E. coli.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04970-09 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Biochemistry of Cellular Transformation by Avian Tumor Viruses

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

PI:	J. P. Bader	Research Microbiologist	LMO	NCI
Others:	D. A. Ray	Chemist	LMO	NCI
	F. A. Donovan	Chemist	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	0.6	1.8

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Proteins encoded by tumorigenic retroviruses may be synthesized as hybrid proteins, containing both viral structural and transformation domains, or as homologs of cellular proteins devoid of any viral structural elements. Cells infected with MC29 virus produce such a hybrid protein, p110 *gag-myc*, which contains a viral domain (*gag*) fused to a cellular domain (*myc*). This protein migrates to the nucleus soon after synthesis, and can be found associated with the nuclear matrix, and possibly with nucleoli. The protein has a short half-life in the cells, and other protein and RNA factors have been shown to be involved in its degradation. The p110 was shown to exist in both monomeric and dimeric forms intracellularly. A cellular protein, p55, can be immunoprecipitated from avian cells using antisera against the *myc* domain. The p55 also was found in the nuclear matrix, and could associate into dimers. Another transforming virus, avian myeloblastosis virus, similarly produces a protein, p48, which is found in the nuclear matrix as a dimer and has a short half-life.

Cells transformed by MC29 virus and related viruses have enlarged nucleoli which are not a consistent feature of other transformed or nontransformed cells. These *myc*-transformed cells incorporated a high proportion of radioactive uridine into nucleolar RNA. Transcription in isolated nuclei was more resistant to an inhibitor of RNA polymerase I than non-*myc* cells, demonstrating an enhanced level of ribosomal RNA synthesis. Several other differences in the *in vitro* transcription characteristics of *myc*-transformed and other cells have been noted. Also, a specific protein, p98, has been found associated with nucleoli of cells transformed by *myc* viruses. The basis for increased nucleolar transcription is under investigation.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

J. P. Bader	Research Microbiologist	LMO	NCI
D. A. Ray	Chemist	LMO	NCI
F. A. Donovan	Chemist	LMO	NCI

Objectives:

To determine the intracellular location and stability of proteins encoded by the avian viruses, MC29 virus and avian myeloblastosis virus (AMV), and of normal cellular proteins homologous to these viral proteins. To analyze the association of these proteins with themselves or with cellular constituents. To analyze the factors responsible for enlargement of nucleoli in cells transformed by MC29 and related viruses.

Methods Employed:

1. Transformation of cells in culture by avian viruses, morphological resolution of transformed cells by microscopy, resolution of cellular organelles.

2. Isolation of cellular organelles, including microsomes, cell surface membranes, nuclei, nucleoli and other nuclear components.

3. Quantitative chemical determinations of protein, DNA, and a variety of enzymes.

4. Immunoprecipitation, immunoaffinity chromatography.

5. Column chromatography of various types.

6. Density gradients, zonal centrifugation and analysis.

7. Polyacrylamide gel electrophoresis, agarose gel electrophoresis, autoradiography, fluorography.

8. Uptake of radioactive molecules into cells, incorporation of radioactive precursors into macromolecules.

9. Synthesis of macromolecules in isolated cellular organelles.

10. Chemical and enzymatic treatment of proteins and nucleic acids.

Major Findings:

The synthesis and stability of the protein responsible for transformation by MC29 virus was examined by radioactive labeling of transformed cells, and immunoselection of the virus-coded p110. The p110 migrated rapidly to the nucleus after synthesis, and sequential extractions revealed that it was

associated with the nuclear matrix. Experiments inhibiting RNA synthesis showed that the viral messenger RNA for p110 was stable for several hours. The p110 under natural conditions decayed with a half-life of about 30 minutes, but inhibition of RNA synthesis extended the half-life. When protein synthesis was inhibited for several hours, then released, the p110 was synthesized with only a few minutes delay. Subsequent inhibition of protein synthesis resulted in a stabilization of the protein. Also, when nuclei were isolated from pulse-labeled cells, the p110 degraded at a reduced rate compared to intact cells. We conclude from these and other studies with antimetabolites that another labile protein, presumably encoded by a cellular gene, is responsible for the degradation of p110. In addition, interaction of p110 with a newly-synthesized RNA is required for maximal turnover of p110.

The intracellular molecular form of p110 was examined by centrifugation through sucrose gradients. When labeled cells were lysed and extracted with a nondenaturing detergent, the p110 sedimented primarily as monomers and dimers. No larger oligomers were obvious, but were not excluded. The pr76 of RAV-1 infected chick embryo cells, selected by the same anti-p27 serum used to detect p110, sediments as a monomer. This demonstrated that dimerization occurs through the _myc_ domain of the viral p110. When the extract was treated with a partially denaturing solution (0.2% sodium dodecyl sulfate), all of the p110 sedimented as monomers. Increasing the salt concentration in the gradients to 0.5 M or 1.0 M NaCl did not dissociate the dimers, and β-mercaptoethanol had no effect on sedimentation rate. Likewise, treatment of extracts with DNase or RNase had no effect on the sedimentation of p110.

Antibodies against two separate domains of the p110 immunoprecipitated monomeric and dimeric forms, but not other proteins in common. This demonstrated that p110 was not firmly associated with a cellular protein. The occurrence of p110 dimers has been observed directly. Bands about twice the molecular weight of p110 have been observed after immunoprecipitation and gel electrophoresis. The identity of these dimeric bands with p110 was made by the ability of the peptide used to induce antibody to compete with the immunoprecipitability of the dimer.

A peptide projected from the nucleotide sequence of the _myc_ genome was used to elicit antibody capable of immunoprecipitating p110. This antibody immunoprecipitates a p55 from noninfected quail or chick embryo cells growing in culture, and the p55 was shown by T. Chen and T. Papas to have tryptic peptides in common with p110. This putative $p55^{myc}$ was examined for its intracellular localization, and, as in the case of p110, it was found exclusively in the nucleus as part of the nuclear matrix. Sedimentation studies revealed the p55 to be primarily a dimer in nondenaturing solutions. The protein also is phosphorylated intracellularly, as is virus-coded p110. A major difference between p55 and p110 is the relative stability of the p55, having an intracellular half-life of over four hours. This p55 is found in decreased amounts in quail or chick embryo cells transformed by MC29, raising the possibility that p55 synthesis is repressed by p110.

The transforming protein, p^{myb}, encoded by AMV, has been identified as a p48 using antiserum directed against a peptide from the _myb_ region of the viral

genome. The p48 is intranuclear, and is unstable, having a half-life similar to that of p110 myc of about 30 minutes. The p48 sediments as a dimer in nondenaturing solutions. The remarkable similarities of $p48^{myb}$ to $p110^{myc}$ suggest a similar physiological function. This, however, is unlikely in a specific sense, since AMV and MC29 have distinctly different biological effects on cells, and the peptide sequences have no commonality.

Avian cells transformed by MC29 and related viruses (CMII, OK10, MH2) containing the myc gene exhibit enlarged nucleoli compared to nontransformed cells or cells transformed by other avian viruses. The size and integrity of nucleoli in these cells was directly dependent on continued RNA synthesis, but not protein or DNA synthesis. Addition of exogenous radioactive uridine to cells resulted in a relatively greater incorporation into nucleolar RNA than the rest of cellular RNA. To more exactly examine the possibility of differential rates of RNA synthesis, nuclei were isolated from myc-transformed and other cells, and transcription was analyzed directly after addition of ribonucleotide triphosphates. The drug, α amanitin, was used to selectively inhibit messenger RNA synthesis, and preliminary studies suggested that ribosomal transcription was proportionately greater in myc-transformed cells. Also, several differences in transcription patterns were found between nontransformed and MC29-transformed cells, including the time course of transcription, dissociation of the transcription system, and susceptibility to inhibition by ATP. Analysis of nucleolar proteins showed a prominent p98 in myc-transformed cells, which was inapparent or absent in other cells.

Significance to Biomedical Research and the Program of the Institute:

The intracellular lability of MC29 and AMV proteins suggests that the continued synthesis of these proteins is required for the maintenance of the transformed state, and antimetabolite studies suggest that a cellular protein(s) is involved in the degradation of the transforming protein. The association of cellular proteins with myc p110 probably is of short duration, since no other protein was found associated with p110 in direct examination of cellular extracts. The MC29 and AMV transforming proteins are found as monomers and dimers in cellular extracts. In determining the physiological function of these proteins, it is necessary to consider the molecular form which is active, and to be certain that the conditions required for the stabilization of that form are maintained. The similarities and differences between the MC29, p110 and cellular p55 may lead to the resolution of the physiological function of the normal cellular myc protein, and the changes which give the protein malignant potential.

Studies on nucleoli and nuclear transcription are attempts to define at a subcellular level the functional activity of the myc transforming protein. Since the myc protein is a nuclear protein, and almost certainly affects transcription of cellular genes, a direct analysis of transcription could define the activity resulting in malignancy.

Proposed Course:

The instability of MC29 and AMV proteins may be directly related to their biochemical functions. We shall investigate the factors involved in degradation by preparing fractional cellular extracts and studying the effects of cellular

components in stability. The binding of DNA and RNA to monomers and dimers will be examined, and if found, attempts to determine possible nucleotide sequence specificity for the binding will be made. Attempts will be made to establish conditions for ready associations and dissociation of molecular aggregates, so that differential functions can be more readily analyzed. It may be necessary to purify these proteins from bulk cellular extracts in order to obtain enough material to determine a biochemical function. Bacterial expression vectors containing portions of the myc protein have been obtained and will be used to produce large amounts of the polypeptide for examination of aggregate formation. As other expression vectors containing more of the myc genome, or other segments of the genome, become available, we shall examine these expressed proteins as well.

Nuclear and nucleolar transcription studies will be pursued. Cloned DNA inserts of regions of ribosomal DNA have been obtained, and will be used to isolate the promoter region for ribosomal RNA synthesis. Factors involved in the regulation of rRNA synthesis will be assessed in myc-transformed and other cells.

Publications:

Bader, J. P. and Ray, D. A.: MC29 virus-coded protein occurs as monomers and dimers in transformed cells. J. Virol. 53: 509-514, 1985.

Balaban, R. S. and Bader, J. P.: Studies on the relationship between glycolysis and $(Na^{+}+K^{+})$-ATPase in cultured cells. Biochim. Biophys. Acta 804: 419-426, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05120-06 LMO

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression of Retroviral and Oncogene Proteins in Bacterial and Mammalian Vectors

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

PI:	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
Others:	F. Wong-Staal	Biologist	LTCB	NCI
	L. Ratner	Senior Staff Fellow	LTCB	NCI
	S. Josephs	Chemist	LTCB	NCI
	S. J. O'Brien	Acting Chief	LVC	NCI
	T. S. Papas	Acting Chief	LMO	NCI
	K. P. Samuel	Visiting Associate	LMO	NCI

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(L. Levinger)

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Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	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 HTLV-I envelope sequence has been expressed by means of an open reading frame vector that allowed rapid identification of colonies making insert protein. The resulting protein was a fusion of a portion of the envelope and *E. coli* beta-galactosidase. The junction between the HTLV-I DNA and the vector was established by sequence analysis of the expression plasmid. Since the protein reacted with antibodies in patient sera, it has potential use in diagnostic assays. Since the segment of the envelope that is expressed is completely defined by the DNA sequence of the insert, this protein may also be used to map epitopes recognized by monoclonal antibodies directed against the HTLV-I envelope.

The DNA sequence of HTLV-III was analyzed for similarity with other retroviruses in the *pol* and *env* regions. The *pol* region was found to have a high degree of sequence homology with other retroviruses, but did not closely resemble any particular retrovirus whose sequence homology was available. The envelope region had little or no sequence homology with other retroviruses, but analysis of the hydrophilicity profile revealed a region that may correspond in structure to the transmembrane envelope protein of other retroviruses.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
F. Wong-Staal	Biologist	LTCB	NCI
L. Ratner	Senior Staff Fellow	LTCB	NCI
S. Josephs	Chemist	LTCB	NCI
S. I. O'Brien	Acting Chief	LVC	NCI
T. S. Papas	Acting Chief	LMO	NCI
K. P. Samuel	Visiting Associate	LMO	NCI

Objectives:

The scope of this investigation is to delineate the relationship between oncogene expression and the conversion of cells from a normal to a malignant state. The nature of the protein products of the oncogenes is especially emphasized in this study because they remain more elusive than the DNA sequences that constitute the genes themselves. Expression of the proteins in bacteria should facilitate the analysis of their biochemical properties and their effect on living cells. This is because these proteins can be easily produced in large quantities from bacteria. These techniques, when coupled with directed mutagenesis, can provide greater insight into the relationship between the structure and function of the protein products of oncogenes.

A further role of the expression system is to produce proteins from the viruses HTLV-I and HTLV-III, both of which are pathogenic in humans. The synthesized proteins can be used as diagnostic reagents or as the basis of subunit vaccines.

Methods Employed:

1. Preparation of plasmid DNA. Plasmid DNAs were prepared as described by Birnboim and Doly (*Nucleic Acids Res.* 7: 1513-1523, 1979) from 5-ml cultures for screening or from one-liter cultures for preparation of restriction fragments. Plasmid DNA from the larger cultures was further purified by ethidium bromide-CsCl equilibrium density centrifugation.

2. Plasmid construction techniques. Plasmid DNAs were cleaved by the appropriate restriction enzymes and subjected to electrophoresis on polyacrylamide gels. Fragments were eluted from the gels by the method of Maxam and Gilbert (*Methods Enzymol.* 65: 499-560, 1980). Vector DNA was prepared for ligation by cleavage with the appropriate restriction enzymes and treated with calf intestinal alkaline phosphatase. Ligation of each isolated fragment (0.5 µg) to vector DNA (1.6 µg) was performed in 66 mM Tris-HCl, pH 7.4, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.4 mM ATP, and 2.5 units/ml T4 DNA ligase (New England Biolabs). The reactions (0.04 ml) were incubated at 4°C for 18 h. Calcium chloride-treated *E. coli* DC646 cells were transformed as described by Cohen *et al.* (*Proc. Natl. Acad. Sci. USA* 69: 2110-2114, 1972) and ampicillin-resistant colonies were screened for plasmids as described above. To study gene expression, plasmid DNAs prepared from the transformed DC646 cells were introduced into N4830 or N4831 cells by the same procedure.

3. Radiolabeling and electrophoresis of bacterial proteins. *E. coli* cells were grown at 32°C in M56 minimal media supplemented with 0.5% glucose, 0.1% each of all amino acids except methionine and cysteine, 0.01% biotin, 0.01% thiamine, and 50 μg/ml of ampicillin. When the OD_{590} of the cultures reached 0.2, the temperature was shifted to 41°C. Aliquots (150 μl) of the cells were taken 5 min before and at 10 min and 30 min after the temperature shift. These cells were added to 15 μl of media containing 2.5 μCi [^{35}S]-cysteine and incubated for 1.5 min. After labeling, some cultures were chased by adding unlabeled cysteine to a final concentration of 0.5 mM. Cellular protein was precipitated with 10% (w/v) trichloroacetic acid, washed with acetone, and resuspended in 1.0% SDS/0.1% β-mercaptoethanol. The proteins were then resolved by electrophoresis on 10% SDS-polyacrylamide gels and visualized by autoradiography.

4. Preparation of bacterial extracts. Unfractionated extracts were prepared from cells grown at 32°C in supplemented M56 media to an OD_{590}=0.3. The cultures were then induced by shaking another 60 min at 41°C. The cells were pelleted by centrifugation, resuspended in 1/40 volume of supplemented M56 media and heated for 20 min at 95°C after being brought to a final concentration of 0.7% SDS and 0.07% β-mercaptoethanol. Alternatively, the cells were pelleted by centrifugation and resuspended in 50 mM Tris-HCl, pH 8.0, in 25% sucrose. Lysozyme was added to a final concentration of 2 mg/ml. After 5 min at 0°C, $MgCl_2$ was added to a 5 mM final concentration, followed by DNase I to 60 μg/ml. The cells were lysed by the addition of 1% NP40/0.5% sodium deoxycholate/0.1 M NaCl/0.01 mM Tris-HCl, pH 7.2/1 mM EDTA and centrifuged at 12,000 g for 10 min. The pelleted material was washed with 1 M NaCl/10 mM Tris HCl, pH 7.2/1 mM EDTA and resedimented. The resultant pellet was resuspended in 1% SDS/0.1% βmercaptoethanol by being heated for 10 min at 95°C.

5. Immunodetection of proteins by Western blotting. Proteins were resolved on SDS-polyacrylamide gels and transferred electrophoretically onto diazobenzyloxymethyl (DBM) paper. After nonspecific protein binding sites were blocked by bovine serum albumin, the paper washed and incubated overnight with antibody. After being washed briefly in a sonicating cleaner, the paper was incubated with ^{125}Ilabeled protein A from *Staphylococcus aureus*. Since protein A binds antibody, the antibody-antigen complex was detected by autoradiography.

6. Computer analysis of DNA sequences. The design of the recombinant expression plasmids made extensive use of the sequences of the DNAs involved. This analysis was greatly facilitated by the use of our Laboratory's MINC 11/23 computer and the NIH mainframe computers using software developed by ourselves and others. Programs we have written for our computer include (1) an editor to create and modify files that contain DNA sequences, (2) a program to search for restriction sites or consensus regulatory sequences, (3) a program to translate DNA sequences into amino acid sequences, (4) a program to create dot matrix homology plots of the type described by Maizel and Lenk (Proc. Natl. Acad. Sci. USA 78: 7665, 1981), (5) a program to plot termination codons and potential initiation codons in DNA sequences, (6) a program that charts the Hopp and Wood (Proc. Natl. Acad. Sci. USA 78: 3824, 1981) hydrophilicity values of deduced amino acid sequences, and (7) a program to identify domains of uncharged hydrophobic amino acids that can penetrate membranes as defined by the criteria of Segrest

and Feldmann (J. Mol. Biol. 87: 853, 1974). Our system induces a voice synthesizer that allows an investigator to proofread sequences without requiring assistance.

Major Findings:

1. Expression of HTLV-I envelope in bacteria. An expression vector was constructed in which 13 codons of the phage λ cII protein were placed adjacent to the E. coli β-galactosidase gene (lacZ) such that the lacZ sequence is out of frame with respect to the lambda cII sequence. The fused gene was placed under the transcriptional control of the strong, well-regulated phage λ p_L promoter.

A restriction enzyme recognition site (NruI) is located between cII and lacZ, allowing insertion of DNA fragments. Inserted open reading frames that are properly aligned with the cII and lacZ sequences can be easily identified, since plasmids containing such sequences confer a lac^+ phenotype on the host bacterium.

This vector was used to express the envelope protein of human T-cell leukemia virus type-I (HTLV-I). The ends of a restriction fragment containing envelope sequences were randomly digested by BAL 31 nuclease and inserted into the NruI site of the vector. A blue colony was isolated that was found to be synthesizing a β-galactosidase with a molecular weight larger than that of the wild type enzyme. This protein was shown to contain HTLV-I envelope sequences since it reacted on Western blots with antibodies in patient sera. The junctions between the HTLV-I envelope DNA and the vector were precisely established by Maxam-Gilbert and dideoxynucleotide DNA sequencing. The protein produced by this plasmid has great promise in diagnostic assays. Since the region of the envelope that is expressed is completely defined by the DNA sequence of the insert, this protein may also be used to map the epitopes recognized by monoclonal antibodies directed against the HTLV-I envelope.

2. Analysis of the HTLV-III pol gene. The sequence of the putative pol gene of HTLV-III was analyzed for homology with other retroviruses. Considerable homology was found in two regions that are the core of the polymerase and the endonuclease proteins. In almost all cases where most other retroviruses were concordant with a consensus sequence, the HTLV-III pol gene was also concordant. However, the HTLV-III sequence does not have an extremely close homology with any of the sequenced pol genes. To study the relationship of the HTLV-III pol gene with other pol genes in a consistent manner, comparable segments of the polymerase and endonuclease regions were aligned with their counterparts in several other retroviruses. A matrix was created from the fraction of matching amino acid residues in each pair of sequences. This data was used with the method of Fitch and Margoliash to create a phylogenetic tree. This tree indicated that HTLV-III evolved in a lineage separate from other retroviruses for which pol sequences are available. The other retroviruses examined fell into three other lineages. They are (1) HTLV-I and BLV, (2) RSV, MMTV, and SMRV, and (3) Moloney MLV, AKV, REV, and endogenous retroviruses.

3. Analysis of the HTLV-III env gene. The sequence of the putative env gene open reading frame (ORF) was analyzed for homology and structural similarity with the env genes of other retroviruses. Very little sequence homology was found. Even in the most conserved region, a region in the transmembrane protein noted by Cianciolo et al. (Nature 311: 515, 1985), the best matches deviated greatly from the consensus sequence. However, analysis of the hydrophilicity profile, as defined by the method of Hopp and Wood, allowed a plausible model to be formed as to the location of the division between the exterior glycoprotein and the transmembrane protein, and as to the structure of a portion of the transmembrane protein. The division point should occur after amino acid residue 518 of the open reading frame. This follows the only occurrence of the sequence (Arg/Lys-X-Arg/Lys-Arg/Lys) in the envelope open reading frame. This region is in a hydrophilic region that precedes a hydrophobic region. This is also true of the division point of other retroviruses. The hydrophobic region next to the clip site is followed by a region of average hydrophilicity. This region contains two cysteine residues that may have a role in joining the transmembrane protein to the exterior glycoprotein. This region is followed by a strongly hydrophobic region containing two uncharged regions separated by a single arginine. These regions may penetrate membranes as they meet the criterion defined by Segrest and Feldmann. There is a longer span from the highly hydrophobic region to the end of the env ORF in HTLV-III than there is in any other retrovirus for which env sequences are available.

4. Purification of bacterially-synthesized c-sis protein. A 33,000-dalton protein was observed when a segment of DNA formed a cDNA clone of c-sis sequences placed in the expression plasmid, pJL6. Induced cells bearing this plasmid were disrupted by sonication and the extracts were pelleted. The sis protein was found in the pellet fraction by Western blot analysis. This protein was purified to near homogeneity by sequencially extracting the pellet with a series of solutions of detergents and denaturants.

Significance to Biomedical Research and the Program of the Institute:

The bacterial protein expression system possesses the potential for the production of three types of useful reagents: (1) enzymatically- or biologically-active proteins, (2) immunogenic proteins, and (3) proteins recognized as antigens. The ras protein produced in E. coli is enzymatically active. It is therefore suitable for structure and function studies based on the site-directed alteration of its sequence. The immunogenicity of the bacterial proteins has been verified in the case of v-myc, since antibodies raised against this protein immunoprecipitate the MC29 p110$^{gag-myc}$ protein. Antibodies produced in this manner should be useful for the study of the intracellular localization and tissue distribution of oncogene proteins. The ability of the bacterial protein to be recognized as an antigen has been shown for both casees of ras and sis. This could lead to diagnostic procedures in which the sera of patients can be analyzed for antibodies that react with the expressed protein. The antigen used is defined by the DNA sequence that is expressed and can be highly purified since it is present in the bacterial lysate as a major component. As an example of this approach, a bacterially-synthesized human T-cell leukemia virus type I (HTLV-I) partial envelope protein was recognized by antibodies in the sera of infected patients. Such synthetic antigens also have the potential of being the basis of a subunit vaccine.

Studies of the bacterially-synthesized p28^{sis} are of special importance since the amino acid sequence predicted from the c-sis sequence is identical to that of human platelet-derived growth factor (PDGF). This homologous relationship suggests that there may be a common mechanism between growth stimulation and carcinogenesis. Furthermore, the observation that PDGF stimulates c-myc mRNA synthesis suggests that carcinogenesis may involve the interaction of several oncogenes.

Proposed Course:

1. Analysis of c-sis gene products. The bacterially-synthesized sis protein will be used to produce monoclonal antibodies against sis/PDGF proteins. These antibodies will be used to study the intracellular localization, tissue distribution (cell type; normal vs. malignant) and the processing pathways of these proteins. It should be possible to conjugate such antibodies with ferritin and visualize the location of proteins that react with the ferritin-antibody complex by electron microscopy. It should also be possible to use these antibodies to observe intermediates in the processing pathways of PDGF. By comparing reducing vs. non-reducing conditions and treating cells with the glycosylation inhibitor, tunicamycin, it should be possible to study how the primary translation product is modified by proteolysis, disulfide bond formation, and glycosylation. These pathways will also be examined in tumor cells to see if they are altered. Of special interest for such studies are the glioblastoma cell line, A172, and the human sarcoma line, 8387. Both of these cell lines have been reported to express high levels of sis mRNA (Eva et al., Nature 295: 116, 1982). The anti-sis antibodies should be useful in the purification and study of the PDGF-like protein excreted by the c-sis transformed cells. Its structure is of interest since the transforming DNA contains the gene for only one subunit of PDGF.

2. Development of improved expression vectors. Several alterations to the bacterial expression vector, pJL6, will be performed. A synthetic oligonucleotide will be cloned into the unique Nde I site such that the sequence up to the cII initiation codon will be preserved, but a unique BamHI site will be introduced adjacent to this codon. The cII translation initiation site will be unaltered and proteins introduced into the BamHI site will not have any codons (other than the initiation codon) derived from phage sequences. In addition, the oligonucleotide used will contain terminators in all three frames such that translation will not be able to proceed past the inserted sequence. Another modification will place a segment of the phage, M13 mp18, into pJL6. This segment contains several restriction sites, including one for Sph I that produces 3' protruding ends. This allows the production of deletions by the procedure of Henikoff (Gene 28: 351, 1984) in which the DNA is removed only in one direction. This method relies on the property of E. coli exonuclease III in which it degrades recessed 3' ends, but not protruding 3' ends. A piece of DNA containing several genes can be inserted and the sequence upstream from each gene can be selectively removed. This should allow the rapid construction of plasmids suitable for the expression of several genes at once. In addition, this vector will contain appropriate restriction sites such that it will be possible to sequence insert DNA without it being necessary to isolate restriction fragments. This will be done by a scheme similar to that of Rüther (Nucleic Acids Res. 10: 5765,

1982) in which cleavage with a second restriction enzyme produces a very small fragment that does not interfere with the elucidation of the sequence. Thus, a single vector should allow the rapid expression and sequence determination of a relatively uncharacterized segment of DNA.

3. Expression of protein products of HTLV-III. Portions of the envelope gene of HTLV-III will be placed in the expression vector, pJL6, or one of its derivatives. Expressed proteins will be observed in SDS-polyacrylamide gels of induced cells. Furthermore, the SV40 expression system of E.S.P. Reddy will be used to produce the HTLV-III envelope protein in mammalian cells.

4. Production of specific mutants in the MC29 v-*myc* gene. A comparison of the sequences of three transforming avian retroviruses that contain the transforming v-*myc* gene indicated that they differ from the chicken proto-*myc* oncogene only at one common amino acid residue. To determine if that residue is necessary for transformation activity, the MC29 v-*myc* sequence will be converted to the equivalent chicken proto-*myc* sequence at that position. This will be done by oligonucleotide based site-specific mutagenesis. To facilitate this procedure, a portion of the v-*myc* gene will be cloned into M13 in p8. After mutagenesis, this segment will be rejoined with the rest of the MC29 proviral DNA and transformation of chicken embryo fibroblasts will be determined by a helper dependent transfection protocol. Any DNAs found to have lost transformation ability will be reconverted to the v-*myc* sequence by site-specific mutagenesis. This will establish that the residue that was mutated has a specific role in transformation.

Publications:

Flordellis, C. S., Kan, N. C., Lautenberger, J. A., Samuel, K. P., Garon, C. F. and Papas, T. S.: Analysis of the cellular proto-oncogene *mht-raf*: Relationship to the 5' sequences of v-*mht* in avian carcinoma virus MH2 and v-*raf* in murine sarcoma virus 3611. *Virology* 141: 267-274, 1985.

Lautenberger, J. A., Seth, A., Jorcyk, C. and Papas, T. S.: Useful modifications of the *Escherichia coli* expression plasmid pJL6. *Gene Anal. Tech.* 1: 63-66, 1984.

Papas, T. S., Kan, N. C., Watson, D. K., Flordellis, C., Lautenberger, J. A., Psallidopoulos, M. C., Rovigatti, U. G., Samuel, K. P., Ascione, R. and Duesberg, P. H.: Two oncogenes in avian carcinoma virus MH2: *myc* and *mht*. *Anticancer Res.* (In Press)

Papas, T. S., Kan, N. C., Watson, D. K., Lautenberger, J. A., Flordellis, C., Samuel, K. P., Rovigatti, U. G., Psallidopoulos, M., Ascione, R. and Duesberg, P. H.: Oncogenes of avian acute leukemia viruses are subsets of normal cellular genes. In Neth, R., Gallo, R. C., Greaves, M. F., Moore, M. A. S., Winkler, K. (Eds.), *Modern Trends in Human Leukemia VI*, Wilsede, Germany, Berlin/Heidelberg, Springer-Verlag (In Press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05186-05 LMO

PERIOD COVERED
October 1, 1984 to September 30, 1985

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

Expression and Regulation of Viral and Cellular Oncogenes

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

PI:	K. P. Samuel	Visiting Associate	LMO	NCI
Others:	T. S. Papas	Acting Chief	LMO	NCI
	C. S. Flordellis	Visiting Fellow	LMO	NCI
	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
	L. Virgilio	Biologist	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.0	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.)

Construction of bacterial expression plasmids containing envelope and pX-LOR gene sequences of the human T-cell leukemia virus type-I (HTLV-I) and env-LOR sequences of HTLV type III (HTLV-III) has been accomplished. These viral gene fragments were linked to the λcII gene of the high level expression vector, pJL6, or its derivatives, pJLA16 and pCJX. Two such expression plasmids, pKS400, which expresses a 16,000 dalton p21E transmembrane fusion-protein from the transmembrane domain of the HTLV-I env protein, and pKL296C, which produces a fusion-protein representing amino acid sequences from the carboxyl-terminus of the $p42^{LOR}$ protein of the HTLV-I pX gene, have been isolated and characterized, and their fusion-proteins purified to homogeneity. Current and projected studies with these and other expression plasmid constructs will be discussed.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

K. P. Samuel	Visiting Associate	LMO	NCI
T. S. Papas	Acting Chief	LMO	NCI
C. S. Flordellis	Visiting Fellow	LMO	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
L. Virgilio	Biologist	LMO	NCI

Objectives:

(1) To construct chimeric genes of human T-lymphotropic retroviruses HTLV-I and HTLV-III envelope and pX-LOR fragments fused to the λcII gene of the high level expression vector, pJLA16, for the purpose of expressing these genes in abundance in *E. coli* bacteria; (2) purification of the bacterially-produced fusion proteins for use in the biochemical and seroepidemiological analyses of HTLV-I and HTLV-III associated human sera and blood bank products; (3) aid in the development of polyclonal and monoclonal antibodies to these antigens and identification of biologically-active epitopes on their surfaces; and (4) utilization of the viral reagents for the development of diagnostic assays and, eventually, the therapeutic application of this effort to human T-cell malignancies and AIDS.

Methods Employed:

1. Cloning - Vector Construction. The expression vector, pJL6, was constructed by Lautenberger in this Laboratory (see Project Number Z01CP05120-05 LMO). Other derivatives of pJL6 (e.g., pJLA16 and pCJX) were constructed by modification of unique restriction enzyme sites within the parent vector. These vectors harbor the p_L promoter of phage λ and the *c*II translation initiation codon of its protein. The ampicillin-resistant gene of pBR322 is present, conferring a marker which selects for an antibiotic resistance for screening of recombinant plasmids. The pJL6 vector contains the unique restriction enzyme sites, HindIII and ClaI, in phase with the *c*II gene, while pJLA16 contains NruI and HindIII sites, useful for cloning.

2. Bacterial Strains. *E. coli* DC646, a derivative of *E. coli* C600$_{r^-_k m^+_k}$ and lysogenic for λ, was utilized for initial transformation, following plasmid construction, for expression of the envelope and pX gene sequences of HTLV-I. The *E. coli* strain, MZ1, is a temperature inducible mutant which carried the temperature-sensitive λ-phage repressor. The repressor protein is active at 32°C and becomes inactivated at 42°C, the temperature at which protein expression is induced when the bacteria carries the vector containing a fused foreign gene fragment. Both DC646 and MZ1 bacteria are grown in NZYDT media or LB broth and agar plates containing ampicillin at 50 μg/ml.

3. (A) Insert DNA Preparation - Envelope Gene. The HTLV-I subclones, $HTLV_{CR}$-HX and $HTLV_{CR}$-CH, were provided to this laboratory by Dr. Gallo (DCT, NCI). To prepare DNA fragments for construction of expression plasmids, 50 to 100 µg of plasmid $HTLV_{CR}$ DNAs were digested with suitable restriction enzymes under the conditions recommended by the suppliers. The enzymes utilized were (a) PvuII and XbaI, which give a 5.4-kbp fragment containing the entire envelope gene (save for 23 amino acids at its amino-terminus), pX region, LTR, and cellular sequences; (b) PvuII and XmaIII give the envelope gene and about 450 bp of non-envelope sequences; (c) HpaI gives the carboxy-terminal one-half of the env gene and about 500 bp of non-env and pX-I sequences; and (d) XhoI and BamHI, which generate two small DNA fragments of about 300 bp and 400 bp, respectively, and which represent the middle and carboxy-terminal regions of the env gene, respectively. The 300 bp env fragment exclusively codes for the C-terminus of the gp46 domain of the HTLV-I envelope gene, while the 400-bp fragment encodes the transmembrane N-terminus p21E domain. Fragments encoding sequences of the env-LOR gene of HTLV type III included (1) a 569-bp StuI-Sca-I, and (2) ~1000-bp PvuII-HindIII fragments of the DNA sequence, respectively. These fragments cover almost the entire 863 amino acid coding sequences of the HTLV-III env-LOR protein.

(B) Insert DNA Preparation - pX Gene. Several plasmid constructs containing amino acid coding sequences for the pX-gene LOR regions of HTLV-I were constructed. These include those with the fragments (1) MstI-SmaI (683 bp), (2) BalI-BalI (655 bp), and ApaI-ApaI(296 bp) representing sequences from the N-terminus and C-terminus regions of the pX gene.

4. Gel Electrophoresis. To purify DNA restriction fragments, entire reactions were loaded onto a "mini" or "medium" preparatory gel box containing 0.8% or 1.0% regular or low melt agarose, and electrophoresed in a 1X Tris-acetate-EDTA buffer. After UV-shadowing, fragments of the band of interest were cut out and the DNA was extracted by either (a) melting the low melt gel fragment at 65°C or (b) crushing the gel and freezing it with phenol, followed by standard extraction techniques.

Further purification of isolated DNA fragments was achieved by passage over DE52 columns or through Schleicher and Schuell's Elutip columns. The concentrations of the resulting DNA fragments were determined by UV-shadowing of ethidium bromide-stained gels (analytical) in the presence of a known amount of λ phage HindIII and phage ØXRF HaeIII DNA standards. Where necessary, all steps in the purification of restriction fragments involved phenol and chloroform-isoamyl alcohol extractions and ethanol precipitations and washings in 80% ethanol. All DNA fragment pellets were resuspended in sterile deionized water and stored at -20°C until used.

5. Ligation of Synthetic HindIII Linkers and Insert DNA. Where synthetic linker molecules were used, they were first phosphorylated in a reaction volume of 10 µl, exactly as described in Molecular Cloning: Laboratory Manual. Each reaction contained 2.5 µg of phosphorylated linkers (HindIII linker molecules of lengths 8, 10, or 12 nucleotide pairs) and up to 1.0 µg of purified insert DNA fragment and 1 µl T4 DNA ligase. Reactions were at 15°C

overnight. The next day, the DNA reactions are digested with excess HindIII enzyme, and the "sticky-ends" containing DNAs purified from agarose gels as described. Those DNA restriction fragments with protruding 5'- or 3'- ends were made flush (blunt) with E. coli DNA PolymeraseI "Klenow" fragment or T4 DNA polymerase, respectively, using reaction conditions suggested by Maniatis et al. (Molecular Cloning, A Laboratory Manual, 1982).

6. Phosphatase Removal of Terminal Phosphates. All "sticky-end" ligation reactions involving insert and vector DNAs were performed with phosphatase-treated linearized pJLA16 or other vector DNAs to reduce plasmid religation background. A 100 µl reaction contains 10 µg enzyme linearized vector DNA, 50 mM Tris-HCl, pH 8.0, and 2 µl each of bacterial alkaline phosphatase and calf intestinal phosphatase. Reactions were at 37°C for up to 1 hr. followed by incubation at 55-65°C for up to 1 hr. Reactions were stopped by phenol extraction.

7. Construction and Screening of Plasmid Clones. Ligation reactions of insert and vector DNA fragments were stopped at 65°C after 5 min. After equilibration ice, the reactions were brought to a 50 µl volume with 50 mM Tris-HCl, pH 7.5, on and kept on ice. To this was added 100 µl of cold $CaCl_2$-treated bacterial cells (DC646) previously grown from a fresh overnight culture to a density of 0.5 to 0.7 A_{590}nm per ml. After standing on ice for 10 min., the transformed cells were heat-shocked at 37°C for 2.5 min., then brought to a 1.0 ml volume with 850 µl NZYDT or LB broth followed by incubation in a 37°C air incubator for up to 1 hr. Aliquots of 200 µl were removed, spread on ampicillin-containing agar plates, and incubated overnight at 37°C. The next day, colonies were picked, and a single colony was used to inoculate 5 ml of NZYDT broth, containing 50 µg per ml of ampicillin, in 15 ml Corning plastic tubes. After shaking over-overnight at 37°C, mini plasmid preparations were made using the method of Birnboim and Doly (Nucleic Acids Res. 7: 1513-1523, 1979). The final plasmid DNA pellets were brought up in 100 µl of sterile H_2O, and 25 µl of each sample were analyzed with appropriate restriction enzymes. To study bacterial expression, the above-prepared plasmid DNAs were introduced into competent MZ1 cells by the same procedure described above, except that the heat-shock and incubation temperatures were 32°C. In later experiments, a more efficient transformation method was used. This is the Hanahan method (J. Mol. Biol. 166: 557-580, 1983), which gives greater transformation efficiencies than the $CaCl_2$ method described previously.

8. Radiolabeling and Gel Electrophoretic Analysis of Bacterial Proteins. The plasmid clones, prepared in MZ1 cells, as described above, were grown at 32°C in M56 minimal media supplemented with 0.5% glucose according to the method of Gottesman and Yarmolinsky (J. Mol. Biol. 31: 487-505, 1968), and containing 0.01% each of all amino acids (except methionine and cysteine), 0.01% thiamine, 0.01% biotin, and 50 µg/ml of ampicillin. The 10 ml volume of each culture was grown to a density of 0.3 A_{590}nm per ml, at which time 150 µl aliquots were removed for labeling at 32°C as uninduced cells, while the remaining cultures were shifted to 41°C for 20 min. Aliquots of 150 µl of the cells were removed and added to 15 µl of the above media containing 10.0 µCi [^{35}S]-methionine or [^{35}S]-cysteine ($\geq$300 mCi/mmol), and incubated for a further 1.5 min. at 32°C or 41°C.

Following protein labeling, each tube was quick-frozen on dry ice for 5 min. and the proteins were pelleted for 15 min. at 4°C in an Eppendorf microfuge. The protein pellets were each resuspended in 50 µl of lysis buffer (1% SDS, 0.1% β-mercaptoethanol, 0.5% bromophenol blue dye) and resolved on a 10% or 15% SDS-polyacrylamide gel according to the procedure of Laemmli (Nature 227: 680-685, 1970). Protein bands were visualized by fluorography after fixing and vacuum-drying of the gel. Gels run with unlabeled protein extracts were stained with coomassie blue dye and destained for visualization of protein bands.

Major Findings:

Bacterial Expression of HTLV-I Gene Sequences. The human T-cell leukemia virus (HTLV) has been implicated in the etiology of adult T-cell leukemias and other T-cell malignancies, as well as causally associated with the dreaded human AIDS disease. HTLV-associated diseases are endemic in such geographical areas as southwest Japan, the Caribbean, southern United States, South America and Africa. The first HTLV isolates (HTLV-I) in the United States by R. Gallo's group, and in Japan by M. Yoshida's group, have been molecularly cloned and the entire nucleic acid sequence of the Japanese isolate has been determined. Since both HTLV-I isolates show extensive homology at both the nucleic acid and protein levels, the sequence data of the Japanese isolate served as a guide to the construction of expression vectors containing the entire envelope gene, or fragments of it, as well as the pX region of HTLV-I.

Initial attempts to express the entire HTLV-I envelope were unsuccessful, possibly because this protein can interact with the bacterial cell membrane in such a way as to be toxic to the cell. Therefore, individual fragments coding for specific regions of the envelope were inserted into pJLA16 by the use of polynucleotide linkers. Such plasmids were introduced in Escherichia coli MZ1, a strain that contains a partial λ prophage bearing the mutant cI857 temperaturesensitive repressor. At 32°C the repressor is active, and the p_L promoter on the plasmid is repressed. At 42°C the repressor is inactive and the p_L promoter is induced, allowing a high level of expression of genes under its transcriptional control. When lysogens carrying either of the two plasmids containing different portions of the HTLV-I envelope gene were grown at 32°C and induced by shifting the temperature to 42°C, prominent bands were revealed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) that were not found in uninduced cells containing the pJL6 vector alone. These proteins were observed in gels of isotopically-labeled bacterial extracts and in gels stained for total proteins. On the basis of DNA sequence data of the envelope gene fragments, the calculated molecular sizes of the pKS300 and pKS400 proteins are 12.84 and 15.88 kilodaltons (Kd), respectively. These sizes include the 1.56-Kd coding sequence contributed by the amino-terminal codons of the λcII gene. The molecular weights of both proteins determined by SDS-PAGE are consistent with those calculated for a 321-base-pair 9pKS300 insert) and a 397-base-pair (pKS400 insert) coding sequence of the gp46 c-terminus and p21E N-terminal regions of HTLV-I env protein.

The HTLV-I env gene codes for a glycoprotein (gp61), molecular weight 61,000 (61-Kd) that is cleaved into the 46-kd exterior glycoprotein (gp46) and the 21-Kd

transmembrane protein (p21E). The precise site of proteolytic cleavage has been determined by locating isotopically-labeled valine residues with respect to the amino-terminal end of gp21. The cleavage of the env gene precursor is adjacent to the residues Arg-Arg that are also next to the proteolytic cleavage sites in the bovine leukemia virus and mouse mammary tumor virus env precursor. Because the BamHI site separating the inserted fragments is close to the region coding for the proteolytic cleavage site that separates gp46 from p21E, the protein from pKS300 contains sequences corresponding to the carboxyl-terminal portion of gp46, and the protein from pKS400 predominantly consists of sequences from p21E.

Serological Identification of the Bacterially-Produced p21E Envelope Domain. Sera from many patients with HTLV-I-associated ATL and certain other lymphoid malignancies contain antibodies to proteins that have been shown to be the product of the viral env gene. In experiments to determine whether such antibodies can recognize a bacterially-synthesized envelope product, a lysate of induced MZ1 (pKS400) cells containing the p21E transmembrane protein fragment was fractionated by SDS-PAGE and transferred to nitrocellulose by electrophoretic (Western) blotting. Strips containing the transferred proteins were reacted with diluted human sera, and the antigen-antibody complexes formed were detected by incubation of the strips with ^{125}I-labeled Staphylococcus aureus protein A and subsequent autoradiography. Prominent bands corresponding to the reaction of antibody to the 15 Kd bacterial envelope product developed when the sera used was from patients with HTLV-I-associated ATL or from HTLV-I antigen-positive individuals. No such reactions were observed with sera from healthy control individuals. This procedure was used to screen a group of 28 coded sera. Antibodies that recognized the bacterially-synthesized HTLV-I envelope protein sequences were found in all sera that had been shown to have antibodies to HTLV-I by an ELISA (enzyme-linked immunosorbent assay) with disrupted virions as the antigen. None of the normal control sera were found to have reacting antibodies. Antibodies from a patient (Mo) with a hairy cell leukemia, whose disease is associated with HTLV-II, strongly reacted to the protein coded for in pKS400. This indicates that there is a high degree of relatedness between the p21E regions of HTLV-I and HTLV-II.

Because the bacterially-synthesized HTLV-I env protein was recognized by antibodies in sera from an HTLV-II (positive) patient, it was of interest to see if this assay could be used to screen for HTLV-III, an even more distantly related subgroup. Therefore, we examined a number of serum samples from AIDS patients, some of whom were also sero-positive for HTLV-I. The sera positive for AIDS that reacted with HTLV-I in the ELISA contained antibodies that recognized the bacterially-synthesized HTLV-I env protein. None of the sera from AIDS patients that were HTLV-I negative contained antibodies that reacted with this protein. Because antibodies that react with HTLV-III proteins can be found in the sera of more than 90 percent of all AIDS patients, this result indicates that there is little or no cross-reaction between the carboxyl-terminal portion of the envelope proteins of HTLV-I and HTLV-III. We have not completely analyzed the the pKS300 protein product for reactivity in sera of leukemia patients,

since this clone has not induced well, and in many instances would not produce a new protein band upon temperature induction.

DNA Sequence Analysis of the Fusion Junction of λcII and the p21E Domain. Final demonstration that the 397-bp BamHI-XhoI DNA fragment, representing the N-terminal domain of the p21E portion of the HTLV-I envelope gene, is fused in-phase to the open reading frame (ORF) of the λcII N-terminal sequence was shown by Maxam-Gilbert DNA sequencing analysis. The open reading frame of the envelope gene fragment was maintained by adding a 10-nucleotide long HindIII synthetic DNA linker molecule to its blunted ends. Subsequent ligation of its HindIII sticky ends to the corresponding ends on the λcII gene generated the efficiently-expressed pKS400 plasmid.

Expression of the C-terminus of the pX-IV LOR Region of HTLV-I. Initial attempts to express a 683-bp pX-IV fragment of LOR from the MstI site at 7623 to a SmaI site at 8306 of the HTLV-I proviral DNA sequence was unsuccessful. To circumvent this problem, a 296-bp ApaI fragment (ApaI sites at positions 8072 and 8368) was fused to the NruI site of the vector, pJLA16, by blunt-end ligation. The resulting plasmid, pKL296C, expressed a fusion protein with an apparent molecular size of about 15-Kd on SDS-PAGE. Its mobility on SDS-PAGE is slower than that expected for a DNA fragment size of 296-bp (coding capacity of 99 amino acids) and with the putative stop codon (TGA) for pX-4-LOR at position 8357. The pX region, bounded by the envelope gene and 3'-LTR sequence, is approximately 1.6-kbp long and contains four open reading frames (numbered pX-I to pX-IV in HTLV-I and pX-a to pX-c in HTLV-II). The predicted DNA sequence of the LOR of pX-IV in HTLV-I and pX-c of HTLV-II can encode a protein of 357 amino acids (p42) and 337 amino acids (p38), respectively. Specific anti-peptide antisera to these regions have immunoprecipitated proteins of those sizes in HTLV-I and HTLV-II immortalized cell lines and HTLV positive human sera as reported by other investigating laboratories.

Detection of Antibodies to the Bacterially-Produced pX Protein in Human Sera. Western blot analyses of a cellular extract of the bacterially-produced pX-IV C-terminal LOR protein with two positive control sera, #1990 and 4993, (sera shown to be positive for antibodies to the p42 HTLV-I pX-LOR protein by T. H. Lee, M. McLane and M. Essex, and five negative sera, three of which (sera #211, 213, and 223) are from patients with AIDS. The bacterially-produced pX protein was recognized by antibodies to the p42 protein in those HTLV-I positive sera. No serological reactivity was seen with the other sera. In a comparative Western blot experiment, four different human sera were used to test for serological reactivity to both the bacterially-produced p21E envelope and pX proteins of HTLV-I. The results showed that serum D32 reacted with envelope protein in pKS400, but not with the pX protein of pKL296C. This serum and serum MI were from ATL patients with relatively high (MI) and moderate (D32) titers to anti-p24. In contrast, serum A195 reacted with neither protein (low anti-p24

titer). Sera numbers D32, MI, A195 and A201 were provided by S. Kranner and W. Parks. It is of interest to note that only about one-quarter of all ATL-positive patients (HTLV-MA seropositive sera) and healthy seropositive carriers were reported to have detectable antibody to the p42 pX protein. Recent studies have localized the pX gene product to the nucleus of HTLV-I immortalized cell lines, as well as in fresh leukemic cells from ATL patients.

Bacterial Expression of HTLV-III env Gene Sequences. The env-LOR open reading frame of HTLV-III has coding capacity for a protein of 863 amino acids, and a gene product precursor of 160,000 daltons (gp160) which is processed to a 120,000 dalton (gp120) exterior glycoprotein and a 41,000 dalton (gp41) transmembrane protein. Expression of different size fragments of env-LOR sequences in the same bacterial vector systems (pJLA16 or pJL6) have been successful, only insofar as detecting new protein bands of the predicted molecular size weights in ^{35}S-labeling experiments. Serological reactivity of these expressed clones, using sera from patients with AIDS, have not yet demonstrated the specificity of these proteins. It appears that where protein expression was observed by ^{35}S-labeling of a new protein band, a corresponding band was not stained when similar SDS-PAGE gels were stained for protein visualization with coomassie blue, probably due to the low level new protein which was highly unstable. Efforts are underway to optimize the expression systems by using different E. coli temperature inducible strains.

Significance to Biomedical Research and the Program of the Institute:

Since retroviral envelope glycoprotein gene products are known to specifically interact with receptors on the surface of host cells at the initial phase of viral infection, and since specific antibodies against these viral antigens can neutralize the infectivity of retroviral particles, it is important to obtain large quantities of the envelope proteins of the human retroviruses, HTLV-I and -III, to obtain specific antibodies raised against them. The development of diagnostic and clinical reagents for the treatment of HTLV-associated infections would become possible as a result of such an approach. The purified p15E protein of murine and feline retroviruses was reported to have immunosuppressive activity in a variety of systems. It is of significant importance to determine whether our p21E counterpart expressed in the plasmid, pKS400, possesses such activity. The availability of purified $p42^{LOR}$ protein from HTLV-I immortalized cells will be of tremendous importance for conducting studies designed to identify its biological role in HTLV-induced human malignancies. The presence of a similar pX-LOR protein in HTLV-III will suggest a role in the cytopathic effects of this virus on T-lymphocytes. As infectious human type-C retroviruses, HTLV-I and HTLV-III have been linked as the causative agents in such T-cell malignancies as cutaneous T-cell lymphomas (mycosis fungoides, sezary syndrome) and adult T-cell leukemias, and the dreaded disease, AIDS, respectively. Moreover, HTLV-I is endemic in distinct geographical areas of the world, including the southwest region of Japan, the Caribbean, South America, southern United States, and Africa, and thus possesses an epidemiological and clinical problem for many regions of the world. The growing numbers of AIDS cases in the United States and other countries, and the high fatality rate of this dreaded disease, suggest the urgent

need for studies directed toward the development of diagnostic and therapeutic reagents against HTLV-III. The above results thus show the importance of using bacterially-synthesized proteins as one approach to study the properties of antibodies to lymphotropic retroviruses in human serum. Because the structure of the genes for such proteins can be controlled by recombinant DNA techniques, the antigens produced by these methods have a defined structure. Such antigens could also be used in competitive experiments to study the structure of natural antigens and help localize epitopes of interest for use in treating such diseases.

Proposed Course:

This project will continue at several levels which involve both our individual laboratory's effort and a close collaborative effort with scientists in different labs. Of primary importance is the expression of HTLV-III envelope gene fragments in our bacterial expression systems. Initial observation of the DNA sequence of clone BH10 with its predicted amino acid sequence in the region of positions 5703 to 8371 (a 2668-bp KpnI fragment) revealed a number of unique blunt-end restriction enzyme cutting sites. Hence, the env-LOR region within the KpnI fragment was divided into small restriction fragments for direct cloning into the NruI restriction site of the vector, pJLA16, or via molecular linker molecules for fusion to the cII gene at the HindIII cloning sites of both pJLA16 and pJL6 vectors. We have established collaborating contacts with other scientific units who are interested in pursuing several aspects of the biochemistry, immunology, and biology of the HTLV family of retroviruses. The initial strategy is recovery and purification of the bacterially-produced envelope and pX proteins of HTLV-I and -III. Using conventional and state-of-the-art protein purification techniques, we anticipate recovery of abundantly-pure and antigenically-reactive proteins. Consequently, the purified proteins will be used to raise antibodies in animals, assuming that they are immunogenic. We have demonstrated in this report that such antigens can be produced in abundance and purified like the pX protein, and be both highly antigenic and immunogenic. Attempts will be made to purify from human sera or cells immortalized with HTLV-I and HTLV-III producing cell lines, pX-LOR proteins using affinity chromatography prepared from monospecific antibody reagents to our bacterially-produced proteins. The $p42^{LOR}$ has been localized in the nucleus of HTLV-I immortalized cells and in fresh cells from HTLV-I leukemia-lymphoma patients. Isolated, pure $p42^{LOR}$ protein will be used for biological studies (e.g., microinjection into human lymphocytic cells) and for development of diagnostic reagents to study its expression and role in HTLV-induced transformation. The reported "trans-acting" role of this putative HTLV-I immortalizing or HTLV-III cytopathic pX-LOR protein is a unique function for retroviruses. We will use our purified protein to study its interaction (e.g., DNA-binding specificity) to the viral LTR sequence, and to localize the unique sequences with which it interacts. A eukaryotic vector system containing the SV40 promoter sequences and other control regions will be used to fuse the pX-LOR sequences of HTLV-I. This plasmid will then be utilized in a transient expression assay in HeLa cells, and a permanent cell line (L-cells) will be developed for the continuous production of the pX-LOR protein. But more importantly, cotransfection experiments will be designed for transfecting the above SV40-pX-LOR vector into uninfected human T-lymphocytes concomitantly with a pSV50CAT vector containing HTLV-I LTR U_3 sequences. By utilizing the classical CATase assay, the direct role of the pX-LOR protein in transactivation and

transformation can be assessed, thereby enabling us to localize regions (sequences) involved in those functions by site-directed mutagenesis of the plasmids.

Publications:

Papas, T. S., Samuel, K. P., Kan, N .C., Ascione, R., Wong-Staal, F., and Lautenberger, J. A.: Production of oncogene specific proteins and human T-cell leukemia (lymphotropic) retrovirus (HTLV-I) envelope protein in bacteria and its potential for use in human malignancies and seroepidemiological surveys. Cancer Res. (In Press)

Samuel, K. P., Flordellis, C. S., DuBois, G. and Papas, T. S.: High level bacterial expression and purification of human T-lymphotropic virus type-I (HTLV-I) transmembrane env protein. Gene Anal. Tech. (In Press)

Samuel, K. P., Lautenberger, J. A., Jorcyk, C. L., Josephs, S., Wong-Staal, F. and Papas, T. S.: Diagnostic potential for human malignancies of bacterially-produced HTLV-I envelope protein. Science 226: 1094-1097, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05238-04 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

The Transforming Genes of Acute Leukemia Viruses and Their Cellular Homologues

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

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LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

To provide an initial step toward the understanding of the functional relationship between the onc genes of transforming retroviruses and their cellular prototypes, structural comparisons at the nucleic acid and protein levels have been carried out. We have determined the complete nucleotide sequence of the chicken and human c-myc genes and compared them to two members of the myc family of transforming retroviruses: MC29 and OK10. Although a close relationship between the viral and cellular myc genes has been found, these genes are not isogenic. The myc-related genes of MC29, MH2, and OK10, and the myb-related genes of AMV and E26 are genetic hybrids with sequences derived from viral structural genes and parts of essential cellular proto-onc genes. The cellular genes contain additional 5' sequences. The substitution of viral genes for parts of the normal cellular genes may be the most significant difference between these genes, perhaps eliciting functional differences between their gene products.

We have initiated studies of the cellular ets gene to determine whether this pattern of a truncated normal gene in the transforming retrovirus can be extended to other onc genes. The cellular gene transcript is considerably larger than the amount of DNA transduced by the virus. We have determined that the mammalian homologues of v-ets consist of two distinct domains located on different chromosomes. Using chromosome-specific probes, we have shown that both loci (ets-1 and ets-2) are transcriptionally active, producing mRNA species. The sequences homologous to ets-1 and ets-2 are colinear in chicken proto-ets and have possibly become separate and functionally distinct since before the evolutionary divergence of mammalia.

PROJECT DESCRIPTION

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

D. K. Watson	Senior Staff Fellow	LMO	NCI
T. S. Papas	Acting Chief	LMO	NCI
S. J. O'Brien	Geneticist	LVC	NCI
N. Sacchi	Visiting Fellow	LMO	NCI
U. G. Rovigatti	Visiting Scientist	LMO	NCI
C. S. Flordellis	Visiting Fellow	LMO	NCI
L. J. Pribyl	Biologist	LMO	NCI

Objectives:

The purpose of this investigation is to determine the relationship between *onc* genes and their normal cellular homologues. Structural analysis of these genes will allow us to better understand their biological functions. In addition, we plan to evaluate the involvement of proto-*onc* genes in human malignancy.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cells by treatment with proteinase K, followed by phenol-chloroform extraction. Vector DNA was prepared from phage and plasmid derivatives by phenol extraction and CsCl gradient centrifugation.

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Specific DNA fragments were purified by electroelution or by extraction of low melting agarose.

3. Preparation of DNA probes using purified *onc*-specific DNA by nick-translation using *E. coli* DNA polymerase and DNase I.

4. Preparation of nitrocellulose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of *onc* probes.

5. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to cosmid or λ vector DNA followed by production of phage by *in vitro* packaging.

6. Isolation of phage from the libraries containing virus-related sequences by hybridization of *onc*-specific probes to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the procedure of Benton and Davis (*Science* 196: 180-182, 1977). Isolation of plasmid DNA from colonies lifted from plates by the method of Grunstein and Hogness (*Proc. Natl. Acad. Sci. USA* 72: 3961-3965, 1975).

7. Subcloning of isolated DNA fragments into pBR322, pBR325, or pBR328, as required.

8. DNA sequencing analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol. 65: 499-560, 1980). In addition, uniquely-labeled DNA will be sequenced following RNA-directed primer extension.

9. Total cellular RNA from cultured cells or tissues was prepared by the guanidine hydrochloride method. In addition, RNA was prepared after cellular fractionation in the presence of RNase inhibitors. Separation of polyA+ and polyA- RNA by two cycles of purification through oligo(dT) cellulose. Characterization of RNA by electrophoresis on formaldehyde-agarose or methylmercury-agarose and Northern analysis.

10. Construction of cDNA library: Double-stranded cDNA was prepared from polyA+ RNA and ligated into λgt10 vector DNA for amplification.

11. Immunoprecipitation of labeled cell lysates with onc-specific antisera and analysis by SDS-polyacrylamide gel electrophoresis. The specific onc gene-related polypeptide was purified for further analysis by electroelution.

12. S^{35}-methionine and -cysteine labeling of expressed protein in bacteria utilizing the plasmid, pJL6 (vector constructed by Dr. James Lautenberger).

13. Tryptic peptide analyses of viral and cellular onc-related polypeptides by two-dimensional, thin-layer electrophoresis and chromatography.

14. Microsequence analysis of onc-related polypeptides to define the site of initiation of translation.

Major Findings:

1. We have determined the nucleotide sequence of the OK10 provirus. The myc sequence of OK10 is the only known onc-specific sequence that is part of two different genes, a large gag-Δpol-myc hybrid of 200,000 daltons and a smaller myc-related protein of 57,000 daltons. It is interesting that the p200 is partially encoded by proto-myc intron-sequences.

2. Comparison of the OK10 sequence with those of RSV pol and env, and chicken proto-myc defines the border functions between these genes in OK10. The recombination between the helper retrovirus and proto-myc that generated OK10 probably occurred at a 2-nucleotide overlap with the pol sequence and a 7-nucleotide overlap with the env sequence and proto-myc.

3. The comparison between OK10 and RSV provides a measure of the defectiveness of OK10; Δpol of OK10 lacks 348 nucleotides at its 3' end and Δenv lacks 1076 nucleotides at its 5' end.

4. A rainbow trout genomic library has been screened using 5' and 3' v-myc-specific sequences and multiple, overlapping clones have been isolated. Restriction enzyme and Southern blot analyses using defined chicken c-myc probes have been carried out to characterize these clones. Sequence analyses have shown that the fish myc is almost 70% homologous to chicken c-myc at the amino acid level.

5. Sequences related to ets (one of the two cellular genes present in the avian retrovirus, E26) have been detected by restriction enzyme digestion and Southern blot analysis using genomic DNA derived from avian and mammalian species. Chicken and human genomic libraries were screened and specific clones have been isolated.

6. DNA prepared from panels of hamster X human and mouse X human somatic cell hybrid clones, which have lost specific human chromosomes, were used for analyses to determine the chromosomal localization of ets. The v-ets homologous sequence is dispersed as two genetic loci, ets-1 and ets-2. These loci contain sequentially distinct domains of v-ets and are mapped to chromosomes 11 and 21.

7. The assignment of ets-1 to chromosome 11 is consistent with the recent study of de Tainshe et al. (Nature 310: 581-583, 1984), who reported the assignment of ets to 11q23-24. Our in situ hybridization of an ets-2 clone to normal human chromosome preparations confirmed the assignment of ets-2 to chromosome 21 and regionally localized to HSA 21q22.1-22.3.

8. When viral probes related to ets-1 and ets-2 are hybridized to a single chicken c-ets clone, we find that sequences related to both loci are present in this clone.

9. Portions of the human ets-1 and ets-2 loci have been sequenced, demonstrating a strong conservation of amino acids (over 90%), suggesting that this gene performs an important function.

10. Using a previously characterized panel of mouse X hamster hybrids (Kosak et al., J. Virol. 49: 297-299, 1984), we have been able to assign ets-1 and ets-2 to murine chromosomes 9 and 16.

11. The domestic cat homologues of the ets proto-oncogenes were found to be D1 (ets-1) and C2 (ets-2).

12. Both genetic loci are transcriptionally active in man yielding distinct products.

Significance to Biomedical Research and the Program of the Institute:

Transformation of cells by acute leukemia viruses is of great importance in defining the gene responsible for leukemogenesis. Analysis of the genomic structure of viral genes and their cellular homologues is of immense importance to better understand the mechanism of the leukemia process.

Proposed Course:

1. Chicken and human genomic ets clones will be further characterized by restriction enzyme and sequence analyses. cDNA libraries prepared from polyA+ RNA will be used to isolate several ets cDNA clones. Special emphasis will be placed on that portion of the cellular ets genes 5' to the v-ets-specific sequences.

2. Portions of the genomic ets clones will be subcloned into expression vectors (pJL6 and/or pUC plasmid derivatives), and the expressed polypeptides will be used as antigens for the preparation of ets-specific antibodies. In addition, synthetic peptides derived from the predicted amino acid sequences of v-ets and human c-ets will also be used as antigens to elicit antibody production.

3. Cellular transformation assays will also be conducted using v-ets DNA in order to define sequences essential for tumorigenesis.

4. DNA from human cell lines and solid tumors will be analyzed for polymorphism and gene rearrangement of c-ets. Using RNA isolated from these sources, the overexpression of the ets gene will also be investigated.

5. In collaboration with L. Pribyl, we will attempt to isolate cellular ets clones from lower eukaryotes to facilitate our understanding of their function.

Publications:

Duesberg, P. H., Nunn, M., Kan, N., Watson, D., Seeburg, P. H. and Papas, T.: Which cancers are caused by activated proto-onc? In Furmanski, P., Hager, J-C. and Rich, M. A. (Eds.): RNA Tumor Viruses, Oncogenes, Human Cancer and AIDS: On the Frontiers of Understanding. Boston, Martinus Nijhoff Publishing (In Press)

Duesberg, P., Nunn, M., Watson, D., Kan, N., Seeburg, P. and Papas, T. Are cellular proto-onc genes structural or functional equivalents of retroviral transforming genes? In Levy, J. (Ed.): Virus in Human Malignancy in AIDS. Toronto, American Cancer Society of Clinical Oncology and American Association of Cancer Research, 1984, pp. 402-404.

Papas, T. S., Kan, N. C., Watson, D. K., Flordellis, C., Lautenberger, J. A., Psallidoulos, M. C., Rovigatti, U. G., Samuel, K. P., Ascione, R. and Duesberg, P. H.: Two oncogenes in avian carcinoma virus MH2: myc and mht. Anticancer Res. (In Press).

Papas, T. S., Kan, N. C., Watson, D. K., Lautenberger, J. A., Flordellis, C., Samuel, K. P., Rovigatti, U. G., Psallidopoulos, M. C., Ascione, R. and Duesberg, P. H.: Myc, a genetic element that is shared by a cellular gene (proto-myc) and by viruses with one (MC29) or two (MH2) onc genes. In Furmanski, P., Hager, J-C. and Rich, M. A. (Eds.): RNA Tumor Viruses, Oncogenes, Human Cancer and AIDS: On the Frontiers of Understanding. Boston, Martinus Nijhoff Publishing (In Press)

Papas, T. S., Kan, N. C., Watson, D. K., Lautenberger, J. A., Flordellis, C., Samuel, K. P., Rovigatti, U. G., Psallidopoulos, M., Ascione, R. and Duesberg, P. H.: Oncogenes of avian acute leukemia viruses are subsets of normal cellular genes. In Neth, R., GAllo, R. C., Greaves, M. F., Moore, M. A. S., Winkler, K. (Eds.), Modern Trends in Human Leukemia V. Wilsede, Germany, Berlin/Heidelberg, Springer-Verlag (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05239-04 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Structural Analysis of the Avian Carcinoma Virus MH2

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

PI:	N. C. Kan	Visiting Associate	LMO	NCI
Others:	T. S. Papas	Acting Chief	LMO	NCI
	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
	C. Flordellis	Visiting Fellow	LMO	NCI

COOPERATING UNITS (if any)

Department of Biology, University of California, Berkeley, CA (P. H. Duesberg)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	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 integrated proviral genome of the avian carcinoma virus, MH2, has been molecularly cloned and its nucleotide sequence has been determined. Our data indicate that MH2 contains two potential transforming genes: Δgag-mht and myc. In order to determine which role each potential onc gene of MH2 plays in oncogenesis, we have constructed deletion and frameshift mutants of each of the two MH2 genes, and have tested these mutants for their virus production and transforming function in cultured primary chicken and quail cells. We have found that the myc gene transforms primary cells by itself without the second potential onc gene, and that the Δgag-mht gene is without detectable transforming function in the primary cell assay. However, further work is necessary to determine whether the Δgag-mht gene has a role in oncogenesis in the animal. In order to understand the molecular mechanisms involved in the transduction of cellular mht genes (c-mht) into the MH2 virus, we have molecularly cloned the chicken c-mht from a phage lambda library containing genomic chicken sequences. Nucleic acid hybridization and heteroduplex and DNA sequence analyses indicate that the v-mht sequence captured by the MH2 virus is spread over 25 kilobases (kb) of chicken genomic DNA. The c-mht locus contains 11 exons which are homologous to the v-mht sequence. Thus, the v-mht onc gene is a subset of its normal cellular homolog in that it lacks introns, and possibly lacks 5' coding sequences. Because there is no sequence homology between c-mht and retroviral helper sequences, the viral transduction of the cellular mht gene occurred through recombination. Finally, we have used the temperature-inducible bacterial expression vector developed in our laboratory to express large amounts of the v-mht protein. Purification of antibodies against the bacterially-synthesized mht protein is in progress.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

N. C. Kan	Visiting Associate	LMO	NCI
T. S. Papas	Acting Chief	LMO	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
C. Flordellis	Visiting Fellow	LMO	NCI

Objectives:

To characterize the MH2 virus containing the dual oncogenes, Δgag-mht and myc, and to elucidate the nature and functions of the encoded oncogene products in both normal and malignant cells. In particular, the following lines of investigation were addressed: (1) molecular cloning and nucleotide sequencing of the MH2 proviral genome to determine its precise genetic structure and the proteins it may encode, and to compare the MH2 virus with other mht- and myc-containing retroviruses; (2) examination of the oncogenic potential of the mht gene in MH2 and the apparently normal function encoded by its counterpart in the normal cell, the proto-mht gene; (3) utilization of high-level expression of the mht gene cloned in an E. coli vector to elicit specific antisera against the mht proteins and to effect the isolation and characterization of in situ mht proteins in normal and MH2-transformed cells.

Methods Employed:

1. Construction of deletion mutants: (a) digestion of recombinant plasmid DNA with restriction enzymes; (b) preparation of DNA fragments with blunt termini by repairing the ends with E. coli DNA polymerase large fragment or by removing the protruding nucleotides with S1 nuclease; (c) preparation of randomly-shortened DNA fragments by treatment with Bal-31 nuclease; (d) dephosphorylation of DNA termini by treatment with calf intestinal alkaline phosphatase to prevent self-ligation of DNA fragments; (e) ligation of DNA fragments, either with blunt ends, or with sticky ends, using T4 DNA ligase; (f) use of small preparations of recombinant DNA (mini-lysates) to identify the desired DNA clones; (g) colony hybridization using nick-translated DNA probes to screen for desired recombinant DNA clones; (h) gel electrophoresis analysis of recombinant DNA fragments on agarose or polyacrylamide gels; and (i) DNA sequence analysis of deletion mutants by the method of Maxam and Gilbert (Methods Enzymol. 65: 499-560, 1980) to determine the precise nature of the deletions.

2. Insertion of viral oncogenes into the expression vectors, pJL6 or pJLA16, using the methods described above.

3. Polyacrylamide gel electrophoresis analysis of radioisotope-labelled or unlabelled bacterial proteins containing oncogene sequences.

4. Preparation of recombinant plasmid DNA by precipitating chromosomal DNA with high salt, followed by CsCl-ethidium bromide banding of supercoiled DNA.

5. Computer analysis of DNA and protein sequences. The Queen and Korn program was used to list DNA and protein sequences, locate restriction sites, predict amino acid sequences, and find repeated sequences and secondary structures. The Maizel program and the modified program by J. Lautenberger were used for graphic demonstrations of sequence homology. The Davehoff program was used to find DNA and protein sequence homology in the available data bank.

Major Findings:

1. The MH2 virus contains two unrelated cell-derived sequences, mht and myc.

2. The genome of MH2 encodes two proteins: (a) the p100 protein consists of the partial retroviral gag sequence at its 5' half and the mht sequence at its 3' half, and (b) the p59 protein consists of six codons from the gag sequence and the myc coding region.

3. The v-mht sequence in MH2 is 94% homologous to the oncogene, v-raf, of the murine sarcoma virus, 3611. We conclude from this data that c-mht and c-raf represent chicken and mouse homologs of the same gene, and that only a limited number of cellular genes can, on retroviral transduction, function as transforming genes.

4. Studies on the deletion mutants of the MH2 virus indicate that the myc gene transforms chicken primary cells by itself, and that the Δgag-mht gene is not required in the primary cell transformation assay.

5. The v-mht sequence captured by the MH2 virus is spread over 25 kb of chicken genomic DNA and is distributed in 11 exons. Thus, the v-mht onc gene is a subset of its normal cellular homolog in that it lacks introns, and possibly lacks 5' coding sequences.

6. The transduction of the cellular mht/raf sequence by an avian helper virus occurred through illegitimate recombination.

Significance to Biomedical Research and the Program of the Institute:

It is clear that the formation, the maintenance and the expression of proviruses are the central features of the life cycle of RNA tumor viruses. Many questions related to these aspects are unresolved. To elucidate the process of oncogenesis induced by these viruses, it is important to understand the structural organization of the transforming genes within the host chromosome and the process by which these oncogenes are expressed and regulated. Resolution of these processes should effect the development of diagnostic and therapeutic reagents designed to detect and interdict the neoplastic event(s).

Proposed Course:

We will focus on answering two questions in the following year: (1) Is mht an oncogene? (2) What are the functions of mht-encoded protein products in normal and cancerous cells?

To answer the first question, whether mht alone is capable of inducing cellular transformation, both in tissue culture cells and in animals, will be demonstrated. In the initial experiment, MH2 proviral DNA will be introduced into chicken embryo fibroblasts (CEF) by the method of DNA-mediated gene transfer or DNA transfection. Upon superinfection with a helper virus, viral particles from the supernatant of transformed CEF will be harvested and characterized. The MH2 virus recovered this way will be injected into chickens and the diseases and tumors caused by the virus will be observed. After this DNA transfection assay has been established, regions from mht, or myc, or gag of the MH2 provirus will be deleted and the resulting DNAs will be introduced into CEF to assess the biological activities of these genes. Furthermore, the deletion mutants of MH2 recovered from the supernatant of transformed CEF will be injected into chickens to determine the types of tumors caused by the MH2-related oncogenes.

To answer the second question, purified mht proteins derived from MH2 and cellular mht proto-oncogenes synthesized in E. coli will be used to raise antibodies. These antibodies will provide a handle to determine the subcellular locations of the proteins and to help elucidate their biological functions in normal and cancerous cells. These mht proteins will also be used in microinjection experiments so that their effects on the cells can be observed directly. Furthermore, deletions in the v-mht and proto-mht genes, which result in truncated forms of the protein molecules, will be constructed and their protein products will be synthesized in E. coli. Microinjection of these proteins into appropriate target cells will demonstrate the biological activities associated with different portions of the proteins.

Publications:

Duesberg, P. H., Nunn, M., Kan, N., Watson, D., Seeburg, P. H. and Papas, T.: Which cancers are caused by activated proto-onc genes? In Furmanski, P., Hager, J-C. and Rich, M. A. (Eds.): RNA Tumor Viruses, Oncogenes, Human Cancer and AIDS: On the Frontiers of Understanding. Boston, Martinus Nijhoff Publishing (In Press)

Duesberg, P. H., Nunn, M., Kan, N., Watson, D., Seeburg, P. H. and Papas, T.: Which cancers are caused by activated proto-onc genes? In Neth, R., Gallo, R., Greaves, M. F. and Janka, M. (Eds.): Haematology and Blood Transfusion, Modern Trends in Human Leukemia VI. Berlin/Heidelberg, Springer-Verlag (In Press)

Flordellis, C. S., Kan, N. C., Lautenberger, J. A., Samuel, K. P., Garon, C.F. and Papas, T. S.: Analysis of the cellular proto-oncogene mht-raf: Relationship to the 5' sequences of v-mht in avian carcinoma virus MH2 and v-raf in murine sarcoma virus 3611. Virology 141: 267-274, 1985.

Papas, T. S., Kan, N. C., Watson, D. K., Flordellis, C., Lautenberger, J. A., Psallidopoulos, M. C., Rovigatti, U. G., Samuel, K. P., Ascione, R. and Duesberg, P. H.: Two oncogenes in avian carcinoma virus MH2: myc and mht. Anticancer Res. (In Press)

Papas, T. S., Samuel, K. P., Kan, N. C., Ascione, R., Wong-Staal, F. and Lautenberger, J. A.: Production of oncogene specific proteins and human T-cell leukemia (lymphotropic) retrovirus (HTLV-I) envelope protein in bacteria and its potential for use in human malignancies and seroepidemiological surveys. Cancer Res. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05286-04 LMO

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Transforming Genes in Human Tumors and Chemically Transformed Human Cells

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

PI:	M. Park	Visiting Fellow	LMO	NCI
Others:	D. G. Blair	Research Chemist	LMO	NCI

COOPERATING UNITS (if any)
Molecular Mechanisms of Carcinogenesis Laboratory, Basic Research Program, Litton Bionetics, Inc., Frederick, MD (G. Vande Woude, M. Dean & T. Robins)

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SECTION
Microbiology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

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 met transforming gene was isolated from a chemically-treated human osteosarcoma cell line (MNNG-HOS) using the NIH3T3 cell transfection assay. DNA prepared from the tumorigenic N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treated HOS (TE-85) cell line induces foci on NIH3T3 cells, whereas DNA prepared from the non-tumorigenic parental HOS cell line failed to induce foci. Met is unrelated to other known oncogenes or transforming genes and has been mapped to the long arm of human chromosome 7 from 7q21-7q31. The transforming activity of met is contained within 35 kb of human DNA and this region of human DNA is not rearranged within MNNG-HOS, HOS or NIH3T3 transformant DNA, nor is it amplified in MNNG-HOS cells when compared with other human cell lines.

The expression of met within several human cell lines and NIH3T3 transformants has been compared, and the 5' and 3' termini of the met message within NIH3T3 transformants have been localized. Several met-related transcripts are detected in many human cell lines in culture and met expression appears to be controlled in a cell-type or tissue-specific fashion. In particular, these results show that the MNNG-HOS cell line expresses a new met RNA of 6.5 kb when compared to the non-tumorigenic HOS cell line. NIH3T3 transformants express only this aberrant RNA, which is shorter than the met RNAs of 12.0 kb, 11.0 kb, 9.5 kb and 8.0 kb detected in HOS cells. Mapping data suggest that the 6.5 kb RNA has the correct 3' end but has an altered 5' end when compared with the HOS cell RNAs. The met transforming activity, therefore, is associated with the expression of a truncated RNA product.

PROJECT DESCRIPTION

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

M. Park	Visiting Fellow	LMO NCI
D. G. Blair	Research Chemist	LMO NCI

Objectives:

To determine the mechanism by which the transforming potential of the met proto-oncogene is activated within MNNG-HOS cells and to characterize the met proto-oncogene locus from human placenta.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells. Construction of representative genomic DNA libraries and cDNA libraries in lambda replacement vectors, and screening such libraries using radioactive probes prepared from cloned DNAs. Preparation of high molecular weight DNA from both cell lines and tumors. Analysis of DNA and RNA by Southern and Northern hybridization techniques using specific radiolabelled probes. Determination of nucleotide sequence data using the Maxam and Gilbert sequencing technique.

Major Findings:

1. The transforming potential of the met gene is associated with the expression of an aberrant RNA in MNNG-HOS cells and NIH3T3 transformants.

The expression of met within several human cell lines and NIH3T3 transformants has been analyzed by Northern blot hybridization. These results show that the MNNG-HOS cell line expresses a new met RNA of 6.5 kb when compared to the non-tumorigenic HOS cell line. NIH3T3 transformants express only this aberrant RNA which is shorter than the met proto-oncogene RNAs of 12.0 kb, 11.0 kb, 9.5 kb and 8.0 kb detected in HOS cells and MNNG-HOS cells. Utilizing probes throughout the met locus, the hybridization profiles of the 6.5 kb met RNA in NIH3T3 cell transformants and within MNNG-HOS cells are identical. Similarly, the 11.0 kb, 9.5 kb and 8.0 kb, HOS and MNNG-HOS cell RNAs share identical hybridization profiles with probes throughout the met locus. However, the 12 kb met RNA species is distinct from the other HOS cell RNAs.

2. The 5' and 3' termini of the met RNA transcript in NIH3T3 cells have been localized.

Using strand-specific probes, the direction of transcription was determined and from Southern and Northern hybridization studies, the 5' and 3' ends of the 6.5 kb mRNA within NIH3T3 transformants were localized. The 3' end of the 6.5 kb mRNA was identified by S1 hybridization analysis. S1-resistant hybrids of identical size were formed with either RNA prepared from HOS cells, MNNG-HOS cells, or NIH3T3 transformants. This suggests that the three HOS cell messages of 11.0 kb, 9.5 kb and 8.0 kb, plus the 6.5 kb message in transformants, probably share the same 3' end.

3. The 3' end of the 6.5 kb met RNA is confirmed by analysis of corresponding cDNA clones.

A representative cDNA library was prepared from one of the met NIH3T3 cell transformants. Using met probes specific for the 3' end of the 6.5 kb transcript, several cDNA clones were identified. Restriction analysis of one of these clones of 1.8 kb has confirmed the location of the 3' end of the 6.5 kb met RNA derived from S1 analysis.

4. The 6.5 kb met RNA in NIH3T3 cell transformants and in MNNG-HOS cells is expressed as a truncated form of the met proto-oncogene transcripts.

From Northern blot analysis using probes which span the met locus, the 6.5 kb RNAs within MNNG-HOS cells and within NIH3T3 transformants show a similar overall hybridization profile as the 11.0 kb, 9.5 kb and 8.0 kb HOS cell RNAs. However, the 6.5 kb RNA also hybridizes to probes which hybridize to the 12 kb HOS cell RNA and not to the 11.0 kb, 9.5 kb and 8.0 kb HOS cell RNAs. Since the 6.5 kb RNA appears to be 3' co-terminal with the three HOS cell RNAs, the 6.5 kb RNA can be interpreted as representing the 3' portion of the 11.0 kb, 9.5 kb or 8.0 kb RNAs. However, the 5' terminal sequences map within a region of the 12.0 kb HOS cell RNA which represents an intron shared by the 11.0 kb, 9.5 kb and 8.0 kb HOS cell RNAs. These results would suggest that met represents a proto-oncogene whose oncogenic potential can be activated through expression of only a portion of that gene.

5. The met proto-oncogene is expressed in a cell line/tissue-specific fashion.

Expression of met was analyzed in established human epithelial, keratinocytes and hematopoietic cell lines, plus two primary human fibroblast cell cultures. In contrast to the 12.0 kb, 11.0 kb, 9.5 kb and 8.0 kb RNA transcripts detected in HOS cell lines, the majority of human cell lines examined express only the 12.0 kb and 11.0 kb met RNAs. Significantly, 6 out of 7 hematopoietic cell lines examined express only the 12.0 kb met RNA and no RNA species related to the 11.0 kb met RNA.

6. Met has been localized to the long arm of human chromosome 7 from 7q21-31.

By the use of somatic cell hybrids, met had been previously mapped to human chromosome 7 from 7p11.4-7qter. In collaboration with Drs. Janet Rowley and Manuel Diaz (University of Chicago), met has been localized to 7q21-7q31 by *in situ* hybridization.

7. Several restriction fragment length polymorphisms (RFLP) have been localized in the met transforming locus.

Three RFLPs, using the restriction enzymes Taq1 and Msp1, have been found within the met transforming locus. These have been found to segregate within individuals in one family. HOS and MNNG-HOS cell DNAs are heterozygous for one of these RFLPs. Thus the presence of the individual met alleles can be identified within HOS cells, MNNG-HOS cells and in NIH3T3 cells transformed with MNNG-HOS cell DNA. From these analyses, we can conclude that only one met allele is biologically active and this allele is always transferred to NIH3T3 cells with the transformed phenotype.

Significance to Biomedical Research and the Program of the Institute:

Recently, analyses of the mechanisms of activation of proto-oncogenes and the activity and interaction of such proteins have provided enormous insight into the role of such proteins in both the regulation of normal cell growth and differentiation, and in the manifestation of the transformed phenotype. Since the met gene appears to be unrelated to known oncogenes, further characterization of this gene and its protein product will increase available knowledge of potential transforming genes. A comparison of the met gene from MNNG-HOS cells with the parental HOS cell gene also provides a model system with which to study one mechanism of transformation of human cells with the carcinogen, MNNG.

It is now clear that many types of cancer are associated with non-random chromosomal aberrations, and that several known oncogenes have been mapped to the breakpoints of specific translocations, resulting in altered expression of these genes. The presence of met on chromosome 7 is of particular interest since several types of human neoplasia are associated with alterations in chromosome 7. Monosomy of chromosome 7, with deletions involving the long arm of chromosome 7, are the most frequent non-random chromosomal aberrations seen in most subtypes of acute non-lymphocytic leukemia (ANLL), and in particular, in patients with secondary ANLL following chemotherapy or radiation treatment.

Proposed Course:

Since there appear to be no rearrangements of the met gene sequences isolated to date within the MNNG-HOS cell line when compared with HOS or human placental DNA, additional human sequences mapping 5' to those cloned from transformants will be isolated from the existing genomic library. To determine if there are detectable differences between the met gene and the normal gene, the restriction map of met gene sequences will be compared with corresponding sequences isolated from human placenta. The met gene clones will also be compared with clones from human placenta by DNA:DNA heteroduplex analysis.

To isolate overlapping cDNA clones spanning the entire 6.5 kb met RNA in NIH3T3 transformants, a second cDNA library will be prepared using both random priming with calf thymus DNA and specific primer extension from the existing cDNA sequence. The cDNA clones isolated to date will be sequenced and characterized for open reading frames. Suitable open reading frame cDNA sequences shall be expressed in bacteria in order to raise antisera to met gene determinants.

Several of the known oncogenes have been mapped near breakpoints in some of the non-random chromosome aberrations detected in several forms of leukemia. Since the met gene maps to the long arm of chromosome 7, and patients with ANLL frequently have deletions involving the long arm of chromosome 7, DNA from patients with ANLL shall be screened by Southern analysis to determine if the met gene is altered or deleted in any of these cases. For these analyses, the presence of restriction fragment length polymorphisms (RFLP) within the met gene will be utilized.

Publications:

Cooper, C. S., Park, M., Blair, D. G., Tainsky, M. A., Huebner, K., Croce, C. M. and Vande Woude, G. F.: Molecular cloning of a new transforming gene from a chemically-transformed human cell line. Nature 311: 29-33, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05288-04 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Control of Developmental Gene Expression - Chromatin Structure of Active Genes

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

PI:	D. D. Blumberg	Senior Staff Fellow	LMO	NCI
Others:	J. F. Comer	Microbiologist	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Molecular Control and Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.85	0.85	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

A very simple model system, the cellular slime mold, Dictyostelium discoideum, is being used to study the mechanisms which control developmental gene activation during normal differentiation. During growth and the early stages of aggregation Dictyostelium cells express 50-55% of their single copy genome as mRNA and HnRNA. An additional 26% of the single copy genome is expressed only during the late stages of development. Initiation of transcription on the late portion of the genome requires cell-cell interaction and cAMP. Because such a high proportion of this small eukaryotic genome is either constitutively transcribed or developmentally induced, it offers a unique opportunity to study the structural organization in chromatin of transcriptionally active genes. Our results indicate that both the constitutively expressed and developmentally inducible genes are in a DNase I-sensitive, active structure in chromatin regardless of whether the developmentally inducible genes are being transcribed. By contrast, micrococcal nuclease has been used to identify a structural organization unique to genes which are actually in the process of being transcribed. Properties of this organization have been used to resolve oligonucleosomes specifically derived from actively transcribed genes and to determine their protein composition.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

D. D. Blumberg	Senior Staff Fellow	LMO	NCI
J. F. Comer	Microbiologist	LMO	NCI

Objectives:

The aim of these studies is to understand the mechanisms which control developmental gene activation during normal differentiation. A very simple model system, the cellular slime mold, Dictyostelium discoideum, is being employed.

The predominant feature of the developmental cycle of Dictyostelium discoideum is the aggregation of unicellular, free-living amoeba into a multicellular organism. Differentiation of amoeba within the newly formed aggregates generates the three distinct cell types found in the mature fruiting body: spore cells, stalk cells, and basal disks. Dictyostelium exhibits many features of development seen in more complex eukaryotic organisms. Specific cell-cell contacts are found, a homogeneous population differentiates into discrete cell types, and there is specific cell migration and pattern formation. These morphogenetic changes are accompanied by major changes in the pattern of gene expression. In particular, post-aggregation Dictyostelium cells contain 2000-3000 new messenger RNA species that are absent from earlier preaggregation-stage cells. These new aggregation-dependent sequences compose 30% of the mass of the messenger RNA in the late cells and, together with their heteronuclear RNA precursors, represent the transcription products of an additional 26% of the single copy portion of the genome.

The initiation of transcription in post-aggregation cells occurs on a portion of the genome, and appears to be dependent upon cell-cell interaction. Additionally, both the rate of transcription and subsequent stability of the mRNAs transcribed off of this portion of the genome are further regulated by a cAMP-mediated process.

The major objective of our work is to elucidate the molecular mechanisms by which these genes are coordinately activated, and to understand the controls which regulate the transcription rate and stability of these mRNAs in response to environmental stimuli--in this case--cell contact and cAMP. In order to achieve this objective, several closely-integrated approaches are being employed. In the first approach described in this summary, we are exploring the basic aspects of the structural organization of these genes, isolated as chromatin, with the objective of understanding the nucleoprotein interactions which are important in activation of gene expression.

Dictyostelium's chromatin offers a unique opportunity to study the structure of actively transcribed genes or genes which will become active in response to a developmental stimulus. Unlike the chromatin from higher cells, in which less than 20% of the DNA is ever transcribed, nearly 80% of the Dictyostelium genome is transcribed. Fifty percent is transcribed during growth, while an additional 30% is active during late development. Thus, analysis of bulk Dictyostelium

chromatin is essentially an analysis of the domain of active chromatin. Additionally, because of the low complexity of the genome, one can probe the structural organization of single-copy genes in their nucleoprotein forms.

Methods Employed:

Standard molecular cloning techniques are being employed to isolate and map recombinant DNAs encoding constitutively expressed and developmentally regulated genes. Two endonucleases, DNase I and micrococcal nuclease, are being used to probe the structural organization of genes in chromatin, while a variety of agarose and polyacrylamide gel systems are being utilized, in one and two dimensions, in order to resolve nucleoprotein complexes, and in histones, as well as the DNA, and RNA species.

Major Findings:

Description of genes used as probes for analysis of transcriptionally-active and inactive regions of dictyostelium chromatin: In terms of expression levels, the developmentally-regulated and constitutively expressed genes, for which we have individual molecularly cloned cDNA probes, fall into three classes. The constitutively expressed genes represented by the cDNA clones, C5, C3 and pCZ22, are expressed at a moderate level throughout growth and development. Analysis of the transcription of these genes using an isolated nuclei system, indicates that their continued presence throughout development correlates with continued transcription of the genes. The developmentally-regulated genes fall into two classes. The genes represented by the cDNA clones, A3, D7 and D19, are not expressed at all in growing cells, but are induced to high levels of expression late in development. That these genes are transcriptionally silent in growing cells is indicated by the fact that no synthesis of RNA from these genes can be detected in growing cells using run-off transcription in isolated nuclei systems (Landfear and Lodish, 1983). Also, using bulk cDNA probes for genes of this type, we can detect no hybridization of messenger RNA or heteronuclear RNA to these sequences, even under conditions which would detect RNAs present at levels as low as one copy per 50 cells. The second type of developmentally-regulated gene is represented by cDNA clones, C1 and D11. These genes are expressed at very low levels in the RNA of growing and early developing cells and are induced to much higher levels of expression late in development.

Differentiation specific genes are already in a DNase I-sensitive "preactive" chromatin structure in the undifferentiated growing cells where they are transcriptionally inactive: Genes which are being or have been transcribed in a particular cell are found in a structural organization in chromatin which renders them more sensitive to digestion by DNase I than genes which are transcriptionally inactive. Since the late genes which specify the differentiation-specific functions in Dictyostelium are completely inactive in growing cells and require a very specific aspect of cellular interaction to be activated, we would like to to know whether these genes exist in a DNase I-sensitive or insensitive configuration in chromatin. In particular, we would like to know if these genes require a change in the chromatin structure in order to be transcribed. This question takes on added interest since the differentiation pathway in Dictyostelium is a reversible pathway. Disruption of cell-cell contacts leads to a shut-off of transcription of these late genes, as well as to rapid degradation of a late messenger RNA species. So far, there is no known instance of a gene which, upon

ceasing to be transcribed, loses its DNase I-sensitive configuration. However, in most systems where these questions have been addressed, the cells have been undergoing terminal differentiation. Thus, it was of additional interest to see how genes in a pathway of reversible differentiations are packaged with respect to the DNase I-sensitive configuration. Nuclei of growing Dictyostelium cells were digested with increasing concentrations of DNase I. The DNA was extracted, deproteinized and redigested with a restriction endonuclease which generated fragments known to map to the structural region of genes expressed actively in growing cells (clones C5, C3 or pCZ22), or only expressed during late development (clones A3, D7 and D19). The rate at which these fragments disappeared as a function of DNaseI digestion was measured. Under conditions of very low ionic strength where the higher order chromatin structure is lost and the DNA exists as the 10 nanometer fiber--the beads on a string configuration--there is no difference in sensitivity to DNase I between constitutively expressed, actively-transcribed genes and the inactive late genes. Likewise, utilizing higher ionic strength--conditions which preserve the higher order solenoid structures--we still detect no difference in the sensitivity of these two classes of genes to digestion by DNase I. To explore this problem further, we have used spot blots to analyze the rate of DNase I digestion of these genes to nonhybridizable size fragments. We still detect no differences in sensitivity of expressed and non-expressed genes, even when the highly-transcribed ribosomal RNA genes are compared with the inactive differentiation-specific genes. These results indicate that the differentiation-specific genes are in a DNase I-sensitive configuration in the chromatin of growing cells, even though they are not transcriptionally active in these cells. We would predict, therefore, that transcription of these genes late in development does not require the kind of alteration in chromatin structure which is defined by a conversion of a gene from the DNase I-insensitive state to a sensitive state. Experiments to verify this directly are currently in progress.

Use of micrococcal nuclease to detect a chromatin structure specifically associated with gene transcription: The enzyme micrococcal nuclease preferentially digests chromatin at sites in the linker region between nucleosomes. In higher eukaryotes in which the bulk of the DNA (80-90%) is transcriptionally inactive, a precise repeating ladder of bands is observed when the digested DNA is resolved on agarose gels. The lengths of the bands are integral multiples the size of the smallest unit of the repeat, the mononucleosome. Dictyostelium's chromatin is unique in that the bulk is active, and all of the genes are in a DNase I-sensitive configuration (see above). Unlike the very precise nucleosome repeat ladder observed in higher eukaryotes, the Dictyostelium pattern is somewhat more diffuse and irregular. This irregular repeat pattern has been analyzed for individual genes which fall into the three transcriptional classes: (1) genes which are transcribed at a moderate rate throughout growth and development; (2) genes which are not transcribed at all in the growing cells, but are expressed only during late development; and (3) genes which are expressed at a very low level in growing cells and induced to a higher level of transcription at the time when cell contact is formed. The differentiation-specific genes, which are transcriptionally inactive in the growing cells from which the chromatin was prepared, give a very precise repeat ladder with a unit length of 175 nucleotides and resemble the patterns seen for bulk inactive chromatin in higher cells. By contrast, the genes which are transcribed at a very low level in the growing cells show a ladder of bands which are not integral multiples of the 175 base pair repeat. Instead, they appear as an irregular ladder of bands with a spacing of about 80

nucleotides between bands. The genes which are transcribed at a higher rate in the growing cells show the same nonintegral repeat as the genes expressed only at a very low level. However, the irregular ladder of bands is now superimposed on a background smear which is similar to the smear seen in higher eukaryotes when probes for very actively transcribed genes are hybridized to microccocal nuclease digests of chromatin. This irregular ladder of bands that we detect in the transcribed genes probably represents a structural intermediate between the very regularly spaced ladder seen upon microccal nuclease digestion of inactive chromatin and the smear seen in association with genes transcribed at a very high rate. This irregular ladder is probably detected because the genes whose structure are being probed are transcribed at a very low rate. Analysis of the location of these cuts should enable us to identify sites on nucleosomes associated with the actively-transcribed genes which are more accessible and open to cutting by microccocal nuclease. At present this irregular ladder of bands is consistent with at least one of two different interpretations. If the irregular ladder of bands results from the actively-transcribed genes being cut predominantly at sites which are approximately 80 base pairs apart, as opposed to the 175 base pairs seen for the inactive genes, then transcription of the genes is resulting in exposure of specific sites within the nucleosome core, as well as at the usual site of micrococcal nuclease attack in the linker region. At the very low levels of transcription, a single or limited number of sites on the core particle may be accessible. At higher levels of transcription the nucleosome core may become progressively more open, resulting in many additional exposed sites accessible to micrococcal nuclease digestion and giving rise, ultimately, to the smear. The second interpretation is that the irregular pattern of bands arises as a result of interspersion of closely-packed core particles lacking a linker region and histone H_1 with normal nucleosomes containing histone H_1 and the linker region. Thus, the possibility that the irregular length DNA bands arise from closely-packed nucleosome cores cannot be discounted. A model in which one has interspersion of close-packed core particles and core particles with linker regions, possibly resulting from a reduction in the amount of histone H_1 associated with these genes, is entirely consistent with the size of DNA bands that we see. Experiments to distinguish between these possibilities are outlined under the Proposed Course section.

Significance to Biomedical Research and the Program of the Institute:

A remarkable characteristic of the eukaryotic cell is its incredibly efficient mechanism for repression of the activity of a gene. In tissues where it is not expressed, the messenger RNA for a developmentally-controlled gene may not be detectable at levels of even 1 copy per 50-100 cells. This is in marked contrast to bacterial cells where the repressed level of expression of the lac operon, for example, is 1 to 10 messenger RNA transcripts per cell. In a eukaryotic cell where messenger RNAs often have half-lives in excess of 4 hrs, 1 messenger transcript per cell can lead to a substantial accumulation of its protein product. Thus, it is vital for the eukaryotic cell to have such an efficient mechanism for suppression of the activity of genes whose products, if expressed out of developmental time or place, would be deleterious for the cell. Clearly, certain genes with oncogenic potential may fall into such a category. The mechanism by which such efficient suppression is achieved is not known, but a candidate for constraining a gene in an efficiently nonexpressible configuration is its structure in chromatin. We have identified clearly distinct chromatin structures associated with tissue-specific genes whose expression is very efficiently

repressed in growing cells (less than 1 copy of messenger RNA per 50 cells) and genes expressed at low to moderate levels in these same cells. By pursuing our analysis of these structures, we should be able to identify components of the structural organization vital for conversion of a gene from an inactive to an active state.

Proposed Course:

Our studies have identified a structural organization unique to genes which are actually being transcribed. Features of this organization may potentially allow us to specifically resolve, on polyacrylamide gels, oligonucleosomes derived from genes which are being actively transcribed. Mono- and oligonucleosome core particles liberated from Dictyostelium nuclei by digestion with micrococcal nuclease have been analyzed on low ionic strength polyacrylamide gels. Two major and several minor nucleosome species have been detected. The DNA associated with these particles has been analyzed by electrophoresis in a second dimension DNA gel. The two mononucleosome species have been identified as being derived from the core particle and the core particle plus linker region DNA from the regularly spaced micrococcal nuclease cuts between nucleosomes. A very rapidly migrating, minor particle is found and appears to be associated with DNA which is 70-130 base pairs in length. This particle is a candidate for having arisen by an internal cut on the nucleosome core. Likewise, a particle migrating more slowly than the core particle plus linker is found to be associated with DNA lengths from 240 to 315 nucleotides. This is a nonintegral repeat length and is likely to have arisen from a cut within the core particle or from two closely-packed core particles. We are currently trying to devise conditions under which we can electrotransfer the DNAs associated with these particles to a nitrocellulose filter and obtain efficient hybridization with low background so that we can confirm that the particles associated with the nonintegral DNA lengths do indeed arise from the actively-transcribed genes. If this is the case, we will then be able to release the proteins from these different mono- and oligonucleosome particles and analyze the protein composition arising from particles derived specifically from the active genes. In this way we will be able to determine whether additional proteins or modified histone variants are found in association with actively-transcribed genes or whether the passage of polymerase alone is sufficient to distort the structure of the mononucleosome to allow the irregular cuts observed following microccocal nuclease digestion. If the irregular micrococcal nuclease repeat pattern arises from the presence of closely-packed core particles lacking a linker region, then we would expect that nucleosomes derived from these nonintegral DNA lengths would contain the core histones (H_2A, H_2B, H_3, H_4), but not histone H_1. A result such as this could have very interesting implications for the higher order coiling of nucleosomes associated with active genes since histone H_1 is responsible for folding the oligonucleosome chains into the higher order solenoid structure.

We are trying to isolate genomic clones which encode the genes whose structures we have been exploring. These clones should enable us to map the boundaries of the disrupted or irregular chromatin domains which we detect in association with the transcribed genes. Based on the few genes which have been cloned and mapped, Dictyostelium genes tend to be small with constitutively-expressed and developmentally-regulated genes closely interspersed and frequently adjacent to repetitive regions. Also, the coding regions tend to be flanked in both 3' and 5' directions by several hundred nucleotide-long stretches of DNA composed of

95-98% AT sequences. As we map the coding regions in our clones and can genetically determine the control sites, we would like to be able to superimpose upon the functional and sequence maps such structural features as attachment points of the DNA to the nuclear matrix, origins of DNA replication, sites not in nucleosomes, as well as the boundaries of different chromatin structure domains and in this way build a structural picture of the organization of transcribed and nontranscribed genes.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05294-04 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Human Teratocarcinoma Cell Line, PA-1, as a Model System for Malignant Progression

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

PI:	M. A. Tainsky	Senior Staff Fellow	LMO	NCI
Others:	D. G. Blair	Research Chemist	LMO	NCI

COOPERATING UNITS (if any)

The Stehlin Foundation for Cancer Research, Houston, TX (B. C. Giovanella)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.90	0.90	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 dominant transforming gene from late passages (330) of a human teratocarcinoma cell line (PA-1) was isolated as a biologically active molecular clone and is a new isolate of the human N-*ras* locus. Its transforming activity is due to a single G→A point mutation at the codon for amino acid 12, which changes the codon for glycine to an aspartic acid residue. DNA from the PA-1 cell line at early passages (36) does not yield foci in DNA transfection assays and the early passage cells are much less tumorigenic in nude mice. These results, therefore, correlate the presence of an activated N-*ras* locus with the enhanced tumorigenicity of a cell line. The activated N-*ras* gene was, therefore, either selected from a small population of the original, metastatic tumor cells or arose by mutation during passage in culture. N-*ras* DNA from early passage (41) PA-1 cells was cloned. Eight independent clones were inactive in the NIH3T3 transfection assay. This agrees with the genomic transfection data and preclude DNA modification as a mechanism for the inactivity of early passage PA-1 cell DNA. When the cloned, biologically active PA-1 gene was introduced by gene transfer into a non-tumorigenic PA-1 cloned cell, the cell was transformed to induce tumors in nude mice. Therefore, a direct causal relationship between N-*ras* and the malignant phenotype of PA-1 cells was established.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

M. A. Tainsky	Senior Staff Fellow	LMO	NCI
D. G. Blair	Research Chemist	LMO	NCI

Objectives:

The presence of a mutated, biologically active ras^N gene correlated with the malignant phenotype of PA-1 cells. The goal of this work was to determine if there was a direct causal effect of the ras^N and the ability of PA-1 cells to form tumors in athymic nude mice.

Methods Employed:

The early passage PA-1 ras^N gene 9.0 R_I fragment was cloned into the λ phage vector λgtWES·λB. Clones were identified by plaque hybridization to a 5' specific ras^N probe. The 5' R_I ras^N fragment was ligated to the human placental 3' R_I fragment and the biologically active ras^N assayed by transfection into NIH3T3 and looking for foci of morphologically-altered cells. Protoplast fusion was carried out by the method of Yoakum *et al*.

Major Findings:

A transforming gene in DNA isolated from passage 330 of the human teratocarcinoma cell line, PA-1, was detected in the NIH 3T3 cell assay. The cell line, PA-1, was derived from the culturing of ascites fluid cells from a patient with a metastatic, ovarian germ line tumor, and has the properties of embryonal carcinoma cell lines. The cell line shows significant alterations in various malignancy-related phenotypes with extended passage in culture. Early-passage PA-1 cells grow slowly in culture and do not form tumors in athymic nude mice. In contrast, late-passage PA-1 cells grow rapidly in culture and readily form tumors in athymic nude mice in 7-10 weeks. In this study, the presence of an activated ras^N oncogene was correlated with the increased level of tumorigenicity observed in late cell culture passages. The DNA sequences responsible for the transforming activity in PA-1 DNA were cloned and found to be a ras^N gene.

DNA sequence analysis of the first exon revealed a change in a single nucleotide base corresponding to the codon for amino acid 12. Instead of GGT coding for the amino acid glycine, the sequence GAT was found, corresponding to a change to aspartic acid. The mutation giving rise to a change in amino acid position 12 was shown to be responsible for activation of this gene.

The low tumorigenicity reported for early-passage PA-1 cells was investigated as to whether the activated ras^N gene was present in early passages. Early-passage PA-1 cells (passage 36) gave no tumors in athymic nude mice, whereas late-passage PA-1 cells (passage 338) rapidly formed large tumors in nude mice. Therefore, the transforming activity was determined for DNA isolated from early- and late-passage PA-1 cells in both the NIH 3T3 cell DNA-mediated transfection

and nude mouse transformation assays. In the transfection assay, no foci were observed with DNA from early-passage PA-1 cells under conditions which gave foci from late-passage cell DNA. In the nude mouse transformation assay, DNAs from early-passage PA-1 cells, late-passage PA-1 cells, or human placenta were transfected into NIH 3T3 cells which were inoculated into athymic nude mice. Fourteen out of eighteen nude mice developed tumors within four weeks after inoculation of cells receiving a late-passage (338) DNA. All of these tumors contained newly-acquired, human ras^N sequences. In contrast to the lack of focus-forming activity with the early-passage cell DNA, tumors did appear in athymic nude mice receiving cells transfected with early-passage PA-1 DNA after seven weeks; however, DNAs from these tumors do not contain human ras^N DNA sequences. Human placental DNA gave no tumors. These analyses suggest that the majority of early-passage PA-1 cells do not have activated ras^N, as compared to late-passage PA-1 cells. The absence of the activated ras^N may be related to the inability of the early-passage PA-1 cells to clone in soft agar or their lack of tumorigenicity in nude mice.

We presumed that the lack of transforming activity of early passage PA-1 DNA was due to the absence of a point mutation in the ras^N gene. However, it may have been due to some DNA modification which was conserved during preparation of the DNA and transfection (i.e., methylation). In order to eliminate this possibility, the ras^N locus from passage 41 PA-1 cells was cloned. 9.0 kb R_I cut passage 41 DNA was isolated and ligated in R_I arms of the λ phage vector λgtWES•λB. Fifty-three ras^N positive clones were isolated from 2 x 10^6 λ plaques by hybridization to a 5' specific ras^N probe. Eight phages were randomly chosen for further study. DNAs from these phages were analyzed to confirm that they contained the appropriate restriction fragment. This R_I fragment was ligated to the 3' R_I fragment of the ras^N gene isolated from human placenta. These ligations, as well as control samples containing the activated gene from late passage PA-1 cells were transfected into NIH3T3 cells which were checked for focus formation. No transforming activity was observed while positive controls were active. Therefore, the ras^N gene in the majority of early passage (41) PA-1 cells does not contain the point mutation observed in late passage cells.

To determine if the activated ras^N can cause transformation of non-tumorigenic PA-1 cells to form tumors in athymic nude mice, the ras^N gene was cloned onto the plasmid pSV2neo which has a selective gene which allows growth of tissue culture cells harboring this plasmid in the drug, G-418. In addition, the non-mutated ras^N gene isolated from human placental DNA was cloned onto this plasmid. A non-tumorigenic single cell derived clone of PA-1 cells was used as a recipient cell. The parent vector and the normal and activated ras^N genes were introduced into these cells by protoplast fusion. The parent cells, as well as the G-418 resistant cells carrying the pSV2neo or pSV2neo ras^N normal gene, were non-tumorigenic in athymic nude mice, while the cells carrying the activated ras^N pSV2neo construct formed tumors in 7-20 weeks. Therefore, a direct causal relationship of an oncogene and its cell of origin has been established.

Significance to Biomedical Research and the Program of the Institute:

Transforming genes, such as the ras^N which was isolated in these experiments, have been the object of much recent study. A number of such genes have been

isolated from human tumors and some have been found to be related to the *ras* family of well-studied rat oncogenes. It is of great interest that the transforming gene of PA-1 is *ras*-related, implying a similarity in the molecular mechanism involved in carcinogenesis in diverse tumor types such as bladder, lung and colon carcinomas, fibrosarcomas, and now an ovarian teratocarcinoma.

Proposed Course:

This project has been terminated.

Publications:

Tainsky, M. A., Cooper, C. S., Giovanella, B. C. and Vande Woude, G. F.: An activated *ras*^{N} gene: Detected in late but not early passage human PA-1 teratocarcinoma cells. *Science* 225: 643-645, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05295-04 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Studies on the Activation of onc Genes in Viruses and Human Tumors

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

PI:	D. G. Blair	Research Chemist	LMO	NCI
Others:	M. A. Tainsky	Senior Staff Fellow	LMO	NCI
	B. M. O'Hara	Visiting Fellow	LMO	NCI
	R. J. Black	Staff Fellow	LMO	NCI
	K. J. Dunn	Bio. Lab. Tech. (Micro.)	LMO	NCI

COOPERATING UNITS (if any) Mol. Mech. of Carc. Lab., Basic Research Program, LBI, Frederick, MD (G. F. Vande Woude, A. Seth, M. Dean, M. K. Oskarsson); Nucl. Acids & Protein Syn. Lab., PRI, Frederick, MD (M. Zweig, S. D. Showalter, L. A. Eader); Lab. of Cell & Mol. Structure, PRI, Frederick, MD (A. Boyd)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.75	1.75	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 human cellular homolog (c-*mos*-Hu) of the Moloney murine sarcoma virus (MSV) transforming sequence, v-*mos*, could be activated to express a transforming potential in NIH3T3 mouse fibroblasts. Linkage of the MSV-derived long terminal repeat (LTR) sequences approximately 50 base pairs 5' to the start of the conserved *mos* open reading frame allowed c-*mos*-Hu to transform NIH3T3 cells at a low efficiency (~150 focus-forming units/p mole). Cells transformed by c-*mos*-Hu were tumorigenic in nude mice and expressed high levels of a 33,000 dalton protein recognized by human *mos*-specific antisera and broadly reactive anti-v-*mos* sera in both immunoprecipitation and Western blot analysis. These results indicated that the transforming potential of *mos*-related cellular sequences has been conserved between mouse and man.

The genetic lesion responsible for the activation of an N-*ras* sequence isolated from a human gastric adenocarcinoma has been identified. A single base change in the 61st codon results in the substitution of an arginine for glutamine at this position, resulting in an activated N-*ras* product able to transform NIH3T3 cells. No biological activity could be detected in DNA from EBV immortalized lymphocytes prepared from the same individual.

Utilizing a nude mouse tumor assay to screen for transfectable oncogenes, transforming sequences have been detected in DNA from human ovarian carcinoma-derived cell lines and from an MuLV-induced mouse lymphoma. The ovarian carcinoma-derived transforming sequences do not appear to be related to the known human *ras* family of oncogenes. The mouse lymphoma-derived sequence appears to be a murine H-*ras* sequence.

PROJECT DESCRIPTION

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

D. G. Blair	Research Chemist	LMO	NCI
M. A. Tainsky	Senior Staff Fellow	LMO	NCI
B. M. O'Hara	Visiting Fellow	LMO	NCI
R. J. Black	Staff Fellow	LMO	NCI
K. J. Dunn	Bio. Lab. Tech. (Micro.)	LMO	NCI

Objectives:

To understand the mechanism of transformation by murine sarcoma viruses and the function of specific gene products of MSV in this process.

To define the functions of specific portions of the Moloney murine sarcoma virus (MSV) genome in MSV transformation and to identify specific genetic sequences necessary to activate the transformation potential of normal cell sequences of mouse and human origin.

To develop screening and selection systems to identify and isolate human transforming DNA sequences from primary human tumors and tumor cell line DNAs. To identify, isolate and characterize such sequences and their gene products. To characterize the normal cellular homologs of such sequences and to determine the mechanism by which their oncogenic potential is activated.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells, DNA and RNA isolation from tumor tissues and tissue culture, Southern and Northern blotting using radioactive probes prepared from cloned DNAs, calcium-phosphate-DNA transfection, measurement of tumorigenicity of cell lines in nude and other strains of mice, immunoprecipitation and protein gel analysis to detect antibodies to specific cellular proteins.

Major Findings:

1. The human mos homolog, c-mos^{Hu}, can induce morphological transformation in NIH3T3 cells. Previous results had indicated that the human homolog of mos, c-mos^{Hu}, was inactive in DNA transfection assays. We have now inserted a Moloney murine sarcoma virus LTR, containing both enhancer and promoter sequences, 5' to the c-mos^{Hu} coding region within ~0.50 kb of the start of the open reading frame thought to encode the human mos coding sequences. These constructs transform NIH3T3 cells with low, but reproducible, frequencies. The foci induced are noticeably smaller and morphologically distinct from those induced by either viral mos (v-mos) or LTR-activated mouse c-mos constructs. The transforming efficiency of c-mos^{Hu} linked to an LTR is 100-fold less than that observed for v-mos. Foci are difficult to observe unless assays are done

in the presence of 0.125 mM dexamethosone, which flattens the normal cell monolayer and renders small foci of transformed cells more distinguishable. Cells transfected with activated human mos constructs are tumorigenic in nude mice and contain multiple copies of unrearranged transfected DNA. They express high levels of a 33,000 Kd protein detectable by human mos-specific peptide antisera and by broadly reactive anti-v-mos sera directed against bacterially-synthesized v-mos protein. These results indicate that the transforming potential of the c-mos genetic sequences have been evolutionarily conserved between mouse and man.

2. A position 12 activated N-ras is present in a primary human gastric adenocarcinoma, but not in normal tissue from the same patient. DNA isolated from a primary human gastric adenocarcinoma has been shown to be active in DNA transfection, and transfected cells have been shown to contain human DNA homologous to the ras^N oncogene. These sequences were neither amplified nor rearranged in the primary tumor nor in EBV immortalized peripheral blood lymphocytes from the same patient. The ras^N gene was cloned from EcoRI digested DNA from the tumor and in vitro reconstruction experiments using segments of the normal ras^N gene mapped the activating lesion to within a PstI-Bst EII fragment of the second coding exon. Sequence analysis of exons I and II and their immediate flanking areas showed no changes when compared to published sequences of the normal gene, except at codon 61 in exon II. There CGA had replaced the normal CAA coding triplet, which would result in a substitution of arginine for glutamine at this position. This indicates that a position 61 mutation is responsible for activating the transforming activity of this gene.

3. An IL-3 independent mouse lymphoma cell line contains an activated ras^H sequence which can be detected by transfection of NIH3T3 cells. We have examined 10 MuLV induced mouse lymphoma cell lines originally isolated by Dr. J. Ihle (Litton Bionetics) for the presence of activated transforming sequences in DNA transfection assays on NIH3T3 cells. Two of these induced both tumors and foci following transfection. Southern blot analysis of the foci and tumors generated by transfection indicated that those induced by one of the lymphomas (designated DA2) contained new bands which hybridized to a ras^H specific probe. The transfectants derived from the second tumor did not show hybridization to ras-specific probes, and the identification of the active transforming sequence will require further analysis.

Analysis of the DA2 lymphoma cell line indicated that new ras^H hybridizing bands could be detected following DNA digestion with several enzymes, suggesting that at least one allele of ras^H in the original tumor had undergone some rearrangement or other alteration. DA2 cells expressed high levels of a protein of approximately 21,000 daltons recognized by anti-ras specific monoclonal antibodies (J. Ihle, personal communication). Similar high levels were not detected in other mouse lymphoma cell lines. These results indicate that an active ras^H is present in an MuLV-induced mouse lymphoma and that the activation may be associated with a high level of p21 expression in the parental tumor-derived cell line.

4. Human ovarian carcinoma-derived cell lines contain active transfectable sequences which are not ras related. A series of ovarian carcinoma cell lines established by Dr. E. Gelmann, NCI, were screened for active transforming

sequences in NIH3T3 transfection. Following cotransfection with pSV2neo and selection for G418 resistance, resistant cell populations were injected into nude mice. DNA from four cell lines and one primary tumor were tested. Two of four cell lines tested induced tumors with moderate efficiency, while the remaining samples failed to induce tumors or induced them only after very long periods of time. DNA was prepared from three tumors induced by the most active cell line (OVCAR3), were tested for their ability to induce secondary tumors, and two of the three primary tumors induced secondary tumors with a decreased latency relative to that observed in the primary transfection. Tumor induction by OVCAR3 DNA and DNA from primary tumors was reproducible with similar efficiencies in independent transfections. Southern analysis of DNA from the active primary transfectants revealed no human N-, K- or H-ras-specific sequences, suggesting that the active sequences are not ras related.

5. Human diploid fibroblasts cannot be stably transformed by microinjection of cloned MSV sequences. Microinjection of cloned oncogenes and transforming retroviruses leads to stable integration of the injected sequences and the efficient transformation of NIH3T3 cells. Cloned m1MSV, which expresses a p60 gag polyprotein, was used to analyze the ability of microinjected DNA to be expressed in and transform both NIH3T3 mouse cells and human diploid fibroblasts derived from human embryonic muscle tissue (HEMS cells). Approximately 70% of injected 3T3 cells expressed the m1 coded pp60 polyprotein, measured by immunofluorescence within 48 hrs of injection. Approximately 20% of the injected 3T3 cells gave rise to foci of stable transformants. In contrast, approximately 10% of injected HEMS cells expressed p60 Ag following injection; and no foci could be detected, although morphologically-altered, fluorescently stained cells could be detected within 48 hrs of microinjection. Expression of p60 Ag could be detected up to seven days after injection, but the number of stained cells either remained constant or decreased slowly within this period.

The lifespan of the cells in culture was not affected by MSV injection, and no MSV-specific hybridization was observed in the microinjected cells when analyzed approximately 4-8 weeks after microinjection. These results suggest that although MSV can be expressed in HEMS cells following microinjection, either the sequences cannot be stably maintained or are suppressed.

Significance to Biomedical Research and the Program of the Institute:

DNA transfection utilizing genomic DNA from human and animal tumors has led to the identification and isolation of specific genetic sequences capable of transforming established cell lines such as NIH3T3 mouse cells. The presence of such sequences in tumors suggests that they could be involved in the initiation, maintenance or progression of the tumorigenic phenotype. The development of detection techniques based on tumorigenesis, as opposed to morphological transformation provides an alternative, biologically significant, selection system capable of identifying transforming sequences whose morphological manifestations may be difficult to detect. It also allows the use of recipient cells in which morphological transformation cannot be readily identified. The use of transformation assays which detect genes inducing biologically significant transformation phenotypes should increase our knowledge of genes or sequences capable of inducing such changes in normal cells. The extension of these assays to other cell types, particularly human cells, is an important validation for the oncogenic potential of genes isolated through selection and

screening in rodent cells. This represents a further step toward our understanding of the steps in the process of transformation of diverse lineages of normal diploid cells *in vivo*.

The presence of active transforming genes in tumor tissue raises the question of how the oncogenic potential of these sequences has been changed from that expressed by these same sequences in normal, non-transformed cells. Such activation events have been shown to be due to both quantitative changes at the level of gene expression and qualitative changes in gene sequence and arrangement. The increased understanding gained by studying how *in vitro* induced changes in model systems affect the oncogenic potential of known genes should increase our understanding of how such changes occur *in vivo*, either spontaneously or in response to chemical stimuli. This should help to identify and define the range of events which could lead to the activation of normal genes with oncogenic potential.

Proposed Course:

Attempts will be made to identify the nature of the transforming activity identified in the OVCAR3 human ovarian carcinoma cell. The lack of relatedness to *ras* oncogenes will be confirmed in secondary transfectants and a more extensive screening of known oncogenes will be undertaken to determine if the gene can be identified.

The cloning of the transforming sequences will be undertaken either as a biologically active fragment or by utilization of conserved linked *alu* repeat sequences. The exact approach will be determined after further transfection analysis of the restriction sensitivity of the active transforming sequences present in primary and secondary tumors.

The transforming activity associated with the ras^H sequences found in the mouse lymphoma DA2 will be further analyzed to determine if the activation involves the insertion of an MuLV provirus adjacent to the normal ras^H gene. Further restriction analysis, coupled with Northern analysis of transfected cells utilizing MuLV LTR specific probes, should allow this possibility to be differentiated from a point mutation within the ras^H coding sequence. If an MuLV insertion is responsible, the ras^H locus will be cloned directly to characterize the nature of the integration and the exact mechanism of activation.

The ability of cloned oncogenes to transform human diploid cells will be studied more extensively by utilizing protoplast fusion to introduce the *mos*, ras^N, v-*myc* and v-*abl* into normal diploid fibroblasts and fibroblasts derived from individuals with Franconi's anemia, which have shown an increased susceptibility to transformation by DNA and RNA viruses. Transforming sequences will be linked to selectable drug resistance markers to allow the selection of cells carrying the gene in the absence of phenotypic expression of a transformed phenotype. The ability of these sequences to transform cells will also be characterized following their introduction as drug-selectable retroviral vector constructs. Tumorigenesis and morphological transformation will be measured.

The effect of multiple oncogenes on diploid fibroblasts will be studied by introducing a second gene linked to sequences encoding resistance to the drug hygromycin. Selection for both hygromycin and G418 resistance will allow

isolation of human cells carrying pairs of oncogenes. The ability of multiple oncogenes to transform or immortalize diploid human cells will be determined.

The nature of the regions of v-mos and c-mos^{Hu} required for transformation will be analyzed further. Additional deletions will be constructed and tested for biological activity to localize the active mos domains. The ability of human mos protein to function as a serine kinase will be measured to determine if changes in this domain are responsible for its low transforming potential. The ability of c-mos^{Hu} to transform human cells will be measured to determine if its transforming potential is species dependent.

Publications:

Cooper, C. S., Park, M., Blair, D. G., Tainsky, M. A., Huebner, K., Croce, C. M. and Vande Woude, G. F.: Molecular cloning of a new transforming gene from a chemically-transformed human cell line. Nature 311: 29-33, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05342-03 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Expression of mos Gene at Elevated Levels

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

PI:	R. J. Black	Staff Fellow	LMO	NCI
Others:	M. A. Tainsky	Senior Staff Fellow	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

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.)

Characterization of the Moloney murine sarcoma virus (MSV) mos gene product has been hindered by expression of extremely low levels of mos protein in transformed cells. In order to analyze the v-mos gene product, we have attempted to develop a eukaryotic expression system in which v-mos protein will be produced at elevated levels. Specifically, plasmid expression vectors have been constructed in which v-mos has been placed under the control of the murine β-globin promoter in various arrangements with or without additional β-globin coding sequences. This plasmid DNA containing v-mos and plasmid DNA containing the gene coding for the enzyme, adenine phosphoribosyltransferase (aprt), have been simultaneously introduced into murine erythroleukemia (MEL) cells lacking aprt, using calcium phosphate-mediated DNA coprecipitation or spheroplast fusion. Upon induction of differentiation, it was predicted that the exogenously added β-globin promoter would be activated to express v-mos protein at elevated levels. However, no v-mos specific mRNA was detected in induced aprt+ cell populations containing unrearranged β-globin/v-mos sequences. A parallel construct in which the bacterial gene, chloramphenicol acetyltransferase (CAT), was substituted for v-mos in between β-globin promoter and coding sequences, was introduced into MEL cells. Similarly, CAT activity was not observed before or after induction. In conclusion, the lack of expression of v-mos and CAT in this β-globin system provides indirect support for the presence of additional, unidentified elements which may affect control of gene activation.

PROJECT DESCRIPTION

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

R. J. Black	Staff Fellow	LMO NCI
M. A. Tainsky	Senior Staff Fellow	LMO NCI

Objectives:

The goal of this project is to develop a eukaryotic expression system in which the product of the v-mos gene is expressed in large quantities. A secondary aim is to incorporate modifications into this eukaryotic expression system which make it suitable to use for high level expression of other gene products.

Methods Employed:

Using standard in vitro recombinant DNA techniques, the v-mos gene from the HT1 strain of MSV or the CAT gene from pBR325 has been placed under the control of the mouse β-globin^{major} promoter from λgt WES.MβG2 (Tilghman, S. M. et al., PNAS 74: 4406-4410, 1977) in three distinct arrangements. Each of the plasmid DNAs has been introduced simultaneously with the selected marker gene, aprt, into MEL aprt$^-$ cells using the calcium-phosphate mediated DNA coprecipitation technique or spheroplast fusion. MEL aprt$^+$ colonies were isolated using selective media containing azaserine and adenine. DNA was extracted from expanded single cell colonies and used in DNA blotting experiments to identify v-mos-containing clones. Those cells into which v-mos was successfully introduced without rearrangements were induced to differentiate; induction was verified three to four days, after addition of inducer, by benzidine staining for hemoglobin production. Upon activation of the exogenously added β-globin promoter, expression of v-mos was examined at the RNA level by dot blot and Northern analyses. CAT activity was assayed by analysis of ^{14}C-chloramphenicol acetylation by thin-layer chromatography (Gorman, S. M. et al., Mol. Cell. Biol. 2: 1044-1051, 1982).

Major Findings:

Cell colonies with an aprt$^+$ phenotype have been obtained after cotransfection of MEL aprt$^-$ cells with plasmid DNA containing the aprt gene, and plasmid DNA in which the v-mos coding region has replaced the globin coding region immediately downstream of the promoter region of β-globin. Southern blot hybridization analysis indicated the presence of unrearranged β-globin/v-mos sequences in four separate clones. Analysis of poly A selected mRNA populations indicated that similar levels of aprt-specific mRNA is present before and after induction, whereas β-globin specific mRNA is absent prior to induction and expressed at high levels after induction. mRNA species containing v-mos sequences were not detectable before or after induction in these aprt$^+$ cells.

Cell colonies with an aprt$^+$ phenotype have also been obtained after introduction of a plasmid containing v-mos in between the β-globlin promoter and coding sequences, and the selectable marker into MEL cells by spheroplast fusion. Examination of DNA from eight aprt$^+$ isolates demonstrated the presence of unrearranged exogenous v-mos and β-globin sequences. Poly A selected RNA from

uninduced and induced cell populations of a single aprt$^+$ isolate, as well as a pool of eight isolates, was probed for β-globin specific and v-mos specific sequences. Characteristic β-globin 10S mRNA, representing endogenous globin expression, was observed in each induced cell population, while no β-globin specific RNA was detected in preparations from uninduced cells. No v-mos specific mRNA was detected by hybridization of replicate samples with a v-mos probe.

In order to preclude the possibility that lack of detectable expression of v-mos in MEL cells is a reflection of some uncharacterized aspect of v-mos and not a result of a defect in expression of any foreign gene from this β-globin promoter system, the bacterial gene, CAT, was substituted for v-mos in the latter type of construction. Subsequent to introduction of this construct into MEL cells by spheroplast fusion, CAT activity was assayed before and after induction. Upon analysis of 20 aprt$^+$ individual single cell clones, as well as uncloned colonies, no CAT activity was observed after induction.

Significance to Biomedical Research and the Program of the Institute:

By utilizing the inherent biological mechanism of induction of differentiation in MEL cells, it was anticipated that this expression system would have the potential to yield large quantities of a specific eukaryotically-produced and modified protein, the coding region of which is under the control of the β-globin promoter, against an insignificant background of general protein synthesis. Specifically, this system was expected to facilitate *in vitro* biochemical analyses of the v-mos gene product by providing significant quantities of protein with which to work and to permit comparison of the nature of eukaryotically-expressed mos and prokaryotically-expressed mos. Although the primary goal of this project has not been achieved, the inability of the various plasmid constructs to express v-mos has provided indirect evidence for the presence of additional elements that may affect activation of globin expression during induction.

Proposed Course:

This project has been terminated.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05344-03 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Studies of Cellular DNA Sequences Required to Transform Human Cells

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

PI:	B. M. O'Hara	Visiting Fellow	LMO	NCI
Others:	D. G. Blair	Research Chemist	LMO	NCI

COOPERATING UNITS (if any)

Hoffman-LaRoche, Nutley, NJ (T. Curran)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

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.)

A panel of hybrids of HeLa and a normal human fibroblast differing in tumorigenicity in nude mice was screened for RNA levels specific for 21 oncogenes and for human papillomavirus 18. Transcripts for 15 of these oncogenes and for papillomavirus were detected. The levels for three of these oncogenes were found to correlate with tumorigenicity. These were _fos_ and _ets_ transcripts, which were present at a higher level in tumorigenic hybrids than in non-tumorigenic hybrids and _myb_ transcripts, which were higher in the non-tumorigenic hybrids. _Fos_ protein levels correlated with _fos_ transcript levels. A non-tumorigenic hybrid, when infected with murine sarcoma virus, did not show alteration in the levels of _fos_, _ets_ or _myb_ transcripts.

A panel of human SV80 cells transfected for the proviral _mos_ gene has been generated. These transfectants differ in tumorigenic potential in nude mice. Explants of the tumorigenic members of this panel do not show any alteration in the size or amount of immunoprecipitable SV40 T antigen consistent with an immunological selection against T antigen expression during tumor formation. RNA has been prepared from the members of the panel for an examination of the levels of _mos_-specific transcripts.

An activated N-_ras_ gene cloned previously from human gastric adenocarcinoma material has been characterized using DNA sequencing and transfection assays of _in vitro_ ligated DNA fragments.

PROJECT DESCRIPTION

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

B. M. O'Hara	Visiting Fellow	LMO	NCI
D. G. Blair	Research Chemist	LMO	NCI

Objectives:

To develop an assay using human cells which will allow detection of focus and tumor formation following transfection of the human cells with viral onc genes.

To use the assay to test the activity of cellular transforming genes previously isolated using NIH 3T3 cell assays.

To use the assay to screen human tumor cell DNAs for cellular transforming genes.

To determine if identical transforming genes are detectable in cells of different species.

To determine if hybrids of HeLa and a normal human fibroblast show a correlation between the levels of any oncogene-specific transcripts and tumorigenicity.

To determine if such a correlation results from transformation by a route alternative to that operating normally in the hybrids and thus to more closely implicate the correlation as a cause of transformation.

To determine if such a correlation is a cause of transformation by experimentally altering, where possible, the levels of the transcripts involved.

To examine the control of fos transcript levels in members of the panel showing an aberrantly high level of fos RNA.

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 DNA, calcium-phosphate-DNA transfection, measurement of tumorigenicity of cell lines in nude mice, immunoprecipitation and protein gel analysis to detect specific cellular proteins.

Drug selection of transfected cells utilizing media supplemented with G418 (Schering) for neomycin resistance and with mycophenolic acid and xanthine for the presence of the guanine phosphoribosyl transferase (gpt) gene. Parents of the human hybrid cell lines, the hybrids and their derivative lines were obtained from Harold Klinger, Albert Einstein College of Medicine, New York, NY.

Major Findings:

Transformation of an immortalized human cell line:

The mos proviral gene transforms SV80 cells.

We have tested a number of established, non-tumorigenic human cell lines for transfectability. Two, the SV80 cell line and ESH20, a hybrid of HeLa and a normal human diploid fibroblast, were found to be reasonably competent in transfection.

The SV80 line was found to become tumorigenic in BALB/c athymic mice after infection with MSV pseudotyped with FeLV or GALV. Following introduction of pM1 (a plasmid carrying a proviral MSV) by cotransfection with pSV2gpt, two pools of transfectants were obtained and were designated SV80 pM1-1 and pM1-2. Southern analysis clearly showed the presence of unrearranged pM1 plasmid in each pool. This was supported by indirect immunofluorescence tests using an antibody directed against p30 determinants of the $p60^{gag}$ polyprotein encoded by pM1 and by our ability to rescue a virus from the transfectants. Cells reacting with p30 antiserum showed a clearly altered morphology characterized by long cytoplasmic extensions closely resembling the morphology of MSV-transformed rodent cells. When tested at 5×10^6/mouse, these pools were non-tumorigenic in BALB/c athymic mice, as was a combination of the pools (SV80 pM1-1 + 2) which were carried for some time and injected at 10^7/mouse. However, tumors were induced when the two original pools were grown from frozen stocks and injected into irradiated mice at 10^7/mouse.

A tumor was also induced following injection of SV80 cells transfected with a mixture of pM1 and p185, a plasmid carrying the adenovirus E1a gene and the E1b gene fused to the bacterial β-galactosidase gene (provided by G. Weinstock and S. Dusing-Schwartz). Southern analysis did not detect mos sequences in the transfectant pool, consistent with a low proportion of cells positive for the p30 fluorescence stain. Cells derived from the tumor, however, contained a rearranged mos sequence. Presumably, this rearrangement accounts for the absence of p30 in the explant population. p185 was not detected in the pool or its explant. The low level of cotransfection of pMI with pSV2gpt in the pool would be consistent with this and would predict that an even lower proportion of the cells would be likely to pick up both plasmids.

To simplify interpretation of the results found with transfectant pools, single colony clones were taken from SV80 pM1-1 + 2 at 12 months of culture. Approximately 30% of the clones were positive for MSV p30 using the immunofluorescence test, some at 100% of the cells stained and some at lower percentages. Five clones tested had unrearranged pM1 sequences detected by Southern analysis. All but one of these, SNS 12, which was rapidly tumorigenic, showed a low or undetectable level of tumorigenicity similar to the parent pool. In all cases tested (all five subclones and SV80 pM1-1), FeLV infection enhanced tumorigenicity.

Explants derived from five tumorigenic SV80 lines carrying the mos proviral gene following transfection or infection were examined for alterations in the

size or amount of immunoprecipitable SV40 T antigen. When compared to pre-implant cells, no differences were seen in the size of the T antigen. The amount of T antigen precipitated from these lines varied somewhat but without correlation to passage in nude mice. This suggests that the SV40 T antigen in SV80 cells is not immunologically selected against during tumor formation and thus is not a factor precluding the use of these cells in tumorigenicity assays.

Mos-Specific RNA in SV80 Transfectants.

Our results indicate that, like MSV infection, transfection with cloned MSV also transforms SV80 cells. However, not all pools or clones carrying unrearranged pM1 sequences are tumorigenic. This would be consistent with a need for a higher level of mos expression for transformation than is found in all cases tested.

To confirm the presence of mos-specific RNA in the transfectants and to compare levels of expression, RNA was prepared from SV80 pM1-1, pM1-2 and pM1-1 + 2 at 2, 2 and 12 months of culture, respectively. The RNA was electrophoresed in formaldehyde-agarose, transferred to nitrocellulose and probed using a mos-specific probe.

SV80 pM1-1 + 2 had roughly equivalent levels of mos-specific RNA, given that 10 and 5% of their cells were positive for p30 staining, respectively, and assuming that all transfected cells are detected in the fluorescence assay. Though these pools were not equivalently tumorigenic, they both formed tumors. In contrast, SV80 pM1-1 + 2 showed a much reduced, though still detectable, level of mos-specific RNA, despite the high proportion of the cells stained with the p30 antiserum. These cells were not detectably tumorigenic. This analysis suggests a loose correlation between the level of mos-specific RNA and tumorigenesis.

Tumor Formation Selects an SV80 Subpopulation Carrying mos.

As mentioned, SV80 p185/pM1 cells formed a tumor which clearly showed selection of a subpopulation carrying new mos sequences. pM1-2 showed 5% p30-positive before injection but >90%, probably 100%, in the tumor explant. The proportion of cells infected with MSV in SV80-FeLV/M1 was 1% before injection and 50% in one explant, >90% in another. (In this first example 50% of the cells were also negative for p30 and SV40 T antigen. The significance of this observation is being analyzed further.) These results suggest that tumor formation selects cells which express p30 and which therefore, presumably, carry the pml mos gene. It also supports the contention that mos contributes to the tumorigenicity of SV80 cells.

Oncogene RNA expression in tumorigenic segregants from non-tumorigenic hybrids between HeLa and normal human fibroblasts.

As an alternative approach to studying mechanisms of transformation of hybrids of HeLa and normal human fibroblasts, we are collaborating with H. Klinger to determine if such hybrids and their tumorigenic derivatives display patterns of oncogene expression correlating with tumorigenicity. The cell lines were provided by H. Klinger.

Tumorigenic clones were obtained by plating cells in 8-azaguanine to select cells losing a number of chromosomes, including the X chromosome carrying the expressed HPRT gene from the normal parent. One hypothesis explaining the tumorigenic nature of the segregants thus obtained is that some of the hybrids lose chromosomes carrying suppressors of the tumorigenic phenotype. These cells may then express previously silent oncogenes. Total polyadenylated RNA was prepared from each line at confluence and examined by Northern analysis for expression of a variety of onc genes. The amounts of RNA in the lanes could be compared following the successive use of each blot with a number of probes, usually including myc and/or actin probes.

The expression pattern or level of three of the oncogenes tested, fos, ets, and myb, appeared to correlate with tumorigenicity.

Fos expression was clearly higher in the tumorigenic hybrids than in the non-tumorigenic hybrids, whereas the D98 parent was low-intermediate in fos expression. It is not necessary that the same mechanism of transformation should be acting in the segregants as is active in D98. Thus, the correlation between fos expression in the hybrids and tumorigenicity indicates that such expression may be involved in transformation of the segregants. It should be noted that differences in detected levels of mRNA can be caused by transcriptional control or by mRNA half-life.

A number of ets messages were found. The largest of these was found in all lines. The smaller mRNAs were found to be present at a higher level in the tumorigenic lines.

The levels of fos protein in four members of the panel (two tumorigenic and two non-tumorigenic) were assayed using immunoprecipitation of ^{35}S-methionine labeled proteins. The levels detected closely resembled the levels of fos-specific RNA in these hybrids.

One non-tumorigenic hybrid was infected with gibbon ape leukemia virus (GALV) and with murine sarcoma virus (MSV) pseudotyped with GALV. The GALV/MSV-infected cells were rapidly tumorigenic in nude mice, while the GALV-infected cells remained non-tumorigenic. RNA prepared from a population of the former cells in which approximately 50% of the cells were infected with MSV showed that MSV transformation did not alter the levels of fos, ets or myb RNAs.

Significance to Biomedical Research and the Program of the Institute:

SV80 cells represent an immortalized human cell line which can be converted to a tumorigenic phenotype by retroviral infection and, as we have demonstrated, by the transfection with the cloned oncogene, v-mos. The relative inefficiency to tumorigenic growth of pml-transfected cells suggests, however, that multiple, unidentified factors are involved and that the capacity for continuous growth and the expression of a dominant transforming gene like v-mos are not sufficient in this system to lead to efficient tumor formation in the athymic nude mouse. The development of cell systems where the effects of different oncogenes can be compared should allow the elucidation of steps which are common to all tumor formation and which, if any, are specific to the action of specific oncogenes.

The role of normal genes in the suppression of the transformed phenotype is of great significance if we hope to determine how a normal cell progresses towards tumorigenicity. Analysis of tumorigenic and non-tumorigenic segregants of HeLa-normal cell hybrids for the role of known oncogenic sequences may elucidate the mechanism of transformation in this system. These molecular analyses complement detailed karyotypic analysis on the same cells, which is in progress in H. Klinger's lab. Potentially, this system can provide information on how suppressors may affect onc gene expression, an area of great interest which is very difficult to approach. It also may be possible to identify the sequences on cloned proto c-onc genes responding to regulation by introducing variously mutated genes into cells normally repressing their expression.

Transcription of the fos gene and translation of its RNA are among the first events known to occur following serum stimulation of cultured cells. These observations, coupled with its conservation at the DNA sequence level among different species and its expression in a variety of normal tissues, suggest a basic role for the protein in cellular functions. Thus, this gene is of particular interest. The system described here represents an example of aberrant fos-specific RNA-level control. It, therefore, presents an opportunity to study these processes and perhaps gain a greater understanding of normal regulation of this gene.

Proposed Course:

We will examine mos-specific RNA expression in a series of transfectants of low and high tumorigenicity and in their explants, all of which are already in our hands. This should determine if a high level of mos-specific RNA correlates with tumorigenicity.

Attempts will be made utilizing peptide antibodies against the mos gene product to correlate the level of gene product with the tumorigenic phenotype.

Attempts will be made to isolate mos segregants from the transfected, tumorigenic cell lines. These can be used to determine if such cells have undergone other genetic changes which make them more sensitive to mos transformation.

Further transfections will be performed involving in vitro ligated combinations of pSV2gpt, v-myc, E1a, T24 and VH02 (a recombinant of v-mos and human c-mos) using SV80 and ESH20 cells as recipients. Protoplast fusions of these and other oncogenes will also be performed to determine if higher efficiencies of gene transfer can be obtained.

Transfections will be carried out to determine if SV80 cells can be used to detect the transfer of single copy selectable genes using drug selection and of single copy proviral mos genes using tumorigenicity assays.

Analysis of fos, ets and myb expression patterns will be continued. The effect of cell growth and confluency on these patterns will be determined in order to separate proliferative effects from stable changes in the absolute levels of expression.

The cloned viral or cellular homologs of those oncogenes whose expression correlates with tumorigenicity will also be introduced into these normal parental cells and the non-tumorigenic hybrids to determine if expression of these sequences will lead to the appearance of a tumorigenic phenotype.

The serum-stimulated response in _fos_ transcription of serum-starved tumorigenic and non-tumorigenic hybrids will be examined for time of transcription, transcription rate and half-life of the message.

Alterations have been made in a cloned _fos_ gene to allow the differentiation of its message from the endogenous message. This construct will be introduced into tumorigenic and non-tumorigenic hybrids to determine if it responds to the cellular regulatory environment. If so, further alterations can be made to identify the sequences in the clone involved in regulation.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05359-02 LMO

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Characterization of the p21 ras Oncogene Product

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

PI:	S. Hattori	Visiting Fellow	LMO	NCI
Others:	T. Y. Shih	Research Chemist	LMO	NCI
	D. J. Clanton	Senior Staff Fellow	LMO	NCI
	L. S. Ulsh	Microbiologist	LMO	NCI
	Z. Q. Chen	Visiting Fellow	LMO	NCI

COOPERATING UNITS (if any)
Laboratory of Cellular and Developmental Biology, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, NIH (Dr. Suzanne Beckner); Program Resources Inc., Frederick, MD (Dr. Martin Zweig)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	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 v-ras-H oncogene, which is responsible for tumorigenesis, has been cloned into E. coli expression vector, and its gene product was purified in a large amount which is sufficient for biochemical and enzymological analyses. Purified protein showed full biochemical activities of GTP/GDP binding, autokinase and GTPase. In order to determine the active site of p21, the effects of antibodies against p21 and N-ethylmaleimide (NEM) were studied. Among six monoclonals, only Y13-259 inhibited p21 activities as much as 80%, which indicated that Y13-259 affected the active site. NEM inactivated p21 activities, indicating that cysteine residue is involved in the active site. The active site could also be specifically labeled by a photoaffinity analogue, P3-(4-azidoanilido)-5'GTP. It was found that monoclonal antibodies against p21 weakly cross-immunoprecipitated the G-protein of adenylate cyclase labeled by 32P-ADP-ribosylation, suggesting that either there might be weak interaction between p21 and G-protein or there might be structural homology between two proteins. New monoclonal antibodies were obtained after immunization of p21 expressed in E. coli into mice.

PROJECT DESCRIPTION

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

S. Hattori	Visiting Fellow	LMO	NCI
T. Y. Shih	Research Chemist	LMO	NCI
D. J. Clanton	Senior Staff Fellow	LMO	NCI
L. S. Ulsh	Microbiologist	LMO	NCI
Z. Q. Chen	Visiting Fellow	LMO	NCI

Objectives:

Recent progress in DNA-mediated gene transfer techniques (transfection) reveals that mutations in *ras* oncogenes are one of the causes of cellular transformation into a cancerous state. It is also shown that activated *ras* gene products show decreased GTPase activity. Therefore, it is of primary importance to characterize the biochemical activities of *ras* oncogene product (p21), including GTP/GDP binding, autokinase and GTPase. Our group developed the methods to determine the active site of p21 molecule. By changing the essential amino acid residue(s) by site-directed mutagenesis, we will be able to evaluate the role of GTP/GDP binding, autophosphorylation and GTPase activity of p21. These *in vitro* activities will be correlated with *in vivo* function when mutated genes are introduced into mammalian cells by transfection.

Methods Employed:

Binding assay with nitrocellulose, thin-layer chromatography, and SDS-gel electrophoresis are employed to analyze biochemical activities of p21. Immunoprecipitation and western blotting are for the detection of p21 in crude lysate. Tryptic peptide mapping by HPLC is used to isolate the peptide(s) which is essential for biochemical activitites. Site-directed mutagenesis is used to make various mutants which will be introduced into mammalian cells by transfection.

Major Findings:

1. Purification and characterization of p21 expressed in E. coli.

The v-*ras*^{H} oncogene was cloned into *E. coli* expression vector, and p21 was expressed at a high level as a fusion protein. p21 was purified by column chromatography on DEAE-Sephacel and Sephadex G-150. Purified p21 showed biochemical activities of GTP/GDP binding, autokinase and GTPase. Dissociation constants for GTP and GDP were determined as 0.83×10^{-8} M and 1.0×10^{-8} M, respectively; and there was a single class of binding site for both nucleotides as analyzed by Scatchard plots. At maximun binding, the stoichiometry of the complex is one nucleotide bound per p21 molecule. Turnover numbers for autokinase and GTPase were 0.33 mmol Pi incorporated per mol, p21 per min and 0.2 mmol Pi released per mol p21 per min. Phosphoamino acid analysis showed that the same threonine residue is phosphorylated as that of the p21 expressed in mammalian cells.

2. Characterization of the active site of p21.

To determine the active site of p21, the effects of monoclonal antibodies against p21 and the cysteine modifying reagent, N-ethylmaleimide (NEM), on enzymatic activities of p21 were studied. Among six monoclonal antibodies tested, only Y13-259 was capable of inhibiting GTP binding and autokinase as much as 80%, which indicated that Y13-259 affected the active site. Other antibodies were slightly stimulatory due to their protective effect against thermal inactivation. NEM inactivated p21 activities which indicated that the cysteine residue(s) is involved in the active site. p21 was protected from inactivation by NEM when p21 was preincubated with GTP. The GTP binding site could also be specifically labeled by a photoaffinity analogue, P^3-(4-azidoanilido)-5'GTP. These findings will allow us to map the active site within the p21 molecule.

3. Identification of cellular target proteins of the p21.

It is of importance to identify the cellular protein(s) which may interact with p21, because there is some indirect evidence which indicates that p21 may regulate the function(s) of the cellular protein(s). Among cellular hormone receptors and regulatory proteins, there are extensive similarities in biochemical properties of G-protein of adenylate cyclase and p21. We showed that ^{32}P-ADP-ribosylated G-protein could be immunoprecipitated by monoclonal antibodies against p21, which indicated that either there might be interaction between p21 and G-protein or there might be structural homology between two proteins.

4. Development of new monoclonal antibodies against p21.

Purified p21 was used to immunize mice in order to get a series of useful monoclonal antibodies against p21. Characterization of these antibodies are in progress.

Significance to Biomedical Research and the Program of the Institute:

The p21 *ras* oncogene products are shown to be associated with various human cancers. Recently, it was also suggested that p21s are involved in signal transduction from growth hormone receptors. Further characterization of biochemical properties of the p21 will give us more insight into their physiological function in cellular growth control and the mechanism of cellular transformation.

Proposed Course:

Characterization of biochemical properties of p21 purified from *E. coli* will be continued for a thorough description of its biochemical function. Comparative studies among various mutants made by site-directed mutagenesis will be carried out to clarify the difference between normal and activated gene products. The active site of p21 will be determined by the approaches described above. These *in vitro* properties will be correlated with *in vivo* function by introduction of these mutated genes into mammalian cells by transfection.

Publications:

Hattori, S., Ulsh, L. S., Halliday, K. and Shih, T. Y.: Biochemical properties of a highly purified v-*ras*^{H} p21 protein overproduced in *E. coli* and inhibition of its activities by a monoclonal antibody. *Mol. Cell. Biol.* (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05360-02 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Studies on Retroviral and Cellular Oncogenes

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

PI:	U. Rovigatti	Visiting Scientist	LMO	NCI
Others:	J. P. Bader	Research Microbiologist	LMO	NCI
	D. K. Watson	Senior Staff Fellow	LMO	NCI
	N. Sacchi	Visiting Fellow	LMO	NCI
	D. G. Blair	Research Chemist	LMO	NCI
	N. C. Kan	Visiting Associate	LMO	NCI
	T. S. Papas	Acting Chief	LMO	NCI

COOPERATING UNITS (if any) Department of Pathology and Medicine, University of Minneapolis, Minneapolis, MN (J. J. Yunis); Department of Genetics, Mt. Sinai School of Medicine, New York, NY (F. Gilbert); Department of Molecular Biology, University of California, Berkeley, CA (P. H. Duesberg)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	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 are pursuing our studies of oncogene involvement in neoplastic transformation and in cellular differentiation. Toward this goal, we have analyzed *in vitro* transformation of cell cultures and alterations of proto-oncogenes in animal and human tumors. Transformation of embryo cells *in vitro* by MH2 virus has been used as a model system for understanding the differential functions of the two oncogenes contained in this virus (v-*mht* and v-*myc*). A complete MH2 provirus containing all the LTR regulatory elements has been constructed by molecular genetics techniques. This provirus (L5-MH2) is now efficiently used in transfections of chick embryo fibroblasts for the production of an infectious transforming virus. Deletion mutants have been obtained from L5-MH2 which contain one oncogene only (either v-*mht* or v-*myc*). Their differential effects on chick embryo fibroblasts *in vitro* are an interesting correlate of the *in vivo* tumorigenicity of the MC-29 family of viruses.

Three human proto-oncogenes homologous to avian retroviral oncogenes have been studied in animal and human tumors. While no alterations have been detected so far in the human c-*mht* structure, interesting abnormalities in c-*myc* and c-*ets* appear to be present in animal and human neoplasias. We have discovered amplification of c-*myc* in a mouse lymphoma and in a human neuroectodermal tumor. In both cases, c-*myc* amplification might be correlated with tumor progression more than with the onset of the disease. Alterations of the c-*ets* proto-oncogene might be even more interesting because of the peculiar association of viral *ets* with myeloid and erythroid leukemias. Rearrangements and amplification of c-*ets* have been discovered in human leukemias.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

U. Rovigatti	Visiting Scientist	LMO	NCI
J. P. Bader	Research Microbiologist	LMO	NCI
D. K. Watson	Senior Staff Fellow	LMO	NCI
N. Sacchi	Visiting Fellow	LMO	NCI
D. G. Blair	Research Chemist	LMO	NCI
N. C. Kan	Visiting Associate	LMO	NCI
T. S. Papas	Acting Chief	LMO	NCI

Objectives:

To study which role viral oncogenes or cellular proto-oncogenes play during neoplastic transformation or cellular differentiation. We have developed a transfection assay system in order to test in vitro transformation potential of the reconstructed complete MH2 provirus and of deletion mutants obtained from this clone. A strategy has been developed for the identification and study of structural changes in retroviral proto-oncogenes in animal and human tumors. Changes like proto-oncogene rearrangements and/or amplification are now studied for their possible role during development and neoplasia.

Methods Employed:

A combination of molecular genetics and transfection assay techniques allow us to study transformation potential of different retroviral constructs in primary cultures of chicken embryo fibroblasts. Wild type and deletion mutant constructs have been obtained by in vitro manipulation of partially digested clones. We took advantage of plasmids which confer specific drug resistance (such as neomycin resistance) in order to select for transfected cells and therefore be able to study retroviral constructs with unpredictable and unselectable effects. Our previous expertise in the analysis of immunoglobulin gene rearrangement has allowed us to extend this analysis to transfected viral oncogenes and cellular oncogene abnormalities. Southern blotting analysis has been extensively utilized in the study of proto-oncogene rearrangement or amplification which may be associated with neoplastic transformation and/or cell differentiation.

Major Findings:

Our method of identification and ligation of partially digested molecules has led to the construction of a fully transforming MH2 provirus which is capable of reinfection and contains both LTRs. From this molecule, we have been able to isolate deletion mutants containing only one of the two oncogenes present in MH2 (either v-mht or v-myc). Our new method of cotransfection with a selectable marker into primary cultures of chicken embryos allows us to increase the sensitivity of detection and to identify transfected colonies with no dramatic morphological changes. Our v-myc containing constructs induce the formation of monocyte-macrophage colonies, while v-mht containing molecules seem to

have a less dramatic effect on cell morphology and may affect proliferation properties of chicken embryo cultures. Southern blotting analysis demonstrates the presence of different constructs in the transfected chick embryo cells. Detection of oncogene abnormalities in tumor cells has been obtained by different means: we have employed both direct Southern blotting analysis of tumor DNA samples and transfection assays into NIH3T3 cells. We have discovered amplification of the c-myc proto-oncogene in a mouse lymphoma and in a human primitive neuroectodermal tumor. There is, so far, no correlation between abnormalities detected by Southern blotting analysis and oncogenes identified by the NIH3T3 transfection assay. Rearrangement and amplification of the c-ets locus has been identified in different types of human neoplasias.

Significance to Biomedical Research and the Program of the Institute:

About 20 years ago, at this Institute, oncogenes were postulated for the first time to play a fundamental role in carcinogenesis. Today, even after retrovirus studies and molecular genetics techniques have provided several strong indications for their possible involvement in cancer and differentiation, we still lack complete evidence that proto-oncogene alterations are necessary and sufficient causes of neoplastic transformation. Our effort in studying retroviral oncogenes is directed specifically toward avian oncogenes such as myc, mht and ets, since little is still known about their function(s) and effect(s) in malignancy or normal development. A particular aspect of this search is the connection between chromosomal abnormalities and oncogene abnormalities in cancer cells. Finally, these studies can provide a better understanding of the longly-hypothesized involvement of cellular proto-oncogenes in development and differentiation.

Proposed Course:

The obtainment of a transfection assay for different retroviral constructs allows us to analyze particular deletion, point mutations or substitution mutants of the MH2 provirus, or any other retrovirus clone. Our Southern blotting analysis will be associated with studies of retroviral gene expression in the transfected cells. For these studies we can take advantage of specific antisera and monoclonal antibodies against purified viral products, artificially synthesized peptides corresponding to known portions of viral or cellular genes and portions of viral or cellular genes expressed in bacterial vectors. Several of these antisera and monoclonals have been obtained in LMO.

Our analysis of proto-oncogene abnormalities in human tumors has been extended to studies of oncogene expression and--especially in the case of oncogene amplification where higher amounts might be expected--to protein studies. Genomic abnormalities in proto-oncogene structures will be analyzed in detail by cloning the amplified and/or rearranged sequence. In the case of chromosomal translocations, particular attention will be focused on the analysis and characterization of chromosomal breakpoints which may provide hints to the mechanisms(s) of proto-oncogene activation or deregulation.

Publications:

Kitchingman, G., Rovigatti, U., Mauer, A., Melvin, S., Murphy, S. and Stass, S.: Rearrangement of immunoglobulin heavy chain genes in T cell acute lymphoblastic leukemia. Blood 65: 725-729, 1984.

Papas, T. S., Kan, N. C., Watson, D. K., Flordellis, C., Lautenberger, J. A., Psallidopoulos, M. C., Rovigatti, U. G., Samuel, K. P., Ascione, R. and Duesberg, P. H.: Two oncogenes in avian carcinoma virus MH2: myc and mht. Anticancer Res. (In Press)

Papas, T. S., Kan, N. C., Watson, D. K., Lautenberger, J. A., Flordellis, C., Samuel, K. P., Rovigatti, U. G., Psallidopoulos, M. C., Ascione, R. and Duesberg, P. H.: Myc, a genetic element that is shared by a cellular gene (proto-myc) and by viruses with one (MC29) or two (MH2) onc genes. In Furmanski, P., Hager, J-C. and Rich, M. A. (Eds.): RNA Tumor Viruses, Oncogenes, Human Cancer and AIDS: On the Frontiers of Understanding. Boston, Martinus Nijhoff Publishing (In Press)

Papas, T. S., Kan, N. C., Watson, D. K., Lautenberger, J. A., Flordellis, C., Samuel, K. P., Rovigatti, U. G., Psallidopoulos, M., Ascione, R. and Duesberg, P. H.: Oncogenes of avian acute leukemia viruses are subsets of normal cellular genes. In Neth, R., Gallo, R. C., Greaves, M. F., Moore, M. A. S., Winkler, K. (Eds.), Modern Trends in Human Leukemia V. Wilsede, Germany, Berlin/Heidelberg, Springer-Verlag (In Press)

Rothberg, P. G., Erisman, M. D., Diehl, R. E., Rovigatti, U. G. and Astrin, S. M.: Structure and expression of the oncogene c-myc in fresh tumor material from patients with hematopoietic malignancies. Mol. Cell. Biol. 4: 1096-1103, 1984.

Rovigatti, U. G. and Astrin, S. M.: Cellular oncogenes: Enhancement of their expression in animal and human tumors. In Pearson, M. L. and Sternberg, N. L. (Eds.): Gene Transfer and Cancer. New York, Raven Press, 1984, pp. 207-218.

Rovigatti, U. G., Mirro, J., Kitchingman, G., Dahl, G., Ochs, J., Murphy, S. and Stass, S.: Heavy chain immunoglobulin gene rearrangement in acute nonlymphocytic leukemia. Blood 63: 1023-1027, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05361-02 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Cell Interaction, cAMP and Control of Developmental Gene Expression

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

PI:	M. Oyama	Visiting Fellow	LMO	NCI
Others:	D. D. Blumberg	Senior Staff Fellow	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Molecular Control and Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

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 cellular slime mold, Dictyostelium discoideum (NC4), grows as a single-celled amoeba. After food is exhausted, the cells aggregate forming a multicellular organism in which two predominant cell types, prespore and prestalk cells, differentiate. Utilizing the appearance of specific antigens and enzyme activities, it was previously shown that preaggregating cells cannot form clumps and cannot differentiate into prespore cells under liquid shaking culture conditions in media containing glucose-albumin-EDTA and cyclic AMP (GAC). Post-aggregating cells, however, can form clumps and differentiate into prespore and prestalk cells in GAC. Differentiation is dependent on cAMP and cell contact. We have investigated expression of cell type-specific mRNAs under these conditions. Preaggregating cells transferred into GAC did not express prespore or prestalk mRNAs. By contrast, aggregating cells transferred to GAC expressed large amounts of both prespore and prestalk mRNAs. When clump formation was inhibited by rapid shaking, aggregating cells in GAC expressed only very low levels of prespore and prestalk mRNAs. Addition of ammonium sulfate to GAC could partially restore prespore mRNA expression and completely restore prestalk type 1 mRNA expression without clump formation. These results show that expression of both prestalk and prespore mRNAs require a specific differentiation step which does not proceed in GAC media. Additionally, at least part of the effects of clump formation (cell contact) on prespore and all of the effects on prestalk cell differentiation can be replaced by addition of $(NH_4)_2SO_4$.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

M. Oyama	Visiting Fellow	LMO	NCI
D. D. Blumberg	Senior Staff Fellow	LMO	NCI

Objectives:

The aim of these studies is to understand the mechanisms which control developmental gene activation during normal differentiation. A very simple model system, the cellular slime mold, Dictyostelium discoideum, is being employed.

The predominant feature of the developmental cycle of Dictyostelium discoideum is the aggregation of unicellular, free-living amoeba into a multicellular organism. Differentiation of amoeba within the newly-formed aggregates generates the three distinct cell types found in the mature fruiting body: spore cells, stalk cells and basal disks. Dictyostelium exhibits many features of development seen in more complex eukaryotic organisms. Specific cell-cell contacts are found, a homogeneous population differentiates into discrete cell types, and there is specific cell migration and pattern formation. These morphogenetic changes are accompanied by major changes in the pattern of gene expression. In particular, post-aggregation Dictyostelium cells contain 2,000-3,000 new messenger RNA species that are absent from earlier preaggregation stage cells. These new aggregation-dependent sequences compose 30% of the mass of the messenger RNA in the late cells and, together with their heteronuclear RNA precursors, represent the transcription products of an additional 26% of the single copy portion of the genome.

The initiation of transcription on this portion of the genome is dependent upon cell-cell interaction. Additionally, both the rate of transcription and subsequent stability of the mRNAs transcribed off of this portion of the genome are then further regulated by a cAMP-mediated process.

The major objective of our work is to elucidate the molecular mechanisms by which these genes are coordinately activated and to understand the controls which regulate the transcription rate and stability of these mRNAs in response to environmental stimuli--in this case cell contact and cAMP. The object of the experiments described here is to better define the nature of the requirement for cell interaction and to establish conditions for the development of a transformation system which will allow us to reintroduce mutated forms of these genes back into cells in order to define, genetically, those sites and structures essential for both the initial activation of the genes in response to cell contact, as well as for their cAMP-mediated regulation.

Methods Employed:

Standard molecular cloning techniques are being employed to isolate and map recombinant DNAs encoding constitutively-expressed and developmentally-regulated genes. Levels of mRNA expressed at various stages in differentiation have been quantitated by hybridization of gel-separated mRNAs to individual cDNA clones encoding developmentally-regulated or constitutively-expressed mRNAs.

Major Findings:

A) The development of suspension culture conditions that permit cell differentiation--rationale and description.

Liquid shaking culture conditions have been used to further explore the nature of the cell-cell interaction event that, together with cAMP, is essential for synthesis and stability of prespore- and prestalk-specific mRNAs. The use of a liquid suspension culture has several specific advantages over the normal in vivo developmental program. The in vivo developmental program involves depositing starved cells upon a solid substratum where they aggregate together into the multicellular mounds necessary for expression of prespore- and prestalk-specific genes. Within these multicellular mounds, cells are not only induced to differentiate, but they must also choose between the prespore- or prestalk-specific pathway. This decision is governed by two factors: (1) by the interactions between adjacent cells and the location of a cell with respect to gradients of "morphogens" (positional information); and (2) by the percent of the cells entering a particular pathway (proportion regulation). In the absence of a biochemical definition, the term: multicellularity, proportion regulation, and positional information, remain merely descriptive of a phenomenon which may play a major role in determining which pathway of genes will be expressed. Since we have individual cloned genes for both prespore and prestalk pathways (see Project Number Z01CE05288-04 LMO), we wanted to develop a system which would allow us to better define the biochemical nature of these phenomena. Cells are harvested after an initial period of development (2-6 hours) on a solid substratum and dispersed into a liquid culture medium containing glucose, albumin, and cAMP, and are shook slowly. Utilizing the activity of spore- and stalk-specific enzymes and antigens, it has been shown that these cells will form small clumps and undergo prespore and prestalk differentiation, but only in the presence of cAMP. When shaken vigorously, the cells cannot form clumps and cannot undergo differentiation even in the presence of cAMP. These conditions allow one to add back to the rapidly shaken cell suspension various compounds or conditioned media to determine if the requirement for multicellularity can be replaced by elevated levels of diffusible compounds or other sorts of molecules, or whether the cells absolutely must be in physical contact. Secondly, these conditions allow us to search for factors which may push the cells preferentially into the prespore or prestalk pathway. This question is of particular interest since we have previously found that a class of prestalk mRNAs and proteins are expressed in both prespore and prestalk cells at an early stage in differentiation, but then are specifically shut off in the prespore cells and accumulate to a high level in the stalk cells. In order to study the molecular mechanisms controlling this switch, we need to obtain conditions where we can induce all of the cells in the population to enter one pathway or the other.

B) Characterization of pathway-specific cDNA clones.

cDNA clones previously identified as being specific for late development were hybridized to filter blots of gel-separated poly A^+ RNA species prepared from prespore and prestalk cells which had been fractionated and purified on the basis of differences in their buoyant density on percoll gradients. Clones D7, D19, and A3 encode mRNAs specific for prespore cells, while clones C1 and D11 encode prestalk-specific mRNAs.

The analysis of the developmental time course of expression of the messenger RNA species, complementary to these cDNA clones, had initially been carried out in axenically-growing strains of Dictyostelium. While strains that are mutated to grow axenically (in the absence of bacteria) are easier to work with biochemically, they have several developmental abnormalities. First, vegetatively-growing axenic cells often express genes which are not normally expressed until the cells are starved and enter the first stage of the developmental cycle called interphase. Secondly, only about 50%-60% of the axenically-growing cells progress all the way through the developmental cycle. Many axenically-growing cells stop development at the aggregation and mound stages. By contrast, all of the bacterially-grown, wild-type (NC4) cells progress synchronously through the entire developmental cycle. In order to better define the nature of the cell type-specific controls regulating late gene expression, we wanted to utilize the wild-type, bacterially-grown strain. We therefore have reexamined the developmental time course of appearance of the messenger RNA species complementary to our collection of cDNA clones.

Several new and interesting points emerge. The most important of these is that the prestalk-specific messenger RNA species decrease very substantially in their abundance during the culmination stage of development. This decrease is seen at the time when the final differentiation of spore and stalk cells from the prespore and prestalk precursor cells is occurring. The original experiments with the axenically-grown cells showed these messenger RNAs remaining at high levels throughout this stage, presumably due to the large number of axenic cells which do not undergo culmination but arrest their development at the mound stage. The shutoff of expression of these messenger RNAs by culmination stage suggests that the expression of these genes is specific to the prepattern or slug stage and not to the terminal stages of stalk cell differentiation.

C) Identification of factors controlling cell type-specific gene expression.

Liquid shaking culture conditions have been used to identify factors controlling cell type-specific gene expression and to further explore the nature of the cell-cell interaction event, which together with cAMP is necessary for late gene expression. The use of a liquid suspension culture has several specific advantages over the normal in vivo developmental program. The in vivo developmental program involves depositing starved cells upon a solid substratum where they aggregate together into the multicellular mounds necessary for further development and expression of prespore and prestalk-specific genes. A very useful "in vitro" system which allows differentiation of the precursor cells into the prespore and prestalk cells in a liquid shaking culture has been developed by Okamoto (J. Gen. Micro. 127: 301-308, 1981). After an initial period of development on a solid substratum (2-6 hrs), cells are harvested and dispersed into a liquid culture medium containing glucose, albumin and cAMP, and are shaken slowly. Utilizing the activity of spore- and stalk-specific enzymes and antigens, it has been shown that these cells will form small clumps, and over a period of time, undergo prespore and prestalk cell differentiation, but only in the presence of cAMP. When vigorously shaken, the cells cannot form clumps and cannot undergo differentiation, even in the presence of cAMP. These conditions allow one to add back to the rapidly shaken cell suspension various compounds or conditioned media to determine if the requirement for multicellularity can be replaced by elevated levels of diffusible compounds or other sorts of molecules, or whether the cells absolutely must be in physical contact. Secondly, these conditions

allow one to search for factors which may push the cells preferentially into the prespore or prestalk pathway. Utilizing this system, we have been able to define the following series of events as being necessary steps in the pathway leading to prespore or prestalk specific messenger RNA expression.

D) Cells must pass through a specific period during early development in order to express both prespore and prestalk specific messenger RNAs.

Cells starved and plated for development for 2 hrs and then dispersed into the liquid suspension culture fail to differentiate. They do not synthesize any of the enzymes or antigens specific to prespore or prestalk cells, nor are they capable of forming into clumps in the culture (Okamoto *et al*.). These cells also fail to accumulate any of the prespore- or prestalk-specific messenger RNA species. By contrast, cells plated for development for 4-6 hrs (late rippling stage but prior to the formation of mounds) and dissociated into glucose-albumin media (GA) will form clumps and will express both prespore- and prestalk-specific messenger RNA species provided that cAMP is present. These experiments define a specific period in the early aggregation stage, between 2 and 6 hrs of development, during which an essential event occurs which is necessary for the synthesis of the prespore- and prestalk-specific messenger RNA species. This event occurs much earlier in the developmental cycle than the formation of the tight cell-cell contacts that our previous experiments defined as an essential step for expression of late messenger RNAs. This early aggregation stage event may represent the first step in a dependent series of events which must take place in order to express the prespore- and prestalk-specific messenger RNAs.

E) Expression of both prespore and prestalk messenger RNAs depend upon close cell association.

When preaggregation stage cells (6 hrs), capable of expressing the late messenger RNAs in glucose-albumin-cAMP slowly shaken cultures, are instead dispersed into the glucose-albumin-cAMP culture media and are shaken very rapidly so that the cells cannot form into clumps, the expression of both the prespore and prestalk messenger RNA species is severely reduced, even in the presence of cAMP. This experiment is essentially the converse of our original experiment which demonstrated that addition of cAMP to cells disaggregated from clumps could restore gene expression. Here we have shown that by preventing cell association from initially occurring, even in the presence of cAMP, we inhibit late gene expression. Thus, both cAMP and some aspect of the close cell association achieved in these clumps is essential for expresssion of both the prespore- and prestalk-specific messenger RNA species.

F) The nature of the cell association requirement differs for the prespore- and prestalk-specific messenger RNA species.

When the early aggregation stage cells, which can induce both prespore- and prestalk-specific messenger RNA in cell clumps in glucose-albumin-cAMP media, are instead dissociated into phosphate buffer, loose cell clumps also form. In the presence of cAMP, these cell clumps can induce expression of the prestalk-specific messenger RNAs but *not* prespore-specific messenger RNAs. Thus, a step necessary for prespore gene expression can occur in the glucose-albumin-cAMP suspension cell clumps which cannot occur over a similar period of time in the phosphate buffer-cAMP cell clumps. If cells are allowed to develop on filter

pads for a longer period of time, until they have reached the mound stage, and are then dispersed into the phosphate buffer plus cAMP media, they can now express the prespore messenger RNAs, as well as the prestalk messenger RNAs. Thus, an additional event that occurs sometime between the aggregation phase (6 hrs) and the mound stage (12 hours) is necessary for expression of the prespore-specific messenger RNAs, but is not necessary for expression of the prestalk messenger RNAs.

G) Addition of ammonium sulfate can completely eliminate the need for cell association for expression of prestalk specific messenger RNA species and can partially induce the expression of prespore messenger RNAs.

Early aggregation stage cells dispersed from filter pads into glucose-albumin suspension culture and shaken very rapidly in the presence of cAMP, produced little or no prespore messenger RNAs and reduced levels of prestalk mRNAs. Addition of ammonium sulfate to these rapidly-shaken suspensions can induce full levels of expression of the prestalk-specific messenger RNAs. These levels are equal to, or even exceed, the level of expression of these messenger RNA species in the slowly shaken glucose-albumin-cAMP cultures where large clumps of cells form. Thus, for the prestalk messenger RNAs ammonia ion or ammonia can replace the need for close cell association. For these messengers, the need for close cell association probably reflects the elevated levels of a diffusible substance like ammonia that is dissipated to too low a level when the clumps are disrupted. The prespore messenger RNAs show a small elevation above the background level in the rapidly shaken glucose-albumin-cAMP cultures when ammonia is added. While these messengers clearly respond to the ammonia, some additional aspect of the close cell interaction phenomena is still required for full levels of expression of the prespore messenger RNAs. Whether this result is due to ammonia directly, or whether it is due to a change in the ionic environment or a change in the intracellular pH as a result of accumulation of high levels of ammonia, is at this point unclear and under investigation. Ammonia, however, is a logical candidate for the role of a general inducer of differentiation since the energy for differentiation in *Dictyostelium* is derived through catabolic processes. During the early hours of development, one-half to two-thirds of the cellular protein is catabolized with the concomitant release of ammonia. The coupling of expression of the prespore and the prestalk messenger RNAs to elevated ammonia levels provides a means for coordinating developmental gene expression with the metabolic state of the cell. Not only can ammonia serve as a coupling between the metabolic state of the cell and the ability to express genes involved in the prespore and prestalk program, but elevated levels of ammonia have also been shown to provide the stimulus for the pseudoplasmodia or slug to migrate. Under conditions of elevated ammonia, one sees slug migration to areas where the ammonia levels are lower. Once the slug has reached an area of lower ammonia, the culmination process begins. Interestingly, it is during the slug stage, when these prestalk messenger RNAs are present at high levels, and during the culmination stage when the levels of these messenger RNAs decrease.

H) Prestalk but not prespore genes lose their dependence upon exogenously added cAMP for expression in suspension cultures:

If cells are harvested from filters at the slug stage, when they are expressing both prespore- and prestalk-specific messenger RNAs, and are dispersed into phosphate buffer in the absence of cAMP, the prespore-specific messenger RNAs are

rapidly lost from the population; addition of cAMP restores expression. However, the prestalk messenger RNAs are not lost and show no decrease in their level of expression on dispersal from the slug stage into phosphate buffer in the absence of cAMP. This is in marked contrast to their behavior at the mound stage when dispersal into phosphate buffer in the absence of cAMP results in a substantial decrease in the prestalk mRNAs. The presence of cAMP prevents this loss at the mound stage. However, at the slug stage, addition of cAMP to the phosphate buffer suspension has absolutely no effect, or even reduces slightly, the level of expression of the prestalk messenger RNAs. Thus, between the mound stage and the slug stage, the prestalk-specific messenger RNAs have undergone a change in their requirements for exogenously-added cAMP upon dispersal into the phosphate buffered suspension culture. This change in regulation of the prestalk messenger RNAs occurs at an interesting time in the developmental program. Work from Takeuchi's lab has demonstrated that cells in loose mounds differentiate into prespore or prestalk cells at random within the mound of cells and then sort out to form the linear prepattern seen in the slug, with the prestalk cells sorting to a position immediately behind the tip, and the prespore cells remaining in the posterior portion of the slug. The tip of the slug emits the pulses of cAMP during the early stages of development and the area immediately adjacent to the tip would be expected to have a much higher level of cAMP. It is just at the time when the prestalk cells would be sorting to their position immediately behind the tip that we see this change in the requirement for cAMP for expression of the prestalk-specific mRNAs. No such change is seen in the regulation of the prespore mRNAs.

Significance to Biomedical Research and the Program of the Institute:

Because of the unique features of its developmental program, *Dictyostelium* provides a powerful and simple system for exploring the mechanisms by which such phenomena as positional information, proportional regulation, and cellular interaction can control the expression of different groups of co-regulated genes during differentiation. We have identified a series of key components or events in the morphological progression of differentiation which affect the accumulation of tissue-specific messenger RNAs. In order to understand how these events exert their ultimate effect on the synthesis and stability of the tissue-specific messenger RNAs, it is necessary to identify the sites on the genes where they act and to use these sites, in turn, as probes for the factors that effect the regulation.

Our results additionally suggest a complex, regulatory circuit-controlling cell type-specific differentiation. Once cells pass into the early aggregation stage, ammonia and cAMP are sufficient for prestalk gene expression. Ammonia and cAMP are also required for prespore gene expression but a third factor is also essential. cAMP and ammonia only induce the prestalk messenger RNAs to a high level in the 20% of the cells that become the prestalk cells. What then is preventing their induction in the 80% of the cells that become prespore cells? Does the third factor, necessary for prespore gene expression, simultaneously repress expression of the prestalk messenger RNAs in prespore cells or are the ammonia and cAMP signals transduced through different "cellular receptors" to exert their effects on different batteries of genes in the two cell types? This duality of response to a common inducer is not unique to *Dictyostelium*, but is seen in many differentiating systems. For example, the phorbol esters induce differentiation in promyelocytic leukemia cells, e.g., HL60, and in keratinocytes, whereas they

inhibit differentiation in Friend erythroleukemia cells. The strength of the Dictyostelium system is the growing accessibility of these problems to molecular analysis.

Proposed Course:

Our future experiments will be directed toward utilizing these culture conditions in conjunction with a transformation system to reintroduce the control regions of these genes fused to the structural regions of genes encoding such easily assayable enzymes as chloramphenicol acetyl transferase (CAT) or -galactosidase. If we can get the cells to express CAT or -galactosidase in response to cAMP and ammonia, we will then be able to utilize in vitro mutagenesis to identify the sites on the genes where these controls act. In conjunction with the work that we are doing on the structural organization of these genes in chromatin, we would ultimately like to be able to define the nucleoprotein interactions involved in regulating the transcriptional activation of these genes and further define the mechanisms by which a cAMP-mediated process in the presence of high ammonia can coordinately regulate both the transcription rate and the stability of these messenger RNAs.

In order to pursue this direction, three component parts are necessary: a transformation system in which exogenous DNA can be reintroduced into the Dictyostelium cells, genomic clones that encode the prespore- and prestalk-specific messenger RNAs, as well as the adjacent control regions, and an appropriate vector for introducing and expressing exogenous DNA under the developmental culture conditions. Since several transformation systems for introducing DNA into Dictyostelium have been described (Hirth, Edwards and Firtel, Proc. Natl. Acad. Sci. USA 79: 7356-7360, 1982; Barclay and Meller, Mol. Cell. Biol. 12: 2117-2130, 1983; Edwards and Towne, personal communication), our work will be directed toward the latter two parts: identification and characterization of the appropriate genomic clones and the development of an appropriate vector system.

Construction of a genomic library and cloning of prespore and prestalk-specific genes. So far our analysis of controls regulating prespore and prestalk-specific gene expression, as well as the work on chromatin structure of these genes, has been carried out using cDNA clones. These are specific probes for the protein coding regions of the genes, but they do not contain the adjacent control regions. We are therefore constructing an EcoR1 partial library in gt WES B4 and plan to screen it with our various cDNA clones for the prespore and prestalk messenger RNAs. Since the Dictyostelium genome is comparatively small, 30,000 clones containing 10-15 kilobases of Dictyostelium, DNA would provide a 99% probability of our library containing any fragment of interest. We have verified that most of our cDNA clones hybridize to one, or at the most, two EcoR1 fragments which are between 1-9 kilobases in size, which is well within the capacity of the gt WES B4 vector. We hope to obtain genomic clones for several of the prespore messenger RNAs and the prestalk messenger RNAs, as well as one or two genomic clones encoding messenger RNAs which are constitutively expressed throughout growth and development to serve as nonregulated controls. Since the messenger RNAs encoded by our cDNA clones are small (1-2.5 kilobases) and since Dictyostelium genes are not known to have large introns, we feel that the isolation, mapping and sequencing of limited regions of these clones should not represent an overly ambitious effort.

Construction of an appropriate vector. One of the exciting recent developments has been the discovery of plasmids in *Dictyostelium*. Several different plasmids ranging in size from 5 kilobases to 27 kilobases have been isolated from the nuclei of bacterially-growing strains of *Dictyostelium*. These plasmids are present at 50 to several hundred copies per cell. We plan to clone one of the *Dictyostelium* plasmids into the cosmid vector pHSG274. This vector has the G-418 resistant genes under the control of the thymidine kinase promoter, as well as under the control of a bacterial promoter; thus, the cosmid confers kanamycin resistance in bacterial cells and G-418 resistance in eukaryotic cells. Barclay and Meller have previously shown that *Dictyostelium* cells can utilize the thymidine kinase promoter and be transformed to G-418 resistance. We will try to identify the smallest restriction fragment from the *Dictyostelium* plasmid which will allow the pHSG274 plasmid to be efficiently introduced, replicated and stably maintained in the *Dictyostelium* cells, both in the presence and absence of drug selection. *Dictyostelium* cells can be clonally isolated by plating on a lawn of bacteria. The *Dictyostelium* cells will eat the bacteria in their immediate vicinity and as they propagate, they will form a large circular clearing similar to a phage plaque. In order to rapidly assay transformation efficiency and to clonally isolate individual transformants, I have isolated an *E. coli* K12 mutant resistant to high levels of G-418. This mutation was selected to be a ribosomal mutation rather than a plasmid born TN5-like resistance which functions by degradation of the drug. Thus, it will be possible to transform the *Dictyostelium* cells with various fragments of the *Dictyostelium* plasmid cloned in the pHSG274 cosmid and plate the transformants out on a lawn of the G-418-resistant bacterial strain in the presence and absence of drug. This will give us a very rapid estimate of the transformation frequency obtained with different fragments cloned from the Dictyostelium plasmid. In this way we should be able to identify a fragment from the Dictyostelium plasmid that can confer autonomous replication and stable maintenance of exogenously added DNA. Using such a fragment cloned into the pHSG274 cosmid and supplemented with a poly linker, we should then have a suitable vector for introducing the control regions from our genomic clones encoding the prespore- or prestalk-specific messenger RNAs fused to either - galactosidase or CAT structural genes and be in a position to start to reconstruct the regulation of these tissue-specific genes in a transformation system.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05439-01 LMO

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Protein Chemistry of p21 Biosynthesis and Processing

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

PI:	Z. Q. Chen	Visiting Fellow	LMO	NCI
Others:	T. Y. Shih	Research Chemist	LMO	NCI
	L. S. Ulsh	Microbiologist	LMO	NCI
	S. Hattori	Visiting Fellow	LMO	NCI
	D. J. Clanton	Senior Staff Fellow	LMO	NCI

COOPERATING UNITS (if any)

National Heart, Lung and Blood Institute, NIH, Bethesda, MD, (Dr. John J. Pisano); Program Resources, Inc., Frederick, MD (Dr. Garrett DuBois)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	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 primary translation product (pro-p21) of p21, which is found in the cytosol, undergoes post-translational modification and the mature protein subsequently became associated with the inner surface of the plasma membrane and binds lipid tightly. We find that p21, overproduced in *E. coli*, is an equivalent of pro-p21 in mammalian cells. Comparative peptide mapping by high performance liquid chromatography (HPLC) or two-dimensional, thin-layer electrophoresis-chromatography (TLC) between 35S-cysteine labeled p21 from NRK cells transformed by Ha-MuSV and p21 in *E. coli* has shown direct evidence for p21 palmitylation at the C-terminal tetrapeptide, presumbaly through a thioester linkage with cysteine-186. Although p21 of Ha-MuSV transformed NRK cells can be metabolically labeled with either 3H-palmitate or 3H-myristate, the lipid moiety of the hydrophobic peptide is identified as palmitic acid. The mechanism of lipidation of p21 proteins appears to be different from N-terminal myristylation observed in p60 *src* and many other membrane-associated proteins.

PROJECT DESCRIPTION

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

Z. Q. Chen	Visiting Fellow	LMO	NCI
T. Y. Shih	Research Chemist	LMO	NCI
L. S. Ulsh	Microbiologist	LMO	NCI
S. Hattori	Visiting Fellow	LMO	NCI
D. J. Clanton	Senior Staff Fellow	LMO	NCI

Objectives:

The objective of this project is to elucidate the biochemistry of the p21 processing events. It will involve the identification of the nature and the site of fatty acid modification or cleavage of peptide, if there is any. The cellular enzyme systems involved in these processing events will also be explored.

Methods Employed:

The p21 of E. coli and Ha-MuSV transformed cells was labeled with ^{35}S-cysteine. For detecting the fatty acid modification, the p21 was also labeled with ^{3}H-palmitic acid or myristic acid. The labeled p21 was purified by immunoprecipitation with monoclonal antibody and the precipitated proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After autoradiography, the p21 bands were excised from the gel and were eluted. After digestion with trypsin, the peptides were resolved by HPLC or TLC. To identify the peptide modified by fatty acid, we synthesized a tetrapeptide (CVLS) according to the predicted C-terminal tryptic peptide of v-ras^{H} p21 of cys-val-leu-ser. (Genetic studies indicated that the site of acylation of p21 by fatty acid is related to 186 cysteine.) Peptides of tryptic digests, recovered after hydroxylamine cleavage of attached fatty acids, were analyzed by high performance liquid chromatography (HPLC).

Major Findings:

1. The p21 overproduced in E. coli is not labeled by ^{3}H-palmitate as is p21 in Ha-NRK cells. E. coli p21 is not processed like p21 in mammalian cells, as shown by short pulse-labeling experiments. There is no change in the mobility of E. coli p21 over time. Therefore, E. coli p21 serves as a convenient equivalent of pro-p21 for comparison of its peptide map with p21. The E. coli p21 is a fusion protein (24K daltons) with the 4 N-terminal residues of v-ras p21 replaced by 14 residues from the expression vector, but there is no cysteine residue in these sequences to complicate the labeling experiments.

2. In comparative peptide mapping by HPLC between ^{35}S-cysteine-labeled p21 from NRK cells transformed by Ha-MuSV and p21 overproduced in E. coli, we have observed a small peptide in E. coli p21, which is absent in Ha-MuSV p21. Instead, the Ha-MuSV p21 displays a very hydrophobic peptide which is double-labeled with ^{3}H-fatty acid, in addition to ^{35}S-cysteine. The unique E. coli peptide co-chromatographs with synthetic CVLS by HPLC. The identity of the hydrophobic peptide was established by hydroxylamine cleavage. The recovered

peptide also co-chromatographs with CVLS. The p21 acylation site at the C-terminal tetrapeptide presumably is through a thioester linkage with cysteine-186.

3. The fatty acid of the acylated C-terminal tetrapeptide is identified as palmitic acid, not myristic acid, irrespective of in vivo labeling either with ^3H-palmitate or ^3H-myristate.

Significance to Biomedical Research and the Program of the Institute:

The post-translational processing event apparently is very important for the transforming function of the p21. Also the same biosynthetic pathway is shared by viral and cellular p21. Elucidation of the precise nature of this event not only will help us to understand the molecular mechanism of the p21 transforming function, but will also present us a possible step for future intervention of the p21 transforming activity.

Some oncogene proteins, such as p60 src, and several other cellular proteins have recently been shown to be myristylated at their N-termini through amide linkages. Palmitylation of p21 through a thioester linkage near the p21 C-terminus may probably represent a different mechanism for post-translational lipidation of proteins.

Proposed Course:

Not only is biosynthesis and processing of p21 involved in the structure of the p21 protein, but protein structure also is extremely important for its function. Mutagenesis of the p21 oncogene has shown that oncogenic substitutions of 12th or 59th amino acid residues of normal p21, encoded by proto-oncogene, drastically decreases its GTPase activity. Mutated p21 molecules with reduced GTPase activity would be inherently more potent in transformation than normal p21. We will continue to use the oligonucleotide-directed site mutagenesis of the p21 oncogene to look for the mutants that have different biochemical properties (for example, GTPase, GTP/GDP binding, etc.) from normal p21. These comparative studies on the mutant p21 would be important for constructing the p21 molecular model and understanding the functions of p21 in the eukaryotic cell.

Publications:

Chen, Z. Q., Ulsh, L. S., DuBois, G. and Shih, T. Y.: Post-translational processing of p21 ras proteins involves palmitylation of the C-terminal tetrapeptide containing cysteine-186. J. Virol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05440-01 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Site-Directed Mutagenesis of ras Oncogenes

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

PI:	D. J. Clanton	Senior Staff Fellow	LMO	NCI
Others:	T. Y. Shih	Research Chemist	LMO	NCI
	S. Hattori	Visiting Fellow	LMO	NCI
	Z. Q. Chen	Visiting Fellow	LMO	NCI
	L. S. Ulsh	Microbiologist	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Oncology

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INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland, 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	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.)

Oligonucleotide directed site-specific mutagenesis is used to dissect the biochemical basis of oncogenic activation and of enzymatic activity of the ras oncogene. Studies are directed toward an understanding of the interrelationship between the known properties of the ras gene product, p21. These include guanine nucleotide binding, GTP hydrolysis activity, and an autokinase activity of the activated ras protein. Mutagenesis of the ras oncogene in specific regions of the protein have been designed to explore the active center which is believed to be responsible for these properties. It has been shown that replacement of the phosphoryl acceptor in p21 has a profound effect on GTPase activity and GTP binding. Mutations have also been created to study the role of GTP binding in oncogenicity.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

D. J. Clanton	Senior Staff Fellow	LMO	NCI
T. Y. Shih	Research Chemist	LMO	NCI
S. Hattori	Visiting Fellow	LMO	NCI
Z. Q. Chen	Visiting Fellow	LMO	NCI
L. S. Ulsh	Microbiologist	LMO	NCI

Objectives:

The objective of this project is to study the biochemical basis of oncogenic activation and of enzymatic activity of the ras oncogenes and their products by site-directed mutagenesis. Mutant proteins developed in this way can be expressed in a bacterial expression system and the comparative biochemistry of the mutant proteins studied. The oncogenicity of the mutant viruses thus generated can be assessed by *in vitro* NIH3T3 cell transfection and by *in vivo* tumor formation.

Methods Employed:

Two methods have been developed in this laboratory for oligonucleotide-directed, site-specific mutagenesis. The first method (McFarland *et al.*, PNAS 79: 6409-6413, 1982) has been to directly mutagenize the bacterial plasmid, pH-1, which contains the full genome of Harvey murine sarcoma virus. The plasmid is nicked by a restriction endonuclease and partially digested with exonuclease III to generate partial duplex molecules which are then hybridized to synthetic oligonucleotides containing a single base mismatch. The plasmid is repaired with polymerase I and transfected into *E. coli* cells for replication of the mutated plasmid DNA. The second method for mutagenesis has utilized the M13 bacteriophage system (Zoller, M.J. and Smith, M., DNA 3: 479-488, 1984). A fragment of the murine sarcoma virus genome which contains the oncogene for *ras* has been cloned into M13 DNA. Mutagenesis by synthetic oligonucleotides is performed on the single-stranded genome of M13. The molecule is repaired as in the first method and transfected into *E. coli* strain JM 103. Phage plaques are then isolated and tested to verify the mutation. A number of other laboratory techniques are utilized for these studies. The development of site-specific mutations requires the use of DNA hybridization techniques and DNA sequencing methods (both Maxam and Gilbert and the Sanger methods are employed). The mutated *ras* genes are isolated by gel electrophoresis and cloned into a bacterial expression system developed for these genes. Proteins expressed by this system are purified through column chromatography and analyzed by polyacrylamide gel electrophoresis. Biochemical studies of the mutated *ras* proteins employ filter binding assays and immunoprecipitation by specific monoclonal antibodies. Oncogenicity is measured by transfection of NIH3T3 cells and tumor formation in mice.

Major Findings:

The use of oligonucleotide-directed, site-specific mutagenesis has successfully generated a number of valuable mutations of the *ras* gene protein, p21. In order to better understand the interrelationship between guanine nucleotide binding, GTPase activity, and autophosphorylation, mutant p21 viral proteins were

initially constructed which replaced the threonine residue at position 59 with serine (thus differing from the viral protein by a single methyl group) and alanine as in the normal c-ras counterpart. Threonine serves as the substrate for phosphate from GTP by an intramolecular autophosphorylation. We have found that the serine mutant is able to undergo autophosphorylation, but at a level 10 times lower than the normal viral protein. The serine mutation decreases the amount of autokinase activity by an amount inversely proportional to GTP hydrolytic activity. The importance of amino acid 59 to GTPase activity is demonstrated by replacing threonine with alanine. This mutation greatly stimulates GTP hydrolytic activity.

Significance to Biomedical Research and the Program of the Institute:

The p21 protein is a highly conserved protein in its structure. This indicates the importance of the protein structure to its function. Specific mutations at amino acids 12, 59, and 61 drastically activates its oncogenicity. These mutations also affect the p21 enzymatic activities such as GTPase and autophosphorylation. Site-specific mutagenesis on the ras gene and its expression in bacterial vectors will allow us to dissect, in great detail, the structure-function relationship of the p21 protein. This knowledge will be helpful in understanding the mechanism of oncogenic activation of the ras proto-oncogenes.

Proposed Course:

Recent studies have shown that guanine nucleotide binding by p21 might play a crucial role in oncogenicity. It is thought that cellular p21 may be a member of the guanine nucleotide-binding proteins, or G proteins, which can transduce signals from cell surface receptors that interact with factors regulating cell growth. Amino acid sequence homology exists between certain G proteins and the ras gene product. It should be possible to accurately map the amino acid(s) necessary for GTP binding through site-specific mutagenesis of the ras gene. By transfecting these mutated genes into NIH3T3 cells, we can test, directly, the role that GTP binding plays in cell transformation. Also of interest are the p21 processing sites. Attempts will be made through site-specific mutagenesis to precisely delineate these processing sites.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05441-01 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Characterization of the Gene Product of the c-myc Locus

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

PI:	R. J. Fisher	Expert	LMO	NCI
Others:	T. S. Papas	Acting Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Nucleic Acids Protein Synthesis Laboratory, Program Resources, Inc., Frederick, MD (M. Zweig and S. Showalter); Laboratory of Cell and Molecular Structure, Program Resources, Inc., Frederick, MD (M. Gonda)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

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

The protein(s) encoded by the c-myc locus have been examined in specific cell lines and will be investigated in those cells which have their myc gene activated by amplification or by chromosomal translocation. To probe these myc protein(s), polyclonal antibodies have been prepared against peptides representing putative amino acid sequences deduced from the DNA sequence of the myc exons. The myc protein, biosynthetically labeled with radioactive amino acids, has been highly purified by immunoaffinity chromatography. To determine where in the myc exons the protein corresponds, the N-terminal amino acid sequences of myc protein are being analyzed. Other methods for the purification of the myc protein using HPLC are being developed to maintain the myc in its native state. It is important to isolate myc in its non-denatured state so that its molecular weight, subunit structure, location in the cell, and DNA binding properties may be determined.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPITON

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

R. J. Fisher	Expert	LMO NCI
T. S. Papas	Acting Chief	LMO NCI

Objectives:

The objective of the proposed research is to purify and characterize the protein product of the human cellular proto-oncogene, c-myc. According to the progress of this work, we will define the direction in which to look for the function of the human c-myc protein.

Methods Employed:

Several specialized human cell lines are being used in these studies: a clonal culture of human foreskin and a human cell line colo 320 strain, both being maintained by in vitro passage. Cells are routinely labeled using S^{35}-cysteine, methionine or C^{14}-arginine and subfractionated by differential centrifugation procedures. Routine preparations involve separations of nuclei from cytoplasmic fractions and utilization of the specific immunoaffinity selection procedure; immunoblot and immunoaffinity chromatography to defect anti-myc epitopic components. Proteins are identified and purified by polyacrylamide-SDS-gel-electrophoresis and fast protein liquid chromatography (FPLC), a high pressure liquid chromatographic procedure. Proteins are cyanogen bromide treated and resultant fragments isolated by reverse phase HPLC. Use of site-specific fluorescent-labeled antibodies developed against putative amino acid sequences of myc, in conjunction with inhibitors of cell division, enables the cellular localization and unique distribution of c-myc products to be visualized by UV photomicrography.

Major Findings:

Since demonstrating that there are human homologs to the viral myc sequence (Eva, A., et al., Nature 295: 116-119, 1982), there has been considerable effort directed toward understanding the role of myc in cellular transformation. Attention has focused on the regulation of the c-myc gene because the expression of the c-myc may be under cell cycle control (Campisi, J., et al., Cell 36: 241-247, 1984; Kelly, K., et al., Cell 35: 603-610, 1983). In Burkitt's lymphomas, there is a translocation of myc sequences from chromosome 8 to the immunoglobulin heavy-chain region on chromosome 14 (Gelmann, E. P., et al., Nature 306: 799-803, 1983) leading to the deregulation of the gene. The loss of cycle control by translocation of the myc gene or by amplification of the myc gene is important in the activation of myc's oncogenic potential. It is generally held that the regulation of the myc gene was somehow more important than the myc gene product in the transformation process because there are no obvious changes in the protein.

The first exon of Hu-c-myc is not thought to be expressed because it contained many translational stop signals in all of its reading frames (Staton, L. W., et al., Nature 305: 401-406, 1983). Thus, it was inferred that the myc translational initiation codon started at exon 2. However, Papas, T. S., et al., Cancer Cell 2: 153-160, 1984 and Gazin, C., et al., EMBO J. 3: 383-387, 1984, have shown that the first exon indeed contains an open reading frame which could potentially code for a protein of 20,000 daltons. Furthermore, Psallidopoulos and Papas (unpublished results) have cloned the human c-myc first exon in an E. coli expression vector and demonstrated that this first exon open reading frame does indeed code for a protein.

To resolve some of these issues, it is of utmost importance to determine the N-terminal amino acid sequence of Hu-c-myc. In order to purify analytical quantities of c-myc and to detect the proteins(s) synthesized by the myc exons, we have developed a series of site-specific antibodies (Lerner, R. A., Nature 299: 592-596, 1982) against putative amino acid sequences within each of the 3 exons. These site-specific antibodies are used to probe the organization of the myc protein in the various myc overproducing and translocated cell lines available to us, such as colo 320 human cell line and human foreskin fibroblasts.

We have thus far isolated five variants of cellular myc product which react specifically against our epitope specific anti-myc probes. Thus far, by the immunologic cytoplasma analyses, components are uniquely compartmentalized and distributed from those of the nuclear-perinuclear complex. Further, N-terminal sequence analysis will resolve the molecular nature of these differences. Nuclear perinuclear c-myc, moreover, appears to copurify and coelectrophorese with an important cellular structural component which is not observable with the cytoplasmic c-myc product. Further elucidation of the nature of this interaction is needed to determine if a functional interrelationship for these components is biologically significant. Toward this end we have highly purified the 58 kilodalton c-myc protein by using a combination of reverse phase HPLC in conjunction with fast protein liquid chromatography and have treated the polypeptide with cyanogen bromide (CnBr). Several unique CnBr fragments that have been identified are recognized by specific immunologic anti-myc probes which we have made and purified using defined putative myc encoded oligopeptides.

Significance to Biomedical Research and the Program of the Institute:

The myc gene product may well be important in the initiation of events in the transition between the G0/G1 phase of the cell cycle. The myc gene becomes an oncogene when it becomes uncoupled from the cell cycle. This, together with the deregulation of myc synthesis in many B-cell neoplasias, implies (although indirectly) that myc plays a central role in cell transformation. It is important to deduce the functional and biologically significant role(s) that the myc oncoprotein product plays in the role of the cells structural and replicative organization. Resolution of these processes will only come by the biochemical separation and analyses of the purified and non-denatured myc encoded products.

Proposed Course:

In the course of doing this work we will answer the following questions: (i) What is the N-terminal amino acid sequence of Hu-c-*myc*? Is there a difference in the N-terminal amino acid sequence of the *myc* protein in cell lines where the *myc* gene has been deregulated by gene translocation or amplification? (ii) Does the internal amino acid sequence of Hu-c-*myc* correspond to the amino acid sequence predicted by the DNA sequences? (iii) Do site-specific antibodies immunoprecipitate the same protein(s) from the various cell lines with deregulated *myc* synthesis? (iv) Is there post-translational processing of Hu-c-*myc*? Does this processing have only physiological significance? (v) Where is the native c-*myc* located within the nucleus? (vi) What is the molecular weight of Hu-c-*myc*? Does the native protein have any subunit structure? (vii) Does this native Hu-c-*myc* demonstrate sequence-specific DNA binding.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05442-01 LMO

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Involvement of c-*ets* in the Pathogenesis of Human Leukemias

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

PI:	N. Sacchi	Visiting Fellow	LMO	NCI
Others:	D. Watson	Senior Staff Fellow	LMO	NCI
	T. Papas	Acting Chief	LMO	NCI

COOPERATING UNITS (if any)

Istituti Clinici di Perfezionamento, Facolta' di Medicina, Milano, Italy (G. Cattoretti); Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands (A. Hagemeijer)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	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.)

Recently we have mapped the c-*ets* locus in humans onto two separate chromosomes, 11 and 21. For convenience we have named these proto-oncogene loci *ets*-1, located on chromosome 11, and *ets*-2, located on chromosome 21. Given the known correlation of many forms of human cancers to specific chromosomal aberrations, we are using our distinct probes to investigate the involvement of the *ets* proto-oncogenes in the pathogenesis of certain suspect leukemia malignancies. A number of these human leukemias, such as the acute undifferentiated leukemias (AUL) and the acute myeloid leukemia with maturation (AML-M2), show specific chromosomal aberrations involving the chromosomes known to contain the *ets*-1 and *ets*-2 proto-oncogene loci. We have identified, in a number of human cells from these acute leukemia patients, chromosomal aberrations such as deletions and translocations involving the 11q23.3 and 21q22 bands, specifically. The expression of c-*ets* is being investigated in an effort to determine if there are any qualitative and/or quantitative changes. Further, since Down's syndrome is associated with trisomy 21 aberrations, and these individuals also have an elevated incidence of acute leukemia, probing cells isolated from these individuals with the proto-oncogenes *ets* may illuminate any molecular basis for this coincidence.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

N. Sacchi	Visiting Fellow	LMO	NCI
D. Watson	Senior Staff Fellow	LMO	NCI
T. Papas	Acting Chief	LMO	NCI

Objectives:

To study the role of the cellular homologs of the v-ets in the etiology of human leukemias as it relates to the chromosomal abnormalities associated with specific malignant disorders. To understand, at the molecular level, the genetic alterations incurred with specific chromosomal abnormalities associated with the ets oncogene.

Methods Employed:

DNA and RNA analysis of leukemic cells presenting specific chromosomal aberrations, e.g., t(4;11)(q21;q23) and t(8;21)(q22;q22), has been performed by using Southern and Northern blot techniques by utilization of molecular probes diagnostic for the human c-ets-1 (6.0 kb) and c-ets-2 (1.0 kb) oncogenes which have been cloned in this laboratory. The specific location of c-ets-1 and c-ets-2 in translocated chromosomes has been established by analyzing panels of somatic cell hybrids between Chinese hamster cells and human leukemic cells presenting specific translocations and retaining either one of the recombinant chromosomes.

Major Findings:

Most of the chromosomal abnormalities seen in neoplastic cells are believed to result from the clonal proliferation of cells in which a chromosomal abnormality has arisen by somatic mutation. Several specific non-random chromosomal abnormalities have been associated with well-defined malignancies, as in the case of the Philadelphia chromosome derived from the translocation t(9;22) in chronic myelogenous leukemia, the t(15;17) chromosome translocation in acute bromyelocitic leukemia (ANLL-M3), and the t(8;14) rearrangement associated with human Burkitt's lymphoma. It is well known that human chromosomes present a number of fragile sites that have a tendency to form chromosomal or chromatid gaps. Significantly, 20 of the 51 fragile sites, so far identified, map at or close to the breakpoints found to be associated with 16 of 31 specific cytogenetic abnormalities identifiable in leukemias, lymphomas and malignant solid tumors.

The cellular proto-oncogenes that are or may be homologous to known retroviral oncogenes map closely to many fragile sites. Together these findings suggest that specific points in the human genome exist which are susceptible to chromosome breakage and rearrangements involving cellular genes; these c-oncogenes are believed to be important in normal cellular growth and differentiation.

The human cellular homology of ets has recently been mapped to two different chromosomes, 11 and 21, in the 11q23.3 and 21q22 bands, respectively. Constitutive fragile sites have, in fact, been recognized on chromosomes 11 and 21 at

the very same points. A number of known human leukemias present specific chromosomal aberrations (such as translocation, deletion) involving the 11q23.3. and 21q22 bands, e.g., the t(4;11) in acute undifferentiated leukemias (AUL), and the t(8;21) in the acute myeloid leukemia with maturation (AML-M2).

The current investigations of the role of the ets onc genes associated in the pathogenesis of these specific neoplasms allows us to conclude that the c-ets-1 probe reveals that there is a translocation of this gene from chromosome 11 to chromosome 4 in AUL patients presenting t(4;11)(q21;q23) translocation. Similarly, the c-ets-2 gene is entirely translocated from chromosome 21 to chromosome 8 in AML-M2 patients with the t(8;21)(q22;q22) translocation. This event seems to effect the expression of the c-ets-2 gene in leukemic cells of AUL-M2 patients with t(8;21).

Significance to Biomedical Research and the Program of the Institute:

The AUL and AML-M2 associated translocational abnormalities appear to exhibit a surprising degree of specificity in this association with particular stages of differentiation of the leukemic cell. It is quite possible to implicate that specific cytogenetic events involving the ets cellular oncogenes differentiate, thereby activating the process of leukemia transformation in specific hematopoietic cells. Understanding the basic molecular concepts of such genetic alterations and the resultant changes in oncogene expression would assist researchers in deciphering the causal interrelationship between these cellular genes and critical cellular events which may govern the manifestation of malignancy. Furthermore, such work could have diagnostic relevance to developing molecular gene probes to monitor the course of such malignancies.

Proposed Course:

1) Screening human leukemias presenting chromosome anomalies in the 11q23.3 and 21q22 bands at DNA and RNA levels with new c-ets-1 and c-ets-2 specific probes to identify possible rearrangements in the c-ets genes and assess quantitative and qualitative changes in the expression of c-ets which may be a consequence of any rearrangements; 2) determine the expression of c-ets oncogenes in preleukemic chromosomal aberrant states, e.g., trisomy 21 and trisomy 11; and 3) use molecular cloning to characterize the breakpoints involved in specific translocations, e.g., the t(4;11)(q21;q23) and the t(8;21)(q22;q22).

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05443-01 LMO

PERIOD COVERED

January 2, 1985 to September 30, 1985

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

Oncogene Expression During Cell Differentiation and Development

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

PI:	N. K. Bhat	Visiting Fellow	LMO	NCI
Others:	T. S. Papas	Acting Chief	LMO	NCI
	R. Ascione	Research Chemist	LMO	NCI
	M. C. Psallidopoulos	Guest Researcher	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.5	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.)*

Spermatogenesis will be used as a model system to study the role of cellular oncogenes during different stages of cell division (mitosis and meiosis) and cell differentiation. Work is being initiated to study the expression of different oncogenes both at the transcriptional level, using specific oncogene DNA probes, and at the protein level, using antisera against viral or cellular oncogene products. Efforts will be made to analyze molecular mechanisms involved in the expression of oncogenes and their putative role in carrying out normal cellular functions.

The constitutive expression of human-myc (Hu-myc) oncogene products, using mammalian expression vector systems and formation of stable cell lines, overproducing Hu-myc, are being established. Hu-myc DNA fragment containing entire coding sequences (three exons) or part of it (second and third exons), have been cloned into a transient expression vector (t25) or into a protein secretion expression vector system. Work is in progress to identify putative Hu-myc transcripts and polypeptides obtained after transfection of these hybrid constructs into CV-1 cells. Based on these results, appropriate constructs will be chosen to form stable cell lines to overproduce Hu-myc-proteins, using metallothionein-bovine papilloma virus-hybrid (MT-BPV) vectors. Biological functions of Hu-myc proteins and its role in cell differentiation, cell proliferation will be assessed.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

N. K. Bhat	Visiting Fellow	LMO	NCI
T. S. Papas	Acting Chief	LMO	NCI
R. Ascione	Research Chemist	LMO	NCI
M. C. Psallidopoulos	Guest Researcher	LMO	NCI

Objectives:

1) To characterize which oncogenes are expressed during mitosis and meiosis, and also during the transition from tetraploid state to diploid state or to the haploid state. 2) To study the role of oncogene products during sexual maturation. 3) To develop a mammalian expression vector system to express a eukaryotic gene of interest. 4) To purify Hu-*myc* gene products to homogeneity and to study the biological functions of these Hu-*myc* gene products. 5) To identify which amino acid residue(s) involved in phosphorylation of Hu-*myc* protein, employing site-specific mutagenesis of putative amino acid to identify residue(s) involved. 6) Comparing the property of modified Hu-*myc* gene products with that of normal *myc* gene products. 7) Studying the regulation of expression of the *myc* gene, i.e., Under what inducible circumstances is the *myc* gene product expressed? 8) to identify the role or involvement of other cellular gene products in the regulation of Hu-*myc* gene expression.

Methods Employed:

Cloning - vector construction: The transient shuttle expression vector, t25, the protein secretion vector, pM14GH8, and the MT-BPV hybrid vector, PBMTX, were provided by Dr. G. N. Pavlakis (Litton Bionetics, Inc.). The plasmid, pM14GH8c, a derivative of pM14GH8, was constructed in two steps: (i) converting ClaI sites into NruI site by filling ClaI site and by ligation. The resulting plasmid is designated as pM14GH8B; (ii) converting BglII site in pM14GH8B to ClaI site by filling BglII site and by ligation. These vectors were digested partially with suitable restriction enzyme, and appropriate fragments were chosen for cloning. For subcloning of insert DNA fragments, pBR322 vector was used.

Subcloning of Hu-myc-DNA fragments: pMC-41 plasmid containing 8.5 kbp HindIII-EcoRI H-*myc* fragment was obtained from Psallidopoulus of this laboratory. To delete portions of 5' Hu-*myc* upstream sequences, pMC41 was digested partially with SmaI. BamHI (octamers) linkers were added at SmaI site, as described in Molecular Cloning, A Laboratory Manual (Maniatis, T., Fritsch, E. F. and Sambrook, J., 1982). DNA was digested with BamHI. Unused linkers were separated from linker ligated to insert DNA, by electrophoresis. Insert DNA fragments were isolated from gels by electroelution and concentrated further by Elutip-d (Schleicher and Schuell) column chromatography and ethanol precipitation. DNA fragments were ligated using T4 DNA ligase; a portion of the ligated mixture was used to transform HB101 or C600 competent cells. Clones were picked by screening for ampicillin resistance on NZYDT agar plates. Plasmids were prepared from 10 ml overnight cultures by alkaline lysis method (Birnboim and Doly, Nucl. Acids Res. 7: 1513-1523, 1979). Plasmids were characterized and confirmed by restriction endonuclease mapping.

Plasmid pMC-41/BE-3 and pMC-41/BE-2 contains 5870 bp (3 exons) and 3842 bp (2 exons) Hu-myc sequences, respectively. pMC41/FBE-2 is a derivative of pMC41/BE-2 and is obtained by deleting 266 bp DNA fragments from 5' upstream Hu-myc sequences as follows. A 1152 bp SacI-BamHI fragment was obtained after digesting pMC-41/FBE-2 with BamHI and SacI. This fragment was then digested partially with FnuDII, and a 850 bp FnuDII/SacI fragment was isolated. BamHI linkers (octamers) were added at FnuDII site as described above. An 850 bp BamHI/SacI insert DNA, thus obtained, was cloned into the BamHl/SacI site of pMC41/BE-2.

Construction of pMt5872, pMt3600: A 5872 bp fragment and a 3600 bp Hu-myc fragment were isolated from pMC41/BE-3 and pMC41/FBE-2 after digestion with EcoRI and BamHI. Transient shuttle expression vector, t25, contains SV40 genome (for replication in suitable mammalian host cells), part of pBR322 sequences (for replication in bacteria) and a metallothinein gene. The metallothinein gene was removed by partial digestion with EcoRI. BamHI-EcoRI fragments of Hu-myc sequences were cloned into the BglII/EcoRI site of t25. Clones containing Hu-myc sequences in proper orientation was confirmed by restriction endonuclease mapping.

Bacterial strains: *E. coli* HB101 $_{r-m_{k+}}$, or *E. coli* C600 $_{r-m_{k+}}$ or *E. coli* (dam^-) strains were used for most of the subcloning work. Cells were made competent with $CaCl_2$, as described in the Molecular Cloning Manual.

Animal cells: For transfection experiments involving SV40-derived plasmids, CV-1 cells were used.

Gel electrophoresis: DNA fragments obtained after digestion with restriction enzymes were electrophoretically separated on 1% agarose gels using the Tris acetate-sodium acetate-EDTA buffer system. DNA bands were visualized by ethidium bromide staining. The DNA bands of interest were cut out of the gel and electroeluted into a dialysis bag. DNA fragments were further concentrated by Elutip-d column chromatography and ethanol precipitation. Concentration of DNA fragments was estimated by comparing the intensity of ethidium bromide stained DNA bands to that of DNA bands of standard molecular weight markers (phage HindIII cut fragments, or ØX174RF HaeIII cut fragments).

Transfection of pMt5872, pMt3600 into CV-1 cells: CV-1 cells growing in log phase were transfected with pMt5872, pMt3600 by the calcium phosphate precipitation technique (Wigler *et al.*, Cell 16: 777-785, 1979). 10 g of plasmid constructs were mixed with 25 g salmon sperm DNA used per plate. At 48 hours posttransfection, plates were washed with phosphate buffered saline (PBS) and cells were directly suspended in 4M guanidinium isothiocyatate (GuSCN) solution and processed for RNA isolation, as described by Chirgwin *et al.* (Biochemistry 18: 5294-5299, 1979). For labeling of cells with ^{35}S-methionine, cells were washed with methionine-free minimal essential medium containing 3% fetal calf serum and 1% glutamine and incubated with the same medium. After an hour, cells were supplemented with ^{35}S-methionine (100 Ci/ml) and incubation was continued for 3 to 4 hours. Labeled cells were washed extensively with PBS and used for immunoprecipitation, using antisera against Hu-myc 2nd exon specific peptide, which was obtained from Dr. Fisher of this laboratory. Immunoprecipitation was carried out using the procedure described by Kessler (J. Immunol. 115: 1617-1624, 1975). Proteins were resolved on polyacrylamide gels according to the procedure of Laemmli (Nature 227: 680-685, 1970).

Isolation of nucleic acids and Northern and Southern analysis: High molecular weight DNA was isolated by SDS/proteinase K procedure, as described in the Molecular Cloning Manual. DNA was digested with appropriate restriction endonucleases, and the fragments are separated on an agarose gel by electrophoresis. DNA fragments were transferred to NYTRAN membranes by capillary action, as described by Southern (J. Mol. Biol. 38: 503-517, 1975), using 20XSSPE. (1XSSPE = 0.18 M NaCl 0.01M sodium phosphate pH 7.5, 1 mM EDTA). DNA was immobilized on the membrane by baking at 80°C under vacuum. These blots were used further for hybridization with nick-translated onc gene probes.

Total RNA was isolated by homogenizing the mouse testis or the transfected cells in 4M GuSCN solution. DNA was sheared by passing solution through an 18-gauge needle. Cellular debris was removed by centrifugation at 8000 rpm at 15°C for 15 min. 8 ml of clear supernatant was layered on 3.5 ml of 5.7 M CsCl cushion. RNA was pelleted by centrifugation at 200,000xg for 20 hrs at 20°C. RNA pellet was suspended in H_2O and was precipitated with 2 volume ethanol. $PolyA^+$ RNA was selected by oligo dT cellulose chromatography, as described by Aviv and Leder (Proc. Natl. Acad. Sci. USA 69: 1408-1412, 1972).

A portion of total RNA (20 g) or $PolyA^+$ RNA (10 g) was size fractionated on denaturing formaldehyde agarose gel, as described by Melton et al. (Nucleic Acids Res. 12: 7035-7056, 1984), using MOPS buffer system. Size-fractionated RNA was transferred to NYTRAN membrane by capillary action using 20XSSPE. RNA was immobilized on the membrane by baking at 80°C in vacuum. The blots were prehybridized at 42°C for 3-6 hrs in a solution containing 50% formamide, 5XSSPE, 5X Denhardt's solution (1X Denhardt's solution = .02% each of BSA, PVP and Ficol 400), One percent sodium sarkosinate and 100 g/ml denatured salmon testis DNA and then probed with a nick-translated onc gene probe. The blots were washed twice with 1XSSPE and 1% SDS at room temperature and then twice with 0.1XSSPE and 1% SDS at 50°C. RNA hybridizing to the onc gene probe was visualized by autoradiography.

Major Findings:

Preliminary results of transfection experiments using pMt5872 and pMt3600 indicate that the Hu-myc gene is being transcribed and the Hu-myc protein is being made from these constructs. Experiments are in progress to confirm putative polypeptides by immunoprecipitation, peptide mapping and micro-sequencing. Transcripts will be characterized by S_1 nuclease mapping.

Significance to Biomedical Research and the Program of the Institute:

Several proto-onc genes, presumably having normal physiological functions, have been either (i) amplified, (ii) undergone point mutation, or (iii) undergone chromosomal translocation in neoplastic cells. Finding the gene products responsible for initiating and maintaining the metastatic behavior of cancer cells is of great importance towards finding a means to inhibit this process. Understanding the functions of cellular onc gene products in normal cellular metabolism, differentiation and development is also of great significance in order to delineate the basic molecular mechanisms involved in neoplasia.

Proposed Course:

Proposed experiments will involve: 1) Expression of myc, myb, ets, mht, mos, fos, ros, fps, fms, fgr, yes, erbA, erbB, rel, ras, sis, abl and met will be studied in 8 day-old testis (enriched in tetraploid and diploid cells) and will be compared with the expression of these oncogenes in adult testis (enriched in haploid and diploid cells) by Northern analysis. 2) Based on initial results, attempts will be made to study specific oncogene expression in different cell types from testis: Type A spermatogonia (enriched in tetraploid cells); secondary spermatocytes (enriched in diploid cells) and in round spermatids (enriched in haploids). 3) Expression of relevant oncogenes will be studied in some of the tumor cell lines established from Leydig cells, Sertoli cells, peritubular myoid cells, and endothelial cells. 4) The effects of testosterone, luteinizing hormone, and follicle stimulating hormone will be attempted on expression of onc genes during different stages of spermatogenesis.

5) Formation of stable cell lines overproducing Hu-myc gene products will be achieved by transfecting MT-BPV-H-myc hybrid constructs into mouse C-127 cells, using the heavy metal induction selection procedure. 6) These transformed cell lines wll be used to study the role of myc gene products in cellular functions. 7) In collaboration with Dr. Fisher of this laboratory, Hu-myc gene products will be purified to homogeneity using fast protein liquid chromatography. Purified human myc protein will be used (i) to raise monoclonal antisera, (ii) to characterize amino acid residues involved in phosphorylation, (iii) to characterize the role of the putative polypeptide-coded by exon 1 of human myc, and (iv) to study the biological functions of this putative polypeptide, in an in vitro reconstituted system.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP08718-07 LMO

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Transcriptional and Post-transcriptional Control of the Lambda Int Gene Expression

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

PI:	D. L. Court	Research Biologist	LMO	NCI
Others:	H. E. Takiff	Guest Researcher	LMO	NCI

COOPERATING UNITS (if any)
Department of Genetics and Molecular Biology, Centro de Investigacion y de Estudios Avanzados Del IPN, Mexico City, Mexico (G. Guarneros, P. Guzman)
E. I. duPont de Nemours & Co., Inc., Wilmington, DE (S. E. Bear)

LAB/BRANCH
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SECTION
Molecular Control and Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

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 _int_ gene of phage λ encodes a protein involved in site-specific recombination. The gene is transcribed early after infection from one promoter, pL, and later from a second promoter, pI. Each transcription event requires different positive activation factors, λ N and cII proteins, respectively. We have examined pI-promoted transcription in the region beyond the _int_ coding sequence. The _int_ mRNA extends to a site designated tI, which is located 277 nucleotides beyond _int_. Transcription at tI terminates with 75% efficiency _in vitro_, and its efficiency is over 95% _in vivo_.

Polymerases initiating at pL transcribe through tI and into the b segment of λ DNA. The read-through pL transcript is sensitive to cleavage by the endonuclease, RNaseIII, both _in vivo_ and _in vitro_. Two specific cuts are made by RNaseIII in a double-stranded RNA structure about 260 nucleotides beyond _int_ in the location of the tI terminator. Functionally, the processed pL transcript is unable to synthesize the _int_ gene product, whereas the terminated and unprocessed pI transcript expresses _int_. Interestingly, unprocessed pL transcripts made in hosts defective in RNaseIII (_rnc-_) can express _int_. The place where processing occurs is called _sib_, and the control of _int_ expression from this site is called retroregulation. Retroregulation by _sib_ is not restricted just to the _int_ gene; we show that if the _sib_ site is cloned beyond a bacterial gene, the gene is controlled by _sib_ and RNaseIII. The RNaseIII processing occurs in a region of extensive dyad symmetry in the DNA. The shorter pI transcript forms a stable stem and loop structure at its 3' end in the region of symmetry, but lacks the entire dyad symmetry required for RNA processing that is formed in the longer pI transcript. Processing removes the stem and loop structure from the pL transcript and forms a 3' end that we believe is far more sensitive to exonuclease attack than the end of the pI transcript.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

D. L. Court	Research Biologist	LMO NCI
H. E. Takiff	Guest Researcher	LMO NCI

Objectives:

The regulation of the λ int gene has been determined. It can be transcribed from either of two promoters, p_I or p_L. p_I requires λcII protein in addition to RNA polymerase to initiate. It transcribes int and terminates at a site, t_I, 277 bases beyond the gene. This RNA synthesizes high levels of int protein. p_L also transcribes int, but is prevented from terminating at t_I by the λ N gene product, which makes polymerases initiating at p_L (but not p_I) non-terminating. The p_L transcripts do not synthesize int. A site on the p_L transcript inhibits int expression. It is a site for endoribonuclease (RNaseIII) located 260 bases beyond int. RNA processing here sensitizes the int mRNA to a proposed 3'-5' exonuclease in E. coli. The terminated p_I transcript is not processed (the RNaseIII site is not formed) and is not sensitive to the exonuclease. This post-transcriptional control of int from a site located beyond the gene is called retroregulation.

Our primary objective now is to determine why the terminated p_I transcript is stable to mRNA degradation and the processed p_L transcript becomes susceptible. Can the processed p_L transcript be made resistant again by placing upstream a strong stem structure in the RNA which exists at the end of the p_I transcript? Does RNaseIII bind to, but not digest, the stem of the terminated transcript? Does this binding protect against exonucleases?

Methods Employed:

Standard microbial, genetic, biochemical, and recombinant DNA techniques are used. Int protein is assayed in vivo by a biological assay for site-specific recombination of phage molecules and in vitro by ELISA analysis of crude extracts. Antibody was made against pure Int protein. λcII mutants were used to express only the p_L promoter and λnut_L mutants were used to express only the p_I promoter.

RNA hybridizations are performed on cloned int DNA fragments on plasmid or M13 vehicles. Int overexpression has been examined in a p_L vector developed in this laboratory.

Major Findings:

1. cII activates the promoters p_I and p_E to allow RNA polymerase to initiate.

2. Int synthesis occurs from p_I transcripts that terminate at t_I, 277 bases beyond the int gene.

3. Int synthesis is blocked from the p_L transcript that extends beyond the t_I terminator. Inhibition of Int synthesis from this transcript is caused by RNaseIII (endoribonuclease) processing.

4. The int mRNA of the p_L transcript is degraded after endonuclease processing occurs. A 3' to 5' exonuclease has been postulated.

5. We have defined within +/-10 bp the limits of the RNaseIII recognition site by using Bal31 to generate a deletion map.

6. RNaseIII protein cuts the p_L transcript at two points within this site *in vitro*. Sib mutants in the site are not processed.

7. Six new promoters have been identified between p_I and t_I.

8. An element that may be involved in DNA replication has been located near the t_I terminator.

9. RNaseIII does not process the terminated p_I transcript *in vivo*, and only partially processes it *in vitro* at high nuclease concentrations.

10. Sib mutants that enhance Int synthesis from p_L reduce Int synthesis from p_I.

11. Insertion of ~1000 bp of foreign DNA fragments between sib and int prevents retroregulation. We postulate that distance or time may prevent the nuclease from being effective; perhaps specific sites block the progress of the exonuclease.

12. An ELISA assay for int has been developed to monitor, accurately, changes in int levels under different conditions.

13. Preliminary studies indicate that the Xis gene product or its translation affects the level of Int protein made in the cell.

14. The genes for RNaseIII (rnc), polynucleotide phosphorylase (pnp), and RNaseII (rnb) have been cloned on pBR322 and rnc on λ.

15. Genetic analysis indicates that rnc is autoregulated.

Significance to Biomedical Research and the Program of the Institute:

In cancer cells the expression of some genes is permanently turned on by mechanisms that we do not yet understand. Our studies are aimed at understanding the molecular basis of how genes are turned on and off. We are using *E. coli* and λ as model systems. Application of knowledge gained in bacterial systems has been applied to understanding gene control in complex systems and is being used in the LMO to better understand the turn-on and turn-off of cellular oncogenes and related retroviral genes.

Proposed Course:

(1) To determine the effect of *Xis* translation on retroregulation and *int* gene translation. (2) To determine if RNaseIII binds to, but does not process, terminated p_I transcripts or *sib* mutant p_L transcripts. (3) To isolate t_I termination mutants. (4) To analyze the mechanism of post-transcriptional control mechanisms, i.e., endo- and exo-ribonuclease control systems in *E. coli*. (5) To determine why some mRNAs are more resistant than others to degradation by nucleases.

Publications:

Bear, S. E., Colberg-Poley, A. M., Court, D. L., Carter, B. J. and Enquist, L. W.: Analysis of two potential shuttle vectors containing herpes simplex virus defective DNA. *J. Mol. Appl. Genet.* 2: 471-484, 1984.

Bear, S. E., Court, D. L. and Friedman, D. I.: An accessory role for *Escherichia coli* integration host factor: Characterization of a lambda mutant dependent upon integration host factor for DNA packaging. *J. Virol.* 52: 966-972, 1984.

Schmeissner, U., McKenney, K., Rosenberg, M. and Court, D.: Removal of a terminator structure by RNA processing regulates *int* gene expression. *J. Mol. Biol.* 176: 39-54, 1984.

Schmeissner, U., McKenney, K., Rosenberg, M. and Court, D.: Transcription terminator involved in the expression of the *int* gene of phage lambda. *Gene* 28: 343-350, 1984.

ANNUAL REPORT OF

LABORATORY OF MOLECULAR VIROLOGY

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

The Laboratory of Molecular Virology (1) analyzes the mechanism of gene expression in normal and transformed eukaryotic cells; (2) uses viruses as tools for probing cellular regulatory mechanisms; (3) develops and applies biological, biochemical and immunological procedures to obtain evidence for the mechanism by which antigens are recognized by the immune system; (4) plans and conducts research on transforming proteins to define their properties in normal cells and their potential role in the development of neoplasms; and (5) investigates the mechanisms by which cellular gene expression, viral gene expression, and interactions between viruses may influence the transformation of cells.

The Virus Tumor Biology Section (1) characterizes the nucleotide sequence from regions of viral and cellular DNA thought to be involved in gene expression; (2) investigates the properties of cellular and viral transforming genes and their protein products; (3) evaluates the mechanisms by which viral and cellular proteins affect the level of gene expression; and (4) develops eukaryotic viral vectors to study gene expression.

The Cell Physiology Section (1) investigates the molecular elements essential for cellular transformation; (2) studies the properties of cell surface molecules in expression of the cellular phenotype; (3) uses recombinant DNA techniques and molecular genetics to study the elements involved in gene regulation; and (4) employs prokaryotic host-vector-systems to examine sequences involved in efficient gene expression and protein production.

The elucidation of signals associated with gene expression is among the primary objectives of the Laboratory of Molecular Virology. In particular, our interest has been directed toward regulatory events which take place at the level of transcription and processing of RNA. We have been involved in the elucidation and analysis of novel genetic elements, enhancer sequences, which appear to be responsible for controlling the rate at which particular genes are transcribed. We have demonstrated the existence of these enhancer sequences not only in the genomes of DNA viruses such as SV40, JCV and BKV, but also in the long terminal repeats (LTRs) of retroviruses. Using a combination of *in vivo* and *in vitro* assays, we have demonstrated that enhancer sequences show host-cell specificity, and thus may be among the elements involved in controlling the host range of certain viruses as well as the tissue-specific expression of certain eukaryotic genes. A number of laboratories have now shown that enhancers are critical elements in determining the activity of eukaryotic genes and that they function in a tissue- or organ-specific fashion. A major effort in our laboratory will be directed at determining whether or not enhancer sequences play a role in the developmental and tissue-specific regulation of gene expression. In addition, we are interested in mutagenizing regions of enhancer elements to elucidate those sets of nucleotides associated with the general activation phenomenon as well as the cellular specificity. *In vivo* and *in vitro* experiments have been designed in an attempt to examine the mechanism by which the activator/enhancer sequences function.

Currently, we have embarked on a number of experiments to define and characterize the biological macromolecules which interact with these regulatory elements.

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

In studies involving the isolation and characterization of cDNA clones derived from different class I genes, it was observed that class I antigens with different primary structures are expressed from the same class I gene by alternate RNA splicing. This novel finding suggests that there exist different functional subsets of these antigens and offers the opportunity to compare the products of these cDNA clones in presenting tumor antigens to the cytotoxic T-cell. Such analyses would lead to a biochemical definition of immune surveillance.

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

A considerable effort has been directed toward the study of mammalian oncogenes and their counterparts in yeast. It is hoped that this information will contribute to an understanding of the process of cell transformation and tumorigenesis. Studies in progress are designed to investigate the role of oncogenes in inducing cell transformation as well as the cellular factors which contribute to metastases.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05101-07 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

Studies on the Molecular Mechanisms for Malignant Transformation of Cells

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

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Masato Tanaka	Visiting Fellow	LMV	NCI
	George Khoury	Chief	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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

The goal of this project is to investigate the molecular mechanisms underlying the malignant transformation of cells. We have been studying the structure and function of the tumor antigen (T-antigen) encoded by simian virus 40 (SV40). Using either recombinant viruses which contain different parts of the T-antigen gene or proteolytic enzymes which cleave the T-antigen molecule at specific sites, we were able to dissect and identify the roles of the various domains of the multifunctional SV40 T-antigen.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Masato Tanaka	Visiting Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

Studies on the structure and function of the simian virus 40 (SV40) tumor antigen (T-antigen) in infected cells.

Methods Employed:

Proteins were detected by immunoprecipitation using either conventional or monoclonal antibodies.

Major Findings:

The simian virus 40 tumor antigen (SV40 T-antigen) is a multifunctional protein that plays a critical role both in cell transformation and in virus propagation. We have attempted to dissect the T-antigen molecule with the hope of obtaining information on the structure-function relationship which might be helpful for an understanding of the neoplastic process. Using human adenovirus 2-simian virus 40 recombinants, we have previously demonstrated that the carboxy-terminal portion of T-antigen is sufficient to induce SV40-specific tumor rejection. We have now shown, by partial cleavage with proteolytic enzymes, that the amino-terminal portion of T-antigen is sufficient to induce binding to the viral origin of DNA replication. Such experiments involving the molecular dissection of a virally induced transforming protein are particularly pertinent to a clear understanding of the mechanism of cellular transformation by oncogenic viruses.

Significance to Biomedical Research and the Program of the Institute:

The mechanism whereby a virally coded protein induces the uncontrolled proliferation of cells, a process which leads to a transformed state of growth, is at present obscure. The fact that SV40 T-antigen can bind to the viral origin of DNA replication, a sequence which has its counterpart in the cellular genome, suggests a mechanism for a viral transforming protein in altering the replication of host DNA. Our attempt to analyze this macromolecular interaction may have direct bearing on the process by which normal cells lose their growth control upon neoplastic transformation.

Proposed Course:

To define the molecular mode of action of this protein during the process of cell transformation.

Publications:

Rhim, J. S., Jay, G., Arnstein, P., Price, F. M., Sanford, K. K. and Aaronson, S. A.: Neoplastic transformation of human epidermal keratinocytes by the combined action of SV40 and ras transforming genes. Science 227: 1250-1252, 1985.

Rhim, J. S., Sanford, K. K., Arnstein, P., Fujita, J., Jay, G. and Aaronson, S.: Human epithelia cell carcinogenesis: Combined action of DNA and RNA tumor viruses produces malignant transformation of primary human epidermal keratinocytes. In Barrett, J. C. (Ed.): Carcinogenesis: A Comprehensive Survey. New York, Raven Press, 1985. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05214-05 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

Genetic Elements Regulating the Initiation of Transcription

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

PI:	John Brady	Expert	LMV	NCI
Others:	George Khoury	Chief	LMV	NCI
	Laimonis Laimins	Staff Fellow	LMV	NCI
	Mary Loeken	PRAT Fellow	LMV	NCI

COOPERATING UNITS (if any)

Biochemical Virology Section, Laboratory of Molecular Microbiology, NIAID, NIH (A. Khan, M. Martin, A. B. Rabson and P. Steele)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	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 simian virus 40 (SV40) 72-bp repeat is a eukaryotic polymerase II enhancer element capable of significantly increasing the level of transcription of homologous or heterologous genes in a position- and orientation-independent fashion. The location of the SV40 tandem repeats between the early and late SV40 genes suggests that it might function equally well for both transcriptional units. SV40 late genes, however, are normally expressed only after the synthesis of sufficient amounts of tumor antigen (T-antigen) and/or the initiation of viral DNA replication. These transcriptional properties of the late promoter present a conceptual problem in light of the bidirectional function described for the prototype SV40 enhancer element. Recently, we have found that SV40 late gene expression is activated by the SV40 early gene product, T-antigen. RNA analysis demonstrates that activation occurs at the transcriptional level. Analysis of template mutants and in vivo competition experiments demonstrate that the 72-bp repeat is important for T-antigen mediated late gene expression. The mechanism by which the 72-bp enhancer element functions, however, may be different for early and late gene expression. The SV40 early enhancer function can be activated by transcriptional factors which specifically recognize the 72-bp repeats and are endogenous to many cell types. The function of the 72-bp transcriptional control sequence which contributes to late transcription is inducible by T-antigen. In addition, binding of the putative late transcription factor(s) to the enhancer element apparently requires T-antigen or another transcriptional factor which associates with T-antigen sites I and II. It is not clear whether the SV40 72-bp repeats function as a classical enhancer (position- and orientation-dependent) for late gene expression.

PROJECT DESCRIPTION

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

John Brady	Expert	LMV	NCI
George Khoury	Chief	LMV	NCI
Laimonis Laimins	Staff Fellow	LMV	NCI
Mary Loeken	PRAT Fellow	LMV	NCI

Objectives:

This project is directed toward an analysis of the genetic elements required for the initiation of transcription. It should provide information about structure and function of eukaryotic promoters.

Methods Employed:

Construction of deletion mutants and point mutants; mapping of recombinant genomes; RNA analysis; protein analysis; cloning in pBR322 and derivatives thereof; expression of recombinants using transient and long-term assays; *in vivo* competition analysis.

Major Findings:

1. Enhancers are cis-essential elements required for the expression of viral genes. They appear to function in concert with other promoter elements including GC-rich hexanucleotides and the TATA box.

2. Point mutations which affect SV40 early enhancer activity also affect T-antigen mediated SV40 late gene expression. This indicates that the same nucleotide sequences important for SV40 early enhancer function are important for SV40 late enhancer activity.

3. SV40 late gene activity is inducible by T-antigen.

4. Efficient binding of the SV40 late transcriptional factor(s) to the 72-bp repeats requires the presence in *cis* and at a fixed distance of the sequences within SV40 T-antigen binding sites I and II. This suggests a cooperative binding and protein-protein interaction for T-antigen mediated late gene function.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the molecular mechanisms leading to the initiation of transcription of eukaryotic genes is important to the understanding of transforming genes and cellular differentiation. The inducibility of the SV40 enhancer by T-antigen for stimulating late transcription may be similar to the induction of cellular transcriptional regulatory sequences during differentiation, or to the activation of certain genes in response to hormone-receptor complexes. This

concept of "inducible enhancers" may apply to genes which require factors for their activation which are produced only at specific times in a cell cycle. We anticipate that analysis of the mechanism of induction of SV40 late gene expression by T-antigen and other transcriptional factors will provide insight into the regulation of a category of inducible genes.

Proposed Course:

1. This project will continue with an emphasis on the identification of the mechanism by which enhancer elements control gene expression.

2. Using appropriate plasmid constructions, it will be determined whether the SV40 72-bp repeats function as a classical enhancer for late transcription (position- and orientation-independent).

3. Using *in vivo* competition analysis, the binding of limiting transcriptional factors to the "early" and T-antigen mediated late SV40 enhancer sequences will be analyzed and compared to other established enhancer sequences such as murine sarcoma virus (MSV), Rous sarcoma virus (RSV) and mouse mammary tumor virus (MMTV).

Publications:

Brady, J., Loeken, M. and Khoury, G.: The SV40 enhancer as a transcriptional control element in T-antigen mediated SV40 late gene expression. In Gluzman, Y. (Ed.): *Genetic Elements Regulating Initiation of Transcription in Eukaryotes*. New York, Cold Spring Harbor Laboratory, 1985. (In Press).

Kessel, M. and Khan, A. S.: Nucleotide sequence analysis and enhancer function of long terminal repeats associated with an endogenous African green monkey retroviral DNA. *Mol. Cell. Biol.* 5: 1335-1342, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05216-05 LMV

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Ras Oncogene Regulation in Yeast

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

PI:	Ravi Dhar	Visiting Scientist	LMV	NCI
Others:	Diego Breviario	Visiting Fellow	LMV	NCI
	Richard Koller	Biologist	LMV	NCI

COOPERATING UNITS (if any)
Clinical Hematology Branch, NHLBI (M. Ruta); Virus & Cell Biology Research, Merck Sharp & Dohme Research Laboratories, West Point, PA (D. DeFeo-Jones, E. Scolnick)

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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.)

Two ras genes isolated from the yeast strain Saccharomyces cerevisiae have been designated c-rasI and c-rasII. Both genes are transcriptionally regulated. Carbon sources appear to play a major role in the regulation of both genes. The c-rasI transcript is induced by dextrose very early in a growth cycle, whereas the transcript is absent when cells reach mid log phase. There are three major c-rasII transcripts differing only in the location of their 5' ends. This gene appears to be regulated primarily by selection of a particular 5' end.

Yeast cells in which only the c-rasI gene is present have been shown to sporulate in rich media in the presence of dextrose. We believe this is due to the absence of c-ras gene product because there is essentially no c-rasI transcript present in late log phase. Thus, the cells are starved even in rich media. We have shown that cells lacking the c-rasII gene do not grow in synthetic media containing non-fermentable carbon sources (e.g., acetone, glycerol, ethanol).

When cells contain a c-rasII Val-19 gene instead of the wild-type c-rasII gene, growth in synthetic media occurs in the presence of dextrose, but cells begin to die when they reach late log phase. Cells do not grow in non-fermentable carbon sources, but instead die. We have also observed the absence of regulation of c-rasII transcripts in these cells.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Ravi Dhar	Visiting Scientist	LMV	NCI
Diego Breviario	Visiting Fellow	LMV	NCI
Richard Koller	Biologist	LMV	NCI

Objectives:

Our major objectives are:

1. To study the transcriptional and translational regulation of the two ras genes in yeast.

2. To study the biological phenotype of ras mutants and correlate gene regulation with function.

Methods Employed:

Recombinant DNA technology; Southern and Northern blot analysis.

Major Findings:

The two ras-related yeast genes c-rasI and c-rasII encode polypeptides of 40,000 and 41,000 daltons, respectively. We have shown that the two genes are regulated in a wild-type yeast strain, *Saccharomyces cerevisiae*.

With regard to regulation of c-rasI:

1. C-rasI transcription is induced by dextrose during early log growth; during late log phase, the transcripts are absent.

2. C-rasI expression is sensitive to the carbon source. Low levels of the c-rasI transcripts are present when cells are grown in non-fermentable carbon sources like ethanol, acetone and glycerol.

3. C-rasI expression responds to the carbon source starvation only. The transcript disappears when the carbon source is withdrawn during early log growth. There was no major change in the concentration of c-rasI transcripts when nitrogen or sulphur were withdrawn.

4. We have shown that cells in which only c-rasI is present (c-rasI$^+$ and c-rasII$^-$) are able to grow in synthetic media (S-media) only in the presence of a fermentable carbon source like dextrose. These cells overproduce c-rasI transcripts compared to their isogenic strain. The cells did not grow in S-media using acetone or ethanol. It has been shown by others that they do grow in non-fermentable carbon sources when supplied with yeast extract. The cells did not die in S-media, whereas more than 95% of the cells had no buds, a condition seen when cells reach stationary phase. Others have shown

that when c-rasI$^+$, c-rasII$^-$ cells are grown in rich media in the presence of dextrose, the cells sporulate. We believe that when cells reach stationary phase, the absence of ras gene product may stimulate sporulation.

With regard to regulation of c-rasII:

1. The wild-type strain of yeast has three major transcripts for c-rasII differing mainly in their 5' ends. This gene is also regulated:

   A. It responded variably to growth conditions (e.g., when grown in S. dextrose media, three transcripts were seen early in log growth; in late log phase, only the two shorter transcripts were observed; during stationary conditions, only the middle transcript was present).

   B. Cells grown in the presence of non-fermentable carbon sources, like glycerol, synthesized only the shortest transcripts. Cells grown in the presence of ethanol contained the two smaller transcripts; when they reached the stationary phase, only the middle transcript was present.

   C. The c-rasII gene responds to starvation by carbon, nitrogen and sulphur. As soon as any of these elements were withdrawn from the media, only one transcript was synthesized (e.g., in carbon starvation, we found only the middle transcript, whereas in nitrogen starvation, only the smallest transcript was present).

   D. Cells containing c-rasI and c-rasII Val-19 (equivalent to the activated Harvey ras oncogene) grew in S-media only in the presence of dextrose. When cells reached late log phase, they began to die. However, these cells did not grow in the presence of non-fementable carbon sources in S-media. The regulation of c-rasI transcripts in these cells was similar to wild-type. The c-rasII gene was not regulated (e.g., all three transcripts were seen even in stationary phase of the cells grown in the presence of dextrose). This situation resembles active growth and may explain why these cells fail to sporulate.

Significance to Biomedical Research and the Program of the Institute:

Activation of ras genes has been associated with certain human tumors. The study of the regulation of ras genes in yeast should help in understanding its role in cell growth and metabolism.

Proposed Course:

1. We have selected mutants of yeast which bypass the c-rasII gene requirement. These grow in non-fermentable carbon sources. We will study the regulation of c-rasI gene in these mutants.

2. We have inserted the lacZ gene of E. coli downstream from the c-rasII promoter. This will help us establish if different transcripts vary in translation regulation.

3. We will isolate suppressor mutations which allow the growth of c-*ras*I and c-*ras*II Val-19 in different non-fermentable carbon sources, so that we may be able to define the target site of the *ras* gene product.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05217-05 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

Studies on the Regulation of SV40 Gene Expression

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

PI:	Shigeko Nomura	Microbiologist	LMV	NCI
Others:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
	George Khoury	Chief	LMV	NCI
	Ravi Dhar	Visiting Scientist	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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

Transfection of CV-1 cells with DNA from frame shift mutant C1-5, a 2-base insertion at the unique HpaII site within the SV40 agnogene, produced plaques significantly smaller and less efficiently than did wild-type DNA. Because the kinetics of plaque formation suggested that second-site mutations may arise, we analyzed cloned DNA isolated from individual plaques. DNA sequence analysis revealed a high frequency of deletions mapping within the SV40 late leader sequences. These deletion mutants were viable and grew more efficiently than parental mutant C1-5, but less efficiently than wild-type SV40. Although the deletions varied in size and location, we found no correlation between their growth characteristics and their altered DNA sequences (e.g., potential to encode a truncated agnoprotein). These observations suggest that the secondary structure of the RNA transcripts, as determined by the sequence of mutants in this region, may play an important role in SV40 late gene expression.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Shigeko Nomura	Microbiologist	LMV	NCI
Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
George Khoury	Chief	LMV	NCI
Ravi Dhar	Visiting Scientist	LMV	NCI

Objectives:

To study the function of the simian virus (SV40) agnoprotein.

Methods Employed:

Tissue culture; DNA transfection; radiolabeling of DNA, RNA and protein; DNA cloning; preparation and analysis of DNA by cesium-chloride-ethidium-bromide equilibrium centrifugation; restriction endonuclease digestion; agarose and polyacrylamide gel electrophoresis; Southern blotting and hybridization; preparation and analysis of RNA by affinity chromatography; HCHO agarose gel electrophoresis; Northern blotting and hybridization; detection and analysis of protein by immunoprecipitation and SDS-polyacrylamide gel electrophoresis, by Western blotting and hybridization and by immunofluorescence; DNA sequencing by the chemical degradation procedure of Maxam and Gilbert.

Major Findings:

A recombinant SV40 molecule containing a KpnI-EcoRV segment (bases 212 to 686) from mutant Cl-5, and the remainder from wild-type SV40 was constructed. Growth characteristics of this DNA by transfection of CV-1 cells were very similar to those of Cl-5 DNA, indicating that Cl-5 DNA contained no other significant alterations in DNA sequence other than in KpnI-EcoRV segment.

The 2-base insertion at the HpaII site not only removed the HpaII recognition sequence, but also introduced overlapping cleavage sites for ThaI and SstII. These new cleavage sites provided a convenient tool for distinguishing between wild-type and mutant Cl-5 DNA. DNA preparations from individual plaques formed after transfection with DNA from Cl-5 were frequently found to contain SV40 DNA subpopulations resistant to SstII digestion. DNA from one such plaque was cloned in pBR322 and further analyzed by restriction endonuclease digestion and by DNA sequencing. Seven out of 30 recombinant clones tested were resistant to SstII digestion as the result of deletions containing sequences in the SstII recognition site. These deletions were located within the late leader sequence including the coding region of agnogene; some deletion mutants retained SstII site. The deletion mutants were viable and formed plaques more efficiently than did the parental Cl-5 DNA although less efficiently than did wild-type SV40. Although these mutants differed in the size and position of deletions, we failed to find any direct correlation between their growth characteristics and the altered DNA. These observations suggest that the secondary structures of the RNA transcripts

encoded by this region may play an important role in determining how efficiently the mutants function within the cell. On the basis of these findings, other known properties of agnoprotein, and suppression of a VP-1 mutation by second-site mutations in the agnoprotein, it seems likely that the agnoprotein is primarily involved in virion assembly.

Significance to Biomedical Research and the Program of the Institute:

The agnoprotein apparently plays a role in efficient reproduction of SV40. Further investigations using mutants containing a single nucleotide pair change may provide insight into the role of agnoprotein in SV40 gene expression.

Proposed Course:

We will analyze the biological and biochemical properties of the deletion mutants described above.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05219-05 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

Construction of a Prokaryotic Vector for the Expression of Mammalian Proteins

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

PI: Gilbert Jay — Chief, Cell Physiology Section — LMV NCI

Others: George Khoury — Chief — LMV NCI

COOPERATING UNITS (*if any*)

Department of Chemistry, University of New Brunswick, Canada (E. Jay)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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 goal of this project is to investigate the regulatory elements required for recognition (i) by ribosomes during the process of initiation of protein synthesis and (ii) by RNA polymerases in the initiation of transcription. We have generated a general plasmid vector containing a synthetic ribosome binding site and a synthetic promoter that assures the efficient expression of mammalian proteins in bacteria.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

Use of synthetic promoters and ribosome binding sites for the efficient expression of mammalian proteins in bacteria.

Methods Employed:

Recombinant DNA techniques; nucleic acid hybridization; gel electrophoresis; electron microscopy; protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

We have constructed plasmid vectors carrying different chemically synthesized "hypothetical" promoter sites in tandem with one of several chemically synthesized hypothetical ribosome binding sites and have compared each of these vectors for the expression of cloned mammalian genes. While the synthetic promoter sequence provided for recognition by the *E. coli* RNA polymerase (a process that led to transcription of the cloned cDNA sequence), the synthetic ribosome binding site allowed recognition of the resulting mRNA by *E. coli* ribosomes (an event that resulted in the synthesis of the mammalian protein). From such analyses it was possible to identify the optimal combination of the two prokaryotic regulatory elements, one that would induce the highest level of expression of eukaryotic proteins in bacteria. A general expression vector carrying these two recognition sequences has been constructed and has been used successfully in the expression of biologically active human gamma (immune) interferon at an unusually high level.

Significance to Biomedical Research and the Program of the Institute:

One of the major goals of recombinant DNA research is to produce significant quantities of medically and agriculturally important proteins. If these products are to be used as human therapeutics, they must be "authentic" so as not to induce an immune response in the recipients. These two prerequisites have induced a great deal of investigation over the past several years. We have successfully generated a prokaryotic expression vector which not only can express authentic mammalian proteins in *E. coli*, but can do so at high levels. Such a vector is important for future production of human products synthesized in bacteria.

Proposed Course:

We shall continue to improve our expression vector with the goal of being able to induce the bacteria to secrete authentic mammalian proteins.

Publications:

Jay, G. and Liu, D. T.: Production of proteins by recombinant DNA technology: An overview. In Vitro. (In Press).

Jay, E., Rommens, J. and Jay, G.: Synthesis of mammalian proteins in bacteria. In Cheremisinoff, P. N. and Ouellette, R. P. (Eds.): Biotechnology Handbook. Lancaster, Technomic Publishing Co., 1985, pp. 387-399.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05220-05 LMV

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Structure and Function of Cell Surface Antigens

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

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Jonathan Vogel	Medical Staff Fellow	LMV	NCI
	Roberta Reynolds	Research Microbiologist	LMV	NCI
	George Khoury	Chief	LMV	NCI

COOPERATING UNITS *(if any)*
Virology & Cellular Immunology Section, Laboratory of Viral Diseases, NIAID (W. Davidson)

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Cell Physiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	2.2	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.)*
We have cloned and analyzed cDNA sequences derived from genes which encode the classical transplantation antigens. Our findings have led to a better understanding of the structure and function of these cell surface antigens, particularly with regard to their role in the presentation of tumor and viral antigens to the immune system.

PROJECT DESCRIPTION

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

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Jonathan Vogel	Medical Staff Fellow	LMV	NCI
Roberta Reynolds	Research Microbiologist	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

We wish to study the organization and expression of the genes coding for the H-2 histocompatibility antigens by molecular cloning technologies.

Methods Employed:

Recombinant DNA techniques; nucleic acid hybridization; gel electrophoresis; electron microscopy; protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

1. Since the class I genes belong to a multigenic family with 25 to 35 individual members, attempts to study their expression would be futile without the availability of unique DNA probes which will identify specific class I genes. While the coding regions of these genes are greater than 80 percent homologous, we have observed that their 3' noncoding regions are completely divergent. Based upon this observation, cDNA probes derived from this latter region of different class I transcripts have been isolated and used successfully to study the expression of individual members of this gene family. In addition, we have also identified locus-specific nucleotide substitutions within the region which encodes the transmembrane domain of different class I antigens and have synthesized specific oligonucleotide probes to further distinguish among these genes. The availability of such specific probes forms the basis for future studies directed towards defining the mechanism of regulation and expression of this important set of genes. For example, since the presence of certain alleles of class I genes have been tightly correlated with susceptibility to specific types of diseases, the probes that we have generated will allow a molecular definition of such associations with the hope of facilitating diagnosis and identifying the etiological basis.

2. One of the major roles of the class I antigens is the presentation of tumor cells and virus-infected cells to the cytotoxic T-lymphocytes, a process that leads to the destruction of cells displaying the "foreign" antigens. A problem of great concern at the present time is to define the macromolecular interactions between the self class I antigen and the nonself "foreign" antigen. An understanding of this phenomenon will undoubtedly help us combat a broad spectrum of viral diseases. In our study of one of the class

I genes, we have observed alternate splicing of its mRNA which would give rise to class I antigens with different amino acid sequences. The existence of different functional subsets of antigens, encoded by the same class I gene but generated by alternate RNA splicing at the post-transcriptional level, has provided a mechanism for generating diversity and polymorphism. The isolation of cDNA clones derived from the differentially-spliced mRNAs has allowed us to begin a series of experiments to ascertain the functions of the various class I antigens both on the effector T-cells as well as on the "foreign" target cells. Whether the use of alternative splice sites is altered upon malignant transformation, possibly as a mechanism for the escape of tumor cells from immune surveillance, is presently being determined.

3. While the basis for having 25 to 35 class I genes within the genome is not clear, it has been speculated that the expression of multiple class I antigens of different specificities in an animal will increase the probability of effective presentation of a wide range of virus-infected cells or tumor cells to the immune system. The expression of the major class I genes appears to be coordinately but differentially regulated. For example, while the H-2K and H-2D subregion genes are turned on in the same cell, the level of expression of the H-2K genes is significantly higher than those of the H-2D genes. To explain this apparent differential regulation, we have made the observation that the H-2D subregion genes carry an insertion of an Alu type 2 repetitive element within their 3' noncoding regions. We were able to demonstrate that this insertion resulted in a change in the endonucleolytic cleavage site which is necessary for generating a correct 3' end for polyadenylation of the mRNA. While the role of the repetitive elements within the mammalian genome has always been a conjecture, our present study provided the first demonstration of a function for the Alu type 2 sequence. Experiments are underway to determine whether the change in polyadenylation site could have decreased the level of expression of the H-2D subregion genes or decreased the stability of their transcripts. Since the expression of class I genes has been found to be perturbed upon malignant transformation, a clear understanding of what regulates their expression is important.

Significance to Biomedical Research and the Program of the Institute:

In the process of immune surveillance, the cytotoxic T-cell recognizes tumor cells and virus-infected cells in context with the classical transplantation antigens. The consequence of this dual recognition process is the destruction of cells displaying both the "self" antigen and the "foreign" antigen. The molecular mechanism underlying this interaction, while of particular importance to our understanding of the cancer problem, has remained obscure. With the molecular cloning of genes for the classical transplantation antigens and the ability to express them in transfected cells, one can begin to dissect this seemingly complex recognition process at the biochemical level.

Proposed Course:

Our observations with the mouse H-2 system are now being extended to the human HLA system, with the goal of obtaining molecular definitions for different HLA-associated diseases.

Publications:

Davidson, W. F., Kress, M., Khoury, G. and Jay, G.: Comparison of HLA class I gene sequences: Derivation of locus-specific oligonucleotide probes specific for HLA-A, HLA-B and HLA-C genes. J. Biol. Chem. (In Press).

Kress, M., Barra, Y., Seidman, J. G., Khoury, G. and Jay, G.: Functional insertion of an Alu type 2 (B2 SINE) repetitive sequence in murine class I genes. Science 226: 974-977, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05254-04 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

Regulation of Insulin Gene Expression

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

PI:	Laimonis Laimins	Staff Fellow	LMV	NCI
Others:	George Khoury	Chief	LMV	NCI
	Monika Holmgren-König	Microbiologist	LMV	NCI

COOPERATING UNITS *(if any)*

Laboratory of Biochemical Pharmacology, NIADDK (A. Furano)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

We have examined the DNA sequences responsible for regulation of insulin expression in the β-cells of pancreas. A DNA transfection procedure for introducing DNA molecules into established lines of transformed hamster β-cells (HIT) has been established. Using this protocol, a sequence upstream of the rat insulin I gene has been identified which can activate expression from a heterologous promoter to high levels in the HIT cell line. This element shares several properties with viral enhancer elements and is presently being characterized in detail. Current studies also focus on the identification of pancreatic cell-specific factors that bind to the upstream regulatory regions of the rat insulin gene and to identify the particular insulin regulatory sequences that interact with these proteins. Sequences located 1.5-kb upstream of the rat insulin I cap site have been identified as members of a major rat long interspersed repetitive (LINE) sequence. These sequences have a down regulatory or negative effect on enhancer function as shown in a transient assay system using the SV40 enhancer and a heterologous indicator gene. They reduce enhancer-mediated transcriptional activity 10-fold when positioned either 5' or 3' to the indicator gene. The mechanism of inhibition is currently under study.

PROJECT DESCRIPTION

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

Laimonis Laimins	Staff Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI
Monika Holmgren-König	Microbiologist	LMV	NCI

Objectives:

The goal of this study is to understand the regulation of expression of insulin genes in fibroblasts and insulinoma cells.

Methods Employed:

RNA analyses by Northern hybridization and primer extension; recombinant DNA technology; protein gel electrophoresis; transient assay system.

Major Findings:

1. A tissue-specific enhancer region has been identified in sequences upstream of the rat insulin I gene.

2. Upstream transcriptional elements appear to be present in sequences flanking the insulin gene.

Significance to Biomedical Research and the Program of the Institute:

Insulin represents a gene of importance in mammalian metabolic and disease states. Upstream flanking sequence polymorphisms in the human insulin genes have been correlated with the onset of certain types of diabetes. Systems allowing study of these sequences may indicate possible mechanisms of aberrant gene expression.

Proposed Course:

Studies will be pursued using expression vectors for insulin, with emphasis on defining regulatory signals and comparing these signals in fibroblasts and insulinoma cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05354-03 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

Studies on the Activated Form of the Human Proto-oncogene, c-Ha-ras

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

PI:	Rudy Pozzatti	Guest Researcher	LMV	NCI
Others:	George Khoury	Chief	LMV	NCI
	Bruce Howard	Chief, Molecular Genetics Section	LMB	NCI
	Lance Liotta	Chief	LP	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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

We have transfected various viral and cellular oncogenes into primary cultures of rat embryo cells and have obtained lines of morphologically transformed cells. Transformation with the ras oncogene alone was observed; however, a 10-fold increase in the transformation frequency was obtained when ras was cotransfected with the adenovirus E1A gene. We have examined cell lines transformed by the ras oncogene alone, and by ras plus E1A and have observed a striking difference in their metastatic potential as assayed in nude mice.

PROJECT DESCRIPTION

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

Rudy Pozzatti	Guest Researcher	LMV	NCI
George Khoury	Chief	LMV	NCI
Bruce Howard	Chief, Molecular Genetics Section	LMB	NCI
Lance Liotta	Chief	LP	NCI

Objectives:

This study is designed to examine the question of what oncogene(s) are required to transform primary rat embryo cells. We obtained transformed cultures from transfection of the ras gene alone, or by cotransfecting ras and the adenovirus E1A gene. A comparison of the phenotypic properties of the two types of transformants revealed a difference in their metastatic potential.

Methods Employed:

Isolation and cloning of specific fragments; construction of recombinant vector molecules; extraction of mRNA; Northern and Southern blotting analysis of RNA and DNA; immunoprecipitation; DNA transfection; tumorigenesis assays in nude mice.

Major Findings:

The c-Ha-ras oncogene isolated from the human bladder carcinoma cell-line, T-24, is capable of transforming the established mouse cell line NIH 3T3. Conflicting reports exist in the literature about whether or not the ras oncogene alone is capable of stably transforming primary cells in culture. Two groups report successful transformation of human and rodent primary cells using a viral enhancer element to increase the levels of ras gene expression. Others have found that the ras gene alone is incapable of transforming primary cultures of rodent cells. However, addition of a second so-called cooperating oncogene to transfection mixtures resulted in efficient transformation of primary cells. These investigators have used the two-gene requirement for the transformation of primary cells to classify viral and cellular oncogenes into two complementation groups depending upon their ability to cooperate in the transformation of primary cells.

We have transfected oncogenes into second passage rat embryo cells and have examined the frequency of morphological transformation that results from using a single gene (ras) versus two "cooperating" genes (ras and the adenovirus E1A gene). Transfection of the ras gene alone resulted in low frequency of transformation (one cell in 10^5). Transfection of two-genes resulted in a 10-fold increase in the transformation frequency (one cell in 10^4). Cell lines were established from the one and two-gene transformants in order to examine their phenotype properties.

In the course of developing these cell lines, we observed that the two-gene transformants became established in culture at greater than 95% efficiency. In contrast, only about 20% of the transformed foci that arose by transfection of the ras gene alone went on to become established in culture. Growth curves in 10% fetal calf serum showed that two-gene transformants had a more rapid cell doubling time than ras alone transformants (12 hours versus 24 hours). The most striking phenotypic difference between the two classes of transformants was observed when tumorigenicity assays were performed. Both one and two-gene transformants formed rapidly growing tumors in nude mice when injected subcutaneously. However, four of the eight ras alone transformants formed metastases in the lungs of animals bearing subcutaneous tumors. None of six two-gene transformants formed metastases after subcutaneous injection. When the metastatic potential of transformed cell lines was analyzed by intravenous injection of cells, all eight ras alone transformants formed large numbers (> 200) of metastatic nodules in the lungs of IV-injected animals. In contrast, only one of six two-gene transformants gave large numbers of lung metastases (~100); the other five lines showed no metastatic potential. These results indicate that the ras gene alone is capable of converting a primary cell to a transformed cell with high metastatic potential.

Significance to Biomedical Research and the Program of the Institute:

The activated form of the human proto-oncogene, c-Ha-ras, has been isolated from a bladder carcinoma. This DNA is capable of morphologically transforming NIH 3T3 cells, and therefore is very likely to be intimately involved in the formation of the original tumor. The experiments described in this report are designed to determine if the ras gene can transform primary cells in culture, or if additional oncogenes are required for morphological transformation.

Proposed Course:

We will continue the study of the one and two-gene transformed cell lines. To date, all of the tumorigenicity and metastatic studies have been done in nude mice. We will examine the metastatic potential of these lines in a syngeneic host, the Sprague-Dawley rat.

Since five of the six two-gene transformants exhibited low or no metastatic potential, the possibility exists that the E1A oncogene may in some way suppress the metastatic phenotype. E1A will be transfected into several of the ras alone transformants, and their metastatic potential will be re-examined with the advent of E1A expression.

Transformation usually confers the property of growth in low serum concentrations. In addition, recent reports suggest that transformed cells often produce transforming growth factors (TGFs). We will examine the single gene and two-gene transformants for TGF synthesis and secretion. In this way, we hope to draw correlations between the presence of a particular transforming gene (ras or E1A) and expression of a growth factor.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05355-03 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

Regulation of Immune Surveillance Against Tumor Cells

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

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Yves Barra	Visiting Fellow	LMV	NCI
	George Khoury	Chief	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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

Since the class I molecules are self antigens present on the surface of all cells in the body, the immune system must be rendered tolerant to them. Yet, these class I antigens must be recognized by cytotoxic T-cells in the associative recognition of virus-infected and tumor cells. In our analysis of class I genes, we have identified a related gene which may function to regulate this self-nonself recognition. This class I gene is expressed only in the liver and encodes a secreted class I antigen. Our demonstration of the secretion of a class I antigen by the liver has explained a previous observation that liver grafts across histocompatibility barriers were never rejected and has led us to suggest that this molecule serves to modulate class I restriction. We reasoned that a molecule with class I specificity that is constantly secreted into the circulation could act as a "blocking" factor, leading to suppression of class I recognition. The level of expression of such a blocking factor may act directly to modulate self-nonself recognition that will destroy aberrant cell types but not normal cells. This hypothesis has significant implications and suggests a means to modulate the host's response to neoplastic and autoimmune diseases. Attempts are being made to determine what regulates the expression of this particular class I gene.

PROJECT DESCRIPTION

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

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Yves Barra	Visiting Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

To study the mechanisms involved in regulating immune surveillance.

Methods Employed:

Recombinant DNA techniques; nucleic acid hybridization; gel electrophoresis; electron microscopy; protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

Structurally, the major transplantation antigens, designated H-2K, D and L in mice and HLA-A, -B and -C in humans, are classical cell-surface glycoproteins. The presentation of these antigens on the cell surface is a functional prerequisite both for their role in inducing allograft rejection, as well as for their involvement in the associative recognition of viral and tumor antigens.

Sequence analysis of mouse H-2 cDNA clones has suggested the existence of a novel class of H-2-related antigens which, unlike the classical membrane-associated molecules, retains only the extracellular portion and is likely to be secreted. The expression of this class of H-2 related mRNA is tissue restricted; it is detectable in liver, but not in brain, kidney, testis, thymus or spleen. In the liver, its accumulation represents about one-fourth of all the H-2-specific transcripts. This class of transcripts is present in mice of different inbred strains, but the level of expression differs markedly among them. A model is presented in which such a soluble form of the H-2 antigen would play the role of a blocking factor in maintaining peripheral inhibition of H-2 recognition. This would assure tolerance of the H-2 molecule as a self antigen while permitting it to act as a guidance molecule for the associative recognition of viral and tumor antigens by cytotoxic T-cells.

Significance to Biomedical Research and the Program of the Institute:

The transplantation antigens have been shown to be restricting elements that permit T-cells to detect foreign antigens in the context of self. In the process of immunosurveillance, the T-cell receptor on the cytotoxic T-lymphocyte must recognize both the foreign antigen and a self transplantation antigen. This dual recognition process, however, may invoke a conceptual paradox. As self molecules, present on all cells in the body, the immune system must be rendered tolerant to the transplantation antigens. Yet, it is with these same

self antigens that the immune system recognizes foreign antigens. It is tempting to speculate that the putative protein product of this H-2-related gene may function as a "blocking" factor. As a "self" antigen, H-2 should have induced a state of immunological unresponsiveness; but as a "guidance" molecule, H-2 has to be recognized in conjunction with the foreign antigen. It is possible that immunocompetent cells with H-2 reactivity are regulated by some form of suppression in the adult ("active tolerance") instead of the complete deletion of H-2 specific immunoreactive cells during prenatal and/or neonatal life ("passive tolerance"). A molecule with H-2 specificity that is constantly secreted into the circulation may well act as a blocking factor to suppress H-2 recognition. The secretion of such H-2 molecules would then be responsible for maintaining the fine balance between self-nonself recognition of the H-2 antigen present on the cell surface. Any perturbation in the level of expression of these secreted H-2 molecules could serve to tip this delicate balance of immune recognition mediated by membrane-associated H-2 molecules and would have significant physiological implications.

Proposed Course:

Attempts are being made to express this H-2-related antigen in transfected cells and to demonstrate that the secreted H-2 product can specifically block the recognition of virus-infected cells by the cytotoxic T-cell.

Publications:

Barra, Y., Tanaka, K., Davidson, W., Khoury, G. and Jay, G.: Expression of a secreted form of the MHC class I antigen. In Pernis, B. and Vogel, H. J. (Eds.): Cell Biology of the Major Histocompatibility Complex. New York, Academic Press, 1985. (In Press).

Barra, Y., Tanaka, K., Isselbacher, K. J., Khoury, G. and Jay, G.: Stable transfer and restricted expression of a cloned class I gene encoding a secreted transplantation-like antigen. Mol. Cell. Biol. 5: 1295-1300, 1985.

Siwarski, D. F., Barra, Y., Jay, G. and Rogers, M. J.: Occurrence of a unique MHC class I gene in distantly related members of the genus Mus. Immunogenetics 21: 267-276, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05390-02 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

How Do Tumor Cells Escape Immune Surveillance?

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

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Kenichi Tanaka	Visiting Associate	LMV	NCI
	Hiroaki Hayashi	Visiting Fellow	LMV	NCI
	George Khoury	Chief	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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

The classical transplantation antigens (the major histocompatibility complex class I antigens) play a key role in host defense against cells expressing foreign antigens. Several naturally occurring tumors and virally transformed cells show an overall suppression of these surface antigens. Since the class I molecules are required in the presentation of neoantigens on tumor cells to the cytotoxic T-lymphocytes, their absence from the cell surface may lead to the escape of these tumors from immunosurveillance. To test this possibility, a functional class I gene was transfected into human adenovirus 12-transformed mouse cells which do not express detectable levels of class I antigens; the transformants were tested for expression of the transfected gene and for changes in oncogenicity. The expression of a single class I gene, introduced by DNA-mediated gene transfer into highly tumorigenic adenovirus 12-transformed cells, was sufficient to abrogate the oncogenicity of these cells. This finding has important implications for the regulation of the malignant phenotype in certain tumors and for the potential modulation of oncogenicity through derepression of the endogenous class I genes.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Kenichi Tanaka	Visiting Associate	LMV	NCI
Hiroaki Hayashi	Visiting Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

Molecular analysis of the escape by tumor cells from immune surveillance.

Methods Employed:

Recombinant DNA techniques; nucleic acid hybridization; gel electrophoresis; electron microscopy; protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

The immune system is involved not only in defense against infections but also against "spontaneously derived" aberrant cells. This latter immune function appears to be essential for the removal of autonomous cell variants that presumably arise frequently in all multicellular organisms.

The development of malignant tumors, therefore, represents not only neoplastic transformation, but the failure of host resistance to eliminate certain abnormal cells. Transformation of a cell is insufficient to ensure its escape from immune surveillance. Cells transformed in culture very often do not induce tumors when transplanted back into immunocompetent syngeneic hosts. It is those properties of certain tumor cells allowing them to resist immune recognition which are ultimately responsible for their tumorigenicity.

The major histocompatibility complex class I (H-2) antigens (designated K, D and L in mice) are indispensable for the presentation of cells bearing "foreign" antigens to the cytotoxic T-lymphocytes. The finding that certain malignant tumors, including teratocarcinomas, eccrine porocarcinomas and cervical carcinomas, have markedly reduced or nondetected levels of cell-surface class I antigens (in contrast to their normal cellular counterparts) suggests a possible mechanism for their escape from immune surveillance.

In support of this hypothesis is the recent finding that cells transformed by the highly oncogenic strain of human adenovirus (Ad12), in contrast to the non-oncogenic strain (Ad5), also express reduced levels of class I antigens on their surfaces. This observation with Ad12 provides an experimental system for demonstrating that the absent or reduced expression of class I antigens is directly responsible for oncogenicity.

We now show that transfection of a functional class I gene into a highly tumorigenic Ad12-transformed cell line that expresses no detectable class I surface antigens resulted in its complete loss of oncogenicity. This finding indicates one possible mechanism for the escape of certain tumors from immune surveillance and suggests future therapeutic approaches for the reversal of certain malignancies.

Significance to Biomedical Research and the Program of the Institute:

The inability of the immune system to combat "aberrant" cells is basic to the problem of neoplasia. However, the mechanism(s) whereby tumor cells escape immune surveillance has yet to be defined. Since the classical transplantation antigens (designated class I antigens) are expressed on the surface of virtually all cells in the body and are indispensable for cell-cell interactions to assure integrity of tissues, a cell that has "accidentally" turned-off the expression of these class I antigens is expected to proliferate within a differentiated tissue without control from its neighbors. These "aberrant" cells have the added facility to escape recognition by cytotoxic T-cells which act on target cells only in context with class I antigens. The finding that expression of class I genes is dependent upon hypermethylation at the DNA level, together with the observation that DNA from primary tumors is hypomethylated, suggests a mechanism whereby the expression of class I antigens is repressed in neoplastic cells and hence escapes surveillance.

Proposed Course:

These studies have suggested the possibility of reversing the neoplastic state of growth by derepression of class I antigens. We will attempt to define the molecular mechanism for the repression of class I gene expression in tumor cells.

Publications:

Tanaka, K., Isselbacher, K. J., Khoury, G. and Jay, G.: Reversal of oncogenesis by the expression of a MHC class I gene. Science 228: 26-30, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05391-02 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

In Vitro Transcription Analysis of the SV40 Late Promoter

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

PI:	John Brady	Expert	LMV	NCI
Others:	Janet Duvall	Biological Aid	LMV	NCI
	Kamel Khalili	Visiting Fellow	LMV	NCI
	Jeffrey Green	Medical Staff Fellow	LMV	NCI
	George Khoury	Chief	LMV	NCI

COOPERATING UNITS (if any)

Laboratory of Biology of Viruses, NIAID (H. Mishoe)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	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.)

We have previously shown that DNA sequences located within the simian virus 40 (SV40) GC-rich, 21-bp repeats constitute an important transcriptional control element of the SV40 late promoter (Brady et al., Mol. Cell. Biol. 4: 133-141, 1984). To gain further insight into the mechanism by which the SV40 GC-rich repeats function, we have analyzed the transcriptional properties of several recombinant DNAs. These results suggest that the SV40 GC-rich sequences can function as independent RNA polymerase II transcriptional-control elements. In vitro competition studies demonstrated that sequences within the GC-rich 21-bp repeats, in the absence of either the SV40 early or late -25 transcriptional control signals (TATA box) or the major RNA initiation sites, efficiently competed for transcription factors required for SV40 early and late RNA synthesis. Our transcription studies also demonstrated that in the absence of contiguous SV40 transcription control sequences, these GC-rich sequences stimulated initiation of bidirectional transcription from proximally located sequences. We further demonstrated that the 21-bp repeat region can stimulate in vitro transcription from the heterologous adenovirus 2 major late promoter.

We have also analyzed the transcriptional properties of a series of mutant templates which contain DNA insertions between the SV40 21-bp tandem repeats and the early Goldberg-Hogness TATA box. Our results suggest that the SV40 early G-H sequences require the presence, within a fixed distance, of upstream regulatory elements in order to function efficiently. When this interaction is disrupted by inserts of increasing distance, SV40 early RNA initiation sites are switched upstream to the "late-early" RNA initiation sites.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Name, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

John Brady	Expert	LMV	NCI
Janet Duvall	Biological Aid	LMV	NCI
Kamel Khalili	Visiting Fellow	LMV	NCI
Jeffrey Green	Medical Staff Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

To identify transcription regulatory sequences of eukaryotic polymerase II promoters.

Methods Employed:

Recombinant DNA techniques; construction of deletion and point mutants; in vitro transcription; electrophoretic analysis of RNA transcripts; nucleic acid hybridization; DNA sequencing; DNA transfection.

Major Findings:

1. The SV40 21-bp repeat, independent of other transcriptional control elements, effectively binds transcriptional factors required for early and late transcription.

2. The 21-bp repeats can stimulate transcription from a heterologous adenovirus-2 major late promoter.

3. In the absence of contiguous transcriptional control sequences, the 21-bp repeats are capable of initiating bidirectional transcription from proximally located sequences.

4. The SV40 early G-H sequence and/or associated transcriptional factors must physically interact with the upstream 21-bp repeats and/or 72-bp repeats in order to function efficiently.

Significance to Biomedical Research and the Program of the Institute:

During cellular transformation, the expression levels of numerous RNA polymerase II transcribed genes are increased or decreased. The mechanism by which eukaryotic genes are regulated at the transcriptional level is fundamental to our understanding of oncogenic transformation. Our in vitro transcriptional analysis of RNA polymerase II genes is designed to identify transcriptional regulatory sequences at the nucleotide level. Ultimately, the interaction of RNA polymerase II and other transcriptional factors with those sequences will elucidate basic mechanisms of eukaryotic gene regulation.

Proposed Course:

1. A series of base substitution mutants will be constructed in the SV40 21-bp repeats. The mutants will then be tested by *in vitro* and *in vivo* transcriptional analysis to identify critical regulatory sequences. In the same context, these studies will determine if the two tandem and one non-tandem 21-bp units are functionally identical or represent distinct transcriptional regulatory units.

2. The *in vitro* competition experiments indicate that individual polymerase II transcriptional sequences can efficiently bind transcriptional factors and/or RNA polymerase. Following cloning of appropriate DNA fragments, regulatory proteins which bind specifically to the polymerase II transcriptional units will be isolated, characterized and tested for biological function.

3. Insertion mutants between the SV40 21-bp repeats and 72-bp enhancer will be constructed. The mutants will be tested *in vitro* and *in vivo* to analyze their effect on SV40 early-early and early-late transcription.

4. SV40 promoter elements will be chemically synthesized and connected in various positions and orientations to assess the requirements for efficient *in vivo* and *in vitro* transcription.

Publications:

Mishoe, H., Brady, J. N., Radonovich, M. and Salzman, N. P.: Simian virus 40 guanine-cytosine-rich sequences function as independent transcriptional control elements *in vitro*. *Mol. Cell. Biol*. 4: 2911- 2920, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05392-02 LMV

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transcriptional Regulation of SV40 Late Transcription by Large T-Antigen.

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

PI:	John Brady	Expert	LMV	NCI
Others:	George Khoury	Chief	LMV	NCI
	Mary Loeken	PRAT Fellow	LMV	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	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 demonstrated that SV40 late gene expression is trans-activated by the SV40 early gene product, T-antigen, in the absence of DNA replication. RNA analysis demonstrates that activation occurs at the transcriptional level. Using deletion and point mutants, two important domains from the SV40 T-antigen-induced late gene expression have been identified. One of these includes T-antigen binding sites I and II (SV40 m.p. 5171-0), while the other is located in the SV40 72-bp repeat (SV40 m.p. 128-272). To determine how the two upstream control elements interact to effect T-antigen dependent trans-activation, we have used template competition analysis. In the presence of increasing levels of competitor DNA fragments, which are capable of binding limiting trans-acting factors, a decrease in expression from a fixed amount of template was observed. In vivo competition with recombinant plasmids containing the entire SV40 late regulatory region and promoter sequences (m.p. 5171-272) results in quantitative removal of limiting trans-acting factor(s). Deletion of either the T-antigen binding sites (m.p. 5171-5243) or the 72-bp tandem repeat (m.p. 128-272) from the competitor plasmid results in markedly less efficient binding of the trans-acting factor. Cotransfection of two separate plasmids, one containing the T-antigen binding sites I and II and the other the 72-bp repeats, fails to induce competition for the trans-acting factors. Insertion of DNA sequences between the T-antigen binding sites and the enhancer sequences also dramatically reduces the efficiency of competition. These results suggest that efficient binding of trans-acting factors requires the presence, in cis, of at least two SV40 regulatory domains. Our studies further suggest that the distance separating these two transcriptional signals is important.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

John Brady	Expert	LMV	NCI
George Khoury	Chief	LMV	NCI
Mary Loeken	PRAT Fellow	LMV	NCI

Objectives:

Identify mechanisms by which eukaryotic genes, transcribed by RNA polymerase II, are regulated at the transcriptional level.

Methods Employed:

Recombinant DNA techniques; eukaryotic cell transfection; nucleic acid hybridization; electropheretic immunoblot analysis of protein; gel electrophoresis; construction of deletion and point mutants; *in vivo* competition assays.

Major Findings:

1. SV40 large T-antigen stimulates transcription from the SV40 late promoter.

2. Analysis of promoter mutants and *in vivo* competition experiments indicate that binding of T-antigen to sites I and II is important for efficient induction of late transcription.

3. T-antigen activation of the SV40 late promoter may mediate removal of negative transcriptional factors.

Significance to Biomedical Research and the Program of the Institute:

We have shown that SV40 large T-antigen stimulates expression of the SV40 late genes at the transcriptional level. The transcriptional activation appears to be mediated, through a trans-acting mechanism, by the binding of SV40 large T-antigen to the SV40 late promoter. This contribution to transcriptional activation is fundamentally different from transcriptional control by cis-acting promoter and enhancer elements. It should provide an insight into regulatory mechanisms by which SV40 T-antigen stimulates transcription of cellular genes during viral infection and oncogenic transformation.

Proposed Course:

1. Regulatory sequences within SV40 T-antigen binding sites I and II are critical for trans-activation of late gene expression. Site-specific mutagenesis of the DNA control region will be used to determine the critical sequences. The effect of the mutations will be analyzed by the use of altered templates and by *in vivo* competition analysis.

2. Amino acid substitution mutants will be examined to determine the critical domains of T-antigen required for interaction with host cellular transcriptional factors.

3. *In vivo* competition experiments suggest that SV40 T-antigen might activate late transcription by removing repressor proteins bound to the late transcriptional unit. The purification of putative host cell repressors by affinity binding to cloned SV40 sequences will be pursued. The function of such factors and their interaction with SV40 T-antigen will be examined using the *in vitro* transcription systems.

4. SV40 T-antigen stimulates transcription of eukaryotic cellular genes, perhaps in a manner similar to activation of the SV40 late promoter. Several of these genes have now been cloned, including a number of genes which are activated in the transformed cell. Transcriptional analysis of these eukaryotic DNA sequences will be performed to determine the mechanisms of gene activation.

Publications:

Brady, J. and Khoury, G.: Trans-activation of polymerase II promoters. In Leive, L., Bonventre, P. F., Morello, J. A., Schlesinger, S., Silver, S. D. and Wu, H. C. (Eds.): *Microbiology - 1985*. Washington, D.C., ASM Publications. (In Press).

Brady, J. and Khoury, G.: Trans-activation of the simian virus 40 late transcription unit by T-antigen. *Mol. Cell. Biol.* 5: 1391-1399, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05393-02 LMV

PERIOD COVERED

October 1, 1984 through September 30, 1985

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

Tissue-Specific Activation of Gene Expression

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

PI:	Laimonis Laimins	Staff Fellow	LMV	NCI
Others:	George Khoury	Chief	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.7	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.)

This study examines the role of enhancer elements in the expression of the Prague strain of Rous sarcoma virus, PrRSV. Viral enhancer elements have been shown to promote high level expression of viral genes as well as cellular genes adjacent to the integrated proviral DNA. Previous studies have associated the 3' LTR and immediate upstream sequences (called the XSR) with the disease spectrum of the tumor defective (td) variants of PrRSV. It is the intent of this study to identify and characterize the enhancer elements in this region and to correlate their properties with the disease spectrum of the virus. One enhancer element has been identified which contains 90 nucleotides of the 3' LTR as well as adjoining upstream sequences. This enhancer exhibits a host preference for expression in chicken cells. An additional enhancer sequence is found entirely within the LTR. We suggest that multiple enhancer domains are present in the 3' terminus of PrRSV and that the presence of at least two of these domains is required for enhancer function.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Laimonis Laimins	Staff Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objectives:

To identify enhancer-like sequences in the Prague strain of Rous sarcoma virus.

Methods Employed:

Transfection of eukaryotic cells; enzymatic assay for chloramphenicol-acetyl-transferase (CAT); S1 analysis of RNA; cloning and restriction endonuclease analysis of DNA.

Major Findings:

1. An enhancer element has been identified in the 3' end of the Prague B strain of Rous sarcoma virus. The enhancer element overlaps the 5' end of the long terminal repeat (LTR) and encompasses upstream sequences.

2. An additional enhancer is located entirely within the LTR region.

3. Multiple domains are required for the function of the PrRSV enhancer.

Significance to Biomedical Research and the Program of the Institute:

The identification of enhancer elements in the LTRs of RNA tumor viruses should provide an insight into the role of retroviruses in activation of cellular genes adjacent to the integrated provirus. This is a fundamental property of retro-viruses associated with their pathogenic potential.

Proposed Course:

The *in vitro* (CAT) assay will be used to study whether other enhancer elements are present in the 3' LTR of Rous sarcoma virus. In addition, point mutations will be constructed to identify specific sequences involved in enhancer activity.

Publications:

Laimins, L. A., Tsichlis, P. and Khoury, G.: Multiple enhancer domains in the 3' terminus of the Prague strain of Rous sarcoma virus. Nucleic Acids Res. 12: 6427-6442, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05394-02 LMV

PERIOD COVERED

September 1, 1984 through September 30, 1985

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

Enhancer Elements in B-Lymphocytes and T-Lymphocytes

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

PI:	Chou-zen Giam	Visiting Fellow	LMV	NCI
Others:	Michael Nerenberg	Medical Staff Fellow	LMV	NCI
	George Khoury	Chief	LMV	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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.)

This project is directed toward an understanding of enhancer elements and positive regulatory proteins that interact with enhancers. Recent focus of the project has been the possible role of the 3' long open reading frame of the human T-cell leukemia virus type-I (HTLV-I) to encode a 40-kd protein factor (pX) that positively regulates transcription directed by the HTLV-I long terminal repeats, a phenomenon known as trans-activation. Our objective is to produce pX in large quantity in E. coli and to study the molecular mechanism of trans-activation in vitro and in vivo.

PROJECT DESCRIPTION

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

Chou-zen Giam	Visiting Fellow	LMV	NCI
Michael Nerenberg	Medical Staff Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI

Objective:

This project concerns the mechanism of enhancer function in B-lymphocytes and T-lymphocytes. It should yield information about the molecular interaction between enhancer elements and protein factors that positively regulate their activity. The present approaches include:

1. Construction of bacterial expression vectors that give rise to overproduction of pX in *E. coli*.

2. Purification of the pX gene product and introduction of this protein into somatic cells by protoplast fusions or microinjection.

3. Nitrocellulose filter binding assays and DNA footprinting assays to establish the interaction between pX and the HTLV-I-LTR.

4. Construction and mutagenesis of HTLV-LTR sequences to study the effect of sequence alteration on the activity of the LTR.

5. Introduction of BPV-transforming genes and the MOPC-41 κ-light chain gene simultaneously into L-cells to address the effect of the BPV-transforming genes on the expresssion of the κ-gene.

Methods Employed:

Recombinant DNA techniques; bacterial expression vectors; somatic cell fusion techniques; protein purification; gene expression using transient and permanent assays; RNA and protein analysis.

Major Findings:

We have successfully expressed the pX coding sequences in *E. coli*. Currently, we are purifying pX for *in vivo* and *in vitro* studies. Methods are being devised to directly deliver pX protein from overproducer strains of *E. coli* into somatic cells by protoplast fusion. Further studies will be developed in the area of the structure and function of pX and the molecular mechanism of *trans*-activation.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the mechanism of action of pX is important to the understanding of leukemogenesis and may also serve as a model system for the study of the

AIDS virus (HTLV-III). The elucidation of the molecular interaction between pX and HTLV-I-LTR is also important in understanding how gene activities are regulated in eukaryotic cells.

Proposed Course:

The project will continue to stress the role of enhancers and enhancer-specific proteins in gene regulation in T-cells and B-cells.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF TUMOR VIRUS BIOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

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 Onocology Section (1) studies the molecular biology and gene regulation of the papillomaviruses and mechanisms of papillomavirus-induced transformation and carcinogenic progression; (2) develops techniques for DNA mediated gene transfer; and (3) carries out studies on extrachromosomal plasmid replication, recombination, and partitioning in mammalian cells.

The Cellular Regulation and Transformation Section (1) examines the role of DNA tumor viruses in carcinogenesis and neoplasia; (2) characterizes the cellular and biochemical alterations associated with the oncogenic transformation; (3) analyzes the specific interactions between cellular and viral proteins during oncogenesis; and (4) examines the effect of specialized cellular differentiated functions on viral gene expression.

Among the primary objectives of the Laboratory of Tumor Virus Biology is the evaluation of the role of the human papillomaviruses in human carcinogenesis and an examination of the molecular biology of this group of viruses. A major focus of the laboratory is the study of the molecular biology of the bovine papillomavirus type 1. This virus is able to transform rodent cells in tissue culture and, as such, provides a model for the systematic study of the molecular biology and genetics of this group of viruses. The viral genome remains as a stable multicopy plasmid in transformed cells and is partitioned faithfully to daughter cells. As such cellular transformation and plasmid replication in the transformed cells provide models for studying the proliferative functions encoded by the papillomaviruses and latent viral infection by these viruses, respectively. A subgenomic segment, consisting of 69% of the genome, contains all of the necessary sequences required for transformation or for stable extrachromosomal plasmid maintenance and replication. This subgenomic transforming segment contains a non-coding region (NCR) consisting of a 1000 base region which sits 5' to a series of eight open reading frames (ORFs) which are transcribed in transformed cells. We have shown that this NCR contains elements of a transcriptional promoter required for the expression of these open reading frames in transformed cells. We have also shown that this NCR contains a transcriptional regulatory element with the properties of a transcriptional enhancer which is specifically transactivated by a specific viral gene product, the E2 gene product. Full-length cDNA cloning of

the viral specific messenger RNAs in transformed cells has permitted the structural and functional analyses of a subset of the viral RNAs in transformed cells. These studies have revealed that all of the RNAs are transcribed from a single strand and that they are polyadenylated at a common site. Multiple viral RNA species can be detected in transformed cells and are generated by differential splicing. Two independent species of cDNA clones contained in an Okayama and Berg expression vector are independently able to transform mouse C127 cells indicating that BPV-1 contains two transforming genes. Mutational analysis in the wild-type viral DNA background reveals that the E6 and E5 ORFs must be intact in the respective cDNAs to affect transformation, suggesting that the gene products of the E5 and E6 ORFs are the transforming proteins of the bovine papillomavirus. Of principle interest to this laboratory in the future will be the identification of these putative transforming gene products within transformed cells as well as within productively infected cells. Mutational analysis indicates that expression of an intact E2 gene product is important for transformation as well as for stable plasmid maintenance. The E2 product is involved in the transactivation of a transcriptional regulatory element present within the NCR. Thus, mutations in the E2 gene products affect the transcription of the early papillomavirus genes involved in plasmid maintenance and in transformation. Thus, the E2 gene product may be indirectly involved in transformation and extrachromosomal plasmid maintenance. Mutations within the E1 gene product have a minimal effect on transformation frequency but result in the integration of the viral DNA in the transformed cells. Thus, the E1 gene product is required for stable plasmid maintenance. Studies are also being directed at the expression of the E1 and E2 gene products in bacterial as well as mammalian cell vector systems in order to study these gene products and their potential interactions with the *cis* regulatory sequences of the bovine papillomavirus.

We have developed the papillomavirus into a mammalian cell cloning vector, taking advantage of the characteristic of these viruses to remain as stable extrachromosomal plasmids in mammalian cells. We have shown that genes devoid of enhancer elements, which are functional within mouse cells, must be positioned adjacent to a BPV-1 enhancer element in order to be expressed in BPV-1 based vectors. In this study, we have also shown that sequences exist in the prokaryotic plasmid vector, pBR322, which effectively "block" this enhancer effect. Using a bovine papillomavirus vector, we have expressed a portion of a proviral DNA of HTLV-1 and expressed the small 21 Kd envelope protein for this virus. The generation of a nontransforming extrachromosomal papillomavirus vector should be useful for the systematic study of the expression of oncogenes.

The papillomaviruses are associated with naturally-occurring tumors and experimentally-induced tumors in a variety of animal systems. In addition, evidence is mounting associating human papillomaviruses with specific human carcinomas. The most compelling evidence involves the association of one group of specific HPVs with cutaneous carcinomas in patients with epidermodysplasia verruciformis and the association of another group of HPVs with cervical carcinoma. Specifically, HPV-16 and HPV-18 have been found in a high percentage of biopsy specimens of cervical carcinomas in several laboratories in Europe and the United States. We have investigated a series of human carcinoma cell lines for the presence of HPV DNAs. Of eight cervical carcinoma cell lines examined, six have been shown to contain integrated HPV-16 or HPV-18 DNA. Of the six positive lines, five have been shown to be transcriptionally active for the papillomavirus sequences. Studies from other laboratories have shown the association of integrated HPV-18 DNA sequences in cervical carcinoma cell lines.

Our studies are the first to report the integration of HPV-16 DNA sequences in cervical carcinoma cell lines. In addition, our studies are the first to report the expression of HPV-16 RNA in cervical carcinoma cell lines. These integrated genomic sequences are now cloned and the cloning and expression of the cDNA copies of the viral transcripts in these cells are a high priority for this laboratory.

The genomic organization of the human papillomaviruses is quite similar to that of the bovine papillomavirus. Thus, the bovine papillomavirus serves as a useful model for predicting the putative gene products of the human papillomaviruses that may be associated with cellular proliferation and possibly carcinogenic progression. Efforts to develop a cell culture system to directly assess these cell proliferation functions are underway. Mouse epidermal cells have been transformed to a tumorigenic state by both bovine papillomavirus and polyoma virus. Cellular transformation using tumorigenesis in nude mice as an assay in 3T3 cells has been useful in demonstrating that some cervical carcinoma cell lines contain active oncogenes. The characterization and further identification of these oncogenes are underway. HPVs have been introduced into a variety of immortalized fibroblasts and epithelial cell lines using cotransfection with other selective markers. These studies will permit the expression of the HPV genes within cell lines.

Studies of the early events in virus-host cell interaction have utilized vesicular stomatitis virus as a model for looking at the uptake of virus into cells. Using synthetic peptides, it has been possible to show that the aminoterminus of the VSV spike protein mediates membrane fusion and interacts specifically with acidic phospholipids. In addition, this peptide is a pH dependent hemolysin, hemagglutinin, and cytotoxin. Further studies will extend to examine early interactions of the papovavirus, simian virus 40 (SV40) with cell membranes.

Considerable effort has been focused at studying transformation by the murine polyoma virus. The polyoma virus is, along with the papillomaviruses, grouped together as papovaviruses which are small DNA tumor viruses. The polyoma middle T antigen has been identified as playing a central role in polyoma virus induced oncogenesis. This protein is associated with a protein kinase activity which can be detected by _in vitro_ phosphorylation of a tyrosine residue. This protein does not possess _intrinsic_ protein kinase activity and is thought to associate with the cellular protein pp60c-src (the cellular homolog of the Rous sarcoma virus transforming gene). It is proposed that the polyoma virus middle T antigen protein kinase activity represents a property of the associated pp60c-src. The potential importance of this protein kinase activity in polyoma mediated oncogenesis is suggested by the finding that viral mutants which are deficient in transforming potential also lack this associated kinase activity. Elevated levels of pp60c-src kinase activity have also been demonstrated in human tumor lines, particularly of neuroectodermal origin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP00543-07 LTVB

PERIOD COVERED
October 1, 1984 to September 30, 1985

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:	C. Baker	Medical Staff Fellow	LTVB	NCI
	B. Spalholz	Guest Researcher	LTVB	NCI
	M. Rabson	Guest Researcher	LTVB	NCI
	Y.-C. Yang	Visiting Associate	LTVB	NCI
	P. Lambert	Guest Researcher	LTVB	NCI
	P. Hermonat	Guest Researcher	LTVB	NCI
	J. Byrne	Biologist	LTVB	NCI

COOPERATING UNITS (if any)
Department of Physiological Chemistry, University of Wisconsin, Madison, WI (E. Schenborn, J. Dahlberg); Laboratory of Molecular Genetics, National Institute of Child Health and Development, NIH (H. Okayama)

LAB/BRANCH
Laboratory of Tumor Virus Biology

SECTION
Viral Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.7	4.2	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.)

The papillomaviruses are a group of small DNA viruses associated with benign and malignant proliferative lesions in a variety of higher vertebrates. Currently, there are recognized to be 31 distinct human papillomaviruses (HPVs) and six bovine papillomaviruses (BPVs). The lytic expression of these viruses is linked to the state of differentiation of squamous epithelial cells and to date no tissue culture system exists for their propagation in the laboratory. The bovine papillomavirus type 1 (BPV-1) is one of a subgroup of papillomaviruses which is capable of inducing fibroblastic tumors when innoculated into hamsters and is capable of inducing morphologic transformation of certain rodent cells in tissue culture. To date, transformation of rodent cells remains the only _in vitro_ assay for the systematic study of the papillomaviruses. Because of this property, BPV-1 has become the prototype for unravelling the molecular biology of the papillomaviruses. A unique feature of this papillomavirus transformation system is that the viral DNA does not integrate into the host chromosome. The DNA remains extrachromosomal as a stable multiple copy plasmid. The factors involved in stable transformation as well as for stable plasmid maintenance are being extensively studied. A second characteristic associated with the papillomavirus infection is the propensity of certain viruses to be associated with lesions which may progress to carcinomas. What factors, either viral or host, which are involved in such a progression from a benign lesion to a carcinoma are as yet unknown. Our studies are designed to unravel the molecular biology of the normal virus infection of cells as well as for understanding the viral and cellular factors involved in carcinogenic progression. We have determined that BPV-1 encodes at least two genes which can independently transform mouse cells. Also, we have mapped a transcriptional regulatory element in the non-coding region (ncr) of BPV-1 which is _trans_-activated by the viral E2 gene product.

PROJECT DESCRIPTION

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

P. M. Howley	Chief	LTVB	NCI
C. Baker	Medical Staff Fellow	LTVB	NCI
B. Spalholz	Guest Researcher	LTVB	NCI
M. Rabson	Guest Researcher	LTVB	NCI
Y.-C. Yang	Visiting Associate	LTVB	NCI
P. Lambert	Guest Researcher	LTVB	NCI
P. Hermonat	Guest Researcher	LTVB	NCI
J. Byrne	Biologist	LTVB	NCI

Objectives:

1. To analyze the molecular biology of the papillomaviruses using the bovine papillomavirus as a model system.
2. To localize and characterize the transforming regions of the bovine papillomavirus.
3. To localize and characterize the regions of the papillomavirus involved in latent plasmid replication.
4. To determine whether or not the transforming functions encoded by the bovine papillomavirus can complement other known viral or cellular oncogenes in transforming primary rodent cells.
5. To develop a tissue culture system for the propagation of papillomaviruses.
6. To analyze the viral mRNAs expressed in BPV-1 transformed cells as well as in productively infected fibropapillomas excised directly from cattle.
7. To localize the transcriptional promoters functional in productively and non-productively infected cells.
8. To determine the factors, both viral and cellular, involved in the control of "late" or virus specific gene expression for the papillomaviruses.
9. To analyze the "early" papillomavirus protein products expressed in transformed cells.
10. To determine the cis and trans functions required for autonomous extrachromosomal plasmid replication.
11. To determine the nature of the molecular events involved in the progression of a benign papillomavirus lesion into a malignant lesion.
12. To characterize the virally encoded gene products involved in the transcriptional control of the papillomavirus genome.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of papillomavirus hybrid plasmids.
2. Tissue culture.
3. Transcriptional analysis including Northern blotting, cDNA cloning into expression vectors, and nuclear run-off experiments.
4. DNA sequencing.
5. The generation of synthetic peptides based on DNA sequence information.
6. Immunoprecipitation.

7. Immunoblotting and immunofluorescence of viral proteins.
8. Transfer of DNA into mammalian cells using standard calcium precipitation, DEA dextran or electroporation technology.

Major Findings:

1. Using genetic studies, we have localized regions of the BPV-1 genome required for transformation and plasmid replication. The transforming region of the BPV-1 genome had previously been localized to a specific subgenomic fragment comprising 69% of the viral genome. All of the sequences in cis and in trans required for transformation as well as for autonomous replication are localized within this 69% of the genome. By constructing defined deletion mutants and by engineering premature termination codons into the open reading frames (ORFs), it has been possible to dissect the genome and to define the various open reading frames which are essential for transformation and autonomous plasmid replication. Deletion mutants affecting the E2 ORF, particularly the aminoterminus, are significantly impaired in their ability to transform as well as in their ability to remain as plasmids, indicating that the E2 gene product has an important role for transformation and for autonomous plasmid replication. Further studies (see below) have indicated that this effect may be indirect and that the E2 ORF gene product is required for the transactivation of a transcriptional regulatory element located in the non-coding region of the BPV-1 genome. Additionally, mutants lacking the E6 and E7 ORFs are still able to induce transformation at a lower efficiency and the transformants have altered characteristics. The expression of the E6 ORF is required for the full transformed phenotype in mouse C127 cells and is required for anchorage-independence and for tumorigenicity. Mutations located within the E1 ORF do not significantly affect the transforming functions but result in the integration of the viral genome in transformed cells implicating the E1 gene product in stable plasmid replication and maintenance. A premature termination codon engineered into the carboxy terminus of the E5 ORF after the first AUG in the E5 ORF indicates that this region is also required for transformation. Thus, BPV encodes at least three genes that have an impact on transformation. The E5 and E6 ORFs appear to be directly involved in transformation and the gene product of the E2 ORF may be indirectly involved. A manuscript describing these transforming functions based on these genetic studies has been published in the Journal of Virology (Sarver et al, J. Virol. 52: 377-388, 1984). Additional studies with the premature termination codon engineered by Dr. Michael Rabson have permitted a finer dissection of these functions and a manuscript is in preparation.

2. A cDNA library made from RNA from BPV-1 transformed C127 cells was constructed using the Okayama-Berg expression plasmid to further define the structure of the RNAs in transformed cells and to characterize the function of individual viral transcripts. Sequence analysis of these cDNAs revealed that these viral transcripts are generated by differential splicing. In conjunction with the ORFs deduced from the BPV-1 DNA sequence, it has been possible to predict the structure of the potentially encoded proteins. The vector used to generate these cDNA clones contains mammalian cell transcriptional regulatory elements, facilitating their functional characterization. We have identified two distinct classes of cDNAs that can each independently transform mouse C127 cells. One class of the cDNAs contains the E6 ORF intact. The second class of cDNAs contains the 3' ORFs including E2, E3

E4, and E5 intact. Thus, BPV-1 encodes two viral functions which are each able to transform mouse cells. Cotransformation experiments indicate that these two gene products appear to act synergistically in this transformation. Premature termination codons engineered into the cDNAs containing the 3' ORFs indicate that the E5 ORF is primarily responsible for the transformation functions from that cDNA. The paper describing the cDNA library and the two independent viral transforming genes has been published in the Proceedings of the National Academy of Sciences (Yang, et al, PNAS 82: 1030-1034, 1985). An additional paper describing the introduction of premature termination codons into each of the 3' ORFs is in preparation.

3. We have mapped a transcriptional regulatory sequence within the 1 kb non-coding region of the BPV-1 genome using an enhancer dependent expression vector for chloramphenicol acetyltransferase (CAT). This transcriptional regulatory element works in a position and orientation independent manner, and its function is significantly augmented in BPV-1 transformed cells and in monkey CV1 cells acutely cotransfected with plasmids expressing BPV-1 early gene products. Using defined deletion mutants of the BPV-1 genome and full-length viral cDNAs expressed from an SV40 early promoter, we demonstrate that the expression of this transactivating factor maps to the 3' ORFs of the viral transforming region. A premature termination codon engineered into the E2 ORF eliminates the expression of this diffusable transactivation function, establishing the E2 gene product as the diffusable transactivating factor. These studies are in press in Cell by Spalholz, Yang and Howley.

4. A full-length cDNA library has been made in a Okayama-Berg vector from the RNA from a productively infected bovine fibropapilloma. Analysis of these cDNAs has indicated that the late transcripts are generated off a separate promoter located in the non-coding region. There is a sequence GGTACACATCC (n. 7212 to 7222) just upstream to the late leader cap site which has striking homology to the SV40 late promoter. The wart specific RNAs are polyadenylated just downstream from the AATAAA sequence at base 7175-7177. Preliminary experiments with nuclear run-offs from transformed cells indicate that there is active transcriptional termination within the several hundred bases upstream from this polyadenylation site.

5. In collaboration with the Dahlberg laboratory at the University of Wisconsin, we have constructed a mutant of BPV-1 DNA that lacks a transcriptional enhancer located 3' to the polyadenylation site of the early viral RNAs expressed in transformed cells. This enhancer has previously been described by the Botchan laboratory at the University of California at Berkeley. This mutant DNA, lacking the distal enhancer, transformed mouse cells with an efficiency comparable to that of the full BPV-1 genome and it existed as a stable multiple copy plasmid in transformed cells. We conclude, therefore, that the BPV-1 distal enhancer is not cis-essential for the expression of viral genes involved in cellular transformation or plasmid maintenance. A manuscript describing these results has been submitted to Molecular and Cellular Biology.

Significance to Biomedical Research and the Program of the Institute:

The papillomaviruses are associated with a number of human carcinomas. Molecular studies of the human papillomaviruses are limited because no reproducible *in vitro*

transformation system has been developed for the human papillomaviruses and no *in vitro* replication system has been developed for any of the papillomaviruses. BPV-1 has been the prototype for the papillomaviruses for unravelling the molecular biology of the papillomaviruses because of the convenient efficient transformation system using rodent cells. The genomic organization of BPV-1 and the human papillomaviruses associated with cervical carcinoma are quite similar suggesting that the biologic properties described for the bovine papillomavirus will have analogous functions within the human papillomaviruses. Therefore, studies with the bovine papillomavirus are crucial for unravelling the mechanistic functions that the papillomaviruses may encode that may be involved in carcinogenic progression.

Proposed Course of Research:

1. To continue a defined and systematic evaluation of the genetics of BPV-1 using defined deletion and insertion mutations.
2. To define the early and late viral promoters functioning in papillomavirus infected cells.
3. To examine the temporal expression of papillomavirus RNAs following viral infection of cells.
4. To map the transcriptional enhancer in the non-coding region using defined deletion mutants and a nested set of linker scanning mutants.
5. To analyze BPV-1 replication in transformed cells as well as in productively infected epithelial cells.
6. To generate antibodies against each of the gene products of the transforming region of the bovine papillomavirus using synthetic oligonucleotides and proteins expressed in prokaryotic expression vectors.
7. To express the transforming gene products of the bovine papillomavirus in mammalian cells using adenovirus and SV40 virus based vectors.
8. To determine the sequences in the papillomavirus which are responsible for transcriptional termination in transformed cells.

Publications:

Howley, P. M., Yang, Y.-C., and Rabson, M. S.: The molecular biology of the bovine papillomavirus. In Rigby, P. W. J. and Wilkie, N. M. (Eds.): *Viruses and Cancer*. Cambridge, Cambridge Univ. Press, 1985, pp. 67-81.

Howley, P. M., Yang, Y.-C., Spalholz, B. and Rabson, M. S. Molecular aspects of papillomavirus-host cell interactions. In vur Hausen, H. and Peto, R. (Eds.): *Origins of Female Genital Cancer: Virological and Epidemiological Aspects*. New York, Cold Spring Harbor Laboratory. (In Press)

Sarver, N., Rabson, M.S., Yang, Y.-C., Byrne, J.C., and Howley, P.M.: Localization and analysis of bovine papillomavirus type 1 transforming functions. *J. Virol.* *52*: 377-388, 1984.

Spalholz, B. A., Yang, Y.-C., Howley, P. M. Identification of a new enhancer within the non-coding region of BPV-1 which is *trans*-activated by BPV-1 early gene products. In Howley, P. and Broker, T. (Eds.): *UCLA Symposia Volume 32, Papilloma Viruses: Molecular and Clinical Aspects*. (In Press)

Spalholz, B. A., Yang, Y.-C. and Howley, P. M.: Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. Cell. (In Press)

Yang, Y.-C., Okayama, H., and Howley, P. M.: Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA 82: 1030-1034, 1985.

Yang, Y.-C., Okayama, H., and Howley, P. M. Structural analysis of viral cDNAs from bovine papillomavirus transformed mouse cells. In Howley, P. and Broker, T. (Eds.): UCLA Symposia Volume 32, Papilloma Virsues: Molecular and Clinical Aspects. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP00547-05 LTVB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

The Use of Papillomavirus DNAs as Eukaryotic Cloning Vectors

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

PI:	P. M. Howley	Chief	LTVB	NCI
Others:	J. C. Byrne	Biologist	LTVB	NCI
	G. Khoury	Chief	LMV	NCI
	D. Levens	Medical Staff Fellow	LP	NCI
	R. Muschel	Senior Investigator	LP	NCI
	M. Reitz	Senior Investigator	LTCB	NCI

COOPERATING UNITS (if any)

Revlon Health Care Research and Development, Springfield, VA (N. Sarver).

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.7	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 bovine papillomavirus (BPV) is capable of transforming certain rodent fibroblast lines in which the viral DNA remains as a stable extrachromosomal plasmid. These properties have been exploited in developing BPV into a stable extrachromosomal mammalian cell vector. The complete genome cloned into pML2 which is a deletion derivative of pBR322 is capable of serving as a shuttle vector which can replicate as a plasmid in mouse C127 cells or in bacteria. We have studied the expression of the rat preproinsulin gene in BPV vectors in C127 cells and have shown that the expression of the gene is enhancer dependent. An "enhancer" element is located in the BPV-1 genome at the 3' end of the transforming region, downstream from the polyadenylation recognition sequence. Using this vector system, a variety of exogenous genes have been expressed. A portion of the human T-cell lymphotropic virus type 1 (HTLV-1) has been expressed off of the mouse metallothionine promoter in a BPV vector. The extrachromosomal state of the DNA should provide a physical characteristic to permit the purification of chromatin complexes of mammalian genes. Using the lac operator, we have developed a technique for rapid purification and identification of sequence specific binding proteins. This technique, combined with the extrachromosomal papillomavirus plasmid vector system, should facilitate the identification of important viral and cellular gene regulatory proteins.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

P. M. Howley	Chief	LTVB	NCI
J. C. Byrne	Biologist	LTVB	NCI
G. Khoury	Chief	LMV	NCI
D. Levens	Medical Staff Fellow	LP	NCI
R. Muschel	Senior Investigator	LP	NCI
M. Reitz	Senior Investigator	LTCB	NCI

Objectives:

This project is directed toward the development of the papillomaviruses as effective and useful mammalian cell vectors. In studying the expression of a variety of foreign genes in papillomavirus based vectors, information is also generated concerning the molecular biology and genetic organization of papillomaviruses.

Methods Employed:

Recombinant-DNA techniques; nucleic acid hybridization; gel electrophoresis; tissue culture; RNA analysis; DNA transfection techniques; radioimmunoassays; immunoprecipitation.

Major Findings:

1. The effect of position in a BPV-1 vector on foreign gene expression was assessed using the rat preproinsulin gene as a model. This gene was inserted at each of the two BPV-1/pML2 junctions in either transcriptional orientation in the pdBPV-1(142-6) shuttle vector previously described by this laboratory. Mouse cells containing this plasmid shuttle vector were selected solely on the basis of morphological transformation and then assayed for rat preproinsulin gene expression. Cells containing this gene at the 3' end of the BPV-1 transforming region expressed the rat preproinsulin gene, whereas cells that contain the gene at the 5' end of the non-transforming region did not. Variability in the plasmid copy number, or the extent of DNA rearrangements could not account for this difference. We conclude that the expression of the rat preproinsulin gene in mouse cells using BPV-1 vectors depends on the transcriptional activation afforded by viral (enhancer) sequences located at the 3' end of the transforming region. An enhancer element has previously been localized to this region. Intervening BPV-1 or pML2d sequences appear to block this enhancer mediated gene activation. In agreement with this viral enhancer dependent activation, a rat preproinsulin gene in the "blocked" position at the 5' end of the non-transforming region could be activated by the insertion of a DNA fragment containing the SV40, MSV, or BPV-1 enhancer element adjacent to the rat preproinsulin gene. Thus, a gene which is normally not expressed in a particular cell may be activated when placed adjacent to a viral enhancer in a BPV-1 vector. A manuscript describing these results has been submitted to Molecular and Cellular Biology.

2. A bovine papillomavirus vector has been used to express a portion of a proviral DNA of a human T-cell leukemia (lymphotropic) virus (HTLV-1). The 3' end of the proviral DNA was inserted into a BPV-1 vector and expressed off a mouse metallothionein promoter. Mouse cells were transfected with this vector and selected solely by virtue of morphologic transformation. These cells were analyzed by Northern blot analysis and indirect immunofluorescence and shown to express HTLV-1 viral gene products. These mouse cells were injected into Balb/C mice and a monoclonal antibody was recovered which specifically recognizes a 21 Kd protein present in HTLV-1 virions indicating that the 3' end of the proviral DNA utilize encodes the small envelope protein for the virus. A manuscript describing these results, done in collaboration with Mary Beth Eiden and Marvin Reitz of the Laboratory of Tumor Cell Biology, is in press in Molecular and Cellular Biology.

3. We have developed a general method for the enrichment and identification of sequence specific DNA binding protein. This method utilizes the cloning of the DNA sequence of interest adjacent to the lac operator and using a lac repressor-beta-galactosidase fusion protein to fish out this DNA and any associated proteins from crude cellular extracts or fractions thereof. The utility of this technique has been demonstrated in bacteria to purify the lambda repressor and in purified preparations of the yeast mitochondrial RNA polymerase to identify a 70,000 MW peptide which binds specifically to the promoter region of the yeast mitichondrial 14S rRNA gene. This approach should have general utility in fishing out extra-chromosomal plasmids and their associated proteins and should have direct utility with BPV-1 based plasmids. A manuscript describing these results is in press in Molecular and Cellular Biology.

Significance to Biomedical Research and the Program of the Institute:

We have developed the DNA of the bovine papillomavirus into a mammalian cell cloning vector. The ability of these vectors to remain extrachromosomal in mammalian cells permits the analysis of exogenous genes in a uniform sequence environment and also provides a physical characteristic that should permit the purification of the genes as DNA or a chromatin. This vector system will be extremely useful in the systematic analysis of gene expression of a number of important genes including oncogenes, human interferon, and hormone genes. The development of the technology to permit the rapid recovery of extrachromosomal molecules directly from mammalian cells should permit the rapid identification of regulatory proteins associated with these specific genes.

Proposed Course of Research:

1. To develop a non-transforming extrachromosomal papillomavirus vector for the study of oncogene expression.
2. To examine the regulation of the human beta-interferon gene in papillomavirus virus vectors.
3. To determine and characterize the proteins involved in the induction of the human beta-interferon gene using rescue of BPV-1 minichromosomes containing the lac operator.
4. To assess the ability of this vector system to deliver a gene to a cell in such a manner as to effect site-specific integration.

5. To determine factors that are necessary to maintain papillomaviruses extra-chromosomally in specialized cells such as lymphocytes and epithelial cells.

Publications:

Howley, P. M.: Bovine papillomavirus vectors. In Fields, B. and Martin, M. (Eds): Genetically Altered Viruses and the Environment. New York, Cold Spring Harbor Laboratory. (In press)

Howley, P. M.: Papillomavirus Vectors. In Lieve, L. (Ed): Microbiology-1985. American Society for Microbiology, 1985, pp. 224-228.

Eiden, M., Newman, M., Fisher, A., Mann, D., Howley, P., and Reitz, M.: HTLV-1 small envelope protein is expressed in mouse cells using a bovine papilloma virus-derived shuttle vector. Mol. Cell. Biol. (In Press)

Levens, D. L., and Howley, P. M.: A novel method for identifying sequence specific DNA binding proteins. Mol. Cell. Biol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP00564-03 LTVB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Early Events in Virus/Host Cell Interaction

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

PI:	C. R. Schlegel	Senior Investigator	LTVB	NCI
Others:	J. Bolen	Senior Staff Fellow	LTVB	NCI
	M. Wade	Biologist	LTVB	NCI
	R. Blumenthal	Chief	LTB	NCI

COOPERATING UNITS (if any)

Laboratory of Biochemistry and Metabolism, Division of Intramural Research, NIADDK (J. Hanover).

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Cellular Regulation and Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	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.)

Many animal viruses enter their respective host cells by the process of adsorptive endocytosis. This route of internalization is also used by many hormones and ligands which bind to specific receptors on the cell plasma membrane. After binding to the cell surface, viruses can follow different pathways dependent upon their particular structure and organization. For example, vesicular stomatitis virus (an enveloped, RNA virus) enters an acidic endosomal vesicle and, consequent to exposure to the acidic pH, is believed to fuse with the membrane of the vesicle. Fusion results in the release of viral nucleic acid into the cell cytoplasm. In contrast to VSV, SV40 (a non-enveloped, DNA virus) appears to translocate quickly from the endosomal vesicle to the nucleus where it is uncoated and its DNA released. The mechanism of this specific and rapid translocation is not known. Our laboratory is studying the mechanism by which two viruses (VSV and SV40) are "targeted" to different and specific sites in the host cell. Specifically, we have asked: (1) are these specific binding sites for viruses at the cell surface; (2) what are the cellular and viral components which participate in binding; (3) do the binding sites contribute to the final "destination" of the virus; (4) how do viruses cross cell membrane barriers; and (5) do viruses contain "signals" to direct their intracellular translocation? Multiple approaches will be used in these studies. Radiolabelled virus will be used to permit the detection and characterization of specific viral binding as well as the fate of internalized virus. Cell fractionation, electron microscopy, and immunofluorescence microscopy will also be used to follow the pathway of viral infection. Fusion of virus with cell membranes will be monitored by fluorescence energy transfer and EM of in vitro fusion models. Attempts will be made to delineate specific, biologically-active domains of viral spike and capsid proteins by use of both synthetic peptides and their corresponding antibodies.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

C. R. Schlegel	Senior Investigator	LTVB	NCI
J. Bolen	Senior Staff Fellow	LTVB	NCI
R. Blumenthal	Chief	LTB	NCI

Objectives:

1. Define the number and composition of cell binding sites for VSV and SV40.
2. Identify domains of VSV spike protein and SV40 capsid proteins involved in:
   a) cell binding
   b) membrane fusion or disruption
   c) targeting to the nucleus
3. Determine whether both VSV and SV40 can fuse or interact with cell membranes.

Methods Employed:

1. S^{35}-VSV binding assays.
2. Electron microscopy (EM).
3. Immunofluorescence microscopy (IF).
4. Liposome and cell fusion assays monitored by energy transfer fluorescence and fluorescence quenching.
5. Detection of G-protein conformation changes by circular dichroism and infra-red spectroscopy.
6. Density gradient centrifugation.
7. Hemolysis assays.

Major Findings:

1. The spike protein of VSV can be reconstituted functionally into liposomes.
2. The spike protein of VSV mediates membrane fusion.
3. VSV spike protein interacts specifically with acidic phospholipids.
4. The NH_2 terminus of VSV spike protein is a pH-dependent hemolysin, hemagglutinin, and cytotoxin.
5. The terminal 6 amino acid peptide of VSV spike protein is the smallest known peptide hemolysin.
6. The terminal 6 amino acid peptide of VSV spike protein causes membrane destabilization and alters membrane permeability to ions.

Significance to Biomedical Research and the Program of the Institute:

1. The NH_2 terminus of VSV spike protein may be an extremely useful molecule for mediating membrane/membrane fusion and could have important applications in clinical (e.g., drug-delivery systems) as well as research investigations.
2. The delineation of the mechanism of virus internalization will be helpful in designing inhibitors of virus infection.
3. Knowledge of the mechanism of membrane fusion is important for understanding viral infection as well as the many cellular processes involved in endocytosis and exocytosis.

4. Tissue tropism may be dependent upon specific viral protein/cell surface interactions.

Proposed Course of Research:

1. Use purified, radiolabelled virus to study the binding and uptake of virus.
2. Study virus translocation in cells by EM, IF, and cell fractionation techniques.
3. Use fluorescence energy transfer and EM to study virus/membrane fusion events.
4. Use synthetic peptides and corresponding antibodies to delineate specific domains of viral spike and capsid proteins.

Publications:

Schlegel, R.: Membrane-active peptides of the vesicular stomatitis virus glycoprotein. Microbiology. (In press)

Schlegel, R. and Wade, M.: Biologically active peptides of the vesicular stomatitis virus glycoprotein. J. Virol. 53: 319-323, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP00565-03 LTVB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Cell Immortalization and Transformation by Papovaviruses

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

PI:	C. R. Schlegel	Senior Investigator	LTVB	NCI
Others:	J. Bolen	Senior Staff Fellow	LTVB	NCI
	Y. Zhang	Visiting Fellow	LTVB	NCI
	J. Bubb	Visiting Fellow	LP	NCI
	S. Schlegel	Expert	LHC	NCI
	M. Wade	Biologist	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 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.25	0.5	1.75

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.)

Cell transformation by papovaviruses requires the expression of several early viral gene products. Our laboratory is currently investigating the mechanism by which papillomaviruses (human and bovine) and polyomavirus perturb cell growth control and participate in tumorigenesis. The main research focus will be on human papillomaviruses (HPV). Although the role of HPV in benign human tumors (warts) is well established, it is only recently that an association between HPV and cervical cancer has been defined. More than 31 types of HPV exist (by DNA hybridization). Only a few of these HPVs are associated with cervical cancer, however. For example, type 16 appears to be found in various stages of cervical dysplasia (or "flat warts") as well as in carcinoma-in-situ and invasive carcinoma. Type 18 HVP is found only in invasive cervical carcinoma. The biological role of these viral genomes in tumor cells is unknown, but the ability of related bovine papillomaviruses (BPV) to transform cells <u>in vitro</u> suggests that HPV may have a role in either initiating or maintaining the transformed state. We will attempt to determine whether human cervical carcinoma lines contain dominant transforming genes using transfection techniques and will also attempt to transform cells <u>in vitro</u> with HPV DNA. Selected, formal studies with BPV will be performed for comparison of HPV and BPV transforming properties. Related to our attempts to transform cells with HPV is an effort to transform epithelial cells <u>in vitro</u>. Our laboratory has an interest in defining the progressive stages of carcinogenesis and, as part of this study, to establish an <u>in vitro</u> system for the transformation of epithelial cells. We have decided to focus on human and murine epidermal cells since much is known about the murine model for the induction of squamous cell carcinoma and since these cells can be propagated <u>in vitro</u>. Culture conditions for human epidermal cells are also well established. Our initial attempt will include transfecting murine epidermal cells with BPV and polyoma DNA and human epidermal cells with HPV-16 and HPV-18 DNA.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

C. R. Schlegel	Senior Investigator	LTVB	NCI
J. Bolen	Senior Staff Fellow	LTVB	NCI
Y. Zhang	Visiting Fellow	LTVB	NCI
J. Bubb	Visiting Fellow	LP	NCI
S. Schlegel	Expert	LHC	NCI
M. Wade	Biologist	LTVB	NCI

Objectives:

1. Generate specific antisera against HPV-1 and HPV-16 proteins using synthetic peptides and open reading frame bacterial expression vectors.
2. Transfect human (or murine) cells with HPV DNA. Use both fibroblasts and epidermal cells as recipients.
3. Transfect murine epidermal cells with polyoma and BPV DNA and characterize their altered behavior.
4. Assay for oncogenes in human cervical carcinomas.

Methods Employed:

1. Keratinocyte cultures with fibroblast feeder layers.
2. Electrofection of epithelial cells with plasmid DNA.
3. Bacterial plasmid construction of HPV and BPV.
4. Plasmid preparation and purification.
5. Immunoblotting and immunofluorescence of viral proteins.
6. Agarose and agar tissue culture techniques.
7. FACS analysis of cellular DNA content.
8. Immunodetection of epidermal keratins, involucrum, and envelope protein.
9. Oncogene assays using cervical carcinoma DNA.

Major Findings:

Mouse epidermal cells can be transformed to the tumorigenic state by both BPV and polyoma. Such tumorigenic cells lack several features of transformed fibroblasts: altered cellular morphology and anchorage independence.

Significance to Biomedical Research and the Program of the Institute:

1. To understand the etiology of papillomavirus-induced carcinogenesis.
2. To define the interaction between viral and cellular genes in tumorigenesis.

Proposed Course of Research:

1. Construct bacterial expression vectors containing the early open reading frames of HPV-1 and HPV-16. Use exposed proteins to generate antibodies specific for these early HPV proteins. Screen human cervical carcinomas and HPV-transformed cells for the presence of viral-specific proteins. Characterize the biological and biochemical properties of the HPV early porteins.

2. Cotransfect immortalized non-tumorigenic mouse cells with purified, cloned HPV DNA and a plasmid containing a neomycin resistance gene. Select for neomycin resistance and characterize resistant cells for the presence of HPV DNA. Characterize HPV(+) cells for their phenotype with respect to tumorigenicity, altered morphology, and growth requirements.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP00898-02 LTVB

PERIOD COVERED

October 1, 1984 to September 30, 1985

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
	C. R. Schlegel	Senior Investigator	LTVB	NCI
Others:	C. C. Baker	Medical Staff Fellow	LTVB	NCI
	C. Yee	Biologist	LTVB	NCI
	I. Hewlett	Visiting Fellow	LTVB	NCI
	W. Phelps	Guest Researcher	LTVB	NCI
	V. Bubb	Visiting Fellow	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section and Cellular Regulation and Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

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 papillomaviruses are associated with naturally occurring carcinomas in a variety of species, including man. There are now 31 human papillomaviruses (HPVs) which have been identified in man. Four of these, HPV-6, HPV-11, HPV-16 and HPV-18, have been associated with human genital tract lesions. HPV-6 and HPV-11 have been found to be associated with a high percentage of benign genital warts, including cervical flat warts and dysplasias. HPV-16 and HPV-18 were each cloned directly from cervical carcinoma biopsy specimens and have been found in a high percentage of cervical carcinomas. We have screened a series of human carcinoma cell lines for the presence of human papillomavirus DNA sequences using HPV-6, HPV-11, HPV-16, and HPV-18 DNA probes. Six of eight cell lines which had been derived from human cervical carcinoma lines were found to contain integrated HPV DNA sequences. In five of these lines, HPV specific polyadenylated RNA species could be identified. Two of the cell lines contained integrated HPV-16 DNA and in each of these cell lines the genomes were transcriptionally active. Genomic clones have been made from these HPV-16 positive lines. Sequence analysis of the virus-host junction fragments for identification of the chromsome into which the HPV has integrated and identification of the adjacent genes is underway. While the association between specific HPVs and cervical carcinoma is strong, the mechanism underlying any potential carcinogenic progression is unknown. Our studies are designed to identify potential mechanisms.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

P. M. Howley	Chief	LTVB	NCI
C. R. Schlegel	Senior Investigator	LTVB	NCI
C. C. Baker	Medical Staff Fellow	LTVB	NCI
C. Yee	Biologist	LTVB	NCI
I. Hewlett	Visiting Fellow	LTVB	NCI
W. Phelps	Guest Researcher	LTVB	NCI
V. Bubb	Visiting Fellow	LP	NCI

Objectives:

1. To analyze human squamous cell carcinomas from a variety of sites for the presence of HPV DNAs.
2. To analyze human squamous cell carcinomas for the presence of HPV specific mRNAs.
3. To clone the integrated HPV DNA sequences from positive carcinomas in order to determine the site of integration with respect to the virus DNA as well as with respect to the host cellular DNA.
4. To identify the chromosomes into which the HPV DNAs have integrated.
5. To generate full-length cDNA clones of the viral transcripts from the positive carcinoma lines.
6. To determine which viral genes are being expressed within the cervical carcinomas and cervical carcinoma cell lines.
7. To determine whether hybrid viral-host cell RNAs are expressed within the positive carcinoma lines.
8. To determine whether HVP-16 and HPV-18 contain genes which can transactivate transcriptional regulatory sequences within the viral genome and within the host cell.

Methods Employed:

1. Standard recombinant DNA technology.
2. Northern blot analysis of RNAs.
3. cDNA cloning using expression vectors.
4. Immunoblotting and immunofluorescence of viral proteins.
5. DNA sequencing.
6. In situ hybridization.
6. Transient and stable DNA transfection techniques.

Major Findings:

1. Eight cervical carcinoma cell lines were screened for human papillomavirus DNAs and six of these lines were found to contain integrated HPV DNAs. Two of these lines (SiHa and CaSki) contain integrated HPV-16 DNA sequences. Four of the lines (Hela cells, ME180 cells, MS751 cells, and C-4I cells) contain integrated HPV-18 DNA sequences. Both of the HPV-16 positive cell lines are transcriptionally active and three of the four HPV-18 cell lines are transcriptionally active. Sizes

of the messages present within these cells have been determined by Northern blot hybridization techniques. These results have been published in the American Journal of Pathology.

2. The integrated sequences of the HPV-16 genome have been cloned from the SiHa cell line. There appear to be 10 copies of the viral genome integrated. The integrated pattern indicates that the genome is disrupted within the E2 gene at the site of integration. The genome appears to be integrated and amplified in an approximately 25 Kb repeating unit. Sequence analysis of the flanking host sequences and the junction fragments are in progress. Attempts to make full-length cDNA clones of the viral RNAs within the cell line are in progress.

Significance to Biomedical Research and the Program of the Institute:

The association of certain human papillomaviruses with human cervical carcinoma is now strong. It will be important to determine whether or not other human squamous cell carcinomas of other origins such as esophagus, pharynx, larynx, and lung may also be associated with specific human papillomaviruses. While the association is strong, no mechanism has yet been determined for how a papillomavirus may be associated with the malignant progression of a benign lesion to a carcinoma. It will be important to analyze the specific human carcinoma cell lines for the molecular consequences of the integration of the viral genome into the host chromosome. The human papillomaviruses appear to be major candidates for viruses associated with naturally occurring human cancers.

Proposed Course of Research:

1. To clone the integrated HPV DNA sequences in the cervical carcinoma cell lines already identified.
2. To sequence the junction and flanking sequences of the integrated DNAs in selected cervical carcinoma cell lines.
3. To determine the chromosomes into which the HPVs have integrated in cervical carcinomas.
4. To study the cytogenetics of cervical carcinoma to determine if any specific chromosomal abnormalities are associated.
5. To study human esophageal carcinomas to determine if there are associated human papillomaviruses.
6. To study human squamous cell carcinomas of the lung to determine if there are associated human papillomaviruses.
7. To study oral pharyngeal and laryngeal human carcinomas to determine if there are associated human papillomaviruses.
8. In human papillomavirus positive carcinomas, to determine if there is active viral transcription.
9. To characterize the viral transcripts that are present within human carcinomas to determine the viral gene products that may be encoded by such RNAs and to determine whether or not there are hybrid RNAs that might encode host gene products or fused viral-host proteins.
10. To determine whether or not the associated human papillomaviruses encode transactivating genes which might transactivate integrated viral transcriptional regulatory elements or alternatively host transcription regulatory elements.

Publications:

Yee, C., Krishnan-Hewlett, I., Baker, C. C., Schlegel, R. and Howley, P. M.: Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. *Am. J. Pathol*. 119: 361-366, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05420-01 LTVB

PERIOD COVERED
October 1, 1984 to September 30, 1985

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

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

PI:	J. B. Bolen	Senior Staff Fellow	LTVB	NCI
Others:	S. Amini	Visiting Fellow	LTVB	NCI
	V. DeSeau	Biologist	LTVB	NCI
	M. Israel	Senior Investigator	PB	NCI

COOPERATING UNITS *(if any)*
Department of Microbiology, State University of New York at Stony Brook, Stony Brook, NY (J. S. Brugge); Department of Molecular and Cellular Biology, Pennsylvania State University, University Park, PA (D. Shalloway)

LAB/BRANCH
Laboratory of Tumor Virus Biology

SECTION
Cellular Regulation and Transformation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues X☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

The polyomaviruses comprise a class of small DNA tumor viruses within the papovavirus group of DNA viruses. Members of the polyomavirus class include polyoma virus (Py) of mice, simian virus 40 (SV40) of monkeys, and JC and BK viruses of humans. Of these viruses, Py has been most thoroughly characterized with respect to the genetic elements and proteins involved in oncogenic transformation of mammalian cells. Genetic and biochemical analysis has revealed that the region of the Py genome required for both Py-mediated tumorigenesis in vivo and oncogenic transformation in vitro encodes three nonstructural proteins: the large, middle, and small tumor antigens (T Ags). These proteins have been designated as T Ags because they are recognized by antibodies in sera from animals bearing Py-induced tumors (T sera). Of these three proteins, a central role for the Py encoded middle T Ag (MTAg) in Py-mediated oncogenesis has been established principally through genetic analysis. The MTAg is associated with a protein kinase activity which can be detected by the in vitro phosphorylation of tyrosine on MTAg when T sera immunoprecipitates of Py infected or transformed cell extracts are incubated with gamma-32P ATP and Mg++. The MTAg does not apparently possess intrinsic protein kinase activity and is thought to associate with the cellular c-src gene product, the cellular homolog of the Rous sarcoma virus transforming gene. Since both the viral (pp60v-src) and cellular (pp60c-src) forms of the src gene protein possess intrinsic tyrosine protein kinase activity, it has been proposed that the MTAg associated protein kinase represents a property of pp60c-src. The potential importance of this protein kinase activity in Py-mediated oncogenesis is suggested by the finding that viral mutants which are deficient in transforming potential generally lack this activity and that there are no known transformation-competent Py strains which encode MTAg molecules which do not possess this associated kinase activity.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

J. B. Bolen	Senior Staff Fellow	LTVB	NCI
S. Amini	Visiting Fellow	LTVB	NCI
V. DeSeau	Biologist	LTVB	NCI
M. Israel	Senior Investigator	PB	NCI

Objectives:

1. Biochemical analysis of the interaction between Py MTAg and pp60c-src.
2. Analysis of the biochemical differences between pp60c-src molecules from normal cells and pp60c-src molecules from Py transformed cells.
3. Role of functional alterations in pp60c-src in Py transformation.
4. Role of functional alterations of pp60c-src activities in other viral and non-viral tumors.
5. Role of non-coding sequences within the Py genome in the expression of Py T Ags.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of Py chimeric plasmids.
2. Cell culture.
3. Cloning of mammalian cells.
4. Transfer of DNA into mammalian cells using calcium-phosphate co-precipitation.
5. Monoclonal antibody production.
6. Transcriptional analyses including Northern and S1 analysis.
7. Immunoprecipitation.
8. Protein kinase assays.
9. One and two-dimensional peptide analysis.
10. Phosphoaminoacid analysis.
11. Immunoblot analysis.
12. High pressure liquid chromatography of proteins and amino acids.
13. Medium pressure and low pressure automated ion-exchange and chromatofocusing analysis of proteins and peptides.
14. One and two-dimensional gel electrophoresis techniques.

Major Findings:

The interaction between Py MTAg and pp60c-src was confirmed by (A) an in vitro assay which tested the ability of cellular proteins to associate with and phosphorylate MTAg, and (B) using a series of monoclonal antibodies (MAb) directed against pp60c-src. Infection of mouse cells with transformation-competent strains of Py results in the stimulation of pp60c-src tyrosine kinase activity 8-10 fold over that observed in either uninfected mouse cells or mouse cells infected with transformation-defective strains of Py. Similar levels of pp60c-src kinase activation were found in all Py transformed cell lines tested. No differences were detected in the level of c-src mRNA or pp60c-src synthesis between Py infected and uninfected mouse cells or Py transformed and normal rodent cells. Only a small

fraction of the MTAg molecules are complexed with a small amount of the total pp60c-src in Py infected and transformed cells and it is only the src molecules associated with MTAg which possess elevated kinase activity. These results demonstrated that the association of MTAg with pp60c-src resulted in an enhancement of the specific activity of the pp60c-src phosphotransferase and defined a new mechanism by which cellular oncogenes could become activated in the absence of DNA mediated mutations or increased transcriptional activity; that is, the activation of pp60c-src kinase activity in Py transformed cells proceeds through protein-protein interactions.

The pp60c-src molecules associated with MTAg possess an additional post-translational modification not found on other src molecules in the cell. This modification was determined to be an additional tyrosine phosphorylation site in the first amino-terminal 188 amino acids of the protein. This additional phosphorylation site may be associated with the increased levels of pp60c-src kinase activity observed in Py transformed cells.

Elevated levels of pp60c-src kinase activity can be observed in some, but not all, hamster embryo cells transformed by other DNA tumor viruses including SV40, adenovirus 2, adenovirus 12, and bovine papillomavirus 1. In those clones of transformed hamster cells where elevated levels of src kinase activity were observed, the increased levels were found to be due to an apparent increase in the specific activity of pp60c-src. The c-src gene product was not found to be physically associated with tumor antigens known to be encoded by these viruses and the pp60c-src protein was not phosphorylated on amino-terminal tyrosine residues. These results pointed out that activation of pp60c-src kinase activity may occur through multiple mechanisms and also implied that elevated c-src kinase activity is not required for the transformation of hamster cells by SV40, adenovirus 2, adenovirus 12, and bovine papillomavirus 1.

Elevated levels of pp60c-src kinase activity can also be observed in human tumor cell lines, particularly those of neuroectodermal origin. We found that pp60c-src kinase activity in numerous human neuroblastoma cell lines was elevated 20 to 40-fold over that observed in either human glioblastoma cells or normal human fibroblasts. The level of c-src gene transcripts and pp60c-src proteins synthesis in the neuroblastoma cells and glioblastoma cells was indistinguishable demonstrating that the specific activity of the neuroblastoma-derived pp60c-src kinase was significantly elevated. Peptide analysis and phosphoamino acid analysis of the c-src proteins in these cells revealed that pp60c-src molecules from the neuroblastoma cells possess at least one unique tyrosine phosphorylation site in the aminoterminal portion of the protein not found on c-src molecules from glioblastoma cells.

The synthesis of pp60c-src in Py transformed cells can be regulated by inducible anti-sense c-src RNA. We constructed a recombinant plasmid with the mouse metallothionein-1 promoter upstream from the c-src gene in an anti-sense orientation. This plasmid was co-transfected into Py transformed rat cells with a plasmid containing the neomycin resistance gene. Two hundred G418 resistant colonies were selected and 40 of these showed decreased levels of c-src expression in the presence of Cd++. The clones expressing the anti-sense src RNA were found to grow at reduced rates in soft agar and to form fewer foci on monolayers of normal rat cells when compared to the parental cells. These results demonstrated that elevated levels of pp60c-src kinase activity contribute to the growth potential and phenotypic properties characteristic of Py transformed cells.

We have identified a strain of Py (Py LID) which is unusual in causing acute morbidity and early death after innoculation into newborn mice. The innoculated mice were found to die as a result of kidney failure associated with extensive virus-mediated destruction of renal tissue. This strain of Py was found to grow more efficiently in baby mouse kidney cell cultures than other strains of Py. The molecular basis for this increased growth potential in kidney cell cultures has been localized in preliminary marker rescue analysis. The sequence differences between the non-coding regions of the Py LID and several other strains of Py have been determined and demonstrate that the Py LID strain of Py contains a 26 base pair direct repeat in a region of the DNA which shares close homology to the known adenovirus enhancer sequence.

Signficance to Biomedical Research and the Program of the Institute:

Our discovery that the increased specific activity of the c-src phosphotransferase in Py transformed cells proceeds through protein-protein interactions is significant since this is the first example of proto-oncogene activation that does not require either mutation or increased transcription of the gene. This observation implies that other potential oncogenes may be activated by a similar mechanism and points to the necessity of determining the specific activities of oncogene products in other systems at the protein level rather than relying on assays which test only the relative amount of the oncogene product. Our finding that the specific activity of the pp60c-src kinase may be regulated by post-translational modifications within the aminoterminal portion of the c-src molecule defines a molecular means through which many tyrosine-specific protein kinase activities can be regulated. Since most of the known tyrosine-specific protein kinases in mammalian cells are involved with the control of cellular proliferative events, our observations should contribute to the understanding of biochemical mechanisms of growth regulation. The development of anti-sense c-src RNA technology in this laboratory represents the first demonstration of the regulation of an endogenous proto-oncogene by this method. This technology has already provided clues as to the potential contribution of pp60c-src in Py transformation. The continued utilization of this approach should provide additional insights into the usefulness of this technology in other biological systems.

Proposed Course of Research:

1. To further define the role of pp60c-src in transformation by Py and other DNA tumor viruses.
2. To examine other types of animal and human tumors for pp60c-src kinase activation.
3. To isolate c-src cDNA from human neuronal cDNA library for use in molecular studies and for cloning into expression vector systems.
4. To further define the role of non-coding sequences within the Py genome for the tissue specific expression of Py T Ags.

Publications:

Bolen, J. B., Fisher, S. E., Chowdhury, K., Williams, J., Dawe, C. J. and Israel, M. A.: A determinant of polyoma virus virulence enhances virus growth in cells of renal origin. J. Virol. 53: 335-339, 1985.

Bolen, J. B., and Israel, M. A.: In vitro association and phosphorylation of polyoma virus middle T antigen by cellular tyrosyl kinase activity. J. Biol. Chem. 259: 11681-11694, 1984.

Bolen, J. B. and Israel, M. A.: Middle tumor antigen of polyomavirus transformation-defective mutant NG 59 is associated with pp60c-src. J. Virol. 53: 114-119, 1985.

Bolen, J. B., Lewis, A. M., Jr., and Israel, M. A.: Stimulation of pp60c-src tyrosyl kinase activity in polyoma virus-infected mouse cells is closely associated with polyoma middle tumor antigen synthesis. J. Cell. Biochem. 27: 157-167, 1985.

Bolen, J. B., Rosen, N., and Israel, M. A.: Elevated pp60c-src tyrosyl kinase activity in human neuroblastomas is associated with aminoterminal tyrosine phosphorylation of the src gene product. Proc. Natl. Acad. Sci. U.S.A. (In press)

Bolen, J. B., Thiele, C. J., Israel, M. A., Yonemoto, W., Lipsich, L. A., and Brugge, J. S.: Enhancement of cellular src gene product associated tyrosyl kinase activity following polyoma virus infection and transformation. Cell 38: 767-777, 1984.

Yonemoto, W., Bolen, J. B., Israel, M. A., Lipsich, L. and Brugge, J. S.: Use of monoclonal antibodies to probe the functional activity of the cellular src gene product in polyoma virus transformed cells. In Howley, P. and Broker, T. (Eds.): Proc. 1985 UCLA Symposium on Monoclonal Antibodies and Cancer Therapy. New York, Alan R. Liss, Inc., 1985. (In press)

Yonemoto, W., Jarvis-Morar, M., Brugge, J. S., Bolen, J. B. and Israel, M. A.: Novel tyrosine phosphorylation within aminoterminal domain of pp60c-src molecules associated with polyoma virus middle tumor anitgen. Proc. Natl. Acad. Sci., U.S.A. (In press)

ANNUAL REPORT OF

THE LABORATORY OF VIRAL CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1984 to September 30, 1985

FUNCTIONAL STATEMENT: The Laboratory of Viral Carcinogenesis (LVC) has been charged with the planning, development, implementation, and coordination of research programs on the etiology and mechanisms of carcinogenesis. In the past particular emphasis was placed on delineating the roles, mechanisms and regulation of action of oncogenic viruses, virus-related and virus-associated genetic sequences, and gene products. Research efforts were conducted on virus-host relationships in virus-induced cancers, focusing on the detection and characterization of oncogenic viruses, their mechanisms of genetic integration and expression, and their modes of transmission in animals and man. The rapid technological development of eukaryote genetics and molecular biology has resulted in a synthesis of viral and cellular gene action during neoplastic processes. As a result, the research emphasis of the laboratory has converged on the elucidation of regulatory genetic events which are operative in human cancers and homologous animal models. The multidisciplinary understanding of the neoplastic process, combining knowledge and technology from immunology, pathology, physiology, molecular biology, and genetics, is the common character of the various research projects of the laboratory, with a constant consideration of therapeutic opportunities. Activities of the Laboratory are conducted by in-house research and collaborative agreements with other research organizations.

SCIENTIFIC BACKGROUND AND SIGNIFICANCE: The combination of technological advances, and a number of coordinate, empirical observations, has dramatically altered the thinking of the scientific community on the molecular and genetic mechanisms of human and animal carcinogenesis. The procedures of molecular cloning, DNA sequencing, hybridoma production and somatic cell genetics have pushed the study of eukaryote genetic analysis from a speculative and interpretive discipline to the level of viewing gene action directly. One has only to peruse the popular press to realize that a revolution in biological thinking and analysis is upon us, and that this situation has become 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 30) described to date has attracted considerable research emphasis over the past few years as an experimental opportunity to study neoplastic transformation directly from both genetic and molecular perspectives. Because of these extensive analyses, there are now at

least five documented modes of oncogene activation associated with tumorigenesis. These include: (a) transduction of proto-oncogene transcripts by retroviruses, thereby placing the oncogene under regulatory control of strong promoters in the viral long terminal repeat (LTRs); (b) chromosomal insertion of an infecting retrovirus adjacent to a proto-oncogene similarly altering their control of transcription; (c) translocation of cellular oncogenes to chromosomal regions of differential regulation; (d) amplification of oncogene containing segments, thereby increasing the dosage of the oncogene; and (e) point mutation in the cognate cellular oncogene. The dramatic demonstration that the human "oncogenes" were, in many cases, homologs of the retroviral "oncogenes" which have been studied for decades, unified these formerly disparate gene sets into a single group which immediately became a viable candidate for somatic cell targets of carcinogenic insults. (2) The normal functional role of these oncogenes in normal (or in neoplastic) tissues was for sometime obscure. Within the last few years, by sequence alignment of cloned DNAs of various oncogenes, three putative matches have revealed a functional association of oncogene products. Specifically, the sis oncogene encodes platelet-derived growth factor; the erbB oncogene encodes the receptor for epidermal growth factor and the transferrin receptor is functionally related to the ras oncogene product. In addition, the fms product has recently been shown to encode the cellular receptor for macrophage colony stimulating factor. The functional relationship between the remaining 20± oncogenes thus far described is an area of intense research efforts in many laboratories to date. (3) Virtually all of the human oncogene homologs have now been chromosomally mapped to specific positions on the human gene map. These are in addition to the nearly 1000 different loci comprising the human genetic map. In addition to the proto-oncogene loci, approximately 20 additional described loci are thought to participate in neoplastic transformation in man (e.g., growth factors, cell surface antigens, retroviral receptors, and integration sites, etc.). A dramatic advance has been the demonstration that certain human tumors with specific chromosomal rearrangements can be interpreted to involve the modulated regulation of cellular oncogenes by normally distant, DNA regulatory elements which have been placed adjacent to the oncogenes by chromosomal rearrangement. (4) Despite a long history of unsuccessful attempts to isolate human type C retroviruses in a valiant effort by the Virus Cancer Program, only very recently have two human diseases been associated with exogenous human retroviruses. These are adult T-cell leukemia (ATL), which is etiologically associated with human T-cell leukemia virus-I and -II, and acquired immune deficiency syndrome (AIDS), which has been serologically correlated with the development of antibodies to a type D retrovirus designated HTLV-III or LAV. These isolates are so new that Koch's postulates are yet to be tested in animal models, yet the correlation data is compelling. (5) The study of animal models of certain of the homologous diseases in primates, rodents, and cats has provided terrific opportunities for reconstruction and analysis of the initiation and progress of mammalian tumors. For example, the AIDS models in primates [simian acquired immune deficiency syndrome (SAIDS)] and in FeLV-infected cats [feline acquired immune deficiency syndrome (FAIDS)] have modified significantly our interpretation of retroviral pathology to include broad levels of immune impairment in addition to (and possibly, in combination with) leukemogenesis.

The most significant findings of the LVC during this year are discussed below.

1. Updating the human gene map of proto-onc genes, and other loci which impact on mammalian carcinogenesis. The combined application of principles and techniques of molecular biology and cell genetics has resulted in the identification and characterization of over 1000 human loci, a value that approaches the gene maps of

Drosophila. We have concentrated our efforts on somatic cell hybrid panels and in situ hybridizations to genes related to neoplastic processes including (1) cellular proto-oncogenes, (2) growth factors, (3) growth factor receptors, (4) endogenous retroviral families, (5) integration sites for retroviruses, and (6) restriction genes that delimit retrovirus replication in mammals. Within the last few years, the human gene map has experienced a large increase in the number of neoplasia loci that have been mapped to specific chromosomal positions. Of the 27 specific human loci that have been chromosomally mapped to date, 11 (40%) have been assigned by the Genetics Section scientists and their collaborators. This year, we have specifically concentrated on understanding the genomic organization of several genes: ets, rel, raf, onc-D, fms, DHFR and a Y chromosome gene family in man. The mammalian homologue to the v-ets oncogene of the avian E26 transforming virus has been shown to be encoded by two transcriptionally active, nonoverlapping structural genes (ets-1 and ets-2) located on human chromosomes 11 and 21, respectively. Over 30 endogenous retroviral loci have been chromosomally assigned using the hybrid panels. A hematological discorder, the $5q^-$ syndrome, has been shown to be hemizygous for the fms oncogene by genetic analysis. This proto-onc-gene was shown to be transcriptionally active in hematopoetic cells and has been shown by our collaborator (C. Sherr) to encode to specific receptor for macrophage colony-stimulating factor. The emerging human gene map continues to provide an unprecedented opportunity for molecular genetic analyses of the initiation and progression of neoplastic processes.

2. Mode of transformation of HTLV-I involves dynamic nonspecific integration, interaction with transacting cellular genes and modulation of host cell major histocompatibility complex (MHC) phenotype. The family of retroviruses known as human T-cell lymphotropic retroviruses (HTLV-I, II, and III) has an affinity for infecting T lymphocytes and a similar genomic structure. All three types of HTLV have been transmitted in vitro, molecularly cloned and sequenced. Despite these advances, the mechanism by which infection with these viruses results in malignant transformation or immunosuppression remains unknown. We are focusing on basic mechanisms both on a cellular and molecular level by which these viruses transform or immunosuppress. In order to address whether HTLV-I may induce transformation through an insertional mutagenesis mechanism, we have utilized somatic cell hybrids constructed between rodent cells and HTLV-I infected cell lines to study the processes and consequences of HTLV chromosomal integration. Integration in vitro was shown to be a dynamic process and proviral integration apparently occurs at random in the genome. Studies are in progress to determine whether common integration sites are present in fresh tumor material. Since HTLV-I infected cells often lose their dependence for interleukin-2 (IL-2) this gene may play a role in transformation induced by HTLV-I. The gene was first mapped to chromosome 4 and sublocalized to 4q26-28 in normal lymphocytes. We next demonstrated that the IL-2 gene was not rearranged in several HTLV-I infected cell lines and mapped to chromosome 4 in Hut 102, suggesting that this gene is not operative in HTLV-I induced malignant transformation. We have also utilized the panel of Hut 102X Chinese hamster hybrids to demonstrate that the novel Class I antigenic determinants expressed on HTLV-I infected cells does not result from induction of Class I genes encoded by the cellular MHC locus, but are probably encoded by integrated HTLV-I. The activities of the promoter unit contained within the LTR of both HTLV-I and HTLV-III were examined by transfecting various cells with recombinant plasmids containing the LTR of HTLV-I or HTLV-III linked to the bacterial gene for chloramphenicol acetyltransferase (CAT). We have demonstrated that infected cells contain factors that act in trans on the LTRs of the infecting virus to activate transcription.

3. Description of the genetic organization of the raf/mil gene family in man and mouse. We have continued the molecular and functional characterization of the v-raf oncogene and its cellular homologs and are investigating their involvement in the etiology of human cancers. Four genes homologous to v-raf have now been identified in man: c-raf-1, c-raf-2, δ-raf, and γ-raf. C-raf-2 is a pseudogene in man located on chromosome 4. C-raf-1, the cellular homolog of v-raf, is an active gene located on chromosome 3p25, a site which is specifically altered in small cell lung carcinoma, ovarian carcinoma, and mixed salivary gland tumors. C-raf-1 specifies a protein of 648 amino acids with a calculated molecular weight of 74 kd. The complete c-raf-1 protein was expressed in E. coli using a complete human fetal liver cDNA cloned into an expression vector. We have also demonstrated the normal protein in mouse fibroblasts by immunoblot analyses. The c-raf-1 gene is composed of 17 exons which span over 45 kb of DNA. Northern hybridization analyses with exon- and intron-specific c-raf-1 (genomic) probes has allowed us to construct a transcriptional map for the c-raf-1 locus and has demonstrated two alternatively spliced poly(A)+ mRNAs. One of these mRNAs, containing the most 3' portion of c-raf-1, may be specifically associated with transformation. A second step in the oncogene activation of c-raf-1 appears to be truncation as all three transforming versions of c-raf-1 are amino-terminally truncated; v-raf with 37 kd, v-mil with 40 kd, and an LTR-inserted and activated c-raf-1 with 50 kd. There are additional raf-related sequences in mouse and human DNA. One of them, isolated from a mouse spleen cDNA library using v-raf, corresponds to an active gene, δ-raf, which is located on human chromosome 7 near the centromere. Another, γ-raf, was isolated from a human fetal liver cDNA library using δ-raf as a probe. Homology between δ-raf and c-raf-1 is 69% for DNA and 74% for amino acid seqences. There are two δ-raf transcripts of 2.3 and 4.0 kb. We have investigated the tissue-specific expression of c-raf-1, δ-raf, and γ-raf and have found that, in contrast to c-raf-1 which is fairly ubiquitous in its expression, transciption of δ-raf and γ-raf shows tissue specificity.

4. Synergistic transforming activity of v-raf and v-myc oncogenes have been demonstrated in vivo and in vitro. This provides the first examples for synergistic action of independently active oncogenes in the transformation of lymphoid/hematopoietic and epithelial cells in vivo. The mechanism underlying synergism appears to involve constitutive expression of two independent signals for growth, a "competence" and a "progression" signal. v-myc would provide the former as deduced from our finding that v-myc expression in certain cells abrogates their requirement for specific growth factors, such as IL-2 and IL-3. v-raf has the potential to induce the latter since fibroblast cells transformed by v-raf produce TGF, a mitogen acting through the EGF receptor. As a spinoff from these studies, a variant virus that predominantly induces carcinoma in mice has been isolated, providing the first example of a molecularly analyzed mammalian carcinoma inducing virus.

Cells from tumors induced by the myc or raf + myc transducing viruses can be readily established in culture in regular medium, whereas culture of cells from raf oncogene-induced tumors requires the addition of IL-3. A function for myc in this synergism has been indicated in studies involving infection of a series of IL-2 and IL-3-dependent cell lines with the various viruses. These studies have demonstrated that expression of high levels of v-myc alone can abrogate the growth factor requirements of these cell lines and probably functions in an analogous manner in its synergistic action with raf in the development of hematopoetic/lymphoid tumors in vivo.

A mouse model system was established for the rapid induction of tumors in tissues that are also being transformed by raf or raf/myc oncogene carrying retroviruses. Tumor induction involved transplacental inoculation of mice at day 16 of pregnancy with ethylnitrosourea (ENU) followed by promotion with butylated hydroxytoluene (BHT). Ninety percent of the animals treated with initiator (ENU) and promoter (BHT) developed tumors within 5 to 14 weeks. In the absence of promotion, 40% of the animals developed tumors with latency of 9 to 25 weeks. In preliminary experiments it was observed that vaccination with v-raf protein doubled the life expectancy of mice treated with both ENU and BHT.

5. Adoptive transmission of Simian AIDS with type D retroviral concentrates in macaques. A Macaque type D/Washington virus was isolated from an explant of retroperitoneal fibromatosis (RF) in a rhesus monkey with SAIDS characterized by persistent diarrhea, weight loss, lymphocytopenia, opportunistic infections and RF. To examine viral pathogenicity, four juvenile colony-born and four feral young adult *Macaca nemestrina* were inoculated with 2 X 10^6 viable viral particles (assayed by end-point dilution of infectivity in cell culture) from a 500X viral concentrate of filtered culture medium of dog thymus cells infected with the Macaque type D/Washington retrovirus. At 5 weeks, one colony-born juvenile macaque died with suggestive RF, and with generalized lymphoid depletion. At 10 weeks, retroperitoneal nodules were palpated in a colony-born macaque that at laparotomy at 18 weeks were histologically diagnosed as RF. After 13 months, the macaque with RF and the one that died at 5 weeks are the only animals with viremia, as determined by isolation of Macaque type D/Washington virus from blood plasma, and are the only animals without seroconversion following virus inoculation. Eight matched control *M. nemestrina*, inoculated with filtered culture medium from uninfected dog thymus cells, have remained healthy.

6. Development of surgical and hormonal procedures for embryo manipulation; preservation and transfer in mouse, cat and other domesticated animal species. A major effort to develop procedures for optimizing cryopreservation of mouse embryos at NIH has led to a successful service program. In 1984, 33,000 embryos were collected from 12 strains of laboratory mice, and 10,000 of these embryos were permanently banked in liquid nitrogen. These studies resulted in a comprehensive data base on the influence of different cryoprotectants, storage vessels, freezer units, sterilizing methods, freezing protocols, and mouse strains. Optimal cryopreservation procedures are genotype dependent, although routine survival rates of 60 to 80% have been achieved following embryo thawing.

Successful procedures, albeit with different rates of success, have been developed for viable embryo collection and transfer in sheep, minature pig, domestic cat and one rare and endangered species, the Scimitar horned oryx. Each of these species has produced one or more heathy litters following transfer. Development of optimal embryo manipulation procedures follow the lead of the mouse models and offer much hope for cryopreservation of mammalian embryos. We have also developed protocols for in vitro fertilization of the domestic cat and sheep. Viable freezing of spermatozoa for six species has been optimized as well. Progress in these areas would be of more than moderate value in gene therapy protocols in the future.

7. Isolation, molecular cloning, DNA sequencing and biological characterization of murine cellular genes which confer sensitivity to chemically induced-tumor promotion (pro-1 and pro-2). Genes that specify sensitivity to promotion of neoplastic transformation by tumor promoters in mouse epidermal JB6 cells have

been cloned. Two such genes which differ from each other and from known oncogenes, termed _pro_-1 and _pro_-2 have been identified. _Pro_-1 has been sequenced. The sequence is intronless, shows the consensus sequences needed for transcription and translation, and is expected to code for a 7000 dalton protein. Tumor promoter treatment of P^+ cells produces transient increases in _pro_-1 RNA levels. Human nasopharyngeal carcinoma cell (CNE) DNA shows both promotion sensitivity (P^+) activity and homologs of _pro_-1 and _pro_-2. _Pro_-1 homologs have been cloned from a CNE library and initial assays indicate that they are biologically active for P^+ activity. In addition a novel transforming activity detectable in P^+ recipient cells has been found in the DNA of TPA transformed JB6 cells. The future course of these studies will be concerned with understanding _pro_ gene expression and _pro_ gene products, and their significance in human and rodent cells. The regulation of _pro_ gene transcription and mRNA processing by tumor promoters and antipromoters will be studied. The structure and biological activity of human _pro_ gene homologs will be characterized. _Pro_-1 proteins will be purified, characterized and localized. A novel transforming gene will be cloned and its mode of regulation by _pro_ genes will be studied.

8. Demonstration of critical interactive role of superoxide, calcium, and phosphoproteins in tumor promotion by phorbol esters. Recent evidence indicates that the free radical superoxide anion, appears to be an essential mediator of neoplastic transformation by tumor-promoting phorbol esters in JB6 mouse epidermal cells. The superoxide anion is apparently critical during the first two hours after the phorbol ester interaction with its receptor. The enzyme superoxide dismutase (SOD), inhibits promotion of transformation by TPA only when added to cells up to two hours after TPA. Superoxide dismutase activity is substantially decreased by TPA treatment of promotion sensitive (P^+) but not promotion resistant (P^-) cells, suggesting a causal relationship between the enzyme decrease and elevation of superoxide anion, as well as a causal relationship of these events to promotion of neoplastic transformation. Two independent approaches have demonstrated that extracellular calcium provides a required signal transducer for promotion of transformation in JB6 cells. The extracellular calcium required for TPA-promoted transformation appears to enter cells via plasma membrane channels. Calcium channel blockers inhibit TPA-promoted transformation at channel blocking concentrations. Cation binding to cation binding proteins may be essential signal transduction events in promotion of transformation. The trivalent cation lanthanum substitutes for calcium in activating protein kinase C but unlike calcium, functions (1) to promote transformation of JB6 cells in the absence of TPA, and (2) to produce conformational changes in certain protein kinase C substrates. The proposed course of this project will focus on extension of the reactive oxygen, calcium and protein kinase signal transduction studies, as well as on connecting to the promotion sensitivity (_pro_) genes project at the level of gene expression. Identification of lanthanum-binding promotion-relevant proteins will be pursued. Tumor promoter inducible proteins and phosphoproteins will be studied with the aim of finding proteins involved in recognizing signal sequences in _pro_ genes. Whether _pro_ genes can complement the calcium resistant putative initiated phenotype (genotype) will be ascertained, as part of an overall effort to understand gene cooperation during tumor promoter induced preneoplastic progression. Several approaches to characterizing the protein products of _pro_-1 and _pro_-2 will be pursued with the goal of determining intracellular localization, purification, and identification of these _pro_ gene products.

9. Expansion of the feline map to include oncogenes, endogenous retrovirus loci, the MHC and loci responsible for inborn errors. The genetic analysis of the domestic cat began with the assignment of 33 isozyme loci to 16 (of 19) chromosomal linkage groups using a panel of rodent x cat somatic cell hybrids. A domestic cat colony was established at the NIH Animal Center and linkage analysis of morphological loci and biochemical loci was initiated by computing log of the odds values of linked genes in pedigrees. Reciprocal skin grafts and allogenic lymphocyte immunizations were initiated and 15 standard antisera against the feline MHC were derived. These sera have allowed the description and resolution of MHC haplotypes in four major cat colonies. The endogenous RD-114 retroviral family was studied in domestic cats by deriving molecular clones of endogenous RDV1 and performing restriction and chromosome mapping. The feline homologues of the proto-onc genes have been studied using molecular clones of v-onc and c-onc from man or mouse. To date, ten oncogene loci have been genetically mapped and their role in feline tumorigenesis is under study. Eight enzyme loci that encode lysosomal enzymes known to be mutated in human inborn errors and in feline counterpart models have been assigned to specific feline chromosomes. Molecular cDNA clones of human lysosomal enzymes are being ligated to appropriate eukaryotic vectors for possible gene therapy in transgenic felids.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05150-06 LVC

PERIOD COVERED
October 1, 1984 to September 30, 1985

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

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

PI:	Ulf R. Rapp	Research Chemist	LVC	NCI
Others:	John L. Cleveland	Staff Fellow	LVC	NCI

COOPERATING UNITS (if any)
Department of Pathology, University of Helsinki, Finland (J. Keski-Oja)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

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.)

Two approaches have been used by this laboratory for the isolation of new oncogenes: (1) transduction with retrovirus and (2) transformation of cells by retrovirus insertion followed by molecular cloning of flanking DNA. The first approach has yielded the v-raf oncogene carrying virus 3611MSV and several other isolates which will be molecularly analyzed in the future. For the second approach an in vitro system was established for the transformation of rat epithelial cells (RC-E) by C3H MuLV in conjunction with 12-O-tetradecanoylphorbol-13-acetate (TPA). Transformed cell clones obtained from soft agar generally were virus non-producers. The purpose of these experiments was to sequence-label TPA-promotable cellular tumor genes, and thus, make possible their isolation by molecular cloning. RC-E cells that had been transformed with MuLV and TPA are being examined for expression of novel RNA transcripts initiating in the viral LTR. Those non-producers which contain novel virus-cell hybrid transcripts are being used for the molecular cloning of LTR-linked cellular DNA. As an example for the principle effectiveness of such a strategy, we have demonstrated for NIH 3T3 cells transformed by transfection with MuLV LTR DNA that the proto-oncogene, c-raf-1, was activated as an oncogene by a promoter insertion mechanism.

PROJECT DESCRIPTION

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

Ulf R. Rapp	Research Chemist	LVC	NCI
John L. Cleveland	Staff Fellow	LVC	NCI

Objectives:

To obtain new oncogenes which might be involved in common human tumors. To develop improved in vitro systems for isolation of genes involved in chemical transformation of epithelial cells; to improve the methodology of the assay systems to permit the processing of large numbers of carcinogen-treated cells and shorten the time required for transformation experiments; to isolate, using techniques of cocarcinogenesis, new cell-derived tumor genes after their linkage with or incorporation into type C viral genomes; and to clone the active tumor gene(s) from a chemically transformed cell.

Methods Employed:

A basic goal of chemical carcinogenesis research is the identification of cellular genes which bring about the transformed phenotype of cells after carcinogen exposure. Assays for quantitative chemical carcinogenesis and cocarcinogenesis with cultured mouse cells have been widely accepted as model processes that occur during the development of spontaneous tumors. While this methodology has been highly successful in delineating details of the metabolic activation of carcinogens and the gradual transformation of cells by such agents and tumor promoters, it has not yielded much information regarding the genes and genetic mechanisms involved.

RNA tumor viruses, on the other hand, while not being serious candidates as causative agents for most human tumors, have provided the first isolates of cellular genes which are apparently sufficient to induce malignant transformation. To combine the advantages of both systems, experiments have been initiated to identify the genes involved in chemical transformation and the regulation of their expression in vitro. Efforts to identify such genes have included (1) screening of chemically transformed cells for the expression of known type C virus-transduced tumor genes, (2) isolation of additional transforming viruses from transduction and cocarcinogenesis experiments to widen the repertory of tumor genes for screening, and (3) attempts to identify genes promotable by 12-O-tetradecanoylphorbol-13-acetate (TPA) by sequence-labeling with murine leukemia virus (MuLV) genomes followed by molecular cloning of genes that were constructed in these cells by LTR promoter insertion.

Major Findings:

The potential of activating proto-oncogenes to transforming genes by LTR promoter insertion has recently been demonstrated by us. In an attempt to establish a novel strategy for the identification of potential cellular oncogenes (c-onc genes) malignant cell lines were isolated after co-transfection of normal NIH 3T3

(carrier) DNA and cloned Moloney leukemia virus long terminal repeat (Mo-LTR) sequences onto NIH 3T3 recipient cells (Muller and Muller, 1984). Theoretically, such an approach can lead to the induction of neoplastic transformation in several ways, including (1) the transcriptional activation of neighboring genes, via the Mo-LTR promoter or enhancer; (2) block of gene transcription as a consequence of Mo-LTR integration; (3) transcriptional activation of carrier DNA sequences by juxtaposition to cellular enhancers or promoters (as suggested by Cooper et al., 1980); or (4) expression of structurally aberrant proteins from truncated, rearranged or mutated carrier DNA sequences. While several of the neoplastic cell lines obtained by this approach apparently contain activated oncogenes that are different from 21 previously described c-onc genes, the transforming gene of one of these clones (designed clone S1) was shown to be homologous to the murine retroviral oncogene v-raf, which in turn is homologous to one of the two oncogenes (v-mil) transduced by the avian leukemia virus MH2. The raf/mil oncogene has thus been identified in three independent ways. Analysis of DNA from 15 secondary (2°) and tertiary (3°) foci derived from clone S1 revealed a single Mo-LTR integration site located on a specific restriction fragment, indicating a linkage between the activated oncogene and Mo-LTR sequences. To clarify the mechanism of oncogene activation in clone S1 we have isolated, by molecular cloning, the Mo-LTR-c-raf complex and analyzed its structure and expression. The results show that the oncogenic activation of c-raf occurred by Mo-LTR integration into the 5th intron of the proto-oncogene leading to the synthesis of high levels of c-raf transcripts starting in the Mo-LTR. Transcriptional activation of c-raf is accompanied by the synthesis of large amounts of cytoplasmic c-raf protein. These findings indicate a promoter insertion mechanism of c-raf activation.

Significance to Biomedical Research and the Program of the Institute:

The overall goal of this work is to characterize cellular genes that are associated with the induction of cancer as it occurs spontaneously or after induction with carcinogens. In consideration of the fact that carcinomas are especially prevalent in humans, special emphasis has been placed on transformation of epithelial cells. A family of cell-derived transforming genes isolated as part of type C viruses or linked to type C virus regulatory sequences might provide the tools necessary for both an understanding of mechanisms of transformation and the development of strategies to neutralize their action.

Proposed Course:

1. Molecular cloning of the gene(s) responsible for oncogenic transformation in mouse cells transformed by MuLV and TPA.

The strategy for these experiments is as follows:

(a) Construction of a gene library containing a collection of recombinant phages representing the entire genome of the transformed cells. The total cellular DNAs from transformed cells were isolated and partially digested with S2u3A. The 15-20 kilobase (kb) DNA fragments were isolated from a gel electrophoresis which separated the partially digested DNAs according to size. A novel derivative of lambda 1059 was used to clone these DNA fragments at BamHI sites. Only the recombinant phages will grow on E. coli strains lysogenic for phage P2. A gene

library consisting of 5 x 10^5 clones could be used to screen for the recombinants containing the transforming gene(s) which should be present with a larger than 99% probability.

(b) Screening the recombinant library for the transforming gene(s). An LTR specific probe will be used to screen the library. A mixture of recombinant DNAs which hybridize to the LTR probe will be isolated from the entire gene library and used to transfect untransformed RC-E cells and to isolate transformed colonies grown in soft agar. A positive result indicates that such a transforming gene(s) has been cloned in vectors along with LTR sequences.

Publications:

Molders, H., Defesche, J., Muller, D., Bonner, T. I., Rapp, U. R. and Muller, R.: Integration of transfected LTR sequences into the c-raf proto-oncogene: Activation by promoter insertion. EMBO J. 4: 693-698, 1985.

Nystrom, M., Knuutila, S., Rapp, U. R. and Keski-Oja, J.: MMC-E Cells - Origin and changes in karyotype accompanying malignant transformation. Cancer Genet. Cytogenet. 15: 243-251, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05180-05 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Evolution and Sequence Organization of Mammalian Retroviruses

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

PI:	Raoul E. Benveniste	Medical Officer	LVC	NCI
Others:	Stephen J. O'Brien	Geneticist	LVC	NCI

COOPERATING UNITS *(if any)*

University of California School of Medicine, Davis, CA (W. Centerwall); Yale University, New Haven, CT (C. Sibley)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.45	0.15	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.)*

Nucleic acid hybridization studies using cloned retroviral DNA were used to examine the evolution and organization of primate and carnivore retroviruses. These data were compared to those obtained on the evolution of unique sequence cellular DNA among these two mammalian orders. DNA fragments related to the gag and pol regions of the endogenous RD-114 and FeLV retroviruses were found in just six closely related species of Felidae. F1 hybrids from crosses of virogene-positive and virogene-negative cats were bred back to the virogene-negative parent. The cellular DNA extracted from 42 of these backcross offspring were analyzed after restriction enzyme digestion with probes specific to the two feline virogenes. The results demonstrate that viral sequences from both retrovirus families are dispersed on multiple chromosomes throughout the cat genome. Genetic mapping of sequences from both families of viruses in the backcross animals will be accomplished by correlating the occurrence of unique viral restriction fragments with feline isozymes previously assigned to specific cat chromosomes.

A phylogenetic tree of carnivore evolution has also been established by molecular hybridization techniques; it differs in many respects from the classical relationships derived by anatomical considerations and the fossil record. Four molecular approaches, including DNA hybridization, isozyme genetic distance, immunological distance and G-banded karyology, were applied to determine the taxonomic status of the giant and lesser panda. A consensus phylogeny is derived that reveals that the lesser panda's ancestors emerged at the time of the procyonid-ursid divergence and that the giant panda diverged from the ursid lineage of the carnivores contemporaneously with the human-gibbon divergence among the primates.

PROJECT DESCRIPTION

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

Raoul E. Benveniste	Medical Officer	LVC	NCI
Stephen J. O'Brien	Geneticist	LVC	NCI

Objectives:

To study the evolution and organization of primate and feline retroviruses within the mammalian genome. To use the time of interspecies viral transfer as a defined marker for the rates of evolution of various species. Recombinant DNA techniques will be employed to develop probes appropriate for the detection of virus-related sequences in various primate and carnivore tissues.

Methods Employed:

The viruses used were the Old World monkey isolates from this laboratory: the baboon type C virus, the colobus-macaque-rhesus class of type C viruses, and the langur type D viruses. The feline viruses included RD-114 and feline leukemia virus (FeLV). Primary cell lines from various feline and ape species were developed and maintained. Replication of retroviruses was detected by assaying the pellet obtained after high-speed centrifugation of supernatant fluid from cells for reverse transcriptase activity. Radioimmunoassays for various type C viral proteins were also employed to characterize new isolates. The cloning of proviral DNA was performed using various plasmids as vectors, as well as phages constructed from lambda. Restriction enzyme maps were generated for various retroviruses. Cloned proviral DNA was used as a probe for cloning viral sequences integrated in the genomes of mammalian species.

Major Findings:

1. Carnivore evolution and phylogeny as derived by DNA hybridization. A phylogenetic tree of the carnivores has been derived from thermal stability measurements of nonrepetitive cellular DNA. The data include 30 species of carnivores, with eight of them as index species. The computer programs developed by Drs. Fitch ("Neighborliness") and Dayhoff ("Mattop") were used to derive phylogenies. The data obtained differ in many respects from those obtained by classical methods such as anatomical comparisons and morphometric measurements. For example, the lesser panda and skunk seem to represent distinct families of carnivores because, from a phylogenetic perspective, they are equidistant from all other carnivores. An in-depth phylogenetic analysis of the giant and red panda using four independent molecular approaches was achieved. The four distance measures (DNA hybridization, Wei's genetic distance, immunological distance, and chromosome banding analysis) converge on a consensus phylogeny. The lesser panda's ancestors emerged at the time of the procyonid-ursid divergence, while ancestors of the giant panda split from the ursid lineage much later, just prior to the radiation that led to modern bears approximately 20 million years ago.

2. The transfer of retroviruses from primates to felines as a marker for studies of evolutionary rates. Earlier work has shown a transfer of viruses from primates to felines sometime during the past several million years. The virus acquired by cats (and six of their descendant species) is called RD-114. We have now shown that this transfer occurred at the time of the gelada-baboon ancestor or about 4 to 6 million years ago. Using this transfer as a marker for evolutionary time, we have studied the rate of accumulation of mutations in those primate and feline species that have diverged since the time of virus transfer. The rate of mutation has been the same in both orders, and the rate of base pair substitutions in DNA is, therefore, independent of generation time in these two mammalian orders. These results allow a direct comparison of recent primate and carnivore divergence times.

3. Characterization of endogenous, retroviral gene sequences in crosses between domestic cat and leopard cat. The domestic cat, Felis catus, possesses multiple copies of gene sequences related to two classes of RNA tumor viruses, FeLV and RD-114 type C viruses. The former class causes lymphoma and myeloproliferative disease, while the latter class has not been shown to cause disease in cats. Both viruses are inherited as stable Mendelian units from one generation to the next.

Molecular hybridization experiments reveal that both RD-114 and FeLV were introduced into the germ line of a common ancestor of six closely related species of the genus Felis: the domestic cat (F. catus), European wildcat (F. silvestris), jungle cat (F. chaus), sand cat (F. margarita), African wildcat (F. libyca) and black-footed cat (F. nigripes). Studies with subgenomic probes from the endogenous RD-114 virus have shown conservation among fragments in the gag and pol viral regions. These same fragments were also found in multiple copies in cell DNA from the other five RD-114-containing cat species. The leopard cat from Southeast Asia contains neither RD-114 nor FeLV viral sequences in its DNA. The offspring of matings between the leopard cat and domestic cats thus afford a unique opportunity to study the segregation of both sets of virogenes in the progeny of backcrosses of F_1 hybrids to the virogene-negative parent. Restriction digests of cell DNAs from backcross offspring reveal that viral sequences from both endogenous families are dispersed on multiple chromosomes throughout the cat genome.

Significance to Biomedical Research and the Program of the Institute:

One of the key questions in evolutionary biology is whether mutations in DNA accumulate as a function of chronological time or as a function of generation time of a species. The only previous "clocks" available were the fossil record or the dates of continental drift, both of which are imprecise. The transfer of retroviruses between species offers a unique method for fixing a point in evolutionary time that is completely independent of any assumptions of geologic time.

The discovery and evolutionary tracing of the numerous primate retroviruses represents an important program of study. Earlier investigations revealed that endogenous retroviral DNA sequences are present in primate cellular DNA and are inherited as stable Mendelian units. In certain cases, these viruses can be transmitted from one species to an evolutionarily distant one and subsequently incorporated into the germ line. There have now been seven documented transfers

of retroviruses between mammalian species. Those transfers that have not resulted in incorporation into the germ line of the recipient species have resulted in retroviruses that are pathogenic to the new species. The AIDS viruses in man, VISNA virus in sheep, equine infectious anemia virus (EIAV) in horses and bovine leukemia virus (BLV) in cattle are all examples of retroviruses that are pathogenic and whose evolutionary origin is also unknown. The elucidation of the species of origin of the AIDS viruses will have important public health consequences.

Proposed Course:

Endogenous viral sequences that are present in all families of carnivores are being cloned and will be used to study their origin from primates and integration sites and as a tool to study carnivore, and especially feline evolution. Since retrovirus DNA sequences evolve at a faster rate than nonrepetitive cellular DNA, these viral sequences could be used to study the phylogeny of closely related carnivore species. The origins of pathogenic retroviruses, such as the AIDS viruses, VISNA virus and EIAV, will be explored by examining cellular DNA from sympatric species for the presence of retroviral sequences.

Publications:

Benveniste, R. E.: The contributions of retroviruses to the study of mammalian evolution. In MacIntyre, R. J. (Ed.): Molecular Evolutionary Genetics (Monographs in Evolutionary Biology Series). New York, Plenum Press (In Press)

O'Brien, S. J., Benveniste, R. E., Nash, W. G., Simonson, J. M., Eichelberger, M. A., Wildt, D. E. and Bush, M.: Constructing a molecular phylogeny of the giant panda, *Ailuropoda melanoleuca*. Bongo (In Press)

O'Brien, S. J., Nash, W. G., Wildt, D. E., Bush, M. E. and Benveniste, R. E.: Riddle of the giant panda phylogeny: A molecular solution. Nature (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05330-03 LVC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Urinary Growth Factors in Human Neoplasia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
PI: Kurt J. Stromberg Medical Director LVC NCI

COOPERATING UNITS (if any)
Division of Endocrinology, Vanderbilt University, Nashville, TN (D. N. Orth); Oncogen, Seattle, WA (D. R. Twardzik); Triton Biosciences, Houston, TX (R. Pardue and J. Dedman)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	0.4	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.)
Our objective was to characterize a growth factor of approximately 30,000 to 35,000 Mr previously reported in the urine of cancer patients. Urine obtained from a patient who had a highly malignant brain tumor (astrocytoma, grade IV) was adsorbed on trimethylsilyl-controlled pore glass (TMS-CpG) beads and eluted with acetonitrile to yield a high molecular weight (HMW) human transforming growth factor (hTGF) with both clonogenic and competing epidermal growth factor (EGF) radioreceptor activities. This HMW hTGF had a molecular weight of 33,000 on SDS-PAGE, identical to that of a highly purified HMW form of human EGF (HMW hEGF) previously reported to be present in trace concentrations in normal human urine. Following surgical resection of the tumor, no appreciable HMW hTGF activity was detectable in urine. HMW hTGF generated a competitive binding curve similar to that of hEGF and parallel to that of HMW hEGF. Both hEGF and HMW hEGF were clonogenic in soft agar, and their clonogenic activity, as well as that of HMW hTGF, were inhibited by hEGF antiserum. Thus, HMW hTGF was indistinguishable from HMW hEGF in terms of apparent molecular size, EGF receptor binding activity, EGF immunoreactivity and clonogenic activity. This urinary HMW hTGF/EGF may be of tumor cell origin or may represent a response of normal host tissues to the tumor or tumor products. In a separate study, an extracellular form of beta-TGF activity was detected in conditioned medium of rat cells after infection with a transformation-defective strain of Abelson leukemia virus and, hence, expression of this growth factor activity was independent of cell transformation. This factor had an approximate Mr of 12,000 and eluted at 37% acetonitrile during high performance liquid chromatography (HPLC). Moreover, the presence of an EGF-dependent, 12,000 Mr, clonogenic activity in extracts of bovine serum alone suggested serum as an origin for the beta-type TGF initially observed in conditioned medium of Snyder-Theilen FeSV-transformed cells. This does not, however, preclude the possibility that beta-TGF is also secreted by the transformed rat embryo cells themselves.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Kurt J. Stromberg Medical Director LVC NCI

Objectives:

To isolate and to characterize members of a family of urinary growth factors in human cancer patients with respect to their histologic type and their correlation with extent of tumor burden, to develop novel growth factor isolation procedures employing hydrophobic adsorption from controlled pore glass beads and to use nu/nu rats bearing human tumors as a model system.

Malignant astrocytoma, grade III or grade IV (glioblastoma multiforme), the most aggressive cancer of the central nervous system, was selected for emphasis because (1) the issue of occult metastasis is avoided (extra-CNS spread of this malignancy is exceedingly rare), (2) repeated tumor debulking occurs (to provide a natural human model for correlation of growth factor activity with tumor burden over an extended period of time), (3) the question of whether or not the high molecular weight growth factor (HMW GF) traverses the blood-brain barrier to reach the kidneys for clearance can eventually be addressed, and (4) high levels of expression of human epidermal growth factor (hEGF) receptors are reported in astrocytomas.

Methods Employed:

The presence of growth factor(s) was assessed by stimulation of growth of normal rat kidney cells (NRK) in soft agar, competition with ^{125}I-labeled mouse EGF (mEGF) for binding to membrane receptors, and radioimmunoassay for hEGF and human alpha-TGF (using C17A fragment reagents). A simple procedure was developed for rapid processing of large volumes of either human or rat urine based on growth factor adsorption to trimethylsilyl-controlled pore glass beads (TMS-CpG beads) and selective elution with appropriate concentrations of acetonitrile. In bulk human urine extractions of up to 26 liters, the use of TMS-CpG beads resulted in a rapid one-step, 225fold reduction of protein with near complete recovery of EGF radioreceptor activity.

Athymic rats were employed to passage human carcinomas of various histologic types and housed in metabolic cages to facilitate efficient urine collection.

Major Findings:

1. Urinary growth factors adsorb to C-1 beads. Growth factors were isolated by a simple new procedure well suited to rapid isolation of trace proteins from dilute solutions, such as large volumes of urine. The procedure is based on hydrophobic adsorption of growth factors to controlled-pore glass beads that have been modified by covalent attachment of a hydrophobic-bonded phase and incremental elution with acetonitrile. This procedure is an adaptation of the method used for rapid isolation of interleukin-2 from conditioned cell culture medium and uses TMS-CpG as an adsorbent.

2. Human brain tumor-associated urinary high molecular weight (HMW) growth factor of 33,000 Mr is identical to human HMW epidermal growth factor. Urine was obtained from a patient who had a highly malignant brain tumor (astrocytoma, grade IV). Following adsorption to TMS-CpG beads and elution with acetonitrile to yield an HMW human transforming growth factor (hTGF), this HMW hTGF promoted clonogenic cell growth in soft agar and competed for membrane receptors with mouse EGF. The growth factor activity eluted from TMS-CpG beads at 25-27% acetonitrile, and after purification by Bio-Gel P-100 chromatography and high performance liquid chromatography (HPLC), had a molecular weight of approximately 33,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), identical to that of a highly purified HMW form of human EGF (HMW hEGF) previously reported to be present in trace concentrations in normal human urine. Following surgical resection of the tumor, no appreciable HMW hTGF activity was detectable in urine. HMW hTGF generated a competitive binding curve similar to that of hEGF and parallel to that of HMW hEGF. Both hEGF and HMW hEGF were clonogenic in soft agar, and their clonogenic activity, as well as that of HMW hTGF, was inhibited by hEGF antiserum. Both HMW hTGF and HMW hEGF had 20 to 25% of the radioreceptor binding activity of hEGF. Thus, HMW hTGF was indistinguishable from HMW hEGF in terms of apparent molecular size, EGF receptor binding activity, EGF immunoreactivity and clonogenic activity. Urinary HMW hTGF/hEGF may be of tumor cell origin or may represent a response of normal host tissues to the tumor or tumor products.

3. Beta-type transforming growth factor from conditioned cell culture medium may derive from serum. During the stepwise isolation of TGF obtained from the conditioned medium of Fischer rat embryo cells transformed by the Snyder-Theilen strain of feline sarcoma virus (ST-FeSV FRE CL 10), it became evident that, paradoxically, increased purification resulted in diminished colony size in soft agar assays. In view of the requirement for the combined presence of TGF and TGF to effect maximal clonogenicity, the possibility was examined that progressive steps in isolation removed a second growth factor necessary for optimum colony growth in soft agar. Accordingly, exogenous mEGF was added to the various fractions during the purification process. This revealed a previously unrecognized growth factor of the TGF -type in that (1) a peak of clonogenic activity was detectable only after mEGF addition, (2) the peak fractions potentiated by mEGF failed to compete with EGF for binding to EGF membrane receptors, and (3) the clonogenic activity eluted during HPLC as a hydrophobic molecule.

To examine whether TGF -like clonogenic activity was related to the transformed phenotype, conditioned medium from transformation-defective Abelson-MuLV-infected rat cells was evaluated. Interestingly, the EGF-dependent clonogenic activity was present in the absence of the viral-induced transformed phenotype. The demonstration of this TGF -like activity in conditioned medium of rat cells nonproductively infected with transformation-defective Abelson-MuLV mutants, which do not secrete TGF s, indicates its presence to be independent of TGF production. Because platelets contain a 10 to 100fold higher concentrations of TGF than other tissues and because serum itself contains a platelet-derived growth factor, 250 ml of fetal bovine serum (FBS) was acid-ethanol extracted. This FBS was equivalent to what would be present in 2.5 liters of Dulbecco's modified Eagle's medium used for cell culture growth of ST-FeSV FRE CL 10 cells or transformation-defective Abelson-MuLV mutant cells. After P-30 Bio-Gel chromotagraphy, a peak of soft agar activity was detected at approximately 12,000 Mr only after an addition of 2 ng exogenous mEGF.

Complete separation of the TGFα and TGFβ from conditioned medium of ST-FeSV FRE CL 10 cells was achieved by HPLC on C_{18} in a Bondapak column using a linear elution gradient of acetonitrile in 0.1% trifluoroacetic acid. The two clonogenic activities present in a fraction pool from the P-30 Bio-Gel chromatography eluted differently in reverse phase HPLC. The EGF-competing activity elutes much earlier (approximately 20% acetonitrile) than the clonogenic activity potentiated by addition of EGF (approximately 37%). This latter activity did not compete with EGF for binding to A431 human carcinoma cell membrane receptors and stimulated colony formation in soft agar only in the presence of exogenously added mEGF (or TGFα). Thus, in its apparent molecular weight (12,000 M_r), HPLC elution (37% acetonitrile), biologic behavior (clonogenicity promoted by very low nanogram amounts of mEGF or TGFα), and its insensitivity to heat, acid, and heparin, the factor resembles TGFβ present in neoplastic and normal cells.

These results confirm recent reports and extend them in that this TGFβ-like factor is also present in the conditioned medium of cells infected with a transformation-defective virus. Moreover, our preliminary evidence indicates its presence in FBS alone. Consequently, because conditioned medium of transformation-defective mutants of rat embryo cells and even serum itself apparently contain this type of EGF-dependent growth factor, expression of this β-type TGF is independent of TGFα production and the transformed phenotype and may derive from serum itself.

4. Nude rats bearing human tumor xenographs have elevated levels of 33,000 Mr growth factor. Growth factors that exhibit EGF receptor competition and stimulate cell growth in soft agar were isolated from the urine of nude rats bearing human tumor xenographs and from the urine of nontumor-bearing controls. A high molecular weight species was purified by a five-step process (including acid precipitation of extraneous proteins, adsorption on TMS-CpG beads, Bio-Gel P-10 and P-100 chromatography and reverse phase HPLC chromatography) to a single band on SDS-PAGE. This protein, which is elevated in tumor-bearing rats, demonstrated the same clonogenic and radioreceptor activities and apparent molecular weight as that protein reported to be elevated in the urine of human tumor-bearing patients. As determined by immunological analysis, the protein from rat urine is unrelated to the HMW hEGF, which it closely resembles in molecular weight and hydrophobicity. Currently, an investigation is being made, using radioimmunoassays (RIAs) to rat EGF and human alpha-TGF, as to whether this growth factor and lower molecular weight urinary growth factors are an expression of host response to tumor burden or are derived directly from the human tumor itself.

Significance to Biomedical Research and the Program of the Institute:

Urinary transforming growth factors may prove to be useful markers of preclinical cancer development in man. Although considerable resources are currently being devoted to the basic molecular biology of TGFs, relatively little emphasis has been placed on developing an approach to their evaluation and possible application to preclinical human cancer detection. Identification of the origin (host or tumor) and correlation with clinical condition (tumor bulk) of the HMW growth factors and other relevant growth factors, are essential steps in this process.

Proposed Course:

Quantitative procedures for large-scale isolation and characterization of growth factors using pooled preoperative urines from patients with various tumors are currently being developed. In the case of the presence of HMW hTGF/EGF in urine of glioblastoma patients, we wish to examine the possibility that this growth factor is actually being transported in the blood and excreted by the kidney, rather than simply being synthesized by the renal distal tubules. Accordingly, we plan to collect for hEGF RIA matched plasma (carefully collected and handled to prevent platelet activation), serum (clotted at room temperature for 3 hours prior to separation), and urine (collected as a spot urine at the same time the blood is drawn) from glioblastoma multiforme patients. In addition, collection of cerebrospinal fluid from the same patients at the same times for hEGF RIA would be instructive in our effort to determine the origin of the HMW hTGF/hEGF.

Until availability and proven suitability of immunologic reagents for human urinary TGF detection are at hand, the long-term objective may have to be to follow individual cancer categories by bulk urine collections and concentration on TMS-CpG beads, followed by eventual purification with HPLC and SDS-PAGE using silver staining of gels to quantify the relative presence of each member of the family of urinary growth factors. Thus, a profile of urinary growth factor expression would be available to relate to the clinical condition during which the urine sample was collected.

Use of the rat model system permits investigation of the relationship between type of tumor (chemical, RNA or DNA virus-induced); tumor histologic type; tumor size; and metastatic progression and the quantity and type of urinary growth factors produced.

Publications:

Stromberg, K. and Twardzik, D. R.: A β-type growth factor, present in conditioned cell culture medium independent of cell transformation, may derive from serum. J. Cell. Biochem. 27: 443-448, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05333-03 LVC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Evolutionary Relationships of the Felidae: A Mitochondrial DNA Approach

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

PI:	Stephen J. O'Brien	Geneticist	LVC	NCI
Others:	Matthew George, Jr.	Guest Researcher	LVC	NCI
	David E. Wildt	Guest Researcher	LVC	NCI
	Lisa Forman	Guest Researcher	LVC	NCI

COOPERATING UNITS *(if any)*
San Diego Zoo, San Diego, CA (O. A. Ryder); Department of Biochemistry, Howard University, Washington, DC (E. T. Butler)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.85	0.55	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 aim of this investigation is to make in-depth comparative studies at the chromosomal, protein and DNA levels among members of the cat family. However, the phylogeny of the cat family (Felidae) is uncertain. Mitochondrial DNA (mtDNA) has been chosen as an evolutionary probe. The molecule evolves rapidly, is maternally inherited and provides a high degree of resolution among closely related species. mtDNA has been isolated from the domestic cat (ten individuals), lion, cheetah (three individuals), and clouded leopard (two individuals). In addition to the four aforementioned species, mtDNA has been isolated from a cougar, a leopard and the South American Geoffrey's cat. The mtDNAs have been digested with 17 different restriction enzymes and cleavage maps have been constructed for five of them (lion, cheetah, domestic cat, clouded leopard and Geoffrey's cat). The intra- and interspecific variations of these five mapped species ("index species") have been measured. The mtDNA of the domestic cat has been molecularly cloned in the plasmid, pBR322. The mtDNA clones will be used as molecular probes of mtDNA from each Felidae species (35 of 37) using "Southern" analysis of high molecular weight DNA from cultured cell lines. The comparative restriction maps of the mtDNA from the various species will be used to construct a molecular phylogeny of the Felidae. This phylogeny will be compared to phylogenetic topologies of the Felidae derived by other molecular and morphometric measurements.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Stephen J. O'Brien	Geneticist	LVC	NCI
Matthew George, Jr.	Guest Researcher	LVC	NCI
David E. Wildt	Guest Researcher	LVC	NCI
Lisa Forman	Guest Researcher	LVC	NCI

Objectives:

1. The construction of mitochondrial DNA (mtDNA) cleavage maps from certain index species (domestic cat, cheetah, lion, clouded leopard, and Geoffrey's cat) of the Felidae, with emphasis on the phylogenetic relationships found in this family. The determination of the extent of genetic variation within and among the different cat species. The development of accurate phylogenetic topologies among extant Felids.

2. Molecular cloning and characterization of mtDNA restriction fragments of domestic cat. The mtDNA clones will be used as molecular probes of the mtDNA found in the high molecular weight DNA derived from 35 Felidae species.

Methods Employed:

Mitochondrial DNA (mtDNA) was isolated and purified from tissue organs by differential centrifugation. This procedure also involves the use of CsCl density gradients. The purified mtDNA was subjected to digestion with 17 different restriction enzymes. The mtDNA fragments were end-labeled with ^{32}P using DNA polymerase I (large fragment) and separated by electrophoresis on vertical agarose slab gels. After autoradiography of the vacuum-dryed gels, the base-pair lengths of the fragments and genome sizes were determined. Construction of the cleavage maps involved multiple enzyme digestions. Other methods included the isolation and preparation of high molecular weight DNA from felid cultured cell lines, preparation and purification of mtDNA fragments for molecular cloning, nick-translation of mtDNA clones, and "Southern" blot analysis.

Major Findings:

1. Construction of mtDNA cleavage maps and analysis of the mtDNA fragment data for five feline species. mtDNA has been isolated from five index cat species: the domestic cat, cheetah, lion, clouded leopard, and Geoffrey's cat. An average of 43 restriction enzymes have been mapped for each of the mtDNAs. The maps reveal that the cat mitochondrial genome is slightly larger than that of human mtDNA. The cat mtDNAs exhibit the same gene order and conservation of restriction enzyme sites (particularly within the rRNA genes) as do other vertebrate species (e.g., mouse and human). When the five different feline mtDNA cleavage maps were examined, a measure of the genetic variation was obtained. The analysis showed that the domestic cat and cheetah differed by 11% in sequence, while the variation between the lion and clouded leopard was 15%. The domestic cat and cheetah mtDNAs showed an average difference of 25% when compared to the mtDNAs of the lion, clouded leopard, and Geoffrey's cat. The Geoffrey's cat mtDNA varied between 13% and 15% when compared to the lion and clouded leopard. The degree of divergence seen was much

higher than that reported from DNA:DNA hybridization studies. An analysis of the intraspecific variation within ten domestic cats examined showed only a small amount of variation. However, the analysis also identified three different mtDNA types or "morphs" within the domestic cats. The three "morphs" were generated through the gain or loss of a restriction site and, in one instance, by the addition of around 15 base pairs. The three cheetah individuals showed no variation; however, two of the individuals were maternally related. The two clouded leopard individuals showed no variation between two restriction enzyme patterns. Fragment analysis of the Panthera (lion, leopard, and cougar) group shows extensive variation. The domestic cat mtDNA, after double digestion with the restriction enzymes BamHI and EcoRI, has been cloned in pBR322. These clones have also been inserted into a pBR322 vector which contains an Sp6 RNA polymerase promoter. Pilot studies have begun to assess the sensitivity of our "nick-translated" probes to the mtDNA found in the cellular DNAs isolated from the index species. With the clones in the Sp6-pBR322 vector, RNA transcripts are also being used as radioactive probes.

Significance to Biomedical Research and the Program of the Institute:

This work complements the in-depth biochemical and genetic studies of the domestic cat (see Project Number Z01CP05385-02 LVC). The phylogeny of this family, which is used as a model system for studies in viral carcinogenesis, is not fully understood. A phylogenetic analysis, using mtDNA as fragment probes, will help resolve some of the uncertainty involving the classification of the Felidae. The domestic cat serves as an important animal model of infectious retroviruses in natural populations (FeLV) and is a fertile system for oncogene isolation. No mammal has produced as many oncogene-containing retroviruses. In addition, FeLV shares many characteristics with human AIDS isolates insofar as more FeLV cats die of immune system impairment than from leukemia. Finally, phylogenetic information is imperative for assessing partners for inter-specific embryo transfers.

Proposed Course:

With the domestic cat mtDNA cloned, the primary main effort will be the derivation of homologous restriction maps using Southern analysis of the 35 additional feline species. A South American cat species has now been added to our "index species". Cleavage maps will also be made for the cougar and leopard mtDNAs. Having representative species will allow our mapping studies to be more accurate and complete. It also aids in our assessment of what types of changes occur in mtDNA from different species. The comparative molecular analysis of mtDNA, in the Felidae with several other molecular metrics being examined in collaboration with this section, promises to produce one of the most comprehensive analyses of molecular evolution in any modern mammalian taxon.

Publications:

O'Brien, S. J., Nash, W. G., Bauer, R. F., Chang, E. H. and Seigel, L. J.: Trends in chromosomal and oncogene evolution in vertebrates. In Patterson, M. K. (Ed.): Uses and Standardization of Vertebrate Cell Cultures. In Vitro Monograph No. 5. Gaithersburg, Tissue Culture Association, 1984, pp. 204-214.

O'Brien, S. J., Seuanez, H. N. and Womack, J. E.: On the evolution of genome organization in mammals. In MacIntyre, R. J. (Ed.): Evolutionary Biology. New York, Plenum Press (In Press)

Wilson, A. C., Cann, R. L., Carr, S. M., George, Jr., M., Gyllensten, U. B., Helm-Bychowski, K., Higuchi, R. G., Palumbi, S. R., Prager, E. M., Sage, R. D. and King, S.: Mitochondrial DNA in relation to evolutionary genetics. Biol. J. Linnaean Soc. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05358-02 LVC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The Role of Retroviruses in Simian AIDS and Retroperitoneal Fibromatosis

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

PI:	Raoul E. Benveniste	Medical Officer	LVC	NCI
Co-PI:	Kurt J. Stromberg	Medical Director	LVC	NCI

COOPERATING UNITS (if any)
Regional Primate Research Center, University of Washington, Seattle, WA (W. E. Giddens, Jr., H. D. Ochs, W. R. Morton, C.-C. Tsai); Program Resources, Inc., Frederick, MD (L. Arthur)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	0.8	1.0

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

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

A Macaque type D retrovirus (Stromberg et al., Science 224: 289-292, 1984) was isolated from an explant of retroperitoneal fibromatosis (RF) in a rhesus monkey with simian immune deficiency syndrome (SAIDS) characterized by persistent diarrhea, weight loss, lymphocytopenia, opportunistic infections and RF. To examine viral pathogenicity, four juvenile colony-born and four feral young adult *Macaca nemestrina* were inoculated with a 500X concentrate of filtered culture medium of dog thymus cells infected with Macaque type D/Washington retrovirus. At 5 weeks, one colony-born juvenile macaque died; it had generalized lymphoid depletion but only suggestive RF. At 10 weeks, retroperitoneal nodules were palpated in a colony-born juvenile macaque that at laparotomy at 18 weeks were histologically diagnosed as RF. After 13 months, the macaque with RF and the one dying at 5 weeks are the only animals with viremia as determined by isolation of the Macaque type D/Washington virus from blood plasma, and are the only animals without seroconversion following virus inoculation. Eight matched control *M. nemestrina*, inoculated with filtered culture medium from uninfected dog thymus cells, have remained healthy. To further examine the interplay between immunization and/or pathogenicity of the Macaque type D/Washington retrovirus isolate, a second viral transmission experiment is underway to achieve a higher incidence of disease using three age groups of *M. nemestrina* inoculated IV with unfrozen Macaque type D/Washington retrovirus isolated from an *M. nemestrina* with SAIDS. To date several macaques have developed nodular abdominal lymphadenopathy, suggestive of retroperitoneal fibromatosis. However, no definite diagnostic, clinical or laboratory evidence of SAIDS has developed in these macaques, which have been continuously caged in a pathogen-free facility.

PROJECT DESCRIPTION

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

Raoul E. Benveniste	Medical Officer	LVC	NCI
Kurt J. Stromberg	Medical Director	LVC	NCI

Objectives:

To study aspects of retroviral association with retroperitoneal fibromatosis (RF) and simian acquired immune deficiency syndrome (SAIDS) in a naturally occurring simian model system (SAIDS), including (1) viral transmission of RF and SAIDS with cell-free filtrates from virus-producing cultures, (2) immunological and biochemical characterization of retroviral proteins from SAIDS viral isolates, (3) epidemiology of virus infection among captive macaques, and (4) prevention of SAIDS through vaccination of susceptible primate colonies.

Methods Employed:

Tissues and fluids from the several species of macaques with clinical signs of RF and SAIDS at the Washington Regional Primate Research Center were cocultivated with heterologous mammalian cells that support the replication of a wide variety of primate retroviruses. Detection of virus release into the conditioned medium from these cocultivations was made by a sensitive reverse transcriptase assay or radioimmunoassay. Characterization of resulting viral isolates was made by standard virological, biochemical, and immunological techniques.

Major Findings:

1. Isolation of Macaque type D/Washington virus has been described (Science 224: 289-292, 1984).

2. Characterization of Macaque type D/Washington viral isolate. The cell lines that support Macaque type D/Washington virus growth remain fibroblastic and no cytopathic effect or morphological evidence of transformation has been observed. However, after infection of several cell lines chosen for their flat morphology and proven ability to detect cell transformation, foci suggestive of a transformed phenotype were observed. The antigenic relatedness of Macaque type D/Washington virus to other type D viruses was determined in specific radioimmunoassays (RIA) for the major core protein (p27) and the major envelope glycoprotein (gp70) purified from Mason-Pfizer monkey virus (MPMV). Lysed virus pellets of MPMV and the endogenous langur virus (PO-1-Lu) effectively competed in the MPMV p27 assay. The extent of competition and similarity of the slopes of the competition curves indicate close immunological relatedness of the core proteins of these three viruses. However, the envelope protein of Macaque type D/Washington virus appears immunologically distinct from MPMV because neither it, PO-1-Lu, nor squirrel monkey retrovirus (SMRV) competed in the MPMV gp70 assay. This virus was also tested and found to be negative in a broadly specific RIA for type C virus core antigens, as well as in an RIA for human T-cell leukemia virus core antigen. In addition, in radioimmunoassays of p10 and glycoprotein antigens

the Macaque type D/Washington isolate from RF tissue can be distinguished from two type D retroviruses isolated at the California and New England Regional Primate Research Centers from the blood of macaques with SAIDS but without RF. Cultures of human T-cell lymphocytes, as well as fresh lymphocytes isolated from macaque peripheral blood, can be infected with Macaque type D/Washington virus.

3. Viral transmission of RF and SAIDS. A Macaque type D retrovirus, (Science 224: 289-292, 1984) was isolated from an explant of RF in a rhesus monkey with SAIDS characterized by persistent diarrhea, weight loss, lymphocytopenia, opportunistic infections and RF. To examine viral pathogenicity, four juvenile colony-born and four feral young adult *Macaca nemestrina* were inoculated with 2 X 10^6 viable viral particles (assayed by end-point dilution of infectivity in cell culture) from a 500X viral concentrate of filtered culture medium of dog thymus cells infected with the Macaque type D/Washington retrovirus. At 5 weeks, one colony-born juvenile macaque died with suggestive RF and with generalized lymphoid depletion. At 10 weeks, retroperitoneal nodules were palpated in a colony-born macaque that at laparotomy at 18 weeks were histologically diagnosed as RF. After thirteen months, the macaque with RF and the one that died at 5 weeks are the only animals with viremia, as determined by isolation of Macaque type D/Washington virus from blood plasma, and are the only animals without sero-conversion following virus inoculation. Eight matched control *M. nemestrina*, inoculated with filtered culture medium from uninfected dog thymus cells, have remained healthy.

To further examine the interplay between immunization and/or pathogenicity of the Macaque type D/Washington retrovirus isolate, a second viral transmission experiment is underway to achieve a higher incidence of disease using three age groups of *M. nemestrina* inoculated IV with 10^7 particles of unfrozen retrovirus isolated from a *M. nemestrina* with SAIDS. To date, several macaques have developed nodular abdominal lymphadenopathy, suggestive of RF. However, no definite diagnostic, clinical or laboratory evidence of SAIDS has developed in these macaques, which have been continuously caged in a pathogen-free facility. This type of containment would eliminate the secondary opportunistic infections that mediate the lethality of SAIDS and illustrates the complexity of performing viral transmission studies of SAIDS in a controlled model system.

In addition, the role of a simian equivalent to HTLV-III as the causative agent of SAIDS, in counterdistinction to RF alone, is under investigation.

Significance to Biomedical Research and the Program of the Institute:

SAIDS represents a paradigm of human AIDS. The recent, ample documentation of retroviral association in human AIDS adds emphasis to the usefulness of this macaque model system, particularly for studies of viral transmission, vaccination and challenge that of course cannot be performed in humans. Secondly, understanding the molecular and cellular mechanisms of lymphoid pathogenesis among SAIDS-affected macaques represents an appropriate research objective, even apart from its correlation to the similar human disease. Thirdly, regarding the origin and natural history of retroviral-induced disease, the SAIDS-related Macaque type D viruses, along with MPMV, appear to represent a third class of infectious retroviruses (in addition to the gibbon-rodent and baboon-feline classes), which are pathogenic following interspecies transfer of endogenous retroviruses among mammalian species that cohabit in a geographic area.

Proposed Course:

The discovery of infectious retroviruses associated with SAIDS and RF appears to be a general finding and may have implications for the study of human AIDS. The availability of specific immunologic and molecular probes will allow a determination of the prevalence and pattern of transmission of this agent among primates. The immediate objective is to determine the nature of the viral pathogenicity of SAIDS. An additional question is to determine if a purely lymphotropic retrovirus can be isolated from the peripheral blood of SAIDS-affected macaques.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05367-01 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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	Geneticist	LVC	NCI
Others:	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	William S. Modi	Staff Fellow	LVC	NCI
	Yoh-Ichi Miyake	Visiting Fellow	LVC	NCI
	Lisa Forman	Guest Researcher	LVC	NCI
	Robert K. Wayne	Biologist	LVC	NCI
	Jo Gayle Howard	Biologist	LVC	NCI

COOPERATING UNITS (if any)

National Zoological Park, Washington, DC (M. Bush, D. E. Wildt, D. Kleiman)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	0.5	1.9

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Syntenic (linkage) maps of over 20 mammalian species and a comparative cytological analysis have indicated a noncontinuous tempo of chromosomal evolution in certain lineages (e.g., primates, felids) that are highly conserved in their chromosomal presentation, while others (rodents, lesser apes, canids) are chromosomally shuffled as if rapid altatory cytological rearrangements occurred during the speciation events. The abundant fibroblast proteins resolved by two-dimensional gel electrophoresis (2DE) in human materials have been studied to (1) identify specific proteins, enzymes and previously characterized gene products; (2) define allelic variation at polymorphic loci for use in pedigree analysis and assessment of genetic structure of outbred populations. A molecular phylogeny of the great and lesser apes and man was derived based on genetic distance of 383 different proteins resolved by 2DE. The South African subspecies of cheetah was shown to be genetically depauperate insofar as it is monomorphic at 52 isozyme loci. Unrelated cheetahs also accepted skin grafts, a situation without precedent among outbred mammalian species. A devestating epizootic of the species by feline infectious peritonitis (FIP and RNA containing corona virus) was hypothesized to result from abrogation of a major histocompatibility complex (MHC) haplotype in T-cell stimulation. A molecular phylogeny of the 37 species of the Felidae was constructed based on immunological distance using serum albumin. Similarly, a consensus phylogeny of the Ursidae Ailuropoda (giant panda) and Ailurus (lesser panda) was derived from distance matrices derived from three distinct molecular measures of genetic distance.

PROJECT DESCRIPTION

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

Stephen J. O'Brien	Geneticist	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
William S. Modi	Staff Fellow	LVC	NCI
Yoh-Ichi Miyake	Visiting Fellow	LVC	NCI
Lisa Forman	Guest Researcher	LVC	NCI
Robert K. Wayne	Biologist	LVC	NCI
Jo Gayle Howard	Biologist	LVC	NCI

Objectives:

1. Development of molecular procedures for assessing genetic status of natural populations and for use in studying heritability of disease susceptibility, both congenital and etiologic.

2. Use of molecular procedures to determine phylogenetic affinities and relationships between extant species of hominoid primates, felids and selected carnivores. The derived topologies have important implications for heterologous embryo transfer and more generally for the ultimate resolution of interacting gene systems that drive development and cancer.

3. The biological resolution of adaptive strategies employed by rarely studied mammalian populations for defense against neoplastic etiologic agents that affect human populations.

Methods Employed:

The following techniques were employed: (1) cell culture procedures, (2) isozyme electrophoresis, (3) two-dimensional gel electrophoresis, (4) microcomplement fixation using heterologous rabbit antisera, (5) high resolution cytogenetics procedures, (6) gene mapping procedures using somatic cell hybrids, (7) virological procedures, (8) surgical skin grafting, and (9) statistical analysis of phylogenetic algorithms.

Major Findings:

1. Resolution of the tempo and mode of genomic and chromosomal evolution in mammals. The cumulative analysis of the synteny gene maps of over 20 mammalian species (see Genetic Maps, 1984) and the comparative cytological appearance of over 100 species has led to the following consensus. Mammalian orders vary tremendously with respect to conservation of chromosome presentation. Certain mammalian families show extreme conservation of linkage and chromosomal integrity. For example, within the cat family, Felidae, 90% of the chromosomes are cytologically identical between each of the 37 member species. A second Carnivore family, the dogs (Canidae) is characterized by extremely variable chromosomal numbers and appearance. With certain dramatic exceptions (e.g., gibbon and owl monkey),

the primates have conserved chromosomal morphologies that have allowed the tracing of chromosome phylogenies as far back as the prosimians. The rodents, like the dogs, gibbons, bears and others, have a highly shuffled or rearranged karyotype within the order, within genera and even within the same species. After reviewing these observations, an inevitable conclusion is that mammalian chromosomal evolution is not continuous; rather, it is punctuated with massive complex rearrangements in certain phyla but with highly conserved arrangements in others. An attempted reconstruction of the cytological rearrangements that have taken place during evolution is in progress and will be compared to nonspecific chromosomal aberrations that are seen in human cancers.

2. Definition and characterization of 27 polymorphic loci in human populations recognized by two-dimensional gel electrophoresis (2DE) and application of 2DE as a metric of phylogenetic inference in hominoid primates. Twenty-seven independent polymorphic loci were detected by 2DE of serum, erythrocytes, and fibroblasts in two large families and analyzed for linkage to classical genetic markers. We detected 7 serum, 4 erythrocyte and 17 fibroblast protein loci that exhibited charge variation in these two families and in a sample of unrelated individuals. The genetic basis of protein variants was confirmed by quantitative gene dosage dependence and by conformance to Mendelian transmission in the two families, except for four rare variants for which transmission analysis was not possible. Linkage analysis demonstrated that each of the variants represent products of independent loci, with the exception of erythrocyte locus (RBC4), which we also detected in fibroblasts (NC27). Two allozyme polymorphisms, glyoxalase-1 (GLO1) and phosphoglucomutase-3 (PGM3), were specifically identified here based on genotypic concordance and molecular mass. Unknown fibroblast protein (NC22) may be linked to apolipoprotein E (Lod score = 2.8 at $\theta_m\theta_f$=0), while a serum protein locus (SER1) may be linked to alpha-haptoglobin (Lod score = 1.9 at θ_m=.1,θ_f=0). These studies indicate a minimum level of average protein charge heterozygosity of approximately 2.2% for the most predominant human cellular proteins and of 5.6% for the most predominant proteins of serum.

A molecular phylogeny for the hominoid primates was constructed using protein genetic distances from a survey of 383 radiolabeled fibroblast polypeptides resolved by two-dimensional electrophoresis (2DE). An internally consistent matrix of Nei-genetic distances was generated on the basis of variants in electrophoretic position. The derived phylogenetic tree indicated a branching sequence, from oldest to most recent, of cercopithecoids (*Macaca mullata*), gibbon-siamang, orangutan, gorilla and human-chimpanzee. Hominid molecular distance measures obtained by 2DE are fairly consistent with those generated with other molecular procedures except that the gorilla and orangutan lineages were found to have diverged from human-chimpanzee at nearly the same time [12 to 16 million years before the present (BP)].

3. Tracing the natural history of an endangered species: The African cheetah. A population genetic survey of over 200 structural loci previously revealed that the South African cheetah (*Acinonyx jubatus jubatus*) has an extreme paucity of genetic variability, probably as a consequence of several population bottlenecks in its recent past. The genetic monomorphism of the species was extended to the major histocompatibility complex (MHC), since 14 reciprocal skin grafts between unrelated cheetahs were accepted. The apparent consequences of such genetic uniformity to the species include (a) great difficulty in captive breeding, (b) a high

degree of juvenile mortality in captivity and in the wild, and (c) a high frequency of spermatozoal abnormalities in ejaculates. The species vulnerability of the cheetah was demonstrated by an epizootic of coronavirus-associated feline infectious peritonitis in an Oregon breeding colony in 1983. Exposure and spread of the coronavirus, which has a very low morbidity in domestic cats (approximately one percent), has decimated a heretofore productive and healthy captive population. The extreme genetic monomorphism, especially at the MHC, and the apparent hypersensitivity of the cheetah to a viral pathogen may be related, and may provide a biological basis for understanding the adaptive significance of abundant genetic variation in outbred mammalian species.

4. Captive and free-ranging golden lion tamarin populations show evidence of genetic depreciation. The golden lion tamarin *Leontopithecus rosalia rosalia*, one of the rarest and most endangered of New World primates, has been the focus of an intensive research and conservation effort for two decades. During that period, managed breeding from 44 founders has brought the captive population to over 400 individuals, a number that equals or exceeds the estimated number of free-ranging golden lion tamarins. The extent of genetic variation among captive and free-ranging golden lion tamarins was estimated by electrophoretic survey of 47 allozyme loci from 83 individuals. The amount of variation was low when compared to 15 other primate species with four percent of the loci being polymorphic (P), and with an average heterozygosity (H) estimate of 0.1. Analysis of captive animals of two allopatric morphotypes, *Leontopithecus rosalia chrysopygus* and *Leontopithecus rosalia chrysomelas* were similar to the *rosalia* findings insofar as they also retained limited genetic polymorphism. Computation of the Nei-genetic distance measurements revealed that the three morphotypes were genetically very similar. These data are consistent with the occurrence of reproductive isolations within recent times possibly contemporaneous with and caused by the predominance of human culture in South America.

5. A molecular phylogeny of the Felidae based on immunological distance. The phylogenetic distances between 34 of the 37 extant species of Felidae were estimated using albumin immunological distances (AID). Albumins from ten cat species were used to prepare antisera in rabbits. A consensus phylogeny was constructed from a matrix of reciprocal AID measurements using four distinct phylogenetic algorithms. A series of one-way measurements using the ten index antisera and those 24 species for which albumins were available (but antisera were not), permitted addition of these "species' limbs" to the previously derived phylogenetic trees. The major conclusions of the derived topology were that (a) the earliest branch of the feline radiation occurred approximately 12 million years BP and led to the small South American cats (ocelot, margay, Geoffroy's cat, etc.); (b) the second branching occurred 8 to 10 million years BP and included the close relatives of the domestic cat (wildcats, jungle cat, sand cat, and black-footed cat) plus Pallas's cat; and (c) the third lineage, the pantherine lineage, which began to radiate 4 to 6 million years BP and included several early branches (cheetah, serval, clouded leopard, golden cats, and puma) and a very recent (two million years BP) split between the lynxes and the modern great cats (*Panthera*). The topology of the Felidae derived from albumin immunological distance is highly consistent with the karyological disposition of these species, as well as with the fossil record of this family.

6. Molecular resolution of the riddle of the giant panda phylogeny. The taxonomic status of the giant panda and the lesser panda, have been a biological puzzle since their description by western naturalists a century ago. Although there has been some agreement that the lesser panda was a member of the raccoon family (Procyonidae), the giant panda has been classified with almost equal frequency in Ursidae (bear family), in Procyonidae, and as a single member of a separate family, Ailuropodidae. The results of four independent molecular genetic approaches, DNA hybridization, isozyme genetic distance, immunological distance and G-banded karyology, were performed and converged on a consensus phylogeny of the pandas based upon the principles of the "molecular clock" hypothesis. The lesser panda diverged from New World procyonids just subsequent to their departure from ursids, while ancestors of the giant panda's split from the ursid lineage just prior to the radiation that led to modern bears. The giant panda's divergence was accomplished by a chromosomal reorganization that can be partially reconstructed from the ursid karyotype, but not from procyonids or the lesser panda. The apparently dramatic, but actually limited distinctions between the giant panda and the bears in chromosomal and anatomical morphology provide a graphic mammalian example of the discordance of molecular versus morphological (and chromosomal) evolutionary change. Genetic markers (isozymes and 2DE) developed for this study were also employed to establish paternity of the baby giant panda born in Washington, DC, in August 1983.

Signifance to Biomedical Research and the Program of the Institute:

Two primary missions of the National Cancer Institute (NCI) are (1) the development of clinical protocols for resisting and treating neoplastic disease and (2) the biological dissection of the molecular-genetic events involved in neoplastic transformation. From the perspective of mission number one, the vigorous pursuit of resolving strategies for developing resistance against cancers or etiologic agents (viruses, carcinogens) employed by free-ranging species is a largely untapped reservoir of biological specialization and information. Ample precedent for this approach is available in the pharmaceutical-treatment industries where scores of chemical treatments (e.g., penicillin, cyclosporin, methotrexate) of unknown mechanisms are derived from free-ranging biological species. The second NCI mission (of resolving the process of transformation) involves the understanding of the genetic programming and/or regulation of the 10^5 genes during development and during carcinogenesis. The structural genes and their regulatory systems are themselves products of a long history of biological evolution. For example, the parallels in evolution of the immune system, the MHC, retrovirus families, and cancer that we see in modern mammals are striking in their antithetical characteristics. It seems as if they have emerged and evolved primarily to counteract each other. In brief, "Nothing in biology makes sense except in light of evolution" (Th. Dobzhansky, 1967), and understanding cancer is probably the best illustration of this statement. For this reason, the resolution of genomic organization among man's relatives during the mammalian radiations holds promise for the ultimate dissection of genetic misprogramming in cancer.

Proposed Course:

We expect to analyze human populations and related primates to determine the extent of genetic variation and association with heritable components of neoplasia. The phylogenetic relationships of the carnivores will be extended to include other

molecular metrics including oncogenes, MHC organization, and endogenous retroviral families.

Publications:

Collier, G. E. and O'Brien, S. J.: A molecular phylogeny of the Felidae: Immunological distance. Evolution (In Press)

Goldman, D., Goldin, L. G., Rathnagiri, P., O'Brien, S. J., Egeland, J. A. and Merril, C. R.: Twenty-seven protein polymorphisms by two-dimensional electrophoresis of serum erythrocytes and fibroblasts in two pedigrees. Am. J. Hum. Genet. (In Press)

Newman, A., Bush, M., Wildt, D. E., van Dam, D., Frankehuis, M., Simmons, L., Phillips, L. and O'Brien, S. J.: Biochemical genetic variation in eight endangered feline species. J. Mammal. (In Press)

O'Brien, S. J.: Genetic and reproductive approaches to an endangered species, the giant panda. In Hubbell, G. (Ed.): Proceedings of the Association of Zoological Parks and Aquariums. Miami, American Association of Zoological Parks and Aquariums, 1984, pp. 169-180.

O'Brien, S. J., Benveniste, R. E., Nash, W. G., Simonson, J. M., Eichelberger, M. A., Wildt, D. E. and Bush, M.: Constructing a molecular phylogeny of the giant panda, Ailuropoda melanoleuca. Bongo (In Press)

O'Brien, S. J., Goldman, D., Knight, J., Moore, H. D., Wildt, D. E., Bush, M., Montali, R. J. and Kleiman, D.: Giant panda paternity. Science 223: 1127-1129, 1984.

O'Brien, S. J., Nash, W. G., Bauer, R., Chang, E. H. and Seigel, L. J.: Trends in chromosomal and oncogene evolution in vertebrates. In Patterson, M. K. (Ed.): Uses and Standardization of Vertebrate Cell Cultures. In Vitro Monograph No. 5. Gaithersburg, Tissue Culture Association, 1984, pp. 204-214.

O'Brien, S. J., Nash, W. G., Wildt, D. E., Bush, M. E. and Benveniste, R. E.: Riddle of the giant panda phylogeny: A molecular solution. Nature (In Press)

O'Brien, S. J., Reeves, R. H., Simonson, J. M., Eichelberger, M. A. and Nash, W. G.: Parallels of genomic organization and endogenous retrovirus organization in cat and man. Dev. Genet. 4: 341-354, 1984.

O'Brien, S. J., Roelke, M. E., Marker, L., Newman, A., Winkler, C. W., Meltzer, D., Colly, L., Everman, J., Bush, M. and Wildt, D. E.: Genetic basis for species vulnerability in the cheetah. Science 227: 1428-1434, 1985.

O'Brien, S. J., Seuanez, H. N. and Womack, J. E.: On the evolution of genome organization in mammals. In MacIntyre, R. J. (Ed.): Evolutionary Biology. New York, Plenum Press (In Press)

O'Connell, C., O'Brien, S. J., Nash, W. G. and Cohen, M.: ERV3, a full length human endogenous provirus: Chromosomal localization and evolutionary relationship. J. Virol. 138: 225-234, 1984.

Wayne, R. K., Forman, L., Newman, A. K., Martenson, J. S. and O'Brien, S. J.: Genetic monitors of captive zoological populations: Morphological and electrophoretic assays. Zoo Biol. (In Press)

Wayne, R. K. and O'Brien, S. J.: The extent and character of morphologic variation in the cheetah (*Acinonyx jubatus*), a genetically uniform species. Evolution (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05382-02 LVC

PERIOD COVERED
October 1, 1984 to September 30, 1985

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	Research Chemist	LVC	NCI
Others:	Michael I. Lerman	Visiting Scientist	LEP	NCI
	Ayako Sakai	Visiting Fellow	LVC	NCI
	Tomiko Shimada	Guest Researcher	LVC	NCI
	Glenn A. Hegamyer	Health Science Officer	LVC	NCI
	Koichi Hirano	Guest Researcher	LVC	NCI
	Thomas D. Gindhart	Expert	LEP	NCI

COOPERATING UNITS (if any) Hunan Med. College, Changsha, Hunan, China(K.-T. Yao); Eppley Inst., Univ. of Nebraska, Omaha, NE(J. Pelling); Cancer Res. Lab., Univ. of W. Ontario, Canada(D. Denhardt); Dept. of Pathol., Harvard Med. Sch., Boston, MA(T. Benjamin); Dept. of Microbiol., Univ. of California, Los Angeles, CA(M. Karin)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.9	2.5	3.4

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

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

The aim of this research is to identify and characterize genes involved in the progression of cells from the normal to the neoplastic phenotype in mice and humans. Evidence suggesting the involvement of such genes in animal and human systems has come from the observations that there are irreversible steps in tumor promotion and that animals can be bred for sensitivity to tumor promotion. Genes that specify sensitivity to promotion of neoplastic transformation by tumor promoters in mouse epidermal JB6 cells have been cloned. Two such genes, termed pro-1 and pro-2, have been identified. These differ from each other and from known oncogenes. Pro-1 has been sequenced. The sequence is intronless, shows the consensus sequences needed for transcription and translation and is expected to code for a 7000 dalton protein. Tumor promoter treatment of promotion sensitive (P+) cells produces transient increases in pro-1 RNA levels. Chinese human nasopharyngeal carcinoma cell (CNE) DNA shows both P+ activity (by transfection) and homologs of pro-1 and pro-2 (by Southern blotting). Pro-1 homologs have been cloned from a CNE library and initial assays indicate that they are biologically active for P+ activity. In addition, a novel transforming activity detectable in P+ recipient cells has been found in the DNA of 12-O-tetradecanoylphorbol-13-acetate (TPA) transformed JB6 cells. In the future, these studies will be concerned with understanding pro gene expression and pro gene products and their significance in human and rodent cells. The regulation of pro gene transcription by tumor promoters and antipromoters will be studied. The structure and biological activity of human pro gene homologs will be characterized. Pro-1 proteins will be purified, characterized and localized. A novel transforming gene will be cloned and its mode of regulation by pro genes will be studied.

PROJECT DESCRIPTION

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

Nancy H. Colburn	Research Chemist	LVC	NCI
Michael I. Lerman	Visiting Scientist	LEP	NCI
Ayako Sakai	Visiting Fellow	LVC	NCI
Tomiko Shimada	Guest Researcher	LVC	NCI
Glenn A. Hegamyer	Health Science Officer	LVC	NCI
Koichi Hirano	Guest Researcher	LVC	NCI
Thomas D. Gindhart	Expert	LEP	NCI

Objectives:

To elucidate the nature of genetically determined events that are causally related to preneoplastic progression in mice and humans. To clone the genes involved in tumor promoter-induced progression to the tumor cell phenotype in JB6 mouse epidermal cells. To characterize the structure, function and mode of regulation of these genes. To clone and characterize human homologs of these promotion sensitivity genes. To characterize genes that specify "cancer proneness" in certain genetically cancer-prone conditions. To clone and characterize a novel transforming gene and its regulation by pro genes.

Methods Employed:

The following techniques are being utilized: (1) gene cloning techniques using sib selection search routines; (2) calcium-phosphate DNA transfection followed by assay sensitivity gain to promotion of anchorage-independence by the tumor promoter, TPA; (3) restriction mapping; (4) sequencing the cloned genes by the Maxam and Gilbert technique; (5) computer-aided analysis of their structure and possible function; (6) Southern and Northern transfer techniques to analyze genome organization and expression of the pro genes; (7) purification of nuclear and messenger RNA; (8) preparation of antibodies to synthetic peptides to pro proteins or to protein synthesized by a bacterial expression vector; and (9) use of such antibodies to purify, localize and identify pro gene products.

Major Findings:

1. Two mouse pro genes (pro-1 and pro-2) specifying sensitivity to induction of neoplastic transformation by TPA in JB6 cells were cloned by sib selection from a size-selected genomic library of clonal cells sensitive to promotion of neoplastic transformation (P^+). By restriction mapping, heteroduplex analysis, direct hybridization and sequence comparisons, we have determined that the two pro sequences are different.

2. Both pro-1 and pro-2 (plasmid clones p26 and p40, respectively) are different from any known oncogenes. Southern blot hybridization under stringent conditions using the active segments of p26 or p40 as ^{32}P-labeled probes showed that pro-1 and pro-2 are not related to abl, fes, fms, mos, myc, Ha-ras, Ki-ras, src, sis, erbA or erbB. This raises the possibility that pro genes, which apparently specify sensitivity to promotion of neoplastic transformation by tumor promoters,

may be very different in structure and function from most of the known oncogenes. Comparison of the complete sequence of pro-1 and 70% of the sequence of pro-2 reveals no homology to any oncogene or any other gene in available data banks.

3. Pro-1 has been sequenced. The structural features of the pro-1 gene are highly unusual in many respects. The gene is relatively small and resides in a highly repetitive (and possibly unstable) genomic segment containing almost all known mouse middle repeated sequences. The pro-1 sequence itself appears as a fusion sequence assembled from two different types of middle repetitive elements, the BAM5 and Alu-type B1 repeats, joined by an apparently unique sequence of 64 bp. The pro-1 sequence has all the landmark consensus sequences involved in accurate and abundant transcription employed by eukaryotic RNA polymerase II genes. The promoter elements ("TATA" and "CAAT" boxes, a typical enhancer cor sequence, and a strong ribosomal binding site) are in an ordered spatial arrangement typical of polymerase II, along with a typical cleavage site downstream to the translation terminator codon. The open reading frame is not contiguous with any introns and predicts a product of 65 amino acid (MW=7,100 daltons) with a highly unusual composition.

4. Tumor promoter treatment of P^+ cells increased the levels of cytoplasmic RNA (cRNA) hybridizing to a pro-1 probe. Basal levels of pro-1 cRNA were about 20 copies per cell, while TPA-induced levels reached 100 to 200 copies per cell. Maximal stimulation was found at 0.5-4 hours of TPA exposure.

5. The pro-2 genomic segment is 3.8 Kbp in size and is a unique sequence except for a small middle repeated element present in the middle of the active gene. The repeated element of 0.6 Kbp is unrelated to known mouse repeats and apparently represents a new class of mouse repeated sequences. Sequencing of the entire 3.8 kb active genomic fragment representing the pro-2 gene is almost complete.

6. Human and baboon DNAs have homologs to both pro genes, indicating conservation during human evolution. Screening of a human sperm library yielded one phage with homology to the entire pro-1 sequence. A human nasopharyngeal carcinoma (CNE_2) cell line genomic library yielded 25 to 30 pro-1 positive phages, reflecting a possible amplification of the pro-1 gene in this tumor line.

7. Genomic DNAs of human Chinese nasopharyngeal carcinoma cell lines CNE_1 and CNE_2 show P+ (promotion sensitivity) biological activity. Transfection of CNE_1 and CNE_2 DNAs into mouse JB6 P- (promotion-resistant) cell lines transferred the sensitive (P+) phenotype, as measured by TPA-induced anchorage-independent colony formation. DNA concentration-response assays revealed the same specific activity (P+ activity/μg DNA) as for mouse P+ genomic DNA.

8. Pro-1 and pro-2 homologs in CNE_2 cells have been cloned. A genomic phage library of DNA from CNE_2 cells was constructed by the replacement of internal segments in charon 4a (EcoRI sites). Six and two clones carrying pro-1 and pro-2 homologs, respectively, were isolated by screening the library using radioactively labeled probes originating from mouse pro-1 and pro-2 genes.

9. Pro-1 homolog(s) in CNE_2 cells appear to be active as donors of P^+ sensitivity to P- cells. DNAs of the phages cloned for pro-1 from the genomic

library of CNE$_2$ showed P^+ activity in the initial transfection assay, suggesting that the P^+ activity of CNE$_2$ total DNA is due at least in part to pro-1 homolog(s). This observation implies that a pro-1 homolog may play a role in the promotion of neoplastic progression of Chinese nasopharyngeal epithelial carcinomas.

10. Normal rodent and human cell DNAs have inactive homologs of pro-1 and pro-2. Although Southern analysis (under stringent conditions) showed the presence of pro-1 and pro-2 homologs in the DNA of mouse liver (several strains) and mouse secondary keratinocytes, as well as human placenta and human lymphocytes, none of these DNAs showed biological activity on transfection into JB6 P^- cell recipients. This suggests that (1) normal pro genes may serve a necessary function in normal cells and (2) activation of pro genes to the P^+ form may involve small structural changes that have little effect on the degree of homology.

11. The phenotypes of promotion-sensitive (P^+) cell lines obtained by transfection of pro-1 or pro-2 show both similarities and differences from those of parental P^+ cell lines. Initial results indicate that, like the parental P^+ cells, the pro-1 and pro-2 P^+ transfectants are sensitive to promotion of transformation by TPA at similar concentrations, but unlike the parental cells, they are relatively insensitive to promotion by epidermal growth factor. These P^+ transfectants are, like the parental P^+ cells, sensitive to the antipromoters -- retinoic acid, ganglioside G_T, superoxide dismutase and EGTA -- but relatively insensitive to the antipromoting glucocorticoid, fluocinolone acetonide. This raises the possibility that the cross-sensitivity to various classes of inducers and inhibitors shown by the original P^+ cells may be specified by a small set of related genes with differing but overlapping specificities.

12. A novel transforming activity has been identified in the DNA of tumor cells derived from JB6 cells by TPA exposure. DNA from TPA-induced, JB6-derived tumor cells, when transferred to JB6 P+ cells, produces anchorage-independent transformation in the absence of TPA. Biologically active DNA has been identified from two independently derived TPA transformants. Transforming activity is not detected after transfection into either JB6 promotion-insensitive (P^-) cells or NIH 3T3 cells. This observation provides the assay needed for cloning a new transforming gene and also suggests that JB6 P+ cells may be useful recipients for detection of some classes of transforming genes that are not detected with the NIH 3T3 assay. This transforming sequence appears to be different from the pro gene sequences. Pro gene activity (assayed in JB6 P^- cells) and transforming gene activity (assayed in JB6 P+ cells) differ from each other on the basis of sensitivity to at least two restriction enzymes, namely BglII and BamHI. This observation, together with the observation that the transforming activity cannot be detected in P^- recipient cells, suggests that this transforming activity is specified by a gene other than the pro genes.

13. Fibroblasts of basal cell nevus syndrome (BCNS) patients, but not agematched normal fibroblasts, can be induced to escape senescence by transfection of pro-1 or v-myc DNA. After transfection of p26 (pro-1) or v-myc plasmid DNAs, BCNS fibroblasts, but not age, race and sex-matched normal fibroblasts, have undergone at least 20 more population doublings than untransfected controls that experienced senescence. This suggests that pro-1 or v-myc genes, in cooperation

with BCNS gene(s), may be able to function in extending longevity of human fibroblasts in culture, which may lead to the establishment of a cell strain, an event postulated to be a component of neoplastic transformation.

Significance to Biomedical Research and the Program of the Institute:

Identification of genes involved in the progression from the normal to the neoplastic phenotype has major implications for cancer prevention. It is entirely reasonable to expect that the functions of such "preneoplastic progression genes," whose activity may be rate-limiting for development of cancer in humans, can be controlled by appropriate diet and exercise, with resultant lengthening of latent periods for cancer development.

Proposed Course:

Pro gene transcription studies will be followed up with the aim of answering the following questions: (1) What are the times and conditions of maximum inducibility of pro-1 and pro-2 RNA by TPA? (2) Do non-phorbol tumor promoters transcriptionally activate pro genes? (3) Do antipromoters block promoter stimulation of pro RNA levels? If so, by what mechanisms? (4) What are the sizes of pro gene transcripts? Do they coincide with those predicted by the coding sequences?

The pro-2 sequence will be subjected to computer-aided analysis of promoter elements, mRNA processing signals and open reading frames as well as comparison with known genes in the data bank. Expression of pro-2 protein will be initiated with bacterial expression vectors as will preparation of antibodies to be used for purification and localization studies.

Cloned human nasopharyngeal carcinoma cell (CNE_2) homologs of pro-1 and pro-2 will be characterized by restriction mapping. Genomic structure and copy number will be compared with normal human pro homologs and with biologically active JB6 mouse pro genes.

Studies to determine P^+ biological activity of CNE_2 pro-1 and pro-2 homologs will be continued. The minimum sequences necessary for biological activity will be determined and these will be subcloned.

Subcloned CNE_2 pro-1 and pro-2 genes will be used as probes for analyzing a variety of human tumor and normal cell DNAs for pro homologs. These human pro probes will also be used to localize pro genes on human chromosomes.

Studies to determine structural differences between normal and activated (P^+) pro gene homologs will be initiated. A series of recombinant plasmids will be constructed from normal and CNE_2 pro-1 homologs and assayed for biological activity in the transfection assay. The sequences critical to biological activity will be subjected to sequence analysis and compared with both normal human and biologically active mouse sequences.

Pro-2 gene structure and pro-2 expression will be studied during mouse skin initiation promotion carcinogenesis in vivo, in collaboration with Dr. Jill Pelling of the Eppley Institute. Cloning of such promotion sensitivity genes from mouse

skin would be difficult if not impossible. An advantage of the JB6-sensitive and -resistant clonal cell lines is that they permit cloning of such genes whose expression in vivo can then be studied. We hope to learn whether pro-2 expression is temporal during stage-wise promotion and whether it may be ruled in or ruled out as causally related to induction or expression of tumors.

This promotion sensitivity gene project will converge with the signal transduction project (Z01CP05383-02 LVC) at the stage of characterizing the protein products of pro-1 and pro-2.

Cloning of the novel transforming gene(s) implicated in TPA-transformed JB6 derivative lines will be initiated. A sib selection approach will be used with a biological assay of DNA transfection followed by (TPA-independent) colony formation in agar. The transforming gene(s) will be characterized with the overall aim of understanding how its expression may be regulated by pro genes and their products.

Studies with the human BCNS cancer-prone human cells will continue in an effort to determine the nature and extent of pro gene integration in the transfectants that have shown extended life span. Pro gene transcription and its induction by tumor promoters in transfectants will be studied. Biochemical events in gene regulation postulated to influence cancer proneness will be studied in BCNS cells in collaboration with Dr. Ray Gantt of NCI.

Publications:

Colburn, N. H., Lerman, M. I., Hegamyer, G. A. and Gindhart, T. D.: A transforming activity not detectable by DNA transfection to NIH 3T3 cells is detected by JB6 mouse epidermal cells. Mol. Cell. Biol. 5: 890-893, 1985.

Colburn, N. H., Lerman, M. I., Hegamyer, G. A., Wendel, E. and Gindhart, T. D.: Genetic determinants of tumor promotion: Studies with promoter resistant variants of JB6 cells. In Bishop, M., Graves, M. and Rowley, T. (Eds.): Genes and Cancer. New York, Alan R. Liss, 1985, Vol. 17, pp. 137-155.

Colburn, N. H., Lerman, M. I., Srinivas, L., Nakamura, Y. and Gindhart, T. D.: Membrane and genetic events in tumor promotion: Studies with promoter resistant variants of JB6 cells. In Fujiki, H. and Sugimura, T. (Eds): Cellular Interactions by Environmental Tumor Promoters. Tokyo, Scientific Societies Press, 1984, pp. 155-166.

Colburn, N. H., Srinivas, L., Hegamyer, G. A., Dion, L. D. and Wendel, E. J.: Role of specific membrane and genetic changes in the mechanism of tumour promotion. Studies with promoter-resistant variants. In Borsonyi, M. (Ed.): Models, Mechanisms and Etiology of Tumour Promotion. Lyon, France, IARC Publications, 1985, pp. 205-215.

Lerman, M. I. and Colburn, N. H.: Pro genes, a novel class of genes that specify sensitivity to induction of neoplastic transformation by tumor promoters. In Cooper, G. M. (Ed.): Viral and Cellular Oncogenes. Boston, Martinus Nijhoff Publishing (In Press)

Lerman, M. I., Hegamyer, G. A. and Colburn, N. H.: Cloning and characterization of putative genes that specify sensitivity to induction of neoplastic transformation by tumor promoters. Proc. Natl. Acad. Sci. USA (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05383-02 LVC

PERIOD COVERED
October 1, 1984 to September 30, 1985

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	Research Chemist	LVC	NCI
Others:	Thomas D. Gindhart	Expert	LEP	NCI
	Bonita Smith	Guest Researcher	LVC	NCI
	Koichi Hirano	Guest Researcher	LVC	NCI
	Donald Court	Biologist	LMO	NCI
	Howard Young	Expert	LMI	NCI
	William Farrar	Senior Staff Fellow	LMI	NCI

COOPERATING UNITS (if any) Univ. Texas Med. Sch., Galveston, TX(R. Fleischman, M. Brysk); Shizuoka College Pharmaceutical Sci., Japan(Y. Nakamura); Dept. Pathobiochemical Cell Res., Institute of Med. Sci., Univ. of Tokyo, Tokyo, Japan(T. Kuroki); PRI, Frederick, MD(M. Zweig); Swiss Inst. for Exper. Cancer Res., Lausanne(P. Cerutti)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.6	2.1	1.5

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 goal of these studies is to determine the required biochemical events that occur between tumor promoter-receptor interaction and the activation of effectors of neoplastic transformation. Candidate second messengers include protein phosphorylation, reactive oxygen generation, and calcium mobilization. Evidence from inhibitor studies indicates that the free radical superoxide anion is an essential early mediator of neoplastic transformation by tumor-promoting phorbol esters in JB6 mouse epidermal cells. Superoxide dismutase activity is substantially decreased by 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment of promotion sensitive (P+) but not promotion resistant (P-) cells, suggesting a causal relationship between the enzyme decrease and elevation of superoxide anion, as well as a causal relationship of these events to promotion of neoplastic transformation. Extracellular calcium acts as a required signal transducer for promotion of transformation in JB6 cells. The extracellular calcium required for TPA-promoted transformation appears to enter cells via plasma membrane channels. Cation binding to cation binding proteins may be essential signal transduction events in promotion of transformation. The trivalent cation lanthanum substitutes for calcium in activating protein kinase C, but unlike calcium, it (1) promotes transformation of JB6 cells in the absence of TPA and (2) produces conformational changes in certain protein kinase C substrates. In the future this project will extend the reactive oxygen, calcium and protein kinase signal transduction studies and converge with the promotion sensitivity (pro) gene project (Z01CP05382-02 LVC) in order to study gene expression. Identification of lanthanum-binding promotion-relevant proteins will be pursued. Tumor promoter inducible proteins and phosphoproteins will be studied in order to identify the proteins that recognize signal sequences in pro genes.

PROJECT DESCRIPTION

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

Nancy H. Colburn	Research Chemist	LVC	NCI
Thomas D. Gindhart	Expert	LEP	NCI
Bonita Smith	Guest Researcher	LVC	NCI
Koichi Hirano	Guest Researcher	LVC	NCI
Donald Court	Biologist	LMO	NCI
Howard Young	Expert	LMI	NCI
William Farrar	Senior Staff Fellow	LMI	NCI

Objectives:

To determine the required biochemical events that occur between the interaction of tumor promoters with the plasma membrane and the activation of intracellular effectors of neoplastic transformation. In the case of phorbol diester tumor promoters, a major aim is to identify promotion-relevant events that are closely coupled to phorbol ester receptor binding. Candidates for such second messengers or signal transduction events include protein kinase C-catalyzed protein phosphorylation, reactive oxygen generation and calcium mobilization. Nucleotide sequences in *pro* genes that constitute recognition signals for 12-O-tetradecanoylphorbol-13-acetate (TPA) and other tumor promoters will be sought. An overall aim is to understand the regulatory interactions between such signal sequences and signal transducers such as C Kinase and active oxygen.

Methods Employed:

The following techniques are being used:

(1) Assay of the effects of various modulators of reactive oxygen on promotion of neoplastic transformation (anchorage independence) by TPA; (2) assay of the effects of these modulators on the expression of anchorage independence by tumor cells; (3) assay of the effects of various modulators of calcium uptake and mobilization on the promotion (by TPA) and the expression (without TPA) of the tumor cell phenotype, as measured by anchorage-independent growth in 0.33% agar; (4) assay of calcium-dependent phospholipid-dependent protein kinase (C kinase) activity (see Project Number Z01CP15273-04 LEP); (5) assay of the effects of TPA on rate of synthesis and phosphorylation of proteins in intact promotion-sensitive (P^+) or -resistant (P^-) cells; and (6) immunoprecipitation and Western blotting with antisera to *pro*-1 peptides.

Major Findings:

1. The free radical superoxide anion appears to be an essential mediator of promotion of neoplastic transformation in JB6 mouse epidermal cells. Superoxide anion activity is critical during the first 2 hours after the interaction of phorbol ester with its receptor (C-kinase). Bovine superoxide dismutase (SOD) or the SOD mimetic copper(II) (3,5-diisopropylsalicylic acid)$_2$ (CuDIPS), which removes superoxide anion, inhibits the induction of transformation (anchorage independence) by TPA in JB6 cells. Delay of the addition of SOD by 1 hour

results in no loss of promotion-inhibitory activity, but delay by 2 hours or more after the exposure of JB6 cells to TPA results in partial to complete loss of promotion-inhibitory activity. This suggests that elevation of superoxide anion is tightly coupled to the phorbol ester receptor binding event on a pathway to transformation.

2. SOD activity is substantially decreased by TPA treatment of promotion sensitive (P+) but not promotion resistant (P-) cells. Ongoing collaborative studies with former UICC fellow, Dr. Y. Nakamura, in Shizuoka, Japan, have yielded this new finding. This decrease in SOD would be expected to be accompanied by elevation of superoxide anion; that such an event should occur in P+ but not P- cells suggests a causal connection to promotion of transformation and reinforces the independent indication of causal relationship that emerged from the above studies (#1) on exogenously added SOD or SOD mimetic CuDIPS antipromoters.

3. Extracellular calcium is required for TPA-induced promotion of transformation in JB6 cells. Lowering the extracellular calcium concentration by either chelation with the divalent chelating agent, EGTA, or use of commercial calcium-depleted medium inhibited TPA-promoted transformation but not the expression of anchorage independence by tumor cells. The inhibition was reversible by the addition of calcium. This suggests that promotion involves a calcium-dependent event such as the activation of a calcium-dependent enzyme.

4. The extracellular calcium required for TPA-promoted transformation appears to enter cells via plasma membrane channels. The calcium channel blockers lanthanum (which decreases calcium entry at the channel mouth) and nifedipine (which binds very specifically to proteins comprising the channel) inhibit TPA-promoted transformation at concentrations consistent with their channel blocking activity. Thus, transformation induction in JB6 cells requires movement of extracellular calcium into the cell via membrane calcium channels.

5. A generalized elevation in cytosolic free calcium levels is, however, not sufficient for the promotion response. The calcium ionophore, A23187, which transports extracellular calcium across cell membranes, did not promote JB6 P^+ cells to a transformed phenotype, nor did it augment TPA-promoted transformation. These findings suggest that the extracellular calcium required for promotion of transformation maintains a compartmentalized calcium pool essential for a calcium-dependent event in promotion; the pool is not specifically affected by elevations in free calcium as produced by ionophores. Thus, A23187 is not able to promote cells or augment TPA promotion, perhaps because numerous cellular calcium-dependent processes are activated in the presence of high free cytosolic calcium.

6. Alterations in cation binding to calcium binding proteins or other cellular constituents may be essential in promotion of transformation in JB6 cells. The pharmacological analogs of calcium, the rare earth elements lanthanum and terbium, promote both JB6 P^+ and JB6 P^- cells (promotion of P^- cells results in fivefold fewer colonies) at high but non-toxic concentrations. Furthermore, the lanthanides alter/affect proteins that are on the pathway in transduction of the phorbol ester signal for promotion of transformation: (1) lanthanum substitutes for calcium in the activation of calcium-dependent, phospholipid-dependent protein kinase C; and (2) several endogenous substrates phosphorylated by protein

kinase C are altered in mobility or resolution in SDS polyacrylamide gels in the presence of lanthanum. In consideration of the above findings in light of the one-to-four order of magnitude greater affinity of the lanthanides for cellular calcium binding sites such as calcium binding proteins, suggests the following conclusions: (1) The lanthanides substitute for calcium or compete with calcium at calcium-binding sites. The alterations in cation binding are necessary and possibly sufficient to induce the transformed phenotype. (2) The lanthanides supply a missing component for promotion sensitivity in JB6 P^- cells, possibly by altering cation binding to a calcium-binding protein essential in promotion of transformation.

7. Eighteen endogenous substrates have been found for the calcium-dependent, phospholipid-dependent protein kinase C in JB6 cells. (See Project Number Z01CP15273-04 LEP). One or more of these substrates is expected to be promotion relevant and might be related to products of genes that specify promotion-sensitivity. (See Project Number Z01CP05382-02 LVC).

Significance to Biomedical Research and the Program of the Institute:

Identification of the events involved in signal transduction during tumor promotion and preneoplastic progression has major implications for cancer prevention. Drugs and dietary supplements that modulate reactive oxygen or calcium are already available. The control of these signals that regulate the expression of "preneoplastic progression" genes may very likely result in increased latent periods for cancer.

Proposed Course:

The initial findings suggesting that lanthanum-sensitive phosphoproteins are promotion relevant will be followed up. The two phosphoprotein bands that change mobility or resolution in the presence of lanthanum will be studied with the aim of identifying the proteins and their role. The question of whether conformational or quantitative changes in particular calcium-sensitive proteins determine promotion sensitivity will be investigated. Tumor promoter-inducible proteins, including phosphoproteins, will be studied by following rates of synthesis and rates of phosphorylation after TPA or lanthanide treatment of intact P^+ and P^- cells. Answers to the following questions will be sought: (1) In which cellular fractions are they found? (2) Is the rate of synthesis or phosphorylation of such promoter-inducible proteins altered in the presence of various anti-promoting agents, such as glucocorticoids, or under conditions that inhibit promotion (calcium-free medium)? Suspected promotion-relevant proteins will be purified and characterized with the aid of monoclonal antibodies.

Lanthanum will be employed as a tool for determining relative stages of preneoplastic development of chemically and spontaneously initiated mouse epidermal cells. Answers will be sought to the following questions: (1) Do the lanthanides promote JB6 prepromotable cells or calcium-resistant cells putative initiated cells of H. Hennings and S. Yuspa (Normal and Abnormal Epidermal Keratinization, University of Tokyo Press, In Press)? (2) Can JB6 prepromotable cells or calcium-resistant cells be made promotion-sensitive by transfection of plasmids containing the active promotion sensitivity genes pro-1 or pro-2? (3) If so, is the transfected sensitivity to TPA or lanthides or both? (4) Are lanthanide-transformed colonies of an irreversible neoplastic phenotype?

This signal transduction project (Z01CP05383-02 LVC) and the promotion sensitivity genes project (Z01CP05382-02 LVC) are converging at the level of regulation of gene expression. It is clear that the extrachromosomal signals, such as active oxygen, calcium and protein kinases, must interact with signal sequences in the untranslated regions of pro genes. Identification of the proteins that link these signal sequences to the protein products of pro genes will be pursued, as will approaches to identifying the protein products of pro-1 and pro-2. A two-pronged approach to obtaining antibodies needed to isolate and characterize pro-1 proteins synthesized in mouse cells will be employed. The first approach will be to utilize antibodies to synthetic pro-1 peptides prepared by Dr. Martin Zweig of Program Resources, Inc. The second approach will involve insertion of pro-1 into suitable bacterial expression vectors. Any pro-1 specific antibodies obtained by these approaches will be utilized for intracellular localization studies as well as for protein purification.

Publications:

Colburn, N. H., Lerman, M. I., Hegamyer, G. A., Wendel, E. J. and Gindhart, T. D.: Genetic determinants of tumor promotion: Studies with promoter resistant variants of JB6 cells. In Bishop, M., Graves, M. and Rowley, T. (Eds.): Genes and Cancer. New York, Alan R. Liss, Inc., Vol. 17, 1984, pp. 137-155.

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Colburn, N. H., Srinivas, L., Hegamyer, G. A., Dion, L. D. and Wendel, E. J.: Role of specific membrane and genetic changes in the mechanism of tumour promotion. Studies with promoter-resistant variants. In Borsonyi, M. (Ed.): The Role of Cocarcinogens and Promoters in Human and Experimental Carcinogenesis. Lyon, France, IARC Publication, 1985, pp. 205-215.

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Gindhart, T. D., Srinivas, L. and Colburn, N. H.: Benzoyl peroxide promotion of transformation of JB6 mouse epidermal cells: Inhibition by ganglioside G_T but not retinoic acid. Carcinogenesis 6: 309-311, 1985.

Nakamura, Y., Colburn, N. H. and Gindhart, T. D.: Role of reactive oxygen in tumor promotion: Implication of superoxide anion in promotion of neoplastic transformation. Carcinogenesis 6: 229-235, 1985.

Smith, B. M., Gindhart, T. D. and Colburn, N. H.: Extracellular calcium requirement for promotion of transformation in JB6 cells. Cancer Res. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05384-02 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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	Geneticist	LVC	NCI
Others:	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	Takis S. Papas	Acting Chief	LMO	NCI
	Ulf R. Rapp	Research Chemist	LVC	NCI
	Leonard J. Seigel	Clinical Associate	LTCB	NCI
	William S. Modi	Staff Fellow	LVC	NCI
	Yoh-Ichi Miyake	Visiting Fellow	LVC	NCI

COOPERATING UNITS (if any) LBI,Frederick,MD(M.Cohen, M.Barbacid, E.Brownell); USUHS, Bethesda,MD(E.H.Chang); CHB,NHLBI,NIH,Bethesda,MD(N.P Anagnou, A.W.Nienhuis); LMM,NIAID,NIH,Bethesda,MD(M.A.Martin); John Hopkins Hosp.,Baltimore,MD(K.Smith); H&W Cytogenetics Serv.,Sterling,VA(W.G.Nash); St.Jude's Hosp.,Memphis,TN(C.Sherr)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.95	1.85	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 combined application of principles and techniques of molecular biology and cell genetics has resulted in the identification and characterization of over 1000 human loci, a value that approaches the gene maps of Drosophila. We have concentrated our efforts on somatic cell hybrid panels and in situ hybridizations to genes related to neoplastic processes including (1) cellular proto-oncogenes, (2) growth factors, (3) growth factor receptors, (4) endogenous retroviral families, (5) integration sites for retroviruses, and (6) restriction genes that delimit retrovirus replication in mammals. Within the last few years, the human gene map has experienced a large increase in the number of neoplasia loci that have been mapped to specific chromosomal positions. Of the 27 specific human loci that have been chromosomally mapped to date, 11 (40%) have been assigned by the Genetics Section scientists and their collaborators. This year, we have specifically concentrated on understanding the genomic organization of several genes: ets, rel, raf, onc-D, fms, DHFR and a Y chromosome gene family in man. The mammalian homologue to the v-ets oncogene of the avian E26 transforming virus has been shown to be encoded by two transcriptionally active, nonoverlapping structural genes (ets-1 and ets-2) located on human chromosomes 11 and 21, respectively. Over 30 endogenous retroviral loci have been chromosomally assigned using the hybrid panels. A hematological discorder, the 5q- syndrome, has been shown to be hemizygous for the fms oncogene by genetic analysis. This proto-onc-gene was shown to be transcriptionally active in hematopoetic cells and has been shown by our collaborator (C. Sherr) to encode to specific receptor for macrophage colony-stimulating factor. The emerging human gene map continues to provide an unprecedented opportunity for molecular genetic analyses of the initiation and progression of neoplastic processes.

PROJECT DESCRIPTION

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

Stephen J. O'Brien	Geneticist	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Takis S. Papas	Acting Chief	LMO	NCI
Ulf R. Rapp	Research Chemist	LVC	NCI
Leonard J. Seigel	Clinical Associate	LTCB	NCI
William S. Modi	Staff Fellow	LVC	NCI
Yoh-Ichi Miyake	Visiting Fellow	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) growth factors, (3) receptors for growth factors and retroviruses, (4) endogenous cellular DNA sequences homologous to retroviral RNA genomes, (5) chromosomal integration sites for chronic transforming retroviruses and (6) restriction genes that delimit retroviral replication.

2. Genetic analysis of cooperative and sequential gene actions in the neoplastic processes and in virogene expression. This consideration involves application of the principles and techniques of microbial genetics to cultured mammalian cells.

3. The development of new approaches to the understanding of genetic control of carcinogenesis. This goal involves the identification and characterization of genetic targets (cellular genes) of carcinogenesis.

4. The resolution of the sequence and tissue specificities of distinct cellular genes in different human malignancies. Thus, neoplastic pathways of genetic networks can be dissected by genetic analysis in protocols reminiscent of dissection of metabolic enzyme pathways in early microbial studies.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide and cellulose acetate; (3) cytological chromosome banding procedures (G and Q, G-11) for specific chromosome identification; (4) virological procedures including radioimmunoassay (RIA), reverse transcriptase assay and viral cloning; (5) immunological assays including RIA, cytotoxicity, fluorescent antibody procedures and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques including cDNA transcription in vitro, solution hybridization, visualization of restricted DNA by the techniques of Southern following agarose electrophoresis and molecular cloning of eukaryotic genes; and (7) in situ hybridization to metaphase chromosomes.

Major Findings:

1. Genetic analysis of human proto-oncogenes. Cellular transforming genes (designed c-onc or proto-onc genes) have been a central object of research for several years. They have been associated with neoplastic transformation in human and animal systems in several respects including (1) transduction of proto-onc genes by retroviruses, (2) integration of nontransforming retroviruses adjacent to cellular onc genes and alteration of their expression, (3) specific transformation of murine 3T3 upon transfection with genomic DNA from human tumors, (4) chromosomal translocation of proto-onc gene adjacent to regulatory elements that alter their transcription, (5) genic amplification of proto-oncogene segments in certain tumors, and (6) point mutation of non-neoplastic gene homologue. Because of their suspected importance in human carcinogenesis, nearly all human homologues have been chromosomally assigned. Our laboratory has reported the chromosomal assignment (using somatic cell hybrid panels) of 10 of the approximately 30 proto-oncogene loci thus far localized in man. These include onc-D, Ha-ras-1, Ha-ras-2, Ki-ras-1, Ki-ras-2, raf-1, raf-2, rel, ets-1 and ets-2. The regional association of each of these loci has been determined and their position with relationship to adjacent loci and with respect to nonspecific chromosomal rearrangement in human tumors has been evaluated. In combination with results of oncogenes and other classes of neoplasia-related loci from our laboratory and others, the oncogene families represent a rich opportunity for dissecting the series of genetic events that we term "neoplastic transformation."

2. Implication of the c-fms gene in the etiology of the $5q^-$ refractory anemia syndrome in man. The c-fms proto-oncogene was shown to be expressed in normal human bone marrow and in fully differentiated blood cells, suggesting that its gene product plays a role in the terminal stages of hematopoietic maturation. Whereas c-fms mRNA was not detected in HL-60 cells, an established promyelocytic line, c-fms expression appeared 48 hours after induction with phorbol ester when most cells had differentiated into adherent, post-mitotic macrophages. An acquired deletion of chromosome 5 ($5q^-$) in bone marrow cells, the human $5q^-$ syndrome, was associated with abnormalities in blood cell production. The normal 5 and $5q^-$ chromosomes were individually segregated by construction of cell hybrids between bone marrow cells and rodent cells. A selective system was used that requires retention of the structural gene for dihydrofolate reductase, located on human chromosome 5. Analysis of DNA from individual hybrid clones revealed that the $5q^-$ deletion had removed the c-fms gene. These results suggest that hemizygosity at the c-fms locus leads to abnormalities in hematopoietic maturation, specifically the $5q^-$ syndrome in man.

3. The c-ets homologue of avian E26 transforming virus is bipartite in man and other mammals, where it is represented by two chromosomally distinct nonoverlapping genes (ets-1 and ets-2). A series of molecular clones of human DNA were selected using a v-ets clone of E26 retrovirus. Restriction and DNA sequence analysis were performed and indicated that two distinct nonoverlapping domains, ets-1 and ets-2, were present in the human genome. Northern blot analysis demonstrated that these two loci are transcriptionally active and encode mRNAs of discrete sizes. The mammalian homologue to the 5' v-ets-domain (ets-1) was mapped using somatic cell hybrid panels to chromosome 11 in man, to chromosome 9 in mouse, and to chromosome D1 in the domestic cat. The mammalian homologue to the 3' v-ets domain (ets-2) was similarly mapped to human chromosome 21, to mouse chromosome

16 and to feline chromosome C2. Both proto-oncogenes fell on syntenic groups of homologous linked-loci that were conserved among the three species (see Project Number Z01CP05385-02 LVC). The occurrence of two distinct functional proto-oncogenes and their conservation of linkage positions in the three mammalian orders indicate that these two genes have been separate since before the evolutionary divergence of mammals.

4. Dispersion of endogenous retroviral families to multiple chromosomes in man. Three independent laboratories, using molecularly cloned probes, have identified three apparently different families of human DNA sequences that are related to replication-competent retroviruses isolated from other mammals. The groups include (1) a small group distantly related to MoMuLV and BaEV, described by M. Cohen et al.; (2) a larger family of 30-50 segments related to Mo-MuLV, described by M. Martin et al.; and (3) a family related to the mouse mammary tumor virus, described by R. Callahan et al. We have collaborated with Dr. M. Cohen on the genetic association of the ERV family and have assigned three members, ERV1, ERV2 and ERV3, to three distinct human chromosomes. One of these, ERV3, is apparently transcriptionally active because specific transcripts can be detected using Northern analysis. The hybrid panels have also been used to examine two distinct families described by M. Martin and his collaborators. The first is a truncated group that lacks LTR sequences. The second is a full-length viral family with approximately 30 members. Both families have been found to be dispersed to multiple chromosomes as well. The chromosomal positions of several of these have been determined using specific env probes, as well as cellular flanking DNA as probes, in the hybrid panel. The genetic associations of these endogenous retroviral sequences and proto-oncogenes or other cis-associated neoplastic loci are under investigation.

5. The Y-3.4 NYS family of repetitive DNA sequences clustered on the human Y chromosome. The human genome is characterized by a variety of repetitive and moderately repetitive DNA families. These moderately repetitive families are not well understood but are generally thought to have important roles in gene regulation, chromosome organization and species diversification in evolution. One family that has been under study is the Y-3.4 NYS family. This family contains a 3.4-kb fragment released from the human Y chromosome by HaeIII. The 3.4-kb fragment contains two different families: (1) a Y-specific family that is repeated 7500 times per haploid genome and comprises over 40% of all Y chromosome sequences; and (2) NYS for non-Y-specific family that is found throughout the autosomes, as well as on the Y chromosomes. We have used specific probes of the NYS domains of this family for in situ hybridization and hybrid panel analysis of material derived from female DNA. Two specific domains (K and D) of the NYS family were found to be clustered on the autosomes and mapped to two specific chromosomes, 15 and 16. The interaction of these families on neighboring genes on these chromosomes is under investigation.

6. Chromosomal organization and racial distribution of the human dihydrofolate reductase gene family. Dihydrofolate reductase catalyzes a key methyl transfer reaction in the de novo synthetic pathway to purines. It is inhibited by methotrexate in situ and is often amplified in drug selection (and in chemotherapy), leading to double minute chromosomes or homogeneously staining regions (HSRs) in cultured cells. The human dihydrofolate reductase (DHFR) gene family is comprised of five distinct loci, a functional gene (hDHFR) and four intronless

genes, three of which (hDHFR-ψ2 to hDHFR-ψ4) are identifiable as pseudogenes because of DNA sequence divergence from the functional gene with introns. The other intronless gene (hDHFR-ψ1) is completely homologous to the coding sequences of the functional gene. Specific, molecularly cloned probes and genomic DNA from two panels of somatic cell hybrids were used to investigate the chromosomal organizations and positions of these genes in the human genome. All five genes are dispersed on different chromosomes.

The functional gene (hDHFR) has been mapped to chromosome 5 using a panel of somatic cell hybrids. Using in situ hybridization, we have localized this gene to a region 5q11-q22 proximal to the centromere. The chromosomal assignment of three pseudogenes has now been determined by genomic DNA analysis of a panel of human x rodent somatic cell hybrids. The hDHFR-ψ1 pseudogene is on chromosome 18, while hDHFR-ψ2 is on chromosome 6 and hDHFR-ψ4 is on chromosome 3. The hDHFR-ψ1 pseudogene exhibits a novel form of polymorphism insofar as the entire pseudogene sequence is present in DNA of some individuals and absent from DNA of others, consistent with a recent evolutionary origin implied by its sequence identity to the coding sequences of the functional gene. The racial distribution of this pseudogene locus has been determined. The allelic frequency defined by analysis of 180 human chromosomes is as follows: Mediterraneans 94%, Asian Indians 77%, Chinese 67%, Southeast Asians 57%, while American Blacks exhibited the lowest frequency (33%). These data indicate that the transposition of this "perfect" pseudogene occurred rather recently and possibly prior to the inception of the human racial groups.

Significance to Biomedical Research and the Program of the Institute:

The characterization of identified loci that participate in cell transformation has two important applications: (1) as the raw material for the dissection of developmental genetic analysis of the cellular events that lead to neoplastic transformation, and (2) as possible targets for carcinogens in screening protocols. The specific understanding of the developmental genetic sequence that characterizes the neoplastic event is necessary to any meaningful attempt to correct and destroy cancerous tissues. The association of newly mapped oncogenes and specific chromosomal arrangements associated with specific malignancies provides a guide by which to approach the genetic basis of these tumors. A comprehensive genetics program, from the molecular to the biological species level, holds promise in the ultimate resolution of the neoplastic process in man.

Proposed Course:

The continued chromosomal placement of new oncogenes, receptors, growth factors and other genes that participate in neoplasia is anticipated. The hybrid panels and the developing procedure of in situ hybridization of single copy DNA probes will be employed in several classes of collaborative genetic experiments. Somatic cell hybrids between specific classes of human T-cells and T-cell leukemias have been developed using murine B- and T-cell lines as rodent partners. These constructs permit tissue-specific regulation of lymphoid function and provide the opportunity to study both trans and cis regulation of cellular oncogene-neoplasia loci in vitro using a combination of genetic and molecular technologies.

Publications:

Anagnou, N. P., O'Brien, S. J., Shimada, T., Nash, W. G., Chen, M. and Nienhuis, A. W.: The chromosomal organization and assignment of the human dihydrofolate reductase genes: Dispersion, selective amplification and polymorphism. Proc. Natl. Acad. Sci. USA 81: 5170-5174, 1984.

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Longo, D. L., Gelmann, E. P., Cossman, J., Young, R. A., Gallo, R. C., O'Brien, S. J. and Matis, L. A.: Isolation of HTLV-transformed B-lymphocyte clone from a patient with HTLV-associated adult T-cell leukemia. Nature 310: 505-506, 1984.

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O'Brien, S. J., Reeves, R. H., Simonson, J. M., Eichelberger, M. A. and Nash, W. G.: Parallels of genomic organization and endogenous retrovirus organization in cat and man. Dev. Genet. 4: 341-354, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05385-02 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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	Geneticist	LVC	NCI
Others:	William S. Modi	Staff Fellow	LVC	NCI
	Yoh-Ichi Miyake	Visiting Fellow	LVC	NCI
	Cheryl A. Winkler	Biologist	LVC	NCI
	David E. Wildt	Guest Researcher	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI

COOPERATING UNITS (if any) Johns Hopkins Hospital, Baltimore, MD (R. H. Reeves); H&W Cytogenetics Services, Inc., Sterling, VA (W. G. Nash); Univ. of CA, San Diego, CA (J. S. O'Brien); Mt. Sinai Sch. of Med., NY, NY (R. Desnick); USUHS, Bethesda, MD (E. H. Chang); LBI, Frederick, MD (E. Brownell); NIAID, NIH, Bethesda, MD (C. Kozak)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.9	2.1	1.8

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The genetic analysis of the domestic cat began with the assignment of 33 isozyme loci to 16 (of 19) chromosomal linkage groups using a panel of rodent x cat somatic cell hybrids. A domestic cat colony was established at the NIH Animal Center and linkage analysis of morphological loci and biochemical loci was initiated by computing log of the odds values of linked genes in pedigrees. Reciprocal skin grafts and allogenic lymphocyte immunizations were initiated and 15 standard antisera against the feline major histocompatibility complex (MHC) were derived. These sera have allowed the description and resolution of MHC haplotypes in four major cat colonies. The endogenous RD-114 retroviral family was studied in domestic cats by deriving molecular clones of endogenous RDV1 and performing restriction and chromosome mapping. The feline homologues of the proto-onc genes have been studied using molecular clones of v-onc and c-onc from man or mouse. To date, 10 oncogene loci have been genetically mapped and their role in feline tumorigenesis is under study. Eight enzyme loci that encode lysosomal enzymes known to be mutated in human inborn errors and in feline counterpart models have been assigned to specific feline chromosomes. Molecular cDNA clones of human lysosomal enzymes are being ligated to appropriate eukaryotic vectors for possible gene therapy in transgenic felids.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Stephen J. O'Brien	Geneticist	LVC	NCI
William S. Modi	Staff Fellow	LVC	NCI
Yoh-Ichi Miyake	Visiting Fellow	LVC	NCI
Cheryl A. Winkler	Biologist	LVC	NCI
David E. Wildt	Guest Researcher	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI

Objectives:

1. The development and expansion of the genetic map of the domestic cat (Felis catus) with particular emphasis on genes of the immune system that have an oncogenic role in leukemia and lymphomas and control developmental processes. The specific classes of genes under study include (1) endogenous cellular DNA sequences homologous to retroviral RNA, (2) chromosomal integration sites for exogenous retroviral insertion and persistence, (3) receptors on cell membranes that interact with viral glycoproteins to determine cell-species compatibility and viral host range, (4) restriction genes that delimit virus replication in various animal species, (5) cellular transforming onc genes, (6) cellular enzyme structural genes and (7) cell surface antigens, including antigens homologous to the major histocompatibility complex (MHC) of other mammalian species.

2. Comparative linkage analysis of the feline gene map with two other major mammalian gene maps, human and mouse, for reconstruction of natural history of chromosomal rearrangements of the three mammalian orders.

3. Development of serological and cellular immunological reagents for analysis of feline lymphoid and myeloid-cell derivatives with special emphasis on correlation of function of T- and B-cell subsets.

4. Molecular and genetic analysis of endogenous feline retroviral families (specifically RD-114 and FeLV).

5. Development of feline embryo manipulation for gene therapy and production of transgenic cats.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide and cellulose acetate; (3) cytological chromosome banding procedures (G and Q, G-11) for specific chromosome identification; (4) virological procedures, including radioimmunoassay (RIA), reverse transcriptase assay and viral cloning; (5) immunological assays, including RIA, cytotoxicity assays, fluorescent antibody procedures, immunoprecipitation, and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques, including cDNA transcription in vitro, solution hybridization, visualization of

restricted DNA by the techniques of Southern following agarose electrophoresis and molecular cloning of eukaryotic genes; and (7) in situ hybridization to metaphase chromosomes.

Major Findings:

1. The latest feline gene map, consisting of over 50 loci, shows a striking syntenic and cytological homology between cat and man, but not between cat (or man) and rodents. Development of the feline genetic map in our laboratory revealed that 50 biochemical loci assigned to 16 of the 19 cat chromosomes were particularly homologous in linkage to the human map using the same loci. Using high resolution G-trypsin banding (1000 bond level of resolution), we were able to demonstrate that 20-25% of the human genome could be aligned band for band to homologous regions. Linkage homologies in other regions were characterized by small intrachromosomal rearrangements. The extensive advances in comparative syntenic and cytogenetic analysis have been utilized to partially reconstruct the chromosomal breaks that have occurred during 100 million years of mammalian evolution. The conservation of linkage association is also considered in identifying mammalian genes (like retroviruses or controlling elements) capable of transposition during species transitions.

2. Comparative genetic analysis of proto-onc genes in the cat and other mammals. Genomic DNAs from a genetically characterized somatic cell hybrid panel of over 60 hybrids has been used to chromosomally map oncogene homologues in the cat. Over 20 molecular clones of v-onc and c-onc equivalents from human, mouse and cat have been used to visualize and map oncogenes. To date, the feline equivalents of Ha-ras-1, Ha-ras-2, Ki-ras-1, Ki-ras-2, sis, fes, rel, ets-1, ets-2 and myc have been chromosomally assigned. The comparative syntenic maps of man and cat provide an unusual opportunity to examine the mobility of oncogenes during mammalian evolution. For example, the ras family consists of three functional loci (Ha-ras-1, Ki-ras-2 and N-ras) and two intronless pseudogenes. The genetic positions of four of the five genes in the cat conform precisely to their predicted positions from the aforementioned chromosomal homologies. The fifth human locus (Ki-ras-1), an intronless human pseudogene, does not exist in the cat, suggesting that its emergence occurred during the primate radiations. On the contrary, the bipartite mammalian ets gene (designated ets-1 and ets-2) maps to two homologous chromosomes in man, mouse and cat, but not in chicken. This result affirms a fusion of two functional genes in avian linkages after their split from the mammalian line. A similar comparison of each of the 30 or so proto-oncogenes is in progress.

3. Definition of FLA, the feline MHC. The major histocompatibility complex responsible for synthesis of antigens that play a key role in graft rejection and in T-cell communication (MHC restriction, associative recognition) have been described in several laboratory animals (and man) with the glaring exception of the domestic cat. The participation of the MHC in the development of the immune response, resistance to leukemias, susceptibility to various diseases, and transplant rejection makes this locus a high priority in the study of feline genetics. We have initiated a closed breeding colony of 100 cats at the NIH Animal Center in Poolesville, MD, for a number of genetic and physiological experiments (see Project Number Z01CP05389-02 LVC). Reciprocal, split-thickness skin grafts were surgically exchanged between over 100 animals over the past three years.

Approximately 75 cats rejected the grafts acutely (representing a difference(s) at the MHC), while 25 cats rejected chronically (representing identity at the MHC and differences at minor histocompatibility loci). Fifteen of the 75 positive cats produced cytotoxic antisera as measured with a two-stage cytotoxic antibody assay. These sera were analyzed by typing over 300 cats from four colonies -- NIH, Cornell University, University of Pennsylvania and University of Colorado -- using pedigree and "cluster" analyses. The derived sera are highly representative because every cat tested was positive with at least one of the alloantisera. Pedigree and cluster analyses have resulted in the definition of specific MHC epitopes and haplotypes in natural populations. Immunoprecipitation experiments revealed that several sera recognized class I determinants (MW=45,000), while others recognized class II molecules (MW=32,000). The development and characterization of these reagents represents a significant advance in the immunobiology of the domestic cat model.

4. Molecular genetic characterization of the RD-114 family of endogenous retroviral genes in the cat. RD-114 is a replication-competent, xenotropic retrovirus that is homologous to a family of moderately repetitive DNA sequences present in approximately 20 copies in the normal cellular genome of domestic cats. In order to examine the extent and character of genomic divergence of the RD-114 gene family, as well as to assess their positional association within the cat genome, we have prepared a series of molecular clones of endogenous RD-114 DNA segments from a genomic library of cat cellular DNA. Their restriction endonuclease maps were compared to each other, and to the map of the prototype, inducible RD-114 virus, which was molecularly cloned from a chronically infected human cell line. The endogenous sequences analyzed were similar to each other in that they were colinear with RD-114 proviral DNA, were bound by long terminal redundancies and conserved many restriction sites in the gag and pol regions. However, the env regions of many of the sequences examined were substantially deleted. Several of the endogenous RD-114 genomes contained a novel envelope sequence that was unrelated to the env gene of the prototype RD-114 env gene but which, like RD-114 and endogenous FeLV provirus, was found only in species of the genus Felis and not in other closely related Felidae genera. The endogenous RD-114 sequences each had a distinct cellular flank and were dispersed on multiple feline chromosomes. The chromosomal locations of four RD-114 virogenes (RDV1-4) were determined using a panel of rodent x cat somatic cell hybrids. One of these, RDV1, is apparently a single locus on feline chromosome B3, which is inducible for replication-competent RD-114 virus.

5. Feline models of human inborn errors in the cat: Gene localization and gene cloning. The domestic cat has provided a number of models of storage diseases that lack specific lysosomal enzymes in both cat and man. We have used a panel of rodent x feline lymphocyte somatic cell hybrids to define the chromosomal positions of several of these enzyme loci in the cat. The specific loci thus far assigned in the cat and the human disease that results in deficiency (in parentheses) are (1) β-glucuronidase (Mucopolysaccharidosis-VII), (2) α-galactosidase (Fabry's disease), (3) α-galactosidase (GM1-gangliosidosis), (4) β-mannosidase (mannosidosis), (5) β-glucosidase (Gaucher's disease), (6) fucosidase-A (α-fucosidosis), (7) β-hexosaminidase-A (Tay-Sach's disease), and (8) iduronidase (Hurler's syndrome). A cDNA library from human tumors grown in nude mice was used to isolate molecular clones of each of these enzyme-genes in the laboratory of Dr. J. S. O'Brien, our principal collaborator on these studies. We have

placed these normal human genes into eukaryotic expression vectors for microinjection into feline embryos. Procedures for recovery, in vitro culture and transfer of feline embryos are being developed in collaboration with Dr. Wildt (see Project Number Z01CP05389-02 LVC) of this section for purposes of gene delivery to murine and feline embryos.

Significance to Biomedical Research and the Program of the Institute:

The development of informative animal models for the study of human neoplasia and metabolic diseases is imperative for understanding their mechanisms and for testing putative therapy. Until recently, the only extensive mammalian genetic maps were for mouse and man. The construction of the gene map of the cat provides a third major mammal with a functional gene map. The occurrence of feline leukemia virus in natural feline populations provides a unique model for the retroviral etiology of leukemia, lymphoma and acquired immune deficiency syndrome. The availability of feline mutants with biochemical deficiencies homologous to human inborn errors makes the feline genetic system even more useful for gene delivery protocols. Despite the rapid advances that have occurred in human molecular biology, the testing of gene delivery systems in animal models remains a clinical and ethical imperative.

Proposed Course:

The continued pursuit of genetic mapping of oncogenes in both cat and man (see Project Number Z01CP05384-02 LVC) is anticipated. FeLV tumors and spontaneous cat tumors will be cytologically monitored for specific chromosomal rearrangements. The immunogenetic analysis of hematopoietic differentiation using monoclonal antibody reagents and functional in vitro assays is anticipated. Gene expression of specific loci that participate in transformation will be approached using hybrids made with murine B- and T-cells. Preliminary injections and vector engineering of eukaryotic genes in developing feline embryos is a high priority.

Publications:

Collier, G. E. and O'Brien, S. J.: A molecular phylogeny of the Felidae: Immunological distance. Evolution (In Press)

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O'Brien, S. J., Nash, W. G., Bauer, R., Chang, E. H. and Seigel, L. J.: Trends in chromosomal and oncogene evolution in vertebrates. In Patterson, M. K. (Ed.): Uses and Standardization of Vertebrate Cell Cultures. In Vitro Monograph No. 5. Gaithersburg, Tissue Culture Association, 1984, pp. 204-214.

O'Brien, S. J., Reeves, R. H., Simonson, J. M., Eichelberger, M. A. and Nash, W. G.: Parallels of genomic organization and endogenous retrovirus organization in cat and man. Dev. Genet. 4: 341-354, 1984.

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Reeves, R. H. and O'Brien, S. J.: Molecular genetic characterization of the RD-114 gene family of endogenous feline retroviral sequences. J. Virol. 52: 164-171, 1984.

Seigel, L. J., Nash, W. G., Harper, M., Wong-Staal, F., Gallo, R. C. and O'Brien, S. J.: Gene for T-cell growth factor: Location on human chromosome 4q and feline chromosome B1. Science 223: 175-178, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05386-02 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Basic Mechanisms in HTLV-Induced Leukemia and AIDS

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

PI:	Stephen J. O'Brien	Geneticist	LVC	NCI
Others:	Leonard J. Seigel	Clinical Associate	LTCB	NCI
	David Derse	Guest Researcher	LVC	NCI
	James W. Casey	Senior Staff Fellow	LVC	NCI
	Robert C. Gallo	Chief	LTCB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	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 family of retroviruses known as human T-cell lymphotropic retroviruses (HTLV-I, II, and III) has an affinity for infecting T lymphocytes and a similar genomic structure. All 3 types of HTLV have been transmitted in vitro, molecularly cloned and sequenced. Despite these advances, the mechanism by which infection with these viruses results in malignant transformation or immunosuppression remains unknown. We are focusing on basic mechanisms both on a cellular and molecular level by which these viruses transform or immonosuppress. In order to address whether HTLV-I may induce transformation through an insertional mutagenesis mechanism, we have utilized somatic cell hybrids constructed between rodent cells and HTLV-I infected cell lines to study the processes and consequences of HTLV chromosomal integration. Integration in vitro was shown to be a dynamic process and proviral integration apparently occurs at random in the genome. Studies are in progress to determine whether common integration sites are present in fresh tumor material. Since HTLV-I infected cells often lose their dependence for interleukin-2 (IL-2) this gene may play a role in transformation induced by HTLV-I. The gene was first mapped to chromosome 4 and sublocalized to 4q26-28 in normal lymphocytes. We next demonstrated that the IL-2 gene was not rearranged in several HTLV-I infected cell lines and mapped to chromosome 4 in Hut 102, suggesting that this gene is not operative in HTLV-I induced malignant transformation. We have also utilized the panel of Hut 102X Chinese hamster hybrids to demonstrate that the novel Class I antigenic determinants expressed on HTLV-I infected cells does not result from induction of Class I genes encoded by the cellular MHC locus, but are probably encoded by integrated HTLV-I. The activities of the promoter unit contained within the LTR of both HTLV-I and HTLV-III were examined by transfecting various cells with recombinant plasmids containing the LTR of HTLV-I or HTLV-III linked to the bacterial gene for chloramphenicol acetyltransferase (CAT). We have demonstrated that infected cells contain factors that act in trans on the LTRs of the infecting virus to activate transcription.

PROJECT DESCRIPTION

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

Stephen J. O'Brien	Geneticist	LVC	NCI
Leonard J. Seigel	Clinical Associate	LTCB	NCI
David Derse	Guest Researcher	LVC	NCI
James W. Casey	Senior Staff Fellow	LVC	NCI
Robert C. Gallo	Chief	LTCB	NCI

Objectives:

1. Determination of the chromosomal integration site(s) of human T-cell lymphotropic retrovirus-I (HTLV-I) in infected leukemic and lymphoma cell lines and fresh tumor tissue. Proviral integration sites will then be compared to the position of certain human loci that are candidates for genetic causes of transformation, including proto-oncogenes, transforming genes active in the DNA transfection assay, genes encoding growth factors or their receptors, as well as translocation breakpoints characteristic of specific neoplasias.

2. Application of somatic cell genetics to the dissection of certain biologic and immunologic phenomena associated with HTLV infection in order to determine their role in the pathogenesis of HTLV-induced neoplasia and immune suppression.

3. Transfection of recombinant plasmids containing HTLV-I and HTLV-III regulatory sequences linked to the chloramphenicol acetyltransferase (CAT) gene in order to elucidate the phenomenon of "trans-activation" observed in HTLV-I-infected cells.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization, (2) starch gel electrophoresis with histochemical isoenzyme analysis, (3) G-11 chromosome staining and G-trypsin chromosome banding, (4) molecular biologic techniques such as Southern and Northern blotting, (5) radioimmune assays, (6) fluorescent antibody staining and use of the fluorescent-activated cell sorter (FACS), (7) DNA transfection, and (8) assays for genomic enhancer elements using the CAT.

Major Findings:

1. HTLV-I integration is monoclonal, dynamic, and chromosomally dispersed in human tumors. Two panels of somatic cell hybrids have been constructed by fusion of rodent cell lines with the HTLV-I-infected cell lines, Hut 102 and MJ. The hybrids were genetically characterized by karyotypic analysis and by examination of 36 isoenzymes previously assigned to specific human chromosomes. High molecular weight DNA was simultaneously extracted from each hybrid for Southern analysis. These panels allow for the chromosomal localization of HTLV proviral integration sites, as well as any human gene for which an assay or cloned molecular probe exists. These hybrids should prove extremely useful in understanding the biology of HTLV-induced transformation on both a cellular and molecular level.

Southern analyses of different passages of the HTLV-infected cell lines, Hut 102 and MJ, with cloned HTLV probes have demonstrated that HTLV integration is dynamic during in vitro cell culture. Cell lines in early passage have one to three detectable proviruses, which increase to greater than 20 in number as the cells are maintained in culture. In addition, proviral integrations are apparently lost as the cells are maintained in culture. Proviral integrations are dispersed among multiple human chromosomes. Two proviral integrations visualized in early passage Hut 102 cells are located on chromosomes 4 and 20. Dynamic integration has also been observed in the cell line MJ.

2. Location of the structural gene for interleukin-2 (IL-2) to chromosome 4. T-cell growth factor (TCGF) or IL-2 is a powerful immunoregulatory lymphokine which is produced by lectin or antigen activated mature T-lymphocytes and in a constitutive manner by certain T-cell lymphoma cell lines. We have utilized a molecular clone of human TCGF and DNA extracted from a panel of somatic cell hybrids (rodent X normal human lymphocytes) to genetically localize the TCGF structural gene to human chromosome 4. In situ hybridization of the TCGF plasmid to human chromosomes resulted in significant labeling of the mid-portion of the long arm of chromosome 4 and sublocalized the TCGF gene to 4q26-28. Genomic DNA from two HTLV-I infected cell lines Hut 102 B2 and MJ, as well as a panel of hybrids prepared with Hut 102 B2, was examined with the same molecular clone. The TCGF gene was apparently not rearranged in MJ or Hut 102 cell lines, and was located on chromosome 4 in Hut 102 B2 as well. These data, as well as the observation by others that the IL-2 gene is not constitutively expressed in HTLV-I infected cells, suggest that the gene encoding IL-2 is not operative in HTLV-I induced maligant transformation. In addition, we assigned the homologous TCGF locus in the domestic cat to chromosome B1 using a somatic cell hybrid panel which segregates cat chromosomes. This data is in support of the linkage and high resolution G-trypsin binding results which indicate that this feline chromosome is partially homologous to human chromosomes.

3. HTLV-I infected tumor cells and in vitro infected cord blood cells express a novel antigenic determinant shared by some HLA class I allotypic antigens. This expression is in addition to and distinct from class I antigens expressed by autologous uninfected cells. We have demonstrated that genomic DNA of infected cells does not contain detectable rearrangements of HLA class I genes nor does it contain specific polymorphic restriction fragments characteristic of the "novel" HLA determinants in normal individuals possessing the HLA genotype. Furthermore, we have shown that the expression of these HLA-related novel antigens in hybrids of HTLV-I infected cells and hamster cells, as measured by a specific monoclonal HLA antibody, did not segregate with the human HLA locus on chromosome six, but rather assorted with multiple chromosomes which contained integrated HTLV-I proviruses. These results clearly indicate that the expression of novel HLA antigens does not result from induction of class I genes encoded by the cellular MHC locus, and support the explanation that the novel HLA antigen expression associated with HTLV-I infection is determined and possibly encoded by integrated HTLV-I. The physical consequences of novel HLA antigen expression on the host immune response to infection is under investigation.

4. Demonstration of functional activity of the promoter unit contained within the long terminal repeats (LTRs) of HTLV-I and HTLV-III by transfecting various

cells with recombinant plasmids containing the LTR of HTLV-I or HTLV-III (pHTLV-I-CAT and pHTLV-III-CAT) coupled to the bacterial gene for CAT. Comparison of the levels of CAT activity in a panel of various cell types transfected with pHTLV-I-CAT has revealed that all cells productively infected with HTLV-I (but not HTLV-III or bovine leukemia virus, BLV) have high levels of CAT activity. Uninfected cells have low levels of CAT activity. This data indicates that infected cells contain factors that act in trans on the HTLV-I LTR to activate transcription. We have also demonstrated that a B-cell line, HS-1, which contains integrated HTLV-I and is able to transmit the virus in vitro, fails to transactivate the HTLV-I LTR. Examination of several cell lines (both B and T) infected in vitro by co-cultivation with HS-I revealed a spectrum of CAT activity that parallels precisely the level of HTLV-I specific mRNA in each cell line. This data suggests that the cellular environment plays a critical role in the regulation of both HTLV transcription and transactivation. Similarly, all cells productively infected with HTLV-III (but not HTLV-I or BLV) yield high levels of CAT activity after transfection with pHTLV-III-CAT. We have demonstrated that a molecular clone of HTLV-III, HXB2, will result in significant levels of CAT activity within 48 hours following co-transfection with pHTLV-III-CAT in the T-cell line, H9. We are currently examining deletions of the HXB2 clone in co-transfection experiments in order to precisely determine the region of the viral genome required for trans-activation. Taken as a whole the data indicate that cells infected with HTLV-I or HTLV-III synthesize factors that enhance viral transcription. A key issue and one on which we are now focussing, is whether these transacting factors are directly encoded by the virus, the cellular genome or both acting in concert.

Significance to Biomedical Research and the Program of the Institute:

HTLV-I is the first retrovirus demonstrated to be strongly associated with, and likely causative of, neoplasia in man. HTLV-III is the etiologic agent of acquired immune deficiency syndrome (AIDS). Characterization of HTLV and its biologic effects may help to elucidate the molecular events that induce malignant transformation of AIDS, as well as those events that permit the transformed cell to elude the immune response. Such advances could lead to the development of reagents useful in the diagnosis, treatment or prevention of certain human lymphomas, leukemias or AIDS.

Proposed Course:

We will determine HTLV-I proviral integration sites in additional fresh tumor specimens to determine whether HTLV integration is random or targeted. Hybrids will be examined with antibodies directed against various segments of HTLV-I *gag* and *env* gene products to determine whether novel HLA antigens observed in HTLV-I-infected cells share epitopes with HTLV gene products. We will also focus on the source of transacting factors, PX or cell. Several types of experiments will be conducted to address this issue: (1) examination of cell lines (from both acute T-cell leukemia patients and AIDS patients) containing various deletions of the HTLV provirus for expression of transacting factors, (2) transfection experiments in which expression vectors containing PX are co-transfected with CAT plasmids containing the HTLV LTR, and (3) transfection of somatic cell hybrids with CAT plasmids in an attempt to incriminate a cellular gene.

Publications:

Seigel, L. J., Nash, W. G., Manzari, V., Wong-Staal, F., Gallo, R. C. and O'Brien, S. J.: A genetic analysis of HTLV integration in HUT 102 and the localization of the structural gene for T-cell growth factor to chromosome 4. In Gallo, R. C. and Essex, M. (Eds.): Cancer Cells. New York, Cold Spring Harbor Press, 1984, pp. 167-180.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05387-02 LVC

PERIOD COVERED
October 1, 1984 to February 28, 1985

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

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

PI:	Pramod Sutrave	Visiting Fellow	LVC	NCI
Others:	Ulf R. Rapp	Research Chemist	LVC	NCI

COOPERATING UNITS (if any)
Max-Planck Institut Fur Molekulare Genetik, Berlin, FRG (K. Bister); Fred Hutchinson Cancer Center, Seattle, WA (M. Linial); Lab. of Cell Biology, NIMH, NIH, Bethesda, MD (T. Bonner)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

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 acutely transforming avian retrovirus, MH2, carries a novel, putative oncogene, v-mil, in addition to the known oncogene, v-myc. We have recently shown by hybridization analysis that v-mil is homologous to v-raf, the transforming gene of the murine retrovirus, 3611-MSV. We have sequenced the v-mil oncogene and compared it with v-raf. The 80% homology between the nucleotide sequences and the 94% homology between the predicted amino acid sequences of the two viral genes clearly indicate that these are the avian and murine forms of the same gene. Comparison of the two sequences with that of the human cellular homolog indicates that v-raf has more 3' untranslated sequences, while v-mil has additional sequences from two 5' exons of the cellular homolog. Although the mil/raf amino acid sequences reveal partial homology to that of the v-src product, no tyrosine-specific protein kinase activity is detected for the gag-mil and gag-raf hybrid proteins. We have also determined the nucleotide sequence of MH2 from an HgiAI site within the coding region of its oncogene v-myc to the KpnI site within the long terminal repeat (LTR). Comparison with published sequences from other retroviruses allowed us to assign the origin of all sequence elements in this region. We conclude that MH2 contains a unique assembly of 3' terminal sequences which includes part of the 3' noncoding (SPC) region of the avian sarcoma virus, Y73, and the complete F3 and F1 segments of Rous sarcoma virus (RSV), strain SR-A. A unique MCF class of recombinant MuLV, associated with alveologenic lung carcinoma in mice was further characterized by sequencing its LTR. The results establish the unique structure of this new MCF class of viruses which has features of both the class I and class II MCF MuLV.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

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

Pramod Sutrave	Visiting Fellow	LVC	NCI
Ulf R. Rapp	Research Chemist	LVC	NCI

Objectives:

Characterization of the human and chicken homologs of the murine oncogene, raf, and MCF recombinant viruses isolated in vitro.

Methods Employed:

The molecular characterization of the homologs of the v-raf oncogene in different species was carried out by sequencing their nucleic acids and comparing them with those of other src family oncogenes. Standard methods of chemical cleavage of Maxam and Gilbert were employed for sequencing. c-raf-2, the human homolog of v-raf, was subcloned in pBR322 and was used for sequencing. The avian acutely transforming retrovirus, MH2, containing two different oncogenes, was subcloned in pBR322 and used for the characterization of the chicken homolog of v-raf. Characterization of the LTR of the recombinant MCF virus was achieved by sequencing the nucleic acid using the chain termination method of Sanger.

Major Findings:

1. MH2 is an acutely transforming avian retrovirus containing two different oncogenes, mil and myc. Earlier hybridization studies had revealed that the v-mil oncogene is closely related to the v-raf oncogene of the murine sarcoma virus, 3611 (3611-MSV). The v-mil oncogene was further characterized by sequencing its nucleic acids. About 80% homology between the nucleotide sequences and 94% homology between the predicted amino acid sequences in v-mil and v-raf together with structural data on the raf related gene δ-raf allowed us to conclude that the v-mil oncogene is most likely the chicken homolog of the v-raf oncogene. Further, in both viruses the mil/raf oncogene product is expressed as a gag fusion polyprotein. Although the mil/raf protein revealed partial homology to the product of v-src, no tyrosine kinase activity was detected in the hybrid mil/raf protein products. Instead, protein kinase activity with specificity for ser/thr was detected.

2. MH2 virus, apart from containing two different oncogenes, has a stretch of sequences related to different isolates of acutely transforming avian retroviruses. The unique sequences are observed between the end of myc and the beginning of the LTR. Sequences were partially homologous to the unidentified 3' noncoding (SPC) region in the avian sarcoma virus, Y-73, and to sequences 3' of the src oncogene up to the U3 region in the LTR of the Rous sarcoma virus (RSV) strain, SR-A. The homology between the SPC region to the 3' end of the src oncogene and MH2 possibly indicates that the helper virus(es) used in the generation of the MH2, RSV SR-A or Pr-C strain was identical.

3. The pathogenic and nonpathogenic MCF recombinant viruses are characterized, not only on the basis of the recombinant env genes, but also on the basis of their LTRs. A unique class of recombinant MuLV, CI-3, associated with alveologenic lung carcinoma in mice was further characterized by sequencing its LTR. It was found that the virus can neither be classified as class I MCF MuLV, which contains recombinant LTRs, a recombinant env gene, and is highly pathogenic in the AKR acceleration assay, nor class II, which contains ecotropic LTRs and is nonpathogenic. Our MCF virus is pathogenic, as is class I, but replicates poorly, a characteristic of class II.

Significance to Biomedical Research and the Program of the Institute:

Examination of the dual oncogene carrying retrovirus, MH2, as to genetic mechanisms that lead to linkage of both genes in this virus is significant because the cellular homologs of both viral oncogenes are involved in common human tumors.

Proposed Course:

This project has been completed.

Publications:

Bister, K., Jansen, H. W., Sutrave, P. and Rapp, U. R.: The oncogenes of avian carcinoma virus MH2. In Bishop, J. M., Greaves, M. and Rowley, J. D. (Eds.): Genes and Cancer. New York, Alan R. Liss, 1984, Vol. 17, pp. 315-320.

Bonner, T. I., Kerby, S., Sutrave, P., Gunnell, M., Mark, G. and Rapp, U. R.: The structure and biological activity of the human homologues of the raf/mil oncogene. Mol. Cell. Biol. (In Press)

Sutrave, P., Jansen, H. W., Bister, K. and Rapp, U. R.: The 3'-terminal region of avian carcinoma virus MH2 shares sequence elements with avian sarcoma viruses Y73 and SR-A. J. Virol. 52: 703-705, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05388-02 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Molecular Basis of Induction of Neoplasia by the Oncogene myc

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

PI: James W. Casey Senior Staff Fellow LVC NCI

COOPERATING UNITS (if any)

Louisiana State University Medical Center, New Orleans, LA (P. Deininger); Program Resources, Inc., Frederick, MD (M. J. Braun)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

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.)

Oncogenes, those DNA sequences that have been selected by conferring a growth advantage to cells due to alteration in sequence or aberrant transcriptional regulation, were originally identified as retroviral transduced cellular genes. We have previously isolated a defective provirus related to FeLV which transduced myc from a cat T-cell lymphoma. Two questions can be addressed regarding the molecular basis of transformation by this FeLV-myc provirus: (1) Are their alteractions in the v-myc portion of the FeLV-myc provirus that confer transforming potential to the provirus? (2) Are their DNA sequences in this feline provirus that determine the apparent T-cell tropism of FeLV-myc provirus? These questions were first addressed by determining the nucleotide sequence of the v-myc and long terminal repeat (LTR) portion of the FeLV-myc provirus. The results show that one nucleotide substitutes in exon III of v-myc causes a loss of serine residue which is normally phosphorylated in chicken c-myc. The possibility that loss of this phosphorylation site allows v-myc to exert a transforming effect is being tested. To precisely quantitate the promotor strength of the FeLV-myc LTR and measure its activity in fibroblast, B-cells and T-cells, a chloramepherical acetyl transferase assay was performed. The results show that the FeLV-myc LTR is 31 times more active in T-cells than the Rous sarcoma virus LTR. Additionally the FeLV-myc LTR is five times more active than the Gardner-Arnstein FeLV LTR on T-cells. The major difference at the DNA sequence level which accounts for T-cell tropism by FeLV-myc is a 44 bp duplication of a core enhancer. In summary, it appears that the transforming capacity of the FeLV-myc provirus is governed both at the transcriptional level and may require point mutations in the v-myc protein which when expressed in concert in T-cells results in lymphomas.

PROJECT DESCRIPTION

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

James W. Casey   Senior Staff Fellow   LVC   NCI

Objectives:

To elucidate the molecular basics of myc oncogene activation and to understand the complex framework of events that in unison give rise to neoplasias. By manipulation of a well-defined retroviral isolate, FeLV-myc, the DNA sequence encoding neoplastic potential can be defined. The specific objectives are (1) to determine if alterations in coding potential of FeLV myc contribute to or are responsible for neoplastic potential, and (2) to determine the mechanism of T-cell tumor tropism displayed by the FeLV-myc provirus.

Methods Employed:

The following techniques were employed: (1) genomic molecular cloning in vectors, (2) DNA-mediated transfection, (3) cDNA library construction, (4) SV40 chloramphenicol acetyl transferase (CAT) assays to measure promoter/enhancer activity, (5) DNA sequence analysis using the Sanger shotgun technique and (6) Southern blotting and mapping of eukaryotic genes.

Major Findings:

An intensive screening by Southern blot hybridization for onc gene rearrangements in 35 feline lymphoid tumors has yielded one c-myc positive tumor. Further characterization of this aberrant feline myc gene showed that it was transduced into FeLV yielding the FeLV-myc provirus. This provirus was isolated from a T-cell lymphoma, and following our report two other laboratories have isolated defective myc containing feline proviruses.

1. Determination of the nucleotide sequence of FeLV-myc. The nucleotide sequence of the myc portion of FeLV-myc has been determined in order to measure the coding sequence status of this new virus. The results show that feline v-myc is very similar to human c-myc and that myc was transduced into the 3' coding portion of p30 and the 5' portion of env. The entire pol gene was deleted in the process of transduction. Further comparison of feline v-myc with human and mouse c-myc shows that both feline and chicken v-myc have lost potential phosphorylation sites in the putative DNA binding region of exon 3. If these sites are phosphorylated in normal c-myc, their loss may alter the affinity of the v-myc protein with DNA. Increased binding affinity of the v-myc protein with a specific promoter could increase the transcription of a number of genes that are controlled by myc.

An additional striking feature is the DNA site specificity of recombination that appears at the 5' and 3' transduction junctions between the parent FeLV provirus and v-myc. The 5' junction of v-myc appears to be within a few nucleotides of the 5' junction of v-fes. More importantly, the viral hexanucleotide CTC CTC is

found immediately 3' to the v-myc gene in our provirus and immediately 3' of the v-fes gene in two feline sarcoma isolates. Together, these data strongly support the notion that, in many cases, site specific recombination can occur in the process of oncogene transduction.

2. The FeLV-myc LTR displays a cell type specific preference for maximal activity. This DNA sequence information stated above provides a sound framework with which to assess the significance of loss of a potential phosphorylation site. However, ultimately the transformed phenotype will depend upon transcriptional, as well as translational activity of the FeLV-myc provirus. One unique feature of our FeLV-myc isolate is that it was obtained from a tumor of T-cell origin. Since FeLV itself is known to infect a wide range of cell types both in vitro and in vivo, it is not unexpected that a T-cell tumor arose. In contrast myc involved transformation in human, mouse and chicken is confined to hemopoietic cells of the B lineage. The question to address in this study is to ask if their is a molecular basis for the appearance of a tumor of T-cell origin.

Since long terminal repeats (LTR) are the transcriptional regulating elements of retroviral proviruses, we concentrated our efforts on the evaluation of the functional level of transcription of the FeLV-myc LTR. The FeLV-myc LTR was linked to the reporter function, the bacterial CAT gene, and for transient expression in different cell types. As a control for specificity, we used the Gardner-Arnstein (GA) FeLV-LTR and the Rous sarcoma virus (RSV) LTR similarly linked to CAT. Our transfection experiments indicate that the FeLV-myc LTR is five times more powerful than the control GA FeLV LTR on T-cells. Stated differently, the FeLV-myc LTR is 31 times more powerful on T-cells than the RSV LTR. These results show that the feline LTRs in general, whether GA FeLV or FeLV-myc, are potent transcriptional elements in cells of lymphoid origin (T- or B-cells) and relatively poor promotor units on fibroblast. Most importantly, the FeLV-myc LTR is even more powerful than the GA LTR on T-cells, the tumor cell type from which it was isolated. The fivefold higher leveled transcription in T-cells could be an essential prerequisite for transformation.

3. A 44 bp duplication is responsible for T-cell preference by FeLV-myc. In order to precisely evaluate the mode in which the FeLV-myc LTR displays cell type transcriptional preference, we have determined its DNA sequence. Comparison of the DNA sequence of FeLV-myc with the GA FeLV LTR shows that the only major differences between these two feline LTRs is a duplication of 44 bp in the U3 region of the FeLV-myc isolate. It is striking that a simple duplication of a short sequence could result in a fivefold elevation in transcriptional activation on T-cells. The core enhancer sequence that is present in most retroviral and cellular enhancers is located within the 44 bp FeLV-myc duplication. These results indicate that the duplication is the major rearrangement responsible for the high levels of transcription of FeLV-myc on T-cells and suggest that T-cells contain higher levels of transacting factor that recognize the 44 bp repeat.

Significance to Biomedical Research and the Program of the Institute:

The correlation of retroviral oncogene structure with biological function is of paramount importance to the understanding of the mechanisms of neoplastic transformation. Retroviruses have been, and continue to be, the best system with which to dissect the numerous events that transpire between infection and tumor

formation. The technology for cataloging transforming events is at hand. Once these processes are fully understood, we can systematically begin to design new approaches to intervene in neoplastic progression.

Proposed Course:

We will evaluate the significance of alteration of the phosphorylation site in FeLV-*myc* by site-directed mutagenesis. Most importantly, we will further characterize the LTR rearrangements that lead to cell type-tumor specificity. With this information, we can re-evaluate the in vitro transforming ability of *myc* and quantitate the level of transcription necessary to effect transformation.

Publications:

Braun, M. J., Deininger, P. L. and Casey, J. W.: Nucleotide sequence of a transduced *myc* gene from a defective feline leukemia provirus. *J. Virol.* (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05389-02 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Development of Reproductive-Endocrine-Genetic Strategies in Animal Species

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

PI:	Stephen J. O'Brien	Geneticist	LVC	NCI
Others:	David E. Wildt	Guest Researcher	LVC	NCI
	Jo Gayle Howard	Biologist	LVC	NCI
	David H. Sachs	Chief	IB	NCI

COOPERATING UNITS (if any)

Department of Animal Health, National Zoological Park, Washington, DC (M. Bush); Veterinary Resources Branch, DRS, NIH, Bethesda, MD (P. M. Schmidt, K. L. Goodrowe, M. C. Schiewe)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	0.7	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 objective of this program is to increase the reproductive-endocrine-genetic data base of nondomesticated, wildlife species. Program approaches emphasize investigation of basic reproductive-endocrine-genetic factors which appear as the most critical prerequisites to the application of artificial breeding strategies. A multidisciplinary approach targeted toward female and male reproduction and genetics is employed. The use of domestic animal models permits testing the efficacy of research concepts before adaptation to rarer, nondomestic species. Areas of effort in the female include hormonal evaluation for correlation of endocrine profiles of pituitary-ovarian function and as an index of the influence of manipulative stress; ovulation induction through the administration of exogenous gonadotropins to optimize the timing of the ovulatory event; and embryo collection, culture, freezing and transfer as techniques for cryobanking genetic stock, improving reproductive potential and eventually allowing microinjection of molecularly cloned genes which participate in transformation and inborn errors. Particular emphasis has been applied to the collection, in vitro culture, freezing, and micromanipulation of embryos of mouse, cat and miniature swine, animal models for rare species and the study of human disease. Areas of effort in the male include semen collection and evaluation to characterize ejaculate norms and correlate these findings to the level of genetic polymorphism in wildlife populations; semen handling and cryopreservation to increase spermatozoal viability and establish optimal methods for chronic storage of genetic material; and hormonal evaluation to improve the understanding of pituitary-gonadal-adrenal relationships with particular emphasis on the marked differences in stress responses among taxonomically related wildlife species. Together, these generalized research concepts permit rapid expansion of physiological-genetic norms for rare species, and also improve methods of assessing fertility potential or genetic status to optimize management efforts for selective natural propagation.

PROJECT DESCRIPTION

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

Stephen J. O'Brien	Geneticist	LVC	NCI
David E. Wildt	Guest Researcher	LVC	NCI
Jo Gayle Howard	Biologist	LVC	NCI
David H. Sachs	Chief	IB	NCI

Objectives:

To increase and integrate the reproductive-endocrine-genetic data base of domestic and nondomestic species, thereby permitting improved propagation and embryological manipulation through natural or artificial breeding or altered approaches to captive animal management.

Methods Employed:

The following techniques were employed: (1) radioimmunoassay for analyses of hormone concentrations in blood, (2) surgical laparoscopy for direct examination of ovarian activity and the atraumatic transfer of embryos, (3) hormonal induction of ovulation and sexual behavior using gonadotropic therapy, (4) surgical procedures for embryo recovery and transfer, (5) programmable freezing for cryopreserving embryos, (6) electroejaculation for semen collection, (7) light and electron microscopy for evaluation of spermatozoal viability and structural integrity, (8) dry ice-pelleting procedures for spermatozoal freezing, (9) in vitro establishment of primary cultures from embryos and skin biopsies, and (10) biochemical genetic procedures for determining extent and character of genetic variation in populations and for monitoring of paternity and maternity exclusion in embryo transfers.

Major Findings:

Research strategies in 1984 were targeted toward female and male reproduction/endocrinology, involving both domestic and nondomestic species. A general overview of major research progress follows:

1. Estrous synchronization and hormonal induction of ovulation. A prerequisite to successful artificial insemination or embryo transfer is the effective inducement of estrous and accurate timing of ovulation. Hormonally controlling estrus and stimulating ovulation frequently results in highly variable responses in domestic species. Consequently, considerable variation also is expected after similar treatment of nondomestic animals. Data collected from domestic species continues to be extrapolated for developing hormonal treatments for wildlife species. Vaginal progestogen-treated pessaries were found to be highly effective for synchronizing sexual receptivity in domestic sheep. Simultaneous studies, however, demonstrated that residual hormone release from the pessary severely retarded sperm transport, thereby reducing fertilization rate. In the sheep, this problem was eliminated by developing a laparoscopic transabdominal insemination approach for direct deposition of fresh or frozen-thawed sperm into the uterine horn allowing the production of both embryos and live-born offspring.

Estrus synchronization efforts also continued using an orally administered synthetic progestogen allyl-trenbolone. This product was tested exclusively in the inbred, minature pig, a valuable NIH model used in tissue transplantation studies. Of particular significance was the finding that estrous intervals induced by withdrawal of allyl-trenbolone administration were fertile, allowing the recovery of viable embryos at six days post-mating.

Gonadotropin regimens continued to be evaluated in several species for effectiveness in stimulating sexual behavior and ovarian activity. Past studies demonstrated considerable variability in behavioral-ovarian response to treatment even among taxonomically related species. Of particular significance was the finding that ovaries of Felidae appear hypersensitive to an excessive dosage of gonadotropin given in a single or infrequent injection sequence. Currently, this problem is receiving major attention with studies in progress to evaluate the effectiveness of low-dosage, frequent injections of follicle stimulating hormone (FSH). Animal models in use include the domestic cat and leopard cat (*Felis bengalensis*). In related studies, the domestic sheep was used in a comparative investigation to evaluate ovarian activity after treatment with various gonadotropic drugs including pregnant mares' serum gonadotropin (PMSG), human menopausal gonadotropin (hMG) and FSH. Response was assessed based on laparoscopic observations of ovarian morphology and embryo quality after artificial insemination. Results indicated that both FSH and hMG gave comparable superovulatory responses with no effect on fertility or embryo quality. An inferior response resulted from the use of PMSG.

2. Embryo collection, culture, freezing and transfer. Embryo techniques, similar to those extensively used in domestic farm stock, could play a valuable role in propagating selected rare species. Of particular importance is the potential of freeze-preserving embryos thereby assuring maintenance of rare gene pools or providing a means of incorporating new genetic material into captive populations with limited genotypic diversity.

In 1984, the program achieved its first embryo recovery in a nondomestic species, the Scimitar-horned oryx. Two high quality embryos were retrieved nonsurgically from a hormonally synchronized and stimulated oryx. Using a novel laproscopic, transabdominal approach, both embryos were transferred into the uterine horn of a hormonally-treated oryx recipient. The latter gave birth to a normal, healthy calf on April 18, 1985.

Comprehensive basic and applied embryo studies also were conducted in a number of domestic animal models. A major collaborative effort continued with the Veterinary Resources Branch of the NIH in continuing an embryo cryopreservation bank for numerous genotypes of inbred and outbred laboratory mice. In 1984, more than 33,000 mouse embryos were collected, 10,000 of these embryos being permanently banked as an invaluable resource and the remainder used in comparative biomedical studies. Major findings in the past year included the determination that genotypic variation, even within a species, plays a major role in the ability of embryos to survive a freezing stress. The vulnerability of embryos to environmental factors was indicated in a study demonstrating that ethylene oxide (a common gas sterilant) has toxic effects on culture rates of embryos in vitro. Also of significance was a comprehensive investigation to elucidate optimal methods for cryopreserving mouse embryos, considering the influence of various cryoprotectants, storage containers, freezer units and freezing procedures. At present, and

depending on genotype, the use of glycerol as the cryoprotectant, ampules for storage and a freezing rate of 0.5°C/min to -80°C before liquid nitrogen storage consistently permits 60 to 85% of all embryos to survive freezing. Current efforts emphasize determining the influence of recipient female genotype on the ability of thawed and transferred embryos to result in live offspring.

Related embryo studies involved the miniature pig, domestic cat and sheep. Of particular importance was the production of live offspring following <u>in utero transfer of miniature pig</u> embryos to standard-sized pig surrogate mothers. Extensive studies to improve embryo techniques in cats continued and were partially successful based on the birth of a litter of kittens after embryo transfer. Even so, this species presented numerous challenges necessitating implementation of <u>in vitro fertilization</u> attempts as an alternative resource for feline embryos. By the end of the year, eight laparoscopically recovered oocytes had cleaved in culture after being inseminated with electroejaculated spermatozoa. In contrast to the cat, sheep embryos were readily available for study due, in part, to more effective superovulation treatments. Live offspring were produced following transfer of fresh embryos to recipients using a laparoscopic transabdominal approach. Subsequent studies concentrated on adapting embryo freezing technology developed in the mouse to the sheep. A comparative study was conducted to determine optimal embryo plunge temperatures into liquid nitrogen, as well as to assess the cryoprotectant ability of glycerol versus propylene glycol. Greatest embryo survivability was detected using the latter reagent when embryos were cooled to at least -30°C before transfer to liquid nitrogen. These embryos were viable; five sheep pregnancies were confirmed from transferring thawed sheep embryos.

3. <u>Hormonal evaluations and the influence of stress</u>. Specific radioimmunologic analyses of blood sera can be used to determine circulating concentrations of numerous protein and steroid hormones. Hormonal information allows the plotting of endocrine profiles which serve as indices of normal or abnormal pituitary-gonadal-adrenal function. These data provide information on duration of reproductive cycles and the influence of environmental factors, including stress, on reproductive-endocrine function. Because many species of wildlife are presumed highly susceptible to stress, it is critical that manipulations imposed on animals (anesthesia, electroejaculation, laparoscopy and laparotomy) be evaluated for acute and chronic effects on general animal health, including reproductive performance. To date, extensive data available on 20 wildlife species (males and females) demonstrated stereotypic adrenal responses among species, even those closely related taxonomically. In general, blood concentrations of glucocorticoids were closely correlated with behavioral aggressiveness of the species, but were not always associated with acute episodic release of pituitary or gonadal hormones. <u>Electroejaculation increased serum cortisol</u> concentrations in the cheetah without affecting either luteinizing (LH) hormone or testosterone secretion. In contrast, elevated cortisol in similarly treated leopards caused an apparent decline in both LH and testosterone. Many female nondomestic species react differently to manipulatory stress and appear particularly sensitive to variations in type and dosage of anesthetic. For example, cortisol levels in cheetahs subjected to laparoscopy are markedly accentuated when using a combination anesthetic consisting of ketamine hydrochloride and halothane gas rather than ketamine HCl alone.

In 1984, adrenal-gonadal hormone studies were expanded into evaluation of wildlife species free-ranging in Kruger National Park. Serial blood samples were collected from elephant, greater kudu, giraffe, wildebeest and lion subjected to specific anesthetic regimens and reproductive manipulations, such as electroejaculation and hormonal challenges with gonadotropin-releasing hormone or adrenal corticotropin hormone. Sera were transported to the U.S. and will be analyzed for adrenal pituitary and gonadal hormone concentrations. Important comparative information will result concerning endocrine function in free-ranging animals compared to captive counterparts. These data also will assist the clinical staff in choosing anesthetic drugs for minimizing manipulatory stress in captive zoo animals requiring immobilization for research or health reasons.

4. Spermatozoal evaluation, freeze-preservation and handling. Basic and applied semen studies continued to emphasize establishing ejaculate norms for many nondomestic species, both captive zoo animals and those free-ranging in the Kruger National Park. Considerable efforts were made to expand observations made that certain wild species of Felidae produce ejaculates with high proportions of abnormal sperm cells. In 1984, 74 individuals representing 21 species were evaluated for standard ejaculate traits, including structural integrity of the spermatozoon. Combining results from previous efforts demonstrated that 20 of the 28 species of Felidae examined averaged greater than 36% pleiomorphic spermatozoa/ ejaculate. Current efforts emphasize evaluating potential biological or environmental factors (genetic variation, stress, mating patterns) for their correlative influence on sperm characteristics. Of similar importance is the relationship between spermatozoal morphology and fertility potential.

Past studies have demonstrated that wildlife spermatozoa frequently react differently to in vitro handling methods compared to domestic animal counterparts. Spermatozoal viability, in particular, declines markedly after collection, sperm usually dying within one to three hours of collection. Considerable progress was made in increasing in vitro longevity by conducting comparative studies to dilute and then separate damaging factors within seminal fluid. To date, the use of Biggars, Stern, Whittingham medium plus 5% heparin followed by low speed centrifugation (1000xg) resulted in domestic cat spermatozoa surviving for as long as 24 hours at room temperature.

Strategies also continued for establishing optimal methods for cryobanking spermatozoa. This concept was developed primarily with the realization that marked differences exist in post-thaw sperm survival when comparing various cryoprotective diluents. The Kruger Park field study provided an excellent opportunity for further testing of semen freezing procedures. Ejaculates collected from elephant, greater kudu and wildebeest were extended with seven different diluents, frozen, thawed and evaluated for spermatozoal motility, longevity in vitro and acrosomal integrity. Optimal diluents were determined for each species. Additional comparative techniques in the elephant demonstrated that the spermatozoa of this species have greater post-thaw survival when the cooling rate is increased during the equilibration interval. To date, comparative cryopreservation studies have resulted in recommendations for banking spermatozoa from seven nondomestic species.

Significance to Biomedical Research and the Program of the Institute:

A sophisticated understanding of the reproductive physiology of domestic animals has evolved only over the past several decades. Related technical advances in this field have permitted propagation through artificial insemination and/or embryo transfer using fresh or frozen-thawed gametic or embryonic material. More recent studies in genetic engineering and embryo macromanipulation have generated additional direction for improved reproductive efficiency in domestic and laboratory animals. Program strategies in progress are designed to methodically solve selected fertility-genetic problems, as well as to establish a reliable data base for organizing innovative propagation-management approaches.

Detailed information on reproductive physiology or molecular genetics is available for only a few wildlife species. It is both reasonable and timely to adapt biomedical technologies now routinely available and effective in human and laboratory/domestic animals to endangered species. These methods permit the collection of data of eventual import to the natural and artificial propagation and management of nondomestic animals. Additionally, the evolution of these results provides continued mutual benefit to the NCI-NIH program goals through ongoing parallel research using domestic animal systems. For example, to develop embryo transfer/embryo freezing capabilities in wildlife species, initial research efforts emphasize use of the laboratory mouse because of its elaborate background in gamete physiology. Technology now has been formulated which permits the recovery of viable mouse embryos and offspring following freezing and liquid nitrogen storage. These methods now are being used to cryobank the multitude of invaluable, murine genetic stocks at the NIH, thus offering biological insurance against genetic drift or natural catastrophe. Interest in developing embryo transfer capabilities in wild species of Felidae and ungulates has concentrated on use of domestic cats and sheep model systems. Experiments with embryo collection and culture and handling procedures in these species, particularly micromanipulation at the cellular level, may eventually permit the microinjection of molecularly cloned genes into appropriately staged embryos which might then be transferred into recipient females. The experimental tracking of the fate of the gene implants would be the beginning of possible gene therapy for genetic diseases and cancer in an animal model system and ultimately in genetically crippled human tissues including neoplastic derivatives.

Proposed Course:

Efforts are scheduled to continue and expand several program areas. Particular emphases will be placed on a comprehensive, comparative evaluation of thawing rates and alternative thawing media for frozen mouse embryos, as well as comparing genotypes of mice designated as optimal embryo recipients; improving technology to permit routine in vitro fertilization and culture of cat oocytes; initiating pilot studies concerning gene injection into mouse embryos and the feasibility of embryo splitting using the mouse and sheep model; adapting embryo transfer technology as developed for sheep to a species of nondomestic hoofed stock; evaluating ejaculate endocrine characteristics of free-ranging species, especially Felidae.

Publications:

Carter, K. K., Chakraborty, P. K., Bush, M. and Wildt, D. E.: Effects of electroejaculation and ketamine-HCL on serum cortisol, progesterone and testosterone in the male cat. J. Androl. 6: 431-437, 1984.

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Schmidt, P. M., Hansen, C. T., Brown, S. S. and Wildt, D. E.: Comparative observations on superovulation and embryo cryopreservation from outbred and inbred mice. In Wagner, W. (Ed.): Proceedings X International Congress on Animal Reproduction and Artificial Insemination. Champaign, University of Illinois, 1984, Vol. 2, pp. 240-241.

Wildt, D. E. and Bush, M.: Prerequisites to artificial breeding: Research approaches deserving high priority. In Fowler, M. (Ed.): Proceedings American Association of Zoo Veterinarians. Davis, American Association of Zoo Veterinarians, 1984, Vol. 199, pp. 163-164.

Wildt, D. E. and Bush, M.: Reproductive physiology studies in zoological species: Concerns and strategies. Zoo Biol. 3: 363-372, 1984.

Wildt, D. E., Chakraborty, P. K., Meltzer, D. and Bush, M.: Pituitary and gonadal response to luteinizing hormone releasing hormone administration in the female and male cheetah. J. Endocrinol. 101: 51-56, 1984.

Wildt, D. E., Howard, J. G., de Vos, V., Chakraborty, P. K. and Bush, M.: Ejaculate characteristics comparative semen freezing and adrenal-testicular relationships in free-ranging African elephants. In Ewing, L. L. (Ed.): Proceedings Society for the Study of Reproduction and Biology Reproduction. Champaign, Society for the Study of Reproduction and Biology Reproduction, 1984, Suppl. 1, Vol. 30, p. 143.

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Wildt, D. E., Montali, R. J. and Bush, M.: Strategies for reproductive physiology studies at the National Zoological Park. In Ippen, R. (Ed.): Proceedings International Symposium for the Diseases of Zoo and Wild Animals. Berlin, Academi der Wissenschaften der DDR, 1984, Vol. 26, pp. 7-11.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05401-01 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Molecular Basics of Retroviral Transcriptional Transactivation

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

PI:	James W. Casey	Senior Staff Fellow	LVC	NCI
Others:	David D. Derse	Guest Researcher	LVC	NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (M. A. Gonda); Litton Bionetics Inc., Frederick, MD (N. R. Rice); Louisiana State University, Baton Rouge, LA (R. Montalaro)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.4	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 bovine leukemia virus (BLV) belongs to a unique class of retroviruses like HTLV-I, II or III in which productive infection is highly restricted both in vivo and in vitro. To examine the molecular basis for the control of BLV expression, we excised the LTRs from cloned proviruses and fused them to the bacterial chloramphenicol acetyltransferase (CAT) gene. Plasmids carrying the CAT gene controlled by the entire BLV LTR or partially deleted LTRs were introduced into a variety of cells. The BLV LTR was inactive as a promoter of transcription in all cell lines tested except those previously established as BLV producers (e.g., FLK-BLV cells). Transcriptional regulatory sequences in the LTR were first identified by deletion mapping. We found that levels of CAT activity in FLK-BLV cells were reduced by 90% when LTR sequences located 100 bp to 170 bp upstream of the CAP site were deleted. Furthermore, removal of LTR sequences downstream of the RNA start site reduced CAT expression by 87%. In further experiments, a 75 bp LTR subfragment encompassing the region 100 bp to 170 bp 5' of the RNA CAP site was cloned into pSV E cat (a pSV2 derivative lacking the 72 bp repeats and requiring "enhancers" for efficient CAT expression). Insertion of the BLV 75 bp fragment into pSV E cat resulted in high level CAT expression only in BLV producer cells. This specific enhancement was orientation but not position independent. A 250 bp fragment containing the long R region of the LTR also activated CAT expression from the heterologous promoter, but only when located immediately 3' of the RNA start site. This activation was not cell-specific and was relatively orientation independent. Thus, the BLV LTR appears to possess two independent elements regulating gene expression. Additionally, experiments have been initiated to understand the mode of gene regulation and antigenic drift of the equine infectious anemia virus (EIAV). Full-length clones of EIAV have been hybridized with HTLV-III and found to be genetically related. Furthermore, DNA sequence analysis shows that the relatedness of EIAV and HTLV-III is substantial, especially in the pol region of EIAV.

PROJECT DESCRIPTION

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

James W. Casey	Senior Staff Fellow	LVC	NCI
David D. Derse	Guest Researcher	LVC	NCI

Objectives:

To understand the mechanism of retroviral gene regulation using bovine leukemia virus (BLV) and equine infectious anemia virus (EIAV) as models. It has long been thought that retroviruses carry all the signals for transcriptional regulation in cis. However, HTLV and BLV appear to carry additional components and to depend upon cellular factors that can complement transcription in trans. Our long-term objective is to understand this mechanism of transactivation by isolating the cellular and viral components and assemble them in an in vitro reaction.

Methods Employed:

The following techniques were employed: (1) genomic molecular cloning in vectors, (2) DNA-mediated transfection, (3) cDNA library construction, (4) SV40 chloramphenicol acetyl transferase (CAT) assays to measure promoter/enhancer activity, (5) DNA sequence analysis using the Sanger shotgun technique, and (6) Southern blotting and mapping of eukaryotic genes.

Major Findings:

1. The BLV long terminal repeat (LTR) contains two distinct regulatory units. Examination of DNA from non-productively infected cell lines showed that BLV provirus formation occurred. These results indicate that the level of observed restriction to productive infection does not entirely reside in envelope determinants. Further analysis of BLV non-productively infected cell lines showed that no RNA transcripts could be detected at a level of sensitivity of one copy per cell. Thus, it is apparent that a block in transcription is a major cause for the lack of a productive infection. To determine the level and mechanism of BLV restriction, we have focused on the LTRs since transcriptional regulatory sequences are located in these segments. The functional activity of the promoter unit contained within the LTR of bovine leukemia virus was examined by monitoring transient expression of a heterologous gene placed under its control. Various cell lines were transfected with recombinant plasmids carrying the bacterial CAT gene coupled to the BLV LTR (pBL-CAT). Transient expression of CAT activity directed by the BLV LTR was observed in the established BLV-producer cell lines derived from FLK cells and bat lung cells, but not in a variety of other cell lines which restrict BLV expression. The amount of CAT activity transiently expressed in FLK-BLV cells was decreased approximately tenfold by deletion of LTR sequences located within a region 100 to 170 nucleotides upstream of the RNA start site. Surprisingly, removal of the region 50 base pairs downstream of the RNA initiation site to the 3'-end of the LTR also reduced the expression of CAT activity by 87%. The BLV LTR thus appears to be an unusual promoter unit, functioning

in a cell type-specific manner and possessing two regulatory sequences, one at the 5' and the other at the 3' side of the RNA start site that influence gene expression.

2. Reconstitution of the BLV cell type specificity in an SV40 promoter core. To more precisely define those sequences essential for control of transcription and most probably reactive with transacting proteins, we have dissected various BLV LTR elements and reconstituted them in an SV40 core promoter, pSV E cat (a pSV2 derivative, lacking the 72 bp repeat). This plasmid's utility lies in the fact that short BLV LTR fragments having enhancer activity will direct cat activity using the SV40 TATAA box. Results of these analyses show that a 75 bp 5' and a 250 bp 3' BLV LTR sequences exist and that they are independent of one another for enhancer function and essential for full transcriptional activity. The location of 5' and 3' BLV LTR sequences and the orientation (plus or minus polarity) must be correct to effect complete transcriptional activation, although they will function to a lesser degree in the minus orientation. The effect of enhancer distance from the RNA cap site on transcriptional activity has been assayed. Transcriptional activity diminished in a nonlinear fashion as distance from the promoter increases. The 75 bp 5' LTR regulatory region is responsive only in infected cell lines and, thus, behave as a cell type specific enhancer. This sequence must be activated by BLV gene products or cellular gene products which themselves are activated by BLV. The 250 bp 3' regulatory region is located entirely within the large R region and is part of a nontranscribed leader. This region is operative in a non-cell type specific manner and will also stimulate SV-40 transcription fourfold. The fact that this sequence enhances a heterologous promotor (SV-40) is consistent with it being a gene activator and not a sequence that simply confers stability to RNA.

3. EIAV is genetically related to HTLV-III. EIAV is a retrovirus which induces anemia in a multi-episode manner and can ultimately compromise the host. These episodes result in generation of apparently altered viruses which induce new immune responses. The changes that occur in EIAV during progression of disease are not entirely clear, although there is evidence that envelope sequences are mutated. To more fully understand this antigenic drift and to correlate DNA changed with pathogenesis, the EIAV has been cloned from the EIAV-infected equine kidney cell line (Wyoming strain). Four proviruses were obtained and all appear to be full-length, since both LTRs are present. A restriction enzyme site polymorphism is present, in that three EIAV provirus are heterogenous with respect to three restriction enzyme sites. These altered sites map to the envelope region of the provirus. EIAV provirus clone 12 was heteroduplexed with HTLV-III and found to be homologous in the pol region. DNA sequence analysis substantiated this finding and indicates that the homology is extensive. Thus, EIAV-like caprine arthritis and visna virus are members of a unique family (Lentiviruses) which show extensive homology to HTLV-III.

Significance to Biomedical Research and the Program of the Institute:

It is imperative to fully understand the mechanism of BLV and EIAV gene regulation before any attempts at pathogenic intervention can be made. By isolating transacting factors and determining their origin and distribution, a framework of events can be constructed and some understanding of neoplastic initiation can be acquired.

Proposed Course:

We will continue to focus on the isolation of transacting factors and development of an in vitro assay system to examine how they activate BLV. We will examine the EIAV LTR to determine whether this virus responds to transcriptional activation by similar factors.

Publications:

Derse, D., Caradonna, S. J. and Casey, J. W.: Bovine leukemia virus long terminal repeat: A cell type-specific promoter. Science 227: 317-320, 1985.

Derse, D., Diniak, A. J., Casey, J. W. and Deininger, P. L.: Nucleotide sequence and structure of integrated bovine leukemia virus long terminal repeats. Virology 141: 162-166, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05404-01 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Growth Modulation of raf Associated Tumors

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

PI:	Ulf R. Rapp	Research Chemist	LVC	NCI
Others:	John L. Cleveland	Staff Fellow	LVC	NCI
	Gunamani Sithanandam	Guest Researcher	LVC	NCI

COOPERATING UNITS *(if any)*

Litton Bionetics, Inc., Frederick, MD (A. M. Schultz)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	0.9	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.)*

A mouse model system was established for the rapid induction of tumors in tissues that are also being transformed by raf or raf/myc oncogene carrying retroviruses. Tumor induction involved transplacental inoculation of mice at day 16 of pregnancy with ethylnitrosourea (ENU) followed by promotion with butylated hydroxytoluene (BHT). Ninety percent of the animals treated with initiator (ENU) and promoter (BHT) developed tumors within 5 to 14 weeks. In the absence of promotion, 40% of the animals developed tumors with latency of 9 to 25 weeks. raf oncogene expression in cell lines established from tumor tissues (lung adenocarcinoma and lymphomas) were initially determined by indirect immunofluorescence, and both tumor types were found to be positive. In preliminary experiments it was observed that vaccination with v-raf protein doubled the life expectancy of mice treated with both ENU and BHT. A significant increase in disease latency upon vaccination was also observed with mice treated with ENU only.

PROJECT DESCRIPTION

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

Ulf R. Rapp	Research Chemist	LVC	NCI
John L. Cleveland	Staff Fellow	LVC	NCI
Gunamani Sithanandam	Guest Researcher	LVC	NCI

Objectives:

To develop regimens for prevention and/or reversal of neoplasms involving raf oncogenes. In order to increase evidence of tumor induction, NIS (female) marked with AKR (male) were used for transplacental inoculation.

Methods Employed:

For induction of lymphomas and lung adenocarcinoma in mice, pregnant females were injected transplacentally with ethylnitrosourea (ENU). To accelerate tumor development in the offspring, weanling age F1 mice were promoted with weekly injections of butylated hydroxytoluene (BHT). Vaccination of newborn mice with oncogene protein followed procedures previously developed for vaccination of high leukemia strains of mice with retroviral structural proteins.

Major Findings:

Ninety percent of the animals treated with both initiator (ENU) and promoter (BHT) developed tumors within 5 to 14 weeks. In the absence of promotion, 40% of the animals developed tumors with a latency of 9 to 25 weeks. The purpose of developing this rapid tumor induction model was to mimick development of tumor types that can also be induced by raf transducing viruses so as to allow us to test for involvement of endogenous raf and other proto-oncogenes in the chemically induced transformation process. At the same time, we intend to employ reagents that show growth inhibitory effects specifically on v-raf transformed cells in vitro for possible modulation of tumor development in vivo.

One possible modulation that we considered was the animals' immune response to the product of oncogenes that may be involved in the development of tumor cells. We, therefore, vaccinated treated and control mice with v-raf protein. In initial experiments with 150 mice, a fairly dramatic effect of this treatment was observed. In the group where animals had been treated with BHT, a doubling of the latency period for development of the earliest tumor was observed. Mice that had only been treated with ENU before vaccination showed an 80% increase in latency. This delay in the onset of tumor development upon vaccination was transient. A comparable incidence of lung adenocarcinoma and lymphoma was observed in treated and control groups 20 weeks after treatment.

Significance to Biomedical Research and the Program of the Institute:

A number of human cancers have been shown to be associated with altered expression and/or structure of certain proto-oncogenes. In the case of raf oncogenes,

altered expression of c-raf-1 appears to be associated with certain lung carcinoma and lymphoma. We expect that treatment regimens, such as the vaccination with oncogene protein, once their efficacy has been established in the animal model systems, will be directly applicable in a clinical setting and may aid the prevention and/or treatment of human cancer. Moreover, a screening for antibodies to oncogene proteins in human sera may lead to the development of diagnostic tests with potential importance for detection and treatment-monitoring of specific tumors.

Proposed Course:

The humoral and cellular immune response to raf oncogene protein and raf protein-expressing tumor cells will be determined. The ENU + BHT induced tumors will be examined for expression of raf and other oncogene products. Depending on these results and especially if other oncogenes appear to be involved which have a transmembrane sequence, their protein products will be included in a cocktail vaccine.

Moreover, we would like to initiate, as an extension of the above described observations, a program for the testing of antibody responses to raf and other oncogene proteins in human lung cancer patients. The bases for this undertaking are (1) the cytogenetic evidence implicating the raf oncogene in human small cell lung carcinoma; (2) our finding using immunocytochemical techniques that 50% of human lung cancer of all histological types express high levels of raf proteins; (3) our ongoing research which suggests that transforming raf proteins may differ from normal raf proteins and provides us with a potential biochemical marker for transformation by this oncogene; and (4) the fact that we have generated, during the past year, a panel of biochemical and immunological reagents for the detection of mouse and human raf proteins and anti-raf antibodies.

We hope to be able to perform screening of human tumor material by RIA and of human sera by Elisa for raf protein and raf AB. We feel that the proposed project is highly significant and timely, and may represent the first of its kind. We are in a position now to do such experiments for the raf oncogene because of our previous investment in the generation of raf specific reagents. We have also made arrangements with Hoffman La Roche to provide us with ras proteins for similar studies in our new animal model, and possibly, in man.

Other factors such as growth factors, interferons and tumor promotion will also be tested for their specific inhibitory effect on raf oncogene transformed cells in vitro.

Such factors will subsequently be included in animal experiments in order to test their potential for modulating the growth of raf oncogene involving tumors.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05414-02 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Etiology and Prevention of Simian Acquired Immune Deficiency Syndrome (SAIDS)

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:	Kurt J. Stromberg	Medical Director	LVC	NCI
	Francis W. Ruscetti	Senior Investigator	LMI	NCI

COOPERATING UNITS *(if any)* Univ. Washington Regional Primate Res. Center, WA (W. Giddens, W. Morton, H. Ochs, C. Tsai); Univ. Wisconsin Primate Res. Center, WI (W. Bridson); LBI, Frederick, MD (L. Henderson, S. Oroszlan); PRI, Frederick, MD (L. Arthur); Univ. Alabama, AL (E. Hunter); Fairfax Hospital, Fairfax, VA (L. Eron, D. Poretz)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	0.8	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.)*

At various primate research centers, several macaque species show an acquired immune deficiency syndrome (simian AIDS, SAIDS) characterized by lymphocytopenia, opportunistic infections, and a fibromatosis tumor (RF). Numerous type D retroviruses (designated SAIDS-D/Washington (SAIDS-D/W) have been isolated from several macaque species by cocultivation of tissues and blood with lymphocyte and monolayer cultures. These isolates morphologically transform certain rodent cell lines. The SAIDS-D/W isolates can be distinguished from all other retroviruses by antigenicity and molecular hybridization, including the related type D viruses isolated from the California and New England Primate Centers, as well as Mason-Pfizer monkey virus (MPMV) and langur monkey type D viruses.

Six structural proteins from SAIDS-D/W and MPMV have been purified and sequenced; these include the gag proteins p4, p10, p12, p14, p27 and a phosphoprotein designated pp18 for MPMV and pp20 for SAIDS-D/W. The gag proteins of these two viruses are distinct but share a high degree of amino acid sequence homology. The phosphoproteins differ entirely in amino acid composition and molecular weight. The N-terminal portion of SAIDS-D/W pp20 shows an unexpected homology to a 28 residue segment of the env precursor polyprotein of Rous sarcoma virus.

Epidemiological studies on approximately 25% of the Washington colony revealed that 57% of the macaques have antibodies to SAIDS-D and 2% to HTLV-I. Twenty animals with RF have SAIDS-D viral sequences in their tumor tissue and SAIDS-D virus has been isolated from all of these animals. Biologically cloned SAIDS-D virus has been inoculated into 19 macaques; four young animals have developed viremia and RF, and another eight animals have developed high antibody titers to various viral proteins. These antibodies neutralize the SAIDS-D virus but not a macaque type C virus. Various antiviral regimens and vaccination protocols are being assessed in an attempt to prevent this disease.

PROJECT DESCRIPTION

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

Raoul E. Benveniste	Medical Officer	LVC	NCI
Kurt J. Stromberg	Medical Director	LVC	NCI
Francis W. Ruscetti	Senior Investigator	LMI	NCI

Objectives:

To identify and characterize the causative agent of simian acquired immune deficiency syndrome (SAIDS) and to propagate the virus to high titers in various cell lines. To examine, by molecular hybridization techniques, the nucleic acid sequence homology between this virus and other primate retroviruses. Biological and molecular clones and a restriction map of the isolate will be obtained. The viral proteins will be purified and antisera to the individual proteins (as well as to the whole virus) will be raised in rabbits in order to develop specific imunological assays.

To examine the prevalence of the SAIDS viral isolate and of any other lymphotropic viruses in the University of Washington primate colony and to correlate clinical status and necropsy reports on the primates with viremia and the presence of viral antibodies. To determine whether the SAIDS-D isolate, including biological and molecular clones, can cause the disease when inoculated into macaques. To prevent SAIDS by vaccination of animals with disrupted virus or purified viral proteins. To determine the pathogenicity of other type D viruses related to the SAIDS isolate, such as Mason-Pfizer monkey virus (MPMV) and the langur type D retroviruses.

To investigate the association of lymphotropic retroviruses and antibodies in human AIDS, as well as the association between other retroviruses and multiple sclerosis and non-A, non-B-hepatitis.

Methods Employed:

Virus isolation was attempted using cell lines that have previously been employed for the isolation of other primate and human retroviruses. Fresh tumors, whole blood or sera obtained from macaques were cocultivated with various cells and the supernatant was assayed weekly to detect reverse transcriptase activity. Molecular cloning was performed with a long terminal repeat (LTR)-gag clone obtained from a related virus. Cloned retroviral DNA will be used as a probe for detection of related DNA sequences in primates. Viral proteins were purified by high pressure liquid chromatography (HPLC) and SDS-gels. Antigens and antibodies were detected by radioimmunoassays developed to the purified SAIDS-D viral proteins.

Major Findings:

1. Isolation of a novel, type D retrovirus from fibromas of macaques with SAIDS and fibromatosis. An acquired immunodeficiency syndrome, similar in certain respects to AIDS, has been observed in macaques at the University of Washington

Regional Primate Research Center (RPRC). Various macaque species show persistent diarrhea, weight loss, anemia, lymphocytopenia, unusual chronic infections (noma and cryptosporidiosis) and a fibromatous tumor termed "retroperitoneal fibromatosis" (RF). This latter tumor often remains localized to the peritoneum, but in over 25% of the cases, it progresses to involve the entire abdominal cavity; a cutaneous form of RF has also been described. Peripheral blood mononuclear cells obtained from monkeys with RF show decreased responses to mitogens as compared to controls and a markedly depressed primary antibody response.

The original SAIDS-D/Washington (SAIDS-D/W) retrovirus was isolated from the RF tissue of an immunodeficient rhesus monkey by cocultivation with a variety of mammalian cell lines known to support the growth of primate viruses. Related viruses have now been isolated from 34 animals (with RF tumors and/or SAIDS) belonging to various species of macaques (*M. nemestrina*, *M. fuscata*, *M. fascicularis* and *M. mulatta*).

2. Characterization of retroviruses isolated from macaques with SAIDS. The SAIDS-D isolates replicate well in a variety of mammalian fibroblasts, as well as in human and primate lymphocytes. The cells that support SAIDS-D virus growth remain fibroblastic with no evidence of transformation. However, after infection with biologically cloned SAIDS-D virus, a rat cell line chosen for its flat morphology, showed foci of proliferating cells that grew well in soft agar. Sixteen transformed single cell clones of varying morphology have been obtained. These clones grow readily in soft agar, exhibit a transformed phenotype and are not producing virus. Cell extracts do not contain the major viral core antigen (p27), although they contain nucleic acid sequences that hybridize to a SAIDS-D viral probe. These sequences are being tested for the presence of oncogenes.

SAIDS-D virus is being cloned in order to obtain restriction maps and to compare this isolate with the related langur virus and MPMV, as well as to the other SAIDS isolates from the New England and California primate centers. An LTR-*gag* clone from MPMV, obtained from Dr. Eric Hunter, is being used to clone the SAIDS-D/W virus into plasmid pBR 322.

3. Purification and characterization of SAIDS-D/W viral proteins. The viral proteins from the SAIDS-D virus have been purified by HPLC on Bondapak C_{18} columns after elution with acetonitrite and propanol gradients. In collaboration with Drs. L. Henderson and S. Oroszlan, and to better establish the relationship of SAIDS-D/W virus to the prototype D-type virus, MPMV, we have purified and compared six structural proteins from each virus. The proteins purified from each D-type retrovirus include p4, p10, p12, p14, p27 and a phosphoprotein designated pp18 for MPMV and pp20 for R-D/W. Amino acid analysis and N-terminal amino acid sequence analysis show that the p4, p12, p14 and p27 proteins of R-D/W are distinct from the homologous proteins of MPMV but that these proteins from the two viruses have a high degree of amino acid sequence homology. The p10 proteins from the two viruses have similar amino acid compositions and are blocked to N-terminal Edman degradation. The phosphoproteins from the two viruses contain phosphoserine but differ in amino acid composition, molecular weight and N-terminal amino acid sequence. The data thus show that each of the R-D/W proteins examined is distinguishable from its MPMV homolog and that a major difference between these two D-type retroviruses is found in the viral phosphoproteins.

The N-terminal amino acid sequences of D-type retroviral proteins were used to search for sequence homologies between D-type and other retroviral amino acid sequences. An unexpected amino acid sequence homology was found between R-D/W pp20 (a gag protein) and a 28 residue segment of the env precursor polyprotein of Rous sarcoma virus. The N-terminal amino acid sequences of the D-type major gag protein (p27) and the nucleic acid binding protein (p14) show only limited amino acid sequence homology to functionally homologous proteins of C-type retroviruses.

4. Immunological characterization of SAIDS-D viral isolates. The purified SAIDS-D viral proteins were inoculated into rabbits in order to raise specific antisera. Competition radioimmunoassays were performed with each of the gag proteins and MPMV, langur virus and various SAIDS-D isolates from the Washington primate colony. The SAIDS-D/W isolate is related in the major core protein (p27) to MPMV, langur virus and to the isolates from the California and New England primate centers. However, SAIDS-D virus can be distinguished from all of these viruses in the p4 and p10 radioimmunoassays. In addition, the phosphoproteins of various isolates from within the colony compete only partially for SAIDS-D/W pp20, suggesting that there is a heterogeneous population of viruses present in the colony. Viruses isolated from animals with lymphoma show the greatest phosphoprotein differences. A detailed restriction map comparison of the multiple isolates will be needed to clarify this point.

5. Epidemiology of SAIDS at the University of Washington Regional Primate Center. In order to examine the correlation between the clinical status of the animals at the primate center and the presence of these retroviruses, fresh blood or tissues obtained at necropsy from animals that had RF or exhibited clinical symptoms of SAIDS (opportunistic infections, progressive weight loss, diarrhea, anemia) were cocultivated in an attempt to isolate viruses. Samples were also examined for antibodies to SAIDS-D p27, HTLV-I p24 and HTLV-III p24. The presence of virus in blood was also detected by assaying for the core viral antigens in ultracentrifuged plasma samples. Feral M. nemestrina (63 animals examined) did not contain virus or antibodies at the time they entered the colony. All 19 animals tested with RF and four with lymphoma had high titers of the SAIDS-D virus. In addition, 63% of animals with symptoms of SAIDS were also naturally infected with SAIDS-D viruses. No virus has been isolated from healthy animals, although 57% of the adult macaques in the colony have antibodies to SAIDS-D and 2% have antibodies to HTLV-I (234 animals, or approximately 25% of the colony, were tested). The SAIDS-D virus is thus endemic in this primate center, and HTLV-I virus might also be present. We are currently attempting to isolate this latter virus from these animals.

6. Inoculation of macaques with SAIDS-D/W virus. In order to establish whether the SAIDS-D viral isolate is the etiologic agent of SAIDS, 19 animals have been inoculated with biologically cloned SAIDS-D/W virus in two separate studies. All 19 animals were virus and antibody negative before the study and were inoculated intravenously with approximately 10^6 virus particles. Five animals have become viremic, one of these has died with symptoms of SAIDS and the other four have developed histologically confirmed RF. These five animals are SAIDS-D antibody negative. An additional nine animals have developed high titers of antibodies to SAIDS-D; none of them are viremic and all are presently healthy. The immune status of these animals is being investigated by Dr. H. Ochs at the University of Washington.

7. Prevention of SAIDS. Naturally occurring and experimentally induced antibodies to the SAIDS-D/W virus neutralize SAIDS-D/W virus in tissue culture but not an endogenous type C virus isolated from macaques. These antibodies are therefore probably protective and in vivo studies are being planned to determine if viremia can be controlled by inoculation with naturally occurring antibodies.

At the University of Washington RPRC, 15% of all deaths during the past three years were caused by SAIDS; 70% of these cases also have an associated RF tumor. We are attempting to limit the spread of the disease by identifying colony animals that are viremic and instituting a quarantine procedure. In addition, we will vaccinate various primates with disrupted, inactivated virus, as well as with purified proteins, in attempts to elicit protective antibody responses to subsequent challenge with live virus.

8. Association of SAIDS with other retroviruses. Although we have been able to induce RF after inoculation of young macaques with biologically cloned SAIDS-D virus, it is not clear that this class of viruses also causes a permanent immunosuppression. The identification of macaques that possess antibodies to lymphotropic viruses (HTLV-I class) suggests that these viruses are also present in the colony; we are attempting isolation in order to investigate their pathogenicity in these animals. In addition, we have recently isolated a new type D retrovirus from a rhesus monkey at the University of Wisconsin Primate Center; this virus will be compared to the other known type D retroviruses.

9. Retroviruses in human AIDS. In collaboration with Drs. Eron and Poretz at the Fairfax Hospital, we have cultured the lymphocytes and other samples from patients with frank AIDS, pre-AIDS, multiple sclerosis and non-A, non-B-hepatitis. Lymphotropic viruses (HTLV-III class) have been isolated from patients with AIDS by cocultivation with several human lymphocyte lines. The host range of these viruses on several primate lymphocyte lines is being examined and the DNA from various mammalian species is being screened for sequences related to this class of viruses.

Significance to Biomedical Research and the Program of the Institute:

The isolation of multiple novel type D retroviruses, the ability to grow the virus to high titers, and the finding that these viruses seem to be present in all animals with RF and in a large proportion of those with symptoms of SAIDS constitutes important preliminary evidence for attributing the etiology of this disease in macaques to this isolate. In addition, the induction of RF in animals inoculated with biologically cloned virus and the recovery of this virus from those animals lends further support to the hypothesis that this class of viruses is the etiologic agent of RF in macaques.

The nucleic acid hybridization data suggest that the natural reservoir for SAIDS in macaques may be Southeast Asia. Sequences partially related to the SAIDS-D virus can be found in all Old World monkey DNAs, suggesting a primate origin for this virus. The highest degree of homology to SAIDS-D viral DNA is obtained with langur DNA, which suggests that the SAIDS-D virus originated from an endogenous langur virus. Our laboratory has previously documented numerous instances of transmission of viruses between species in the same geographic area, with subsequent incorporation into the germ line. For example, the gibbon class of type

C viruses, which is tumorigenic in primates, is believed to have been derived by trans-species infection of these apes with endogenous viruses from various species of Southeast Asian rodents, such as *Mus caroli* or *Mus cervicolor*. Southeast Asia, where both macaques and langurs reside, may thus be a reservoir for the SAIDS-D virus.

It is important to establish a definite viral etiology for SAIDS, both as a model for immunosuppressive diseases and as a primate model for human AIDS. The ability to prevent SAIDS by vaccination will have important implications for the development of similar vaccines in the control of human AIDS. Even if vaccination attempts are initially unsuccessful, the identification and quarantine of animals that are viremic will help control the spread of the disease within the colony. Additionally, a population of SAIDS-free macaques can be identified as source of animals for future experiments with additional human and primate viruses.

The similarities between the immunosuppressive disease of primates (SAIDS) and human AIDS include both the clinical symptoms and various immunological parameters and tissue histology. The identification of a virus associated with SAIDS, the ability to reproduce the disease with cloned virus, and the prevention of disease by vaccination will have strong implications for the human disease and its control.

Proposed Course:

The rodent cells transformed by the SAIDS-D virus will be examined for the nucleic acid sequences and screened for oncogenes. The purified proteins and the cloned viral isolates will be used to inoculate monkeys and to develop effective vaccines against this immunodeficiency disease. The related type D viruses, MPMV and langur monkey virus, will also be inoculated into macaques. Because there may be a species specificity with respect to the manifestation of SAIDS symptoms and pathology, various species of macaques will be inoculated with the SAIDS-D isolates.

We will continue to identify those primates at the University of Washington RPRC that are viremic, contain SAIDS-D nucleic acid sequences in their tissues or have antibodies. The purpose will be to investigate the epidemiology of the disease and establish a correlation between the presence of SAIDS-D virus and the clinical condition of the animals. The primate colony will also be examined for viruses related to HTLV-I and HTLV-III.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05417-01 LVC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular and Functional Characterization of the raf Oncogene Group

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

PI:	John L. Cleveland	Staff Fellow	LVC	NCI
Other:	Ulf R. Rapp	Research Chemist	LVC	NCI
	Mahmoud Huleihel	Visiting Fellow	LVC	NCI
	Robert Nalewaik	Microbiologist	LVC	NCI

COOPERATING UNITS (if any) Laboratory of Cell Biology, NIMH, NIH, Bethesda, MD (T. I. Bonner); Program Resources, Inc., Frederick, MD (S. Kerby and M. Gunnell); Litton Bionetics, Inc., Frederick, MD (A. Schultz); Wistar Institute, Philadelphia, PA (C. Croce and K. Huebner)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

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.)

We have continued the molecular and functional characterization of the v-raf oncogene and its cellular homologs and are investigating their involvement in the etiology of human cancers. Four genes related to v-raf have now been identified in man: c-raf-1, c-raf-2, δ-raf, and γ-raf. C-raf-2 is a pseudogene in man located on chromosome 4. C-raf-1, the cellular homolog of v-raf, is an active gene located on chromosome 3p25, a site which is specifically altered in small cell lung carcinoma, ovarian carcinoma, and mixed salivary gland tumors. C-raf-1 specifies a protein of 648 amino acids with a calculated molecular weight of 74 kd. The complete c-raf-1 protein was expressed in *E. coli* using a complete human fetal liver cDNA cloned into an expression vector. We have also demonstrated the normal protein in mouse fibroblasts by immunoblot analyses. The c-raf-1 gene is composed of 17 exons which span over 45 kb of DNA. Northern hybridization analyses with exon and intron-specific c-raf-1 (genomic) probes has allowed us to construct a transcriptional map for the c-raf-1 locus and has demonstrated two alternatively spliced poly(A)+ mRNAs. One of these mRNAs, containing the most 3' portion of c-raf-1, may be specifically associated with transformation. A second step in the oncogenic activation of c-raf-1 appears to be truncation as all three transforming versions of c-raf-1 are amino-terminally truncated; v-raf with 37 kd, v-mil with 40 kd, and an LTR-inserted and activated c-raf-1 with 50 kd. There are additional raf-related sequences in mouse and human DNA. One of them, isolated from a mouse spleen cDNA library using v-raf, corresponds to an active gene, δ-raf, which is located on human chromosome 7 near the centromere. Another, γ-raf, was isolated from a human fetal liver cDNA library using δ-raf as probe. Homology between δ-raf and c-raf-1 is 69% for DNA and 74% for amino acid sequences. There are two δ-raf transcripts of 2.3 and 4.0 kb. We have investigated the tissue-specific expression of c-raf-1, δ-raf, and γ-raf and have found that, in contrast to c-raf-1 which is fairly ubiquitious in its expression, transciption of δ-raf and γ-raf shows tissue specificity.

PROJECT DESCRIPTION

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

John L. Cleveland	Staff Fellow	LVC	NCI
Ulf R. Rapp	Research Chemist	LVC	NCI
Mahmoud Huleihel	Visiting Fellow	LVC	NCI
R. Nalewaik	Microbiologist	LVC	NCI

Objectives:

The specific objectives of these studies are (1) to define the mechanism(s) by which c-raf-1, a normal proto-oncogene, becomes activated and causes transformation in cells; (2) to molecularly and functionally characterize the members of the raf oncogene group and to determine how these genes are regulated in normal and transformed cells; (3) to define how raf oncogenes are associated with the initiation and maintenance of human cancers and to develop experimental regimens for control of raf-induced transformation; and (4) to identify and characterize genes and gene products which regulate raf expression and to determine the specific targets of "activated" and normal raf.

Methods Employed:

Standard recombinant DNA techniques were used to molecularly clone and subclone raf genomic and cDNA species from bacteriophage λgt10 libraries. raf clones were sequenced using dideoxy- and Maxam and Gilbert techniques and were computer analyzed. Specific cDNA clones were inserted into expression vectors and high levels of synthesis of raf polypeptides in E. coli demonstrated by protein gel and immunoblot analyses. raf-specific transcripts were identified by Northern blot analyses of poly(A)+ RNA preparations from tumors or established cell lines. cDNA libraries from specific cell lines were made by standard techniques, cloned into λgt10, and specific raf cDNAs were isolated. Protein analyses of raf in normal and transformed cells were analysed by metabolic labeling, immunoprecipitation and gel analyses and raf-associated kinase activity demonstrated using our established protocol.

Major Findings:

We have made significant progress in the molecular and functional characterization of the v-raf oncogene and its cellular homologs and are now investigating the role of the raf oncogene group in the etiology of human neoplasia. The major findings are as follows:

1. One of the few carcinoma-inducing viruses is the acutely transforming retrovirus, MH2, which carries the putative oncogene v-mil and the known oncogene, v-myc. Recently, a high degree of homology was discovered between v-mil and v-raf, the transforming gene of the murine retrovirus 3611 murine sarcoma virus (MSV), whereas homology to v-src is low. Both viruses express their oncogenes as the gag-fusion polyproteins p100gag-mil and p75gag-raf (of respective relative

molecular mass [M_r] 100,000 and 75,000), while the myc oncogene of MH2 is expressed by means of a subgenomic messenger RNA. We have recently demonstrated that p100gag-mil is a cytoplasmic protein. We have, using affinity chromatography for viral gag proteins RSV p19 (gag-mil) and MuLV p30 (gag-raf), purified p100gag-mil and p75gag-raf fusion proteins. Both purified proteins exhibit protein kinase activities in vitro which, in contrast to the src-related p130gag-fps of Fujinami sarcoma virus (FSV) and all other characterized oncogene-encoded protein kinases, phosphorylate serine and threonine but not tyrosine. Both types of protein kinases phosphorylate lipids in vitro.

(2) The molecular cloning and sequencing of v-raf-related sequences in mouse and man has identified three active genes belonging to the raf oncogene family and an inactive pseudogene, c-raf-2, present only in man. All four of these genes have been molecularly cloned from human genomic libraries and c-raf-1 additionally from a mouse genomic library. Specific cDNA clones for each gene have been isolated, sequenced, and predicted proteins confirmed by high levels of synthesis in expression vectors in E. coli. Northern hybridization analyses have identified the poly(A)+ RNAs specific for each respective cDNA in both the human and mouse established cell lines and in tissues of the mouse. A transcriptional map of the c-raf-1 locus has been constructed through use of exon and intron-specific probes and has identified two alternatively spliced poly(A)+ RNAs. The expression of one of these RNAs appears specifically associated with transformation as it is not expressed in normal mouse fibroblasts or in mouse tissues analyzed. In addition, an identifier-like 200 bp poly(A)+ RNA has been shown to be transcribed from most of the large introns of the c-raf-1 locus and a cDNA for this RNA also has been isolated and sequenced. The poly(A)+ RNAs for two other raf-related genes and respective cDNAs have been cloned and sequenced, which we have termed δ-raf and γ-raf, have been identified by Northern blot analyses. Both genes exhibit cell type and tissue-specific expression, in contrast to c-raf-1 which is found in all tissues and cell lines analyzed, albeit at different levels. The normal versus ectopic expression of these raf-related genes and their involvement in neoplasia is being investigated by analysis of RNA samples from a variety of tumors and transformed cell lines. All three active raf genes have been mapped to specific chromosomal locations using somatic cell/Southern hybridizations and in situ hybridization of metaphase chromosome spreads in man and mouse. C-raf-1 has been localized to chromosome 3 in man at position p25, a site specifically altered in small cell lung carcinoma, ovarian carcinoma, and mixed salivary gland tumors. δ-raf has been mapped to chromosome 7 near the centromere in the human.

3. Cellular raf polypeptides have been demonstrated by immunoblot analysis using antisera derived from synthetic polypeptides predicted from the sequence of c-raf-1 and δ-raf. Additional sera were obtained against the v-raf protein expressed in E. coli. These studies have demonstrated a c-raf-1 polypeptide of 74 kd present in mouse fibroblasts and two lower molecular weight forms of 50 and 30 kd. A functional examination of the complete c-raf-1 polypeptide produced in E. coli has indicated that amino-terminal processing of the nascent polypeptide may enhance its enzymatic activity, a characteristic reminiscent of a family of proteolytically activated Ser/Thr specific kinases which share 40S ribosomal protein S6 as a target and are enzymatically activated after treatment with specific growth factors.

Significance to Biomedical Research and the Program of the Institute:

Our studies have comprehensively characterized the genomic structure, transcripts, and polypeptides of normal and transforming versions of the raf oncogene family and have functionally characterized these genes as Ser/Thr specific protein kinases which are likely important regulators of the signal transmission pathway of certain growth factors in cells. Definition of normal and transforming versions of raf at all levels are critical in identifying their function, regulation, and association with neoplasia. Several lines of evidence implicate raf in the genesis of human cancers including familial renal cell carcinoma, small cell lung carcinoma, and mixed parotid gland tumors, all of which involve specific rearrangements of chromosome 3, the location of c-raf-1. We have shown that transformation by raf likely involves two events: an amino terminal truncation and alternatively a splicing event which likely generates a different C-terminus for transforming versus normal c-raf-1. We are, therefore, currently performing RNA and protein analysis to see if raf-induced transformation of a variety of cell types and from tumors in vivo demonstrate these same characteristics.

Proposed Course:

We will continue our characterization of the raf oncogene family and its role in human neoplasia. Specifically, we wish to (1) identify the regulatory regions of raf genes which control their transcription, and after identifying these regions, we will select potential raf-regulatory proteins and use these to specifically regulate raf expression; (2) define the targets of raf Ser/Thr kinases in normal and transformed cells and to understand the sequence of events which lead to transformation by raf; (3) define the role of raf genes in the etiology of specific human cancers including small cell lung carcinoma, familial renal carcinoma, and mixed parotid gland cancers; (4) utilize purified raf proteins in development of experimental regimens for the control of raf-induced neoplasia; and (5) identify function of raf/mil gene products in normal cells.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05418-01 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Interaction between raf and myc Oncogenes in Transformation In Vitro and In Vivo

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

PI:	John L. Cleveland	Staff Fellow	LVC	NCI
Others:	Ulf R. Rapp	Research Chemist	LVC	NCI
	Chirabrata Majumdar	Expert	LVC	NCI
	Elizabetta Blasi	Visiting Fellow	LMI	NCI
	Luigi Varesio	Visiting Associate	LMI	NCI

COOPERATING UNITS *(if any)*

Litton Bionetics, Inc., Frederick, MD (J. N. Ihle); Laboratory of Viral Diseases, NIAID, NIH, Bethesda, MD (H. C. Morse)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	0.8	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.)*

We have investigated the transforming activities of the raf and myc oncogenes in hematopoetic and fibroblastic cells using a series of oncogene-transducing murine retroviruses. Fibrosarcomas and, to a lesser extent, erythroid hyperplasia are induced in newborn mice after a latency of 4-8 weeks by 3611 MSV, which contains the raf oncogene. In contrast, newly constructed myc oncogene-transducing murine retroviruses induce T-cell lymphomas after a 9-week latency. A combination of both oncogenes in an infectious murine retrovirus induces hematologic neoplasms 1-3 weeks after inoculation; these neoplasms consist primarily of immunoblastic lymphomas of both T- and B-cell lineage and erythroblastosis. Additionally, we have observed fibrosarcoma and adenocarcinoma of the pancreas, liver and lung. In parallel to the synergistic action of raf and myc oncogenes on hematopoetic and epithelial cell transformation in vivo, we find that raf-induced transformation of fibroblast cell lines in culture is enhanced by the addition of myc, which by itself only weakly morphologically transforms these permanent cell lines. Synergism between raf and myc has also been demonstrated in the immortalization of murine macrophages from fresh bone marrow and in the immortalization of growth factor-independent pre-B cell mast cells and myeloid stem cells derived from infected primary fetal liver cultures. The mechanisms underlying raf + myc synergism have been investigated in vitro. Cells from tumors induced by the myc or raf + myc transducing viruses can readily be established in culture in regular medium, whereas culture of cells from raf oncogene-induced tumors requires the addition of interleukin-3 (IL-3). A function for myc in this synergism has been indicated in studies involving infection of a series of IL-2 and IL-3-dependent cell lines with the various viruses. These studies have demonstrated that expression of high levels of v-myc alone can abrogate the growth factor requirements of these cell lines and probably functions in an analogous manner in its synergistic action with raf in the development of hematopoetic/lymphoid tumors in vivo.

PROJECT DESCRIPTION

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

John L. Cleveland	Staff Fellow	LVC	NCI
Ulf R. Rapp	Research Chemist	LVC	NCI
Chirabrata Majumdar	Expert	LVC	NCI
Elisabetta Blasi	Visiting Fellow	LMI	NCI
Luigi Varesio	Visiting Associate	LMI	NCI

Objectives:

It has previously been demonstrated that oncogenes may act synergistically in specific combinations when assayed for morphologic transformation of primary fibroblastic cells in culture. However, it has not been demonstrated that cell-derived oncogenes act in synergy in vivo in the development of neoplasia, which is generally regarded as a complex disease requiring multiple events. Several types of data indicate interactions between raf and myc oncogenes in some human tumors, including small cell lung carcinoma, which has very poor prognosis. To study synergism between raf and myc, we have made recombinant murine retroviruses containing myc alone or raf + myc and have demonstrated a complementation between raf and myc in hematopoetic, lymphoid, fibroblastic and epithelial cell transformation in vivo. The specific contribution(s) of each oncogene to this synergy are being assessed.

Methods Employed:

Standard recombinant DNA technology was used to construct new murine retroviruses, and high titer stocks of these viruses were obtained after transfection of NIH 3T3 fibroblast cells and rescue with ecotropic Moloney leukemia or amphotrophic 4070 A helper viruses. Blot analysis of DNA and RNA, protein gel analysis and immunofluorescence with v-raf and v-myc specific antisera and infectious cell center and reverse transcriptase assays were used to demonstrate that these cells contain and produce the respective recombinant viruses. The tumor-inducing potential of construct viruses was determined by intraperitoneal inoculation of newborn NFS/N mice. Viruses with the same in vitro and in vivo transforming properties were recovered from tumors induced by the construct viruses. Lesions induced in mice were examined histopathologically, and the cells making up the tumors were typed using cell sorting and fluoresence assays for lineage-specific cell surface markers. To demonstrate recombinant virus in tumors, these neoplasms were analyzed by DNA and RNA blotting, and virus-specific RNA and protein were demonstrated in cell lines established from primary tumors. Infections of primary murine bone marrow and fetal liver cultures, as well as infection of established, growth factor-dependent cell lines with the new construct viruses, were carried out using standard procedures and infection was demonstrated by infectious cell center assay. Cells were morphologically typed by flow microflouremtry and immunofluorescence, and virus was demonstrated by DNA, RNA and protein gel analyses.

Major Findings:

The in vivo and in vitro analyses utilizing these new murine recombinant retroviruses have led to several major findings relevant to oncogene expression and the involvement of the retroviruses as causative agents of neoplasia: (1) For the first time, we have demonstrated a complementation or synergy between two oncogenes (raf and myc) in transformation in vivo, specifically of multiple hematopoetic cell lineages, as well as fibroblasts and epithelial cells. The synergy was not only evident as a wider range of tumor types but also in the rapid induction of neoplasms (latency of 2 weeks for raf + myc versus 9 weeks for raf alone or 4 months for myc alone virus). These studies have also defined neoplasms specific for 3611 MSV, carrying v-raf, as fibrosarcomas, and erythroid hyperplasia, and that of the myc recombinant retrovirus as T-cell lymphomas. (2) Consistent with the synergy between raf and myc observed in vivo, we have demonstrated that the isolation of growth factor-independent cells of several lineages from infected primary cultures of murine bone marrow or fetal liver requires expression of both oncogenes. In contrast to the growth requirements of hematopoietic cells from tumors induced by v-raf transducing virus (requiring interleukin-3, IL-3), cells transformed in vivo by virus carrying either raf + myc or myc alone grow independent of IL-3 or other lymphokine supplement. In vitro infection of fresh bone marrow or fetal liver demonstrated an absolute requirement for both oncogenes in the establishment of immortilized, growth factor-independent lines, indicating that a second event is likely to be involved in v-myc virus-induced tumors. (3) A function for myc in this synergism has come from studies involving infection of a series of IL-2 and IL-3-dependent cell lines with the construct viruses. We observed that v-myc alone could abrogate both growth factor requirements. Thus, it appears that myc acts as a central relay for the transmission of signals from several distinct (competence-inducing) growth factors, including platelet-derived growth factor, IL-3 and IL-2. The function of raf in this synergism is under investigation and available data suggest that v-raf directly or indirectly provides a progression signal for transformed cells. (4) Associated with high levels of v-myc expression in virus-infected fibroblastic and hematopoetic cell lines is the suppression of c-myc transcription. Available data support autoregulatory models of c-myc transcription.

Significance to Biomedical Research and the Program of the Institute:

The majority of human cancers and neoplastic disease in general are not likely to be caused by single "events" or genes. Therefore, the studies described here, in which two "activated" forms of normal proto-oncogenes can function synergistically in the transformation of hematopoetic and epithelial cells in vivo, are the first demonstration that two activated proto-oncogenes acting together are necessary and sufficient for carcinogenesis and correlate with specific tumors. In vitro studies have identified possible mechanisms to explain how the two activated proto-oncogenes function in this synergy, a concept that is fundamental to understanding the multiple events involved in cellular transformation. Finally, the observation that an oncogene, v-myc, can autoregulate the expression of its normal counterpart, c-myc, demonstrated another means by which activated or normal proto-oncogene expression can be regulated in normal and tumor tissue if specific transcriptional regulators for these genes can be identified and utilized to control expression.

Proposed Course:

This project represents an excellent model system for the study of activated and normal proto-oncogenes and their association with specific types of neoplasia. Using recombinant retroviruses carrying a large spectrum of oncogenes, we are able to determine neoplasms associated with these genes in vivo, while in vitro studies allow the identification of the mechanisms by which onc genes confer the transformed phenotype. Future studies will include incorporation of other oncogenes into these viruses and definition of combinations of oncogenes that are not only synergistic in inducing the transformed phenotype but also provide a definition of those oncogenes or normal cellular genes that are antagonistic and perhaps reverse the transformed phenotype. A logical extension of this work will be to identify oncogenes as regulators of cellular transcription, identify their targets and ultimately, to identify regulators of activated and normal proto-oncogenes.

Publications:

Cleveland, J. L., Jansen, H. W., Bister, K., Morse, H. C., Ihle, J. N. and Rapp, U. R.: Interaction between raf and myc oncogenes in transformation in vitro and in vivo. J. Cell. Biochem. (In Press)

Ihle, J. N., Keller, J., Rein A., Cleveland, J. and Rapp, U.: Interleukin 3 regulation of the growth of normal and transformed hematopoietic cells. In Feramisco, P., Ozanne, B. and Stiles, C. (Eds.): Cancer Cells: Growth Factors in Transformation. New York, Cold Spring Harbor Laboratory (In Press)

Ihle, J. N., Keller, J., Rein, A., Pierce, J. and Rapp, U. R.: The effects of transforming retroviruses on mast cell growth and IL-3 dependence. In Befus, D., Bienenstock, J. and Denburg, J. (Eds.): Mass Cell Heterogeneity. Hamilton, Raven Press (In Press)

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Rapp, U. R., Bonner, T. I., Moelling, K., Jansen, H. W., Bister, K. and Ihle, J.: Genes and gene products involved in growth regulation of tumor cells. In Havemann, K. and Sorenson, G. (Eds.): Recent Results in Cancer Research. New York, Alan R. Liss (In Press)

Rapp, U. R., Cleveland, J. L., Brightman, K. L., Scott, A. and Ihle, J. N.: Abrogation of growth dependence from interleukin 3 and interleukin 2 by infection of factor dependent cells with v-myc recombinant murine retroviruses. Nature (In Press)

Rapp, U. R., Cleveland, J. L., Morse, H. C., Frederickson, T., Holmes, K., Jansen, H. W. and Bister, K.: Synergistic oncogenes: Rapid induction of hemopoietic tumors in mice by a raf/myc recombinant virus. J. Virol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05419-01 LVC

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Expression of raf/myc Recombinant Viruses - Cooperative Oncogenes

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

	Name	Title	Lab	Institute
PI:	Chirabrata Majumdar	Expert	LVC	NCI
Others:	Ulf R. Rapp	Research Chemist	LVC	NCI
	John L. Cleveland	Staff Fellow	LVC	NCI
	Gunamani Sithanandam	Guest Researcher	LVC	NCI

COOPERATING UNITS (if any)

Max-Planck Institut Fur Molekulare Genetik, Berlin, FRG (H. W. Jansen and K. Bister); Laboratory of Viral Diseases, NIAID, NIH, Bethesda, MD (H. C. Morse, III)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	1.5	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.)

Murine retrovirus, 3611 MSV, harboring the transduced raf oncogene induces fibrosarcoma in newborn mice. A construct carrying both raf and the avian oncogene, myc (pHWJ-2), induces hematopoetic neoplasms consisting of immunoblastic lymphomas of both T and B lineage cells and erythroblastosis in addition to less prominent fibrosarcomas and adenocarcinoma less than 3 weeks after inoculation. Constructs carrying only the myc oncogene induce tumors after a latency of equal to or greater than 9 weeks. A variant of the myc only virus, J-3, was found to induce altered foci in cultured fibroblast cells. Neonatal mice develop carcinoma in pancreas, liver and lung when inoculated with this variant, with a latency of 2 to 6 months. The raf oncogene induced transformation of hematopoetic and epithelial cells and is enhanced by the presence of the myc gene which apparently does not transform these cells when grown in standard culture media. Thus, simultaneous expression of both raf and myc oncogenes in these cells alters their respective transforming spectra. Southern blot analysis on the DNA from J-3 (myc only)-induced tumors, as well as from cell lines established from these tumors, showed that at least three out of four tumors are clonal in nature, that in one instance part of the viral sequences were deleted, and in another a repair of the nonfunctional v-raf gene in the virus seems to have occurred. In parallel with these results, we detected, by Northern hybridization, that the genomic RNA transcripts of these two viruses, J-3 and J-3* also differ in size as though the deleted v-raf gene in J-3 had been repaired by recombination with the cellular raf-1 gene.

PROJECT DESCRIPTION

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

Chirabrata Majumdar	Expert	LVC	NCI
Ulf R. Rapp	Research Chemist	LVC	NCI
John L. Cleveland	Staff Fellow	LVC	NCI

Objectives:

The natural occurrence of both v-mil (the avian homologue of the murine oncogene v-raf) and v-myc in MH2, the 3p25; 8q23 translocation in mixed salivary gland tumor with possible effects on both raf (3p25) and myc (8q24) and the fact that the small cell/large cell lung carcinoma expresses both these two oncogenes suggest that these two oncogenes might act cooperatively in the development of natural tumors. The objectives of this research are to examine the effect of a combination of these two oncogenes on latency and target cell range, to examine the mechanisms underlying a potential synergism especially as it relates to the induction of carcinoma, and to identify genetic determinants that might direct the preferential transformation of epithelial cells.

Methods Employed:

In order to investigate potential interactions between the v-raf and v-myc oncogenes, we have made constructs between DNA from p3611 MSV, pMH2 and pMC29 such that either or both genes were part of a transmissible viral genome.

For the assembly of pHWJ-1 which contains a raf-mil hybrid oncogene and the 5' half of myc, pBR322 derivatives containing the cloned proviruses of MH2 or of 3611-MSV were cleaved with ClaI and SphI. The appropriate fragments were electroeluted from an agarose gel and ligated. The plasmids obtained after transformation of competent E. coli cells using this reaction mixture were selected for size and for orientation of fragments. The clone obtained, pHWJ-1, contains a gag-raf-mil hybrid oncogene with an in-frame fusion of 5' raf and 3' mil sequences. For the construction of pHWJ-2, which contains the gag-raf-mil hybrid oncogene and a complete myc gene, plasmids containing the cloned provirus of 3611-MSV or the 2.9 kb BamHI fragment (pMC29-B) of the cloned MC29 provirus were cleaved with ClaI and SphI. The appropriate fragments were isolated, ligated and subsequently cleaved with ClaI. The desired fragment was gel-purified and ligated with the 9.4 kb fragment of pHWJ-1 which had been dephosphorylated after cleavage with ClaI. The plasmids obtained after transformation were selected for size and orientation of fragments. The clone obtained, pHWJ-2, contains the same gag-raf-mil hybrid oncogene as pHWJ-1, and in addition, a complete myc oncogene including a splice acceptor signal and the translational initiation and termination codons. Plasmid pHWJ-3 was constructed by deletion of a 257 bp XhoI fragment which spans the gag-raf border in pHWJ-2. This deletion causes an out-of-frame fusion of gag and raf and thus prevents expression of the raf-mil hybrid oncogene. For construction of a murine provirus expressing the myc oncogene as a hybrid protein, p3611MSV-E was cleaved with KpnI and BamHI, pMC29-B was cleaved with EcoRV and BamHI, and pHWJ-1 was cleaved with KpnI and PvuII. The appropriate

fragments were electroeluted from an agarose gel and ligated. The ligation mixture was used for transformation of competent E. coli cells and the resulting plasmids were selected for size and restriction site pattern. The plasmid pHWJ-4 contains an open reading frame encompassing murine and avian gag sequences and the entire myc gene, but it lacks the 3' LTR. To complete the provirus, pHWJ-2 and pHWJ-4 were cleaved with Eco-RI and BamHI. The appropriate fragments were electroeluted from an agarose gel and ligated. After transformation of E. coli cells, the plasmids were selected for size and restriction site pattern. The clone obtained, pHWJ-5, contains an in-frame fusion of gag and myc sequences and is able to direct synthesis of a gag-myc hybrid protein. The viruses obtained after transfection of NIH 3T3 cells with the complete recombinant provirus clones and helper virus DNA were named J-1, J-2, J-3, and J-5. RNA analysis from tumors induced by J-1 through J-5 viruses was performed on oligo-dT-selected polyA RNA by hybridization with appropriate nick translated probes following agarose gel electrophoresis and transfer to nitrocellulose. Southern blot analysis was done by digestion of DNA with appropriate restriction enzymes, gel electrophoresis, transfer and hybridization with radiolabelled probe as described above.

Major Findings:

1. v-raf and v-myc act synergistically as evidenced by (a) a shortened latency period for tumor development, and (b) a wider target cell range for transformation in vivo. A requirement for both oncogenes was also demonstrated for transformation of primary bone marrow and fetal liver cells in culture. Moreover, these findings showed the usefulness of raf/myc recombinant virus for the efficient immortalization of B lineage cells, an ability that may prove useful for the generation of antigen-specific antibody-producing cells in vitro.

2. Viruses carrying only v-myc were shown to induce clonal hematopoetic neoplasms. This is a first demonstration of pathogenicity of this oncogene in mammals.

3. A novel carcinoma inducing virus was isolated as a variant of the recombinant, retrovirus J-3 which contains a mutated raf and a functional myc oncogene. The new virus, J-3*, induces carcinoma in pancreas, liver, and lung of inoculated newborn mice. This is the first molecularly characterized mammalian retrovirus which preferentially induces short latency carcinomas.

Significance to Biomedical Research and the Program of the Institute:

Several lines of evidence suggest that natural malignancies develop in several steps of initiation and promotion. Our findings on the effect of a combination of the oncogenes v-raf and v-myc on pathogenicity provide the first demonstration of a synergistic action of activated proto-oncogenes in vivo. This finding provides us with a model system for the analysis of the multistep nature of tumor development and allows us to examine mechanisms involved in the sequential transformation of cells to malignancy. Moreover, an extension of these studies may lead us to antagonistic combinations of oncogenes and thereby identify gene products which may become useful for the management of tumors that are specifically associated with a particular activated proto-oncogene. The isolation of the novel J-3* virus which preferentially induces carcinoma in various tissues of mice, including lung, will be especially useful for the further characterization of genetic determinants of epithelial cell transformation.

Proposed Course:

1. Examination of tumors induced by raf and raf/myc and myc carrying viruses. In order to determine the contribution of the oncogenes to tumor development, their expression in tissues of virus-infected mice will be determined by in situ hybridization, immunocytochemistry, Northern and quantitative dot blot hybridization for oncogene specific transcripts and immunoprecipitation of oncogene proteins in cell lines derived from tumorous tissues. Moreover, the clonality of tumors will be determined in order to obtain information on the possible involvement of events other than expression of the viral oncogenes in tumor development.

2. Molecular cloning of the carcinoma inducing J-3* virus and identification of genetic changes associated with altered pathogenicity. Nonproducer-transformed rat cells will be prepared for isolation of proviral DNA to be cloned into phage vectors following standard procedures.

3. Other combinations of oncogenes, by themselves or together with growth factors, will be tested for the identification of antagonistic effects on transformation. The basis for this study is the finding that the tumor promoter TPA, which has mitogenic activity for untransformed NIH 3T3 cells, has inhibitory activity on the same cells after transformation with v-raf.

Publications:

Blasi, E., Mathieson, B., Varesio, L., Cleveland, J. L., Borchert, P. and Rapp, U. R.: In vitro transformation of hemopoietic cells by a raf/myc recombinant retrovirus. Nature (In Press)

Cleveland, J. L., Morse, H. C., Ihle, J. N. and Rapp, U. R.: Interaction between raf and myc oncogenes in transformation in vitro and in vivo. In Harris, C. (Ed.): Proceedings of the UCLA Symposia on Biochemical and Molecular Epidemiology of Cancer. Los Angeles, University of California (In Press)

Rapp, U. R., Bonner, T. I. and Cleveland, J. L.: The raf oncogene. In Gallo, R. C., Stehelin, D. and Varnier, O. E. (Eds.): Retroviruses and Human Pathology. Clifton, Humana Press (In Press)

Rapp, U. R., Bonner, T. I., Moelling, K., Bister, K. and Ihle, J.: Genes and gene products involved in growth regulation of tumor cells. In Havemann, K. and Sorenson, G. (Eds.): Recent Results in Cancer Research. New York, Springer-Verlag (In Press)

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ANNUAL REPORT OF

BIOLOGICAL CARCINOGENESIS BRANCH

NATIONAL CANCER INSTITUTE

October 1, 1984 to September 30, 1985

The Biological Carcinogenesis Branch (BCB) plans, develops, directs and manages a national extramural program of basic and applied research concerned with the role of biological agents as possible etiological factors or co-factors in cancer and on the control of these agents and their diseases; establishes program priorities, and evaluates program effectiveness; provides a broad spectrum of information, advice and consultation to individual scientists and institutional science management officials relative to NIH and NCI funding and scientific review policies and procedures, preparation of grant applications and choice of funding instruments; provides NCI management with recommendations as to funding needs, priorities and strategies for the support of relevant research areas consistent with the current state of development of individual research activities and the promise of new initiatives; plans, develops and manages research resources necessary for the conduct of the coordinated research program; develops and maintains computerized data management systems; and plans, organizes and conducts meetings and workshops to further the program objectives, and maintains contact with the relevant scientific community to identify and evaluate new research trends relating to its program responsibilities.

The objectives of the research program are accomplished through a variety of extramural support mechanisms including traditional individual research project grants (R01), program project grants (P01), new investigator awards (R23), conference grants (R13), cooperative agreements (U01), contracts (N01), small business innovative research grants (R43), and outstanding investigator grant awards (R35). Currently, the Branch administers 387 research activities with an annual budget of approximately 64.8 million dollars. The research projects of the Branch divide into five main categories. Research programs on viruses with a DNA core which are known or suspected to be involved in the induction of malignant transformation are included in the DNA Virus Studies components. The Branch program component designated DNA Virus Studies I deals with research on the two main groups of large DNA viruses, the herpesviruses and adenoviruses. The program component designated DNA Virus Studies II supports research on the main groups of small DNA viruses, the polyoma, simian virus 40 (SV40), and papillomaviruses. Research dealing with RNA core viruses which are known or suspected of involvement in the malignant transformation of animal and human cells are covered by RNA Virus Studies components. The Branch program component designated RNA Virus Studies I involves research concerning murine, feline, bovine, primate, and hamster viruses. The program component designated RNA Virus Studies II incorporates research involving avian tumor viruses, pox viruses, myxoviruses, picornaviruses, hepatitis viruses, and plant viruses. The Office of the Branch Chief serves as the focal point for special studies or activities of high visibility. It is currently responsible for traditional projects, conference grants, and

cooperative agreements concerning virological studies on the Acquired Immune Deficiency Syndrome (AIDS) and Kaposi's sarcoma.

To facilitate and support these research activities the research resources component of the Branch is responsible for developing, allocating and maintaining the inventory of biological research resources necessary for the extramural research effort. The planning, initiating and oversight necessary to generate specific research resources are functions of the individual Program Directors who administer each of the research components within the Branch. The research resources component includes a data management element which is responsible for the automated retrieval and inventories of BCB resources, computer-systems planning, and automated analysis and management support. The automated inventories include the research resources virus and antisera inventory, the serum collection, and the human tissue collection. During this period, more than 2,000 sera and tissue specimens and over 500 viral reagents were shipped to research laboratories from the inventory of frozen and stored biological materials.

The BCB resources payback system has been described in detail in previous reports. The payback system is one in which the recipients of resource materials or services reimburse the resource or production contractor directly for the services or materials received, based on a price schedule agreed upon in advance between the NCI and its resource contractor. The contractor in turn credits those funds received from recipients against its production costs and these are shown on monthly vouchers which it submits to the Government for payments under the contract. There are currently six resource contracts functioning in the payback mode. These include two for production of viral reagents, two for animal resources, one for specialized testing services and one for maintenance and distribution of stored frozen biological reagents. The payback system seems to be performing as expected. The demand for high quality biological reagents not readily available from commercial sources has remained fairly constant. Reimbursement for full or partial costs of services has led to more careful use of costly resource reagents, with a subsequent reduced level of effort in several resource contracts or the termination of unnecessary activities.

Table I focuses on mechanisms of support of extramural research and related activities in the area of biological carcinogenesis. The total BCB grant and contract budget in FY85 is estimated to be about 64.8 million dollars. It should be noted in the table that the Branch now administers 22 program project grants at a level of 18.4 million dollars and 20 cooperative agreements at a level of 3.02 million dollars. Of the cooperative agreements, 16 are AIDS-related research projects. Table II provides an estimate of the grant and contract support, respectively, in each of the six Branch components. The Branch currently administers 13 contracts and 374 grants.

During FY85, the Branch was very active in sponsoring a variety of new research initiatives. These consisted of both grant and contract initiatives and reflected emerging areas of research opportunity as well as the recompetition of several productive contract programs. Since the issuance of a Request for Applications (RFA) or a Request for Proposals (RFP) is contingent upon concept approval by the DCE Board of Scientific Counselors (BSC) and, in the

case of the BCB, is often preceded by a workshop, this report discusses the initiatives generated during the fiscal year as individual entities, rather than discussing each initiative separately under the various categories of workshop, concept, and RFA. In terms of scientific areas, five research initiatives concerned retroviruses, one concerned papillomaviruses, one concerned human polyomaviruses, five were for viral products or for virally infected material, and one was for a repository for such materials. During the year, the Branch sponsored three workshops and one discussion group, received clearance for three grant RFAs and one grant program announcement, funded four cooperative agreements from two RFAs issued in FY84, and funded one contract each (5) for five of the six RFPs issued. The sixth contract RFP will be funded in FY86. A large proportion of these initiatives involved retroviruses, specifically the HTLV family, and represents the recognition of these viruses as agents important in the etiology of several human malignancies. The specific details for each of these initiatives is given below.

One RFA for cooperative agreements, originally published by the BCB in FY84, was initiated and funded during this year. Its purpose was to encourage research on human T cell leukemia/lymphoma virus (HTLV) types I and II. The HTLV family of viruses has only recently been recognized. HTLV I and II are associated with human malignancies, and HTLV III with AIDS. Since AIDS research is being funded through a variety of mechanisms, the purpose of this specific RFA was to stimulate research on HTLV I and II in order to elucidate such fundamental issues as the mode of virus transmission, virus-host cell interactions, and the molecular basis of virus transformation by two members of this important group of human retroviruses. Four new cooperative agreements submitted under this RFA were funded in FY85.

Four concepts for new research grant initiatives were presented by the Branch to the BSC in FY85. The first concerned human papillomaviruses (HPV) and was based on input from a BCB workshop held on June 13, 1984. The workshop was chaired by Dr. Marcel Baluda of the BSC. Recent studies have suggested that these viruses have a role in the development of certain human carcinomas, particularly those of the anogenital tract. The consensus of the workshop participants was that research on the role of papillomaviruses in the etiology of cervical cancer needed stimulation and that an RFA should be developed to stimulate basic research on the possible clinical progression of HPV infections to dysplasia and carcinoma in human subjects and to relate this progression to the molecular biology of HPV. It was felt that the goals of such research should include the elucidation of the mechanisms of viral infection, replication and oncogenic transformation; the nature of the host's response to HPV; and the co-presence and possible involvement of other viral agents, such as herpes simplex virus (HSV) and cytomegalovirus (CMV), with HPV in the oncogenic process. This concept was presented to the BSC at their October 1984 meeting and was approved for implementation as an RFA for traditional research grants. The RFA was issued in FY85 for funding in FY86.

A second research grant initiative concerned feline leukemia virus (FeLV) research and was based on input from a BCB workshop held on November 29-30, 1984. Dr. Myron Essex of the BSC chaired the meeting. Other Board members attending were Dr. Marcel Baluda and Dr. Charlotte Friend. Feline leukemia virus is responsible not only for malignant diseases, such as leukemia,

lymphosarcoma, and other neoplastic conditions, but also causes other serious diseases such as anemia, immunosuppression, and reproductive failure. More cats die of complications arising from the immunosuppression caused by FeLV than through tumor induction. In this respect, a parallel exists between FeLV and the recently discovered human retrovirus, human T cell leukemia/lymphoma virus (HTLV), especially HTLV-III. The consensus of the participants was that feline leukemia virus offered important models of oncogenesis/immunodeficiency syndrome in an outbred species and that NCI should stimulate additional studies on the biology, immunology, and molecular biology of feline leukemia virus. This concept was presented to the BSC at their February 1985 meeting and was approved for implementation as a program announcement. This announcement is currently in preparation for issuance; awards in response to it will be funded in FY86. If an insufficient response is received to this announcement, it will be reissued in FY86 as an RFA.

A third research grant initiative concerns basic studies on the development and assessment of retroviral vaccines. It was based on a workshop held on December 10-11, 1984. Dr. Hilary Koprowski of the DCE Board of Scientific Counselors chaired the meeting. Other Board members attending were Dr. Myron Essex, Dr. Marcel Baluda, and Dr. Charlotte Friend. The consensus of the participants was that NCI should stimulate new areas of scientific research on retroviral vaccines such as: development of naturally-occurring retrovirus animal models to systematically evaluate strategies for vaccine development and assessment; basic studies to determine how retroviruses interact with the host immune surveillance system; and utilization of newer methodologies to produce and evaluate retroviral vaccines. This concept was presented to the BSC at their February 1985 meeting and was approved for implementation as an RFA for traditional research grants. This RFA is currently being prepared for release. Awards in response to this announcement will be funded in FY86.

The fourth research grant initiative concerned studies of novel exogenous and endogenous human retroviruses and was based on a discussion group which met on March 11, 1985. Dr. Padman Sarma, Program Director, RNA Virus Studies I, BCB, chaired the meeting. Three members of the BSC, Dr. Charlotte Friend, Dr. Marcel Baluda, and Dr. Myron Essex attended. The consensus of this group was that multiple uncharacterized human type-C viruses and endogenous proviruses may represent a potential source of infectious retroviruses and by analogy with animal model systems may serve as a potential source of new human cancer viruses. The discussion group felt that this area of research needed stimulation and that an RFA should be developed. This concept was presented to the BSC at their May 1985 meeting and was approved for implementation as an RFA for traditional research grants. An RFA is currently being developed for this concept. Awards in response to this announcement will be funded in FY86.

A fifth initiative to stimulate virology research is currently being developed. It will be based on a workshop on human polyomaviruses that was held on March 7, 1985. Dr. Renato Dulbecco of the BSC chaired the meeting. The consensus of the workshop participants was that these human viruses provide unique opportunities to directly study the mechanisms involved in the viral transformation of human cells and that NCI should stimulate basic studies on the mechanisms of transformation of human polyomaviruses and their possible role in the etiology of human cancer. These studies will include

such areas as determining the incidence and structure of polyomavirus DNA in normal and malignant human tissues; functional analysis of polyomavirus DNA isolated from human tumors with regard to the expression of viral genes, transformation activity in transfection assays, and maintenance of viral sequences when tumor cells are serially cultured; isolation and characterization of human B-lymphotropic virus and other new human polyomaviruses; and studies of the mechanism of persistent polyomavirus infections in man and the target cells involved in this interaction. A concept for an RFA based on these issues is being prepared for submission to the BSC in FY86.

Five contracts were awarded in FY85; four were for continuing efforts and one was for a new effort. The continuing efforts were: 1) a contract for the production, characterization and distribution of avian myeloblastosis virus reverse transcriptase. This enzyme is used in the production of cDNA copies of oncornaviral genomes, or parts of such genomes, for use as probes to identify viral sequences in normal or malignant tissues, to compare viral and cellular sequences for homology, to determine the relatedness of viruses, to permit expression of viral sequences in bacterial systems, and for other studies of the molecular biology of viruses; 2) a contract for inter- and intra-species identification of cell cultures. The extensive use and informal cross-supply of cell cultures among investigators has resulted in a major problem of frequent erroneous or mislabeled cell lines. Correctly identified cell lines are of critical importance since research utilizing misidentified cell lines is a waste of time and research funds; 3) a contract to maintain an already existing, NCI-owned breeding colony of cotton-topped marmosets so that appropriate numbers of these nonhuman primates would be available for experimental use by investigators in cancer research; 4) a contract funded by the NCI, but shared with NIAID to breed woodchucks (Marmota monax) to be used as models for primary hepatocellular carcinoma associated with hepatitis B virus infection.

A new contract effort has been initiated for the maintenance of a herd of cattle and sheep infected with bovine leukemia virus (BLV) in order to supply investigators with scarce bovine and ovine resource materials which are not routinely available to investigators. Bovine leukemia is an important model of oncogenesis associated with an exogenous retrovirus. Similarities in aspects such as possible evolutionary origin, biology and special properties between BLV and HTLV make studies of BLV important and worthwhile as a valuable animal model of HTLV-induced human cancer.

Additionally, an RFP was issued during this year for the competetive continuation of a contract for the maintenance of a repository for viruses, sera, reagents and tissues collected under ongoing and previous resource contracts. This effort will be funded in FY86.

Past research sponsored by the Branch has yielded much fundamental information on biological carcinogenesis by studying animal tumor viruses which induce cells to become malignant. These viruses provide tools for understanding how cellular genes and their products convert a normal cell into a tumor cell. Of equal importance, horizontally transmitted particulate human viruses may themselves be directly responsible for some malignant transformations in man. Investigations in the area of biological carcinogenesis have shown that viral

information present as nucleic acid sequences in the genes of normal cells and replicating with them may be intimately involved in the development of cancer. This may occur through either the direct effects of viral transforming genes, through the influence of viral enhancing elements (promotors or long terminal repeats) on cellular oncogenes, or through the synergistic influence of viruses with a variety of environmental factors, such as hormones, chemicals, radiation, and the like. In the research program, attention is given to studies defining the interaction of viruses and cells in both animal and human cancers. The work stresses efforts to identify minute regions of viral nucleic acid and cell chromosomes which are responsible for malignancy; to understand the molecular pathways of viral replication; to identify virus products which may trigger the transformation of a cell to malignancy; and to understand host responses to viruses which ultimately may prevent cancer.

Recent studies have shown that a number of rapidly transforming RNA viruses have derived their ability to cause cancer in animals, and perhaps in humans, from genes found in normal cells. These genetic sequences are unrelated to those necessary for normal viral replication and have been found to produce proteins which are necessary for the initiation of the transformed state. Since the gene products result in transformation, the genes have been termed "oncogenes." Oncogenes have been found to possess the following characteristics: they are essential for certain types of transformation; they are derived from cellular genetic sequences; they have a limited number of specific targets; they act by means of their translational protein products; and, are probably limited in number (approximately 20 have been found). Currently, ongoing research seeks to continue the search for oncogenes in both animal and human tumor systems and characterize these genes; explore the emergent area of human oncogenes and their relationship(s) to viral oncogenes in terms of nucleic acid homology and translational (oncogene) products; purify and characterize the translational gene products of these genes; use these purified products in delineating the mechanism(s) of transformation; and, define the function(s) and mechanisms of regulation of the cellular homologs (c-oncs) of viral transforming genes. The research highlights of the past year are presented here and in greater detail in the various section reports which follow.

A new area of intensive research activity has developed dealing with the HTLV family of retroviruses. HTLV-I and HTLV-II are associated with human malignancies, and HTLV-III with AIDS. Research focusing on any one of these viruses has shed knowledge on the other two members of this group. Thus, comparisons of the similarities and differences among these viruses has the potential to increase our knowledge of three virally induced human malignancies. The linear viral genome of HTLV retroviruses has regions corresponding to the gag, pol and env genes of conventional retroviruses. Adjacent to the 3' end of the env gene is a region designated pX. While the functions of the gag, pol and env genes appear homologous to that of other retroviruses, the pX region does not appear to be homologous with other retroviruses. The viral genome is bracketed on each end by long terminal repeats (LTRs). The LTRs carry regulatory segments known as promoters and enhancers, which activate transcription of viral genes into messenger RNA, the first step in protein synthesis.

Unlike some of the other retroviruses that can transform animal cells, HTLV-I and -II do not contain oncogenes derived from cellular proto-oncogenes and do not have to integrate adjacent to cellular oncogenes in order to activate uncontrolled cell growth. A novel system of cancer induction for HTLV-I and -II, and cell death caused by HTLV-III, has been proposed. It is theorized that HTLV-I and -II are able to cause uncontrolled cell growth, resulting in cancer, by producing a protein that turns on cellular genes responsible for cell division. In the case of HTLV-III, an analogous viral protein may turn off the cellular genes which stimulate cell growth and turn on those that cause cells to stop dividing, resulting in cell death. This protein, designated the 42 kilodalton protein, is encoded in the pX region of the viral genome. It acts upon the enhancers present in the LTR regions and leads to increased transcription. This novel type of regulation has been called trans-activation. Additional studies have shown that the 42 kilodalton protein of HTLV-I was sufficient in itself to mediate the effect of trans-acting transcriptional regulation. Stable cell lines that expressed only this protein displayed a high level of trans-acting transcriptional regulation. The LTR of HTLV-II has also been found to be responsive to HTLV-I encoded trans-acting factors. However, the transcription unit of the HTLV-II LTR will only function in certain cell types, thus suggesting that cellular factors are required, in addition to virus trans-acting factors, for the trans-acting phenomenon.

The retrovirus responsible for the human AIDS has been variously termed human T-cell lymphotropic virus III (HTLV-III), lymphadenopathy associated virus (LAV), and AIDS associated retrovirus (ARV). It was originally thought to be most closely related to the oncogenic HTLV-I which is responsible for a small proportion of human adult T-cell leukemia. However, a recent study of an AIDS retrovirus isolate using molecular hybridization and heteroduplex analysis has suggested that HTLV-III may be more closely related to the visna virus of sheep, a member of the lentivirus subfamily. The AIDS retrovirus isolate appeared to be less closely related to HTLV-I or to a variety of other retroviruses. The ability of the AIDS retrovirus to infect and to persist in brains of AIDS patients, its cytopathic effect on T4 lymphocytes in vitro, and its lack of overt oncogenic potential, suggests similarities with the cytopathic lentiviruses, some of which are neurotropic (visna, caprine arthritis-encephalitis). However, the AIDS retrovirus is lymphotropic for T4 cells in a manner similar to HTLV-I and therefore its designation as human T-cell lymphotropic virus-III (HTLV-III) does reflect a biological relationship with the HTLV family of retroviruses. Thus, the evolutionary origin of HTLV-III remains unknown and is a subject of active research.

Recently, an HTLV-III type agent that naturally infects rhesus monkeys was detected. It is associated with the so-called simian AIDS (SAIDS). The virus was isolated from four monkeys with SAIDS using Hut 78 cells of human origin. Serum from these and other rhesus monkeys in the same cages precipitated proteins of a size identical to those of human HTLV-III. The sera of monkey origin could precipitate proteins of both the monkey virus and the human AIDS virus. Similarly, reference sera from AIDS patients precipitated the proteins of both the monkey virus and the human agent. By morphologic criteria, as well as reverse transcriptase activity, the new monkey virus is clearly very closely related to the human agent. It is postulated that the human agent may have originated from some population of Old World monkeys or apes. These

results are important not only because they may elucidate the origin of the AIDS virus, but also because the rhesus monkey virus could provide a model for AIDS treatment and vaccine development.

Studies in patient populations are providing important information on the natural history of AIDS infection. In a prospective study in Boston, 136 homosexual males have been enrolled and studied for factors related to the pathogenesis of AIDS. Of these, 114 have been followed for prolonged periods, up to 20 months. At enrollment 28/57 healthy homosexual controls, 36/40 patients with AIDS related complex (ARC), and 17/17 patients with AIDS were HTLV-III seropositive (HTLV-III+). HTLV-III has been isolated from peripheral blood leukocytes and semen of several study subjects, the first successful isolations from the latter source. In the course of this study, healthy individuals carrying HTLV-III in their blood and semen have been identified; these individuals are being closely monitored for co-factors that may be related to the development of AIDS.

Investigators in California have isolated an AIDS associated retrovirus (ARV) from over 90 different individuals who have AIDS or are at risk of development of AIDS. These include a large number of patients with Kaposi's sarcoma, lymphadenopathy syndrome and other pre-AIDS conditions. Virus has also been obtained from normal healthy individuals in a "carrier" state. Some of these viruses show differences in replicating ability. Virus has been isolated from two individuals who are antibody negative, but who have a substantial amount of virus in their white cells. These results indicate the possible danger of screening blood for virus by only testing for antibody. Infectious ARV virus has also been isolated from the saliva and semen of about 10% of subjects studied. ARV has also been isolated directly from salivary fluid obtained through cannulation of Stensen's duct. These results suggest that infectious viruses are being produced in salivary glands. These two studies confirm that virus can be found in a number of body secretions and that healthy HTLV-III carriers exist in the population.

Recent studies of the relationship of viral and cellular oncogenes to the transformation process continue to support the hypothesis that carcinogenesis is a multistage process. An in vitro transformation assay for demonstrating the presence of transfectable transforming genes in the DNA of human tumor cells was successful only with previously initiated or immortalized cells such as the established mouse fibroblast cell line, NIH/3T3. It appears that cellular oncogenes cannot transform truly normal cells without the assistance of some type of helper or initiator or a mutational event. Normal, pre-senescent cells appear refractory to transformation, but such resistance completely disappears after immortalization by chemical and physical carcinogens.

Despite great strides in identifying cellular genes with transforming potential, little is yet known about proto-oncogene function or how the altered counterparts of the genes disrupt normal growth regulations. Proteins encoded by the cellular oncogene c-abl apparently help trigger chronic myelogenous leukemia, which affects about 10,000 new victims each year. Investigators have demonstrated that the appearance of the Philadelphia chromosome (involving the translocation of chromosome 9 with the c-abl oncogene to chromosome

22) triggers the abnormal activation of this c-abl oncogene in its new location on chromosome 22. As a result of this translocation, an altered mRNA and a structurally altered c-abl protein, p210 c-abl, are produced. This altered protein has a tyrosine kinase activity not detectable in the normal c-abl protein. It is hypothesized that this transforming protein may in turn cause a chain of abnormal chemical reactions in the growth cycle of the cell, making it malignant.

The transforming genes of small DNA tumor viruses, such as SV40 and mouse polyoma, appear to be very different from the oncogenes of the RNA retroviruses. These DNA oncogenes are totally of viral origin and produce proteins called tumor or "T" antigens which are necessary to the normal replication and maturation of these viruses in lytic infections. In the SV40 system, the large tumor antigen gene, which alone can transform cells, has been extensively studied using biochemical, immunological, genetic and most recently transgenic methods. Transgenic mice have added a new dimension to the use of SV40 as a model for tumorigenesis. Using the new technique of microinjection of recombinant DNA plasmids into fertilized mouse eggs, transgenic mice were produced which carry SV40 T-antigen genes integrated into the genome of every cell of the animals. A high percentage of these transgenic mice developed tumors within the choroid plexus. A line of these mice has been established in which the SV40 DNA is genetically transmitted to progeny. In this line, nearly every affected animal succumbed to choroid plexus brain tumors within five months after birth. Both SV40 T-antigen mRNA and protein were readily detected in affected tissues; however, SV40 T-antigen gene expression was barely detectable in unaffected tissues or in susceptible tissues prior to overt pathology, suggesting that tumorigenesis depended upon activation of the SV40 genes. Cell lines could be readily established from tissues of transgenic mice; such cultured cells contained T-antigen. Examination of DNA extracted from tumor tissue or from cell lines derived from tumors revealed structural rearrangements and changes in DNA methylation of the SV40 DNA when compared to DNA from unaffected tissues in these same mice. The SV40 genes were frequently amplified in tumor tissue, further indicating that their expression is probably involved in tumorigenesis in transgenic mice. These mice may provide an excellent system to examine both tissue specific tumorigenesis by SV40 large T-antigen and the selective expression of genes in developing eukaryotic organisms.

Although the mouse polyoma virus T-antigens are not yet as well characterized, a recent observation involving the middle T-antigen of mouse polyoma suggests a possible common element in the transformation process with the RNA oncogenic retroviruses. The middle T-antigen of polyoma appears to form a specific complex with the cellular tyrosine phosphokinase enzyme, pp60c-src. This protein is the cellular homolog of the viral oncogene from the Rous sarcoma virus, pp60v-src. Thus, the transformation of cells by polyoma may be mediated by a change in the phosphokinase activity of the pp60c-src in vivo. Changes in phosphokinase activity have also been found in the avian and murine retrovirus systems, but are primarily related to changes in the viral oncogenes in these systems.

An alternative hypothesis for the mechanism of phenotypic transformation by polyoma middle T-antigen/pp60c-src complex has recently been suggested by the

discovery of a new enzymatic activity of the complex. Immunoprecipitates of wild type middle T-antigen pp60c-src complexes were found to have a 20-fold increase over background of a phosphorylation activity for the membrane phospholipid phosphatidylinositol. The level of this kinase activity correlated with the ability of the virus to transform. This phosphatidylinositol kinase activity apparently arises from pp60c-src when it complexes with middle T-antigen. It has previously been shown that the purified viral protein homolog of pp60c-src also possesses a similar kinase activity. This finding opens a new line of research on polyoma transformation. Phosphatidylinositol and its phosphorylated derivatives are a source of second messengers in the cell (e.g., diacylglycerol and 1,4,5 inositol triphosphate) that have pleiotropic effects on cell phenotype. The observation of this new kinase activity also supports the hypothesis that some DNA and RNA tumor viruses may share common mechanisms for cellular transformation.

Possible differences exist in the mechanism(s) of transformation by the various members of the herpesvirus family. In the case of Epstein-Barr virus (EBV), the entire genome of the virus persists as an episome in transformed cells. In the case of herpes simplex virus (HSV) types 1 and 2, short regions, designated morphological transforming regions (mtr), have been identified: one morphological transforming region, mtr I, has been identified among HSV 1 strains, and two transforming fragments, mtr II and mtr III, have been found in HSV 2 strains.

Experiments aimed at defining the exact sequences within the mtr II fragment of HSV 2 that have transforming activity have identified a 737 base pair segment that can convert primary rodent cells to a transformed phenotype. There is no evidence that this fragment encodes a polypeptide. Sequence analysis indicates that this molecule can form a stem and loop structure flanked by direct repeats. These findings suggest a mechanism by which HSV 2, in contrast to other DNA tumor viruses, can initiate morphological transformation without the involvement of a viral protein. It is hypothesized that the 737 base pair segment acts as an insertional element and causes changes in the host cell DNA by a hit-and-run mechanism, rather than by insertion of a large portion of the viral genome into the host cell genome, as is characteristic for other transforming viruses. Transformation mediated by small fragments of DNA represents a novel pathway for DNA tumor viruses, but explains many of the unusual phenomena associated with HSV transformation. First, the reason that no viral antigen(s) is consistently expressed in HSV-transformed cells becomes clear: this region does not appear to encode a polypeptide. Second, the observation that viral sequences retained by transformed cells change in quantity and complexity during the passage of the cell lines (again in contrast to many other DNA viruses) probably reflects the fact that there is no selective pressure to retain viral DNA. Third, the finding that the transforming genes of HSV 1 and HSV 2 are not located colinearly despite the genetic similarity of these regions of the genome can be explained by diversity of the genomes at the level of nucleotide sequence, which is not reflected in the gene products encoded.

While the studies described above provide a model for the mode of action of mtr II, the function of mtr III is less clear. Recent experiments provide a possible explanation for the detection of two HSV 2 transforming fragments.

Cells of the rat 3Y1 line were transformed by transfection with HSV 2 DNA from the mtr II fragment which had been inserted into pBR322 plasmids. Transformed foci were isolated and the pBR322 plasmids were rescued from them by propagation in E. coli. The DNA in these plasmids was then examined for HSV 2 containing sequences. The HSV 2 specific direct repeat containing the stem and loop from the mtr II was present as would be expected. Surprisingly, a rat specific sequence having homology to the mtr III sequences of HSV 2 also appeared to be incorporated into the plasmid. This latter observation could offer a partial explanation for the ability of both HSV fragments to transform cells. The mtr II fragment acting as an insertional element may preferentially insert itself near the rat sequences which have homology to the viral mtr III sequences. This would explain the ability to rescue rat sequences. The mtr III element, when transfected alone into cells, may recombine preferentially with its cellular homolog. The end product of both of these reactions would be disrupted cellular sequences or perhaps activated cellular oncogenes and would lead to the transformation of cells. Knowledge of the way that these viruses may cause transformation is important both to the prevention and treatment of neoplasms caused by these agents.

The herpesviruses can be grouped into two subsets on the basis of the target cells involved in primary infection and subsequent latency. Thus, HSV and varicella-zoster viruses are designated as neurotropic, while EBV and CMV are designated as lymphotropic. The region of the HSV 1 genome responsible for neurovirulence has recently been determined. While the primary emphasis of studies supported by the Biological Carcinogenesis Branch relates to the tumorigenic potential of this virus, the fact that HSV 1 can establish a latent infection in nerve cells allows it to persist in the host cell, and may allow it subsequently to act as a cofactor in infection by other viruses. The ability to construct viruses without such a neurovirulence region, may be a first step toward the development of vaccines to prevent HSV infection. Understandably, there has been reluctance to use a live attenuated herpesvirus vaccine possessing a neurovirulence capability which could have the potential to establish latent infection.

Recombinant DNA technology has led to the development of a variety of vectors to carry the genetic material under study. The initial vectors used in this technology were primarily plasmids and bacteriophage. In order to study and express genes in mammalian cells, a number of animal virus vector systems have been developed. These include vaccinia, papillomavirus, SV40 virus, adenovirus, and retrovirus vectors. Adenovirus vectors allow for the infection of monkey and human cells and for the occurrence of the normal process of post-transcriptional modification of these polypeptides. This system will obviously be useful for molecular studies. Because of the mild disease produced by many adenoviruses in humans, adenovirus vectors could prove useful as an in vivo carrier of either molecules from other viruses (and thus function as a vaccine) or corrective molecules for individuals bearing specific somatic cell genetic defects.

Recent studies of the human papillomaviruses (HPV) have led to speculation that these viruses have a pathogenic role in the development of certain human carcinomas, particularly those of the anogenital tract. These viruses have long been associated with a broad spectrum of wart diseases, and their

association with cancer has been noted previously. HPV DNA has been consistently detected in an unusual wart disease, epidermodysplasia verruciformis, which has a high rate of conversion to carcinoma. Studies reported this year have demonstrated the presence of HPV in several types of anogenital tumors, such as cervical and vaginal carcinoma in situ, and verrucous carcinoma. Since many patients with anogenital wart disease do not develop genital malignancies, other factors, such as host immunity, cocarcinogens, and infection with other virus groups, may be involved in the process of malignant conversion.

Another observation which has further implicated HPV in an etiological relationship to cervical neoplasia was the detection of HPV DNA sequences in lymph nodes containing metastases. Samples of enlarged lymph nodes and the primary cervical carcinoma were obtained from thirteen patients. Those lymph nodes which contained metastases were invariably positive for the same HPV sequences present in the primary cancer. This result clearly showed that HPV genes are associated with malignant cells and that the sequences are maintained in these cells for the several generations required to form a metastatic lesion. Thus, the HPV genes are probably necessary to maintain the malignant phenotype. Similar results had been previously obtained with metastases from epidermodysplasia verruciformis patients.

Additional studies are assessing the role of delta agent, a defective RNA virus which requires the persistence and helper function of hepatitis B virus (HBV) for its replication. To assess the influence of delta agent on viral replication in patients persistently infected with both viruses and showing chronic liver disease (CLD), the level of HBV DNA was measured in the serum and liver of hepatitis B surface antigen positive CLD patients who were either positive or negative for delta antigen in their livers. In individuals who were delta antigen positive and had chronic liver disease, HBV DNA was detected transiently in the serum of four of twenty-one cases where it was present in low levels. In contrast, forty-three percent of the delta antigen negative/hepatitis B surface antigen positive CLD patients were HBV DNA positive and five of these had high serum HBV DNA levels in the range observed during acute HBV infection. Serum hepatitis B surface antigen and anti-hepatitis B core titers were significantly lower in delta antigen positive cases; this correlated with the reduced amount of hepatitis B core antigen in the liver as demonstrated by immunofluorescence or immunoperoxidase staining. Fewer delta antigen positive/hepatitis B surface antigen positive carriers showed virion or replicating HBV DNA forms in their liver as compared with delta antigen negative/hepatitis B surface antigen positive carriers. These findings indicate that in delta antigen positive chronic hepatitis, synthesis of hepatitis B virus genomes and gene products remains suppressed, whereas production of delta components continues at high levels. These observations may be of value in understanding the pathobiology of chronic delta antigen infection and suggest that liver disease activity in delta antigen positive/hepatitis B surface antigen positive carriers results primarily from the superimposed chronic delta infection and is possibly associated with continued delta replication rather than with HBV replication.

Thus, the BCB has supported a variety of studies on both RNA and DNA viruses. These studies have demonstrated novel mechanisms by which some of these agents

cause oncogenic transformation and/or cancer. In addition, a number of new scientific initiatives have been developed, and there is an increased appreciation of the role of papillomaviruses as etiologic agents in some human malignancy. Although the seminal questions of how viral oncogenes transform cells and how cellular oncogenes may be related to human cancer have yet to be answered, the research activities carried out by the BCB are providing the fundamental information necessary to their ultimate resolution.

TABLE I

BIOLOGICAL CARCINOGENESIS BRANCH

Extramural Activities - FY 1985 (Estimated)

	No. of Contracts/Grants	$ (Millions)
Research Contracts	1	0.18
Research Grants	374	62.37
Traditional Project Grants (309 grants; $38.62 million)		
Conference Grants (10 grants; $0.04 million)		
New Investigator Research Grants (9 grants; $0.39 million)		
Small Business Innovative Research (1 grant; $0.00 million)		
Outstanding Investigator Grants (3 grants; $1.94 million)		
Program Project Grants (22 grants; $18.36 million)		
Cooperative Agreements (20 grants; $3.02 million)		
Research Resources Contracts	12	2.25
TOTAL	387	64.80

TABLE II

BIOLOGICAL CARCINOGENESIS BRANCH

Contracts and Grants Active During FY 1985

	FY 85 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	$ (Millions)	No. of Grants	$ (Millions)
DNA Virus Studies I	1	0.18	90	16.84
DNA Virus Studies II	0	0	82	13.13
RNA Virus Studies I	0	0	93	15.11
RNA Virus Studies II	0	0	90	14.47
Research Resources	13	2.25	0	0
Office of the Chief	0	0	19	2.82
TOTAL	13	2.43	374	62.37

OFFICE OF THE BRANCH CHIEF

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. BASILICO, Claudio New York University 5 U01 CA 37295-02	Molecular Biology of AIDS Related Tumors
2. DESROSIERS, Ronald C. Harvard University 5 R01 CA 38205-02	Type D Retroviruses and Macaque Immunodeficiency
3. ESSEX, Myron E. Harvard University 5 U01 CA 37466-02	Association Between HTLV and AIDS
4. FINBERG, Robert W. Dana-Farber Cancer Institute 5 U01 CA 34979-03	Animal Models of AIDS
5. GARDNER, Murray B. University of California (Davis) 5 U01 CA 37467-02	Simian Acquired Immunodeficiency Syndrome - A Model for Human AIDS
6. GERMAN, James L., III New York Blood Center 5 U01 CA 37327-02	Chromosome Mutation in the Pathogenesis of AIDS
7. HAYWARD, Gary S. Johns Hopkins University 5 U01 CA 37314-02	Interaction of EBV and CMV in AIDS and Kaposi's Sarcoma
8. HIRSCH, Martin S. Massachusetts General Hospital 5 U01 CA 35020-03	Viruses, Acquired Immunodeficiency and Kaposi's Sarcoma
9. MC DOUGALL, James K. Fred Hutchinson Cancer Research Center 5 U01 CA 37265-02	Cytomegalovirus in AIDS and Kaposi's Sarcoma
10. MULLINS, James I. Harvard University 5 U01 CA 34975-03	Retroviruses and AIDS
11. POIESZ, Bernard J. SUNY Upstate Medical Center 5 U01 CA 37478-02	Acquired Immunodeficiency Syndrome: Association with HTLV
12. RICH, Marvin A. AMC Cancer Research Center 1 R13 CA 38586-01	Conference on RNA Tumor Viruses in Human Cancer

13.	ROSENTHAL, Leonard J. Georgetown University 5 U01 CA 37259-02	Role of HCMV in KS Associated with AIDS
14.	SCHOOLEY, Robert T. Massachusetts General Hospital 5 U01 CA 37461-02	Human T-Cell Leukemia Virus; Virus-Host Interactions
15.	SUMAYA, Ciro V. University of Texas Health Sciences Center 5 U01 CA 37477-02	Epstein-Barr Virus and Chromosomal Aberrations in AIDS
16.	VOLBERDING, Paul A. University of California (San Francisco) 5 U01 CA 34980-03	Studies of Acquired Immune Deficiency Syndrome
17.	VOLSKY, David J. Creighton University 5 U01 CA 37465-02	Studies of the Viral Etiology of AIDS
18.	YOHN, David S. International Association for Comparative Research on Leukemia and Related Diseases 1 R13 CA 39923-01	XII International Symposium on Comparative Leukemia Research
19.	ZAIA, John A. City of Hope National Medical Center 5 U01 CA 34991-03	Role of CMV in the Acquired Immunodeficiency Syndrome

SUMMARY REPORT

DNA VIRUS STUDIES I

The DNA Virus Studies I component of the Branch involves research on two groups of large DNA viruses, the herpes- and adenoviruses. In this component extramural research is supported by both grants and contracts. There are 90 research grants with an estimated total funding level of $16.8 million. These include the traditional research grants, program project grants, conference grants, new investigator grants, and small business innovative research grants. The major research emphasis lies in studies of the mechanism(s) of viral transformation, which include genome structure, function and expression (74%); and virus-cell interaction (26%). In terms of the viruses being studied, 30% involve herpes simplex virus (HSV), 29% involve Epstein-Barr virus (EBV), 9% involve cytomegalovirus (CMV), 9% involve other herpesviruses, and 25% involve adenoviruses. One applied research contract which concerns the development of indicator methods for the diagnosis and prognosis of nasopharyngeal carcinomas is also supported at the level of $176,218.

Investigators supported by this program are attempting to elucidate the mechanism(s) of transformation of herpes- and adenoviruses by a variety of approaches, including localization of transformation function(s) to specific sequences of the viral genome, elucidation of the function(s) of individual viral genes, and determination of the mechanism(s) of regulation of synthesis of viral gene products.

Studies of both the herpes- and adenoviruses have sought to identify the regions of the viral genome involved in the transforming process. Most of the members of the herpesvirus family can transform cells in vitro and all of them can establish latent infections in man and animals. Moreover, many of the herpesviruses have been suspected of having a role in tumor induction in man, either directly or as cofactors. Studies designed to identify the regions of the herpesvirus genome involved in carcinogenesis have used several tactics. These include: investigation of morphological transformation by these viruses in cultured cells; induction of transformation by transfection of cultures cells with fragments of viral DNA; and study of tissue specimens from human tumors in order to detect the presence of viral nucleic acid sequences or viral polypeptides.

Current data indicates the existence of possible differences in the mechanism(s) of transformation by the various members of the herpesvirus family. Although definitive answers are not yet available, one morphological transforming region, mtr I, has been identified among HSV 1 strains, and two transforming fragments, mtr II (BglII-N) and mtr III (BglII-C), have been observed in HSV 2 strains. Experiments aimed at defining the exact sequences within the BglII-N fragment of HSV 2 that have transforming activity have identified a 737 base pair segment that can transform rodent cells. There is no evidence that this fragment encodes a polypeptide. Sequence analysis further indicates that this molecule can form a stem and loop structure flanked by direct repeats. These findings suggest a mechanism by which HSV 2 can initiate morphological transformation without the involvement of a viral protein. It is hypothesized that the 737 base pair segment acts as an insertional element and causes changes in the host cell DNA by a hit-and-run mechanism. A hit-and-run mechanism would involve

viral genetic material causing alterations in the host cell genome without the insertion of a large DNA sequence into the host cell genome and with little or no viral genetic material being detected in the transformed host cell after several cycles of cell replication. Transformation mediated by small fragments of DNA represents a novel pathway for DNA tumor viruses, but explains many of the unusual phenomena associated with HSV transformation. First, the reason that no viral antigen(s) is consistently expressed in HSV-transformed cells becomes clear: this region does not appear to encode a polypeptide. Second, the observation that viral sequences retained by transformed cells change in quantity and complexity during the passage of the cell lines probably reflects the fact that there is no selective pressure to retain viral DNA. Third, the finding that the transforming genes of HSV 1 and HSV 2 are not located co-linearly despite the genetic similarity of these regions of the genome can be explained by diversity of these genomes at the level of nucleotide sequence, which is not reflected in the gene products encoded (19,45).

Recent experiments, however, provide a possible explanation for the detection of two HSV 2 transforming fragments. Cells of the 3Y1 line were transformed by transfection with HSV 2 DNA from the BglII-N fragment which has been inserted into pBR322 plasmids. Transformed foci were isolated and the pBR322 plasmids were rescued from them by propagation in E. coli. The DNA in these plasmids was then examined for HSV 2 containing sequences. As might be anticipated, the HSV 2 specific direct repeat containing the stem and loop from the BglII-N (mtr II) was present. Surprisingly, a rat specific sequence having homology to the BglII-C (mtr III) sequences of HSV 2 also appeared to be incorporated into the plasmid. This latter observation could offer a partial explanation for the ability of both BglII-C and BglII-N fragments to transform cells. The BglII-N fragment may preferentially insert itself near the rat sequences which have homology to the viral BglII-C fragment (and thus are able to rescue these sequences), whereas the BglII-C fragment may recombine preferentially with its cellular homolog. The end product of both reactions would be disrupted cellular sequences or perhaps activated cellular oncogenes (12).

The transforming region of human cytomegalovirus is also being defined. Previous studies using transfection of rodent cells with cloned restriction endonuclease fragments of viral DNA have localized the transforming region to a fragment designated pCM4000 (map units 0.123 to 0.14). In order to determine the minimum size fragment of human cytomegalovirus AD169 required to initiate transformation, primary rat embryo cells were transfected with a series of deletion fragments constructed by digestion of a cloned fragment of pCM4000 with exonuclease III and S1 nuclease. The results indicate that the left-hand boundary of the minimum size sequence possessing transforming activity must reside between 490 and 318 bases from the HindIII site of pCM4000. The right-hand boundary was defined by the EcoRI site which is 20 bases from the HindIII site. This sequence of pCM4000 comprises 2,848 base pairs and has an AT composition of 59.5%. Reading frame analysis of the sequence indicated the longest open reading frame was 118 amino acids in length. Northern blot analysis of polyadenylated and non-polyadenylated RNA extracted from cells at immediate early and late times after infection with HCMV indicated that one 5.0 kilobase RNA species hybridized to pCM4000. Examination of the DNA sequence around this region detected sequences that can be drawn as a stem-loop structure.

Despite the similarity of the HSV 2 and CMV insertional elements, there is no sequence homology between the two; in fact the CMV element is very AT rich (70%), whereas the HSV 2 element is very GC rich (76%). It is possible that this difference in the base composition of the structures may reflect an underlying difference in the precise mechanisms by which these two herpesviruses transform. However, the fact that the fragments of the viral DNA representing less than 0.2% of their respective viral genomes have been identified as active in transformation assays, and the fact that both fragments contain sequences that are able to form stem-loop configurations, provides substantial evidence to suggest the involvement of these structures in transformation (45).

The transforming region of a lymphotropic herpesvirus, Epstein Barr virus, is also under investigation. In contrast to herpes simplex virus, in which it is difficult to consistently detect retention of viral DNA in transformed cells, and in which the viral DNA that is found represents only small portions of the genome, EBV viral DNA usually persists as multicopy circular extrachromosomal elements called episomes. These episomes are the result of a covalent linkage between the terminal repeat sequences at both ends of the linear viral DNA. With continued passage of the latently infected, growth transformed cells, defective viral DNA molecules are found in addition to the prototype form. EBV transformed cells are of B cell origin. Only in one line, an African Burkitt tumor cell line, Namalwa, has integration of the entire viral genome into a single site been shown. The viral DNA does not exist in episomal form in the Namalwa cell line. Because the entire genome of EBV seems to persist in the transformed cell, the search for the transforming regions of the viral genome has had to be pursued by a different tactic with EBV than with HSV or CMV. Investigators have studied the mRNA transcripts made in transformed cells and have tried to correlate these with the regions of the viral genome transcribed and with the corresponding polypeptide products. At least three different mRNAs are regularly detected within latently infected transformed cells. These are encoded by DNA domains designated as LT1, LT2 and LT3. It has been demonstrated that the LT2 encodes the EBNA I polypeptide. EBNA (EBV-induced nuclear antigen) is an early protein complex found in both EBV infected and transformed cells. There now appears to be several polypeptides in this antigen complex. It is not yet certain whether all of them, or only the polypeptide designated as EBNA I, are encoded in the LT2 region. It is speculated that the LT1 region encodes an 82 kilodalton DNA binding polypeptide. The role of each of these three regions in transformation is currently being studied (39,40).

Another lymphotropic herpesvirus is Herpes saimiri. The squirrel monkey is the natural host for this virus. While most wild caught and colony-reared squirrel monkeys are infected with this virus, very few animals exhibit any malignant disease. Experimental infection of other species of new world monkeys with this virus frequently results in malignant lymphoma and/or leukemia. Established tumor cell lines which are T cell in nature can be derived from lymphoid tissues of tumor bearing animals. Such cell lines have been examined for the regions of the viral genome which are consistently maintained and therefore could be involved in transformation. Analysis of a number of cell lines has suggested that 73% of the viral genome is not necessary to maintain the transformed state. The sequences that are most consistently retained are the left most 16 kilobase pairs and the right most 15 kilobase pairs of the L DNA (this is the unique DNA sequence of Herpes saimiri). Further studies to more precisely localize this region involved studying a natural deletion mutant occurring in the region of the H-L junction (that is the left most region of the L DNA) as well as

construction of additional deletion mutants in this region. In vitro and in vivo studies with these deletion mutants, as well as with recombinant viruses in which the deletions had been restored, indicated that the DNA sequences between 0.0 and 4.0 map units are required for the oncogenicity of Herpes saimiri, but not for the replication of the virus. The advantage of the Herpes saimiri system is that it provides an animal model with both similarities and differences to several human herpesvirus infections. As such, it may provide a natural model system to help elucidate a number of key issues relevant to herpesvirus infections such as the mechanism(s) of latency, oncogenesis and pathogenesis (18).

The herpesviruses can be grouped into two subsets on the basis of the target cells involved in primary infection and subsequent latency. Thus, HSV and varicella-zoster viruses are designated as neurotropic, while EBV and CMV are designated as lymphotropic. The region of the HSV 1 genome responsible for neurovirulence has recently been localized to 0.71-0.83 map units. While the primary emphasis of studies supported by the DNA Virus Studies I program relates to the tumorigenic potential of this virus, the fact that HSV 1 can establish a latent infection in nerve cells is the property which allows it to persist in the host cell, and may allow it to subsequently act as a cofactor to infection by other viruses. The fact that such latent viruses have the potential for lifelong recurrences may also allow them to act as long term carcinogens. Thus, knowledge of this putative neurovirulent region of HSV 1 may be highly relevant to its oncogenicity. Moreover, the ability to construct viruses without such a neurovirulence region, may be a first step toward the development of vaccines to prevent HSV infection, as there has been reluctance to use a live attenuated herpesvirus vaccine possessing a neurovirulence capability and thus having the potential to establish latent infection (86).

In the case of the adenoviruses, the regions of the viral DNA necessary for transformation are known. Integration of both the E1A and E1B regions of the adenovirus genome is necessary for complete transformation of cells. However, the E1A region alone can induce immortalization of cells, but not the full transformation phenotype. It is also known that the same E1A and E1B viral products produced during transformation are also produced in lytic infection. In order to distinguish the individual roles in transformation and lytic infection of the E1A gene products which are transcribed as mRNAs of 13S, 12S and 9S, investigators have used several approaches. Some investigators have isolated the corresponding cDNA clones for each of the three transcriptional units and recombined them both into plasmids and viruses. In this way, they can study the function of each of these gene products in both regulation of adenovirus replication as well as in transformation. Other investigators have prepared deletion mutants, temperature sensitive mutants and cold sensitive mutants with the aim of studying alterations in the viral replicative cycle and/or the transformation process under permissive and restrictive conditions. Still other investigators have studied transcriptional regulation of the genes in this region. It should be noted that both the 12S and 13S mRNA are transcribed from a single promoter during early productive infection. They differ only in the size of the intron that is removed during RNA splicing; they possess different 5' donor splice sites and share a common 3' acceptor splice site. The polypeptides encoded by the 12S and 13S mRNAs possess 243 and 289 residues, respectively. They have identical N-termini and C-termini and differ only by an internal 46 amino acid sequence that is unique to the 289 residue polypeptide.

In studies using reconstructed adenoviruses that contain either the 12S or the 13S cDNA region in place of the wild-type E1A, it has been demonstrated that viruses containing a 13S cDNA region are competent for all lytic functions in Hela cells, whereas those containing a 12S cDNA region are defective. In order to study the ability of these reconstructed viruses to transform cells, they have been used to infect baby rat kidney cells in culture. The 12S containing viruses transform these cells with an extremely high efficiency, whereas both the 13S and wild-type virus are cytotoxic in this assay. It has also been demonstrated that transfection with plasmids containing either 12S or 13S cDNAs leads to the establishment of baby rat kidney cells in culture. Both plasmids are also able to cooperate with plasmids carrying either the ras oncogene or the polyoma middle T gene to give stable transformants of baby rat kidney cells. These results suggest that the establishment function of E1A is distinct from the function which stimulates the expression of other adenovirus early genes during lytic infection. Complementary studies using a variety of site-specific mutants of the E1A products have also suggested that the minimal amino terminal sequence of E1A necessary for oncogene cooperation is common to both the 12S and 13S mRNAs and is different from the E1A functions necessary for transcriptional activation of the viral early region genes (2,8,9,67).

Studies by other investigators have examined transcriptional regulation by these same E1A gene products. The transcription from all early adenovirus promoters is stimulated by a 289 amino acid phosphoprotein encoded by the 13S mRNA. To determine if this protein could act on a nonviral gene placed on the viral chromosome, adenovirus recombinants were constructed in which the rat preproinsulin I gene, including its promoter region, was substituted in both orientations for E1A. Preproinsulin mRNA synthesis from these recombinants was greatly stimulated after infection of line 293 cells, which constitutively express E1A protein, compared to HeLa cells which do not. Expression of the preproinsulin gene was also greatly stimulated when HeLa cells were coinfected with the recombinants and wild-type adenovirus or a mutant defective in a second E1A protein, but much less so by coinfection with a mutant defective in the 289 amino acid phosphoprotein. Much of the E1A induced preproinsulin mRNA had a 5' end at the same position as the preproinsulin mRNA isolated from insulinoma cells, but a considerable fraction had 5' ends mapping heterogeneously within several hundred nucleotides of this site. Preproinsulin mRNA was also detected in 293 cells, but not HeLa or HEK cells after transfection of a plasmid containing the preproinsulin gene with no adenovirus sequence. These results indicated that there is no cis-acting adenovirus sequence required for E1A protein stimulation of preproinsulin transcription. Infection of rat cells with adenovirus did not induce detectable mRNA synthesis from the endogenous preproinsulin I gene. These results demonstrate that the E1A protein can induce expression of a nonviral gene when it is newly introduced into mammalian cells by viral infection or transfection, but it does not induce the endogenous cellular gene. All of these studies should help to further elucidate the mechanism of transformation by adenoviruses (2,22).

From studies on the regulation of transcription of viral mRNA and on the mechanism of viral nucleic acid replication, it has become obvious that DNA binding proteins play a major role in these regulatory processes. These proteins can be of either viral or cellular origin. Such proteins initially were identified by purification schemes which sought polypeptides that showed differential binding to such substrates as synthetic polynucleotides and phosphocellulose. The availability of monoclonal antibodies and the recombinant DNA technology has

facilitated isolating other DNA binding polypeptides on a more theoretical basis. That is, polypeptides or portions of such polypeptides that are suspected of binding to DNA on the basis of the time of their synthesis during the replicative cycle, their location in the virion, or the behavior of mutants having defects in the DNA sequences encoding them, can be synthesized using recombinant DNA technology and an expression vector system. Monoclonal antibodies can then be prepared to these peptides and used both in further purification of the entire protein and in biochemical assays of the DNA binding activity. Examples of the isolation of DNA binding proteins of EBV and HSV 1 are given below.

EBNA (EBV-induced nuclear antigen) is one of the two EBV induced proteins that is present both in EBV infected and EBV transformed cell lines. EBV persists in immortalized B cells as an episome. However, the plasmid state is only maintained if the cells are expressing the EBNA protein or a region of the viral DNA (BamHI-K) encoding for a portion of that polypeptide. The recent synthesis of a 28 kilodalton fragment of the BamHI-K EBNA protein has allowed for studies of the biological function of this protein. It has been demonstrated that the protein has DNA binding properties; moreover, that it binds tightly to supercoiled and linear double stranded DNA, and that the C-terminal portion of this fragment binds specifically to two regions in the origin of replication of the EBV genome. The formation of this protein-DNA complex could play a role in the control of both the rate of plasmid replication and the copy number of the plasmid in the cell. Whether the binding also plays a role in repression of the lytic cycle of the virus or in the stimulation of lymphocyte cell division seen in vivo remains to be determined (29,30).

A direct biochemical approach to the isolation of the EBNA complex from EBV infected cells has involved the use of chromatography and chromatofocusing on a variety of affinity columns. These studies have suggested that possibly four forms of EBNA exist: EBNA I, EBNA II, EBNA IIa, and EBNA IIb. EBNA I and EBNA IIa appear to have the same amino acid sequence and to be encoded by the BamHI-K region of the viral genome. This region is also known as LT2 and is transcriptionally active in transformed cells. The EBNA I and EBNA IIa polypeptides differ in their post-transcriptional modification and hence in their isoelectric point and affinity of binding to DNA cellulose and Blue Sepharose columns. These molecules are extremely basic in their amino terminal region and extremely acidic in the last 50 residues of the carboxy terminal region. EBNA IIb is still in the process of being characterized. It appears to be even more firmly bound to DNA substrates. The role(s) of these various forms of EBNA in the regulation of EBV replication remain to be elucidated (39,40,79).

Similar studies with the major DNA binding protein of herpes simplex virus 1 are also underway. These studies involve the ICP8 polypeptide which appears to have a negative regulatory effect on HSV replication in that it forms at least part of the control system that prevents late gene expression from occurring until after replication of the viral DNA. ICP8 also seems to have preferential binding for single stranded rather than double stranded DNA. Understanding the mechanism by which late transcription is inhibited should help in understanding the initiation and maintenance of latency by this virus (42).

Many of the studies described above have relied heavily on recombinant DNA technology. A by-product of these investigations has been the development of a variety of vectors to carry the genetic material under study. The initial

vectors used by this technology were primarily plasmids and bacteriophage. In order to study and express genes in mammalian cells, a number of animal virus vector systems have been developed. These include vaccinia, papillomavirus and adenovirus vectors. Adenovirus vectors allow for the infection of monkey and human cells and for the occurrence of the normal process of post-transcriptional modification of these polypeptides. This system may be useful not only for molecular studies, but because of the mild disease produced by many adenoviruses in humans, adenovirus vectors could prove useful as an in vivo carrier of either molecules from other viruses (and thus function as a vaccine) or corrective molecules for individuals bearing specific somatic cell genetic defects (67).

It has also been proposed to use herpesviruses as vectors for gene therapy. The reasons for this include the fact that relatively large pieces of DNA can be accommodated, that the virus is replication competent, and most importantly, that latent infection of the host may provide the means for continued expression of the gene in the animal. In order for such a plan to be practical, the negative aspects of recurrent lesions would have to be eliminated by appropriately modifying the virus genome (18).

In summary, much progress has been made in elucidating the regions of the genomes of herpes- and adenoviruses involved in transformation in vitro. However, further studies must be done in order to elucidate the mechanisms by which these viruses actually cause oncogenesis in vivo. The final goal of such studies is the control and reversal of this process. An important by-product of some of these studies has been the indication of the possible use of adenoviruses and herpesviruses as possible vectors for gene therapy to modify inborn or acquired somatic cell defects.

DNA VIRUS STUDIES I

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. AURELIAN, Laure Univ. of Maryland (Baltimore) 1 R01 CA 39691-01	Transformation by Restriction Fragments of HSV DNA
2. BERK, Arnold J. Univ. of Calif. (Los Angeles) 5 R01 CA 25235-07	Biosynthesis of Adenovirus Early RNAs
3. BERK, Arnold J. Univ. of Calif. (Los Angeles) 1 R01 CA 41062-01	Transcription Stimulation by Adenovirus E1A Protein
4. BROWN, Nathaniel A. Univ. of Calif. (Los Angeles) 5 R01 CA 35536-02	Human Lymphocytes Clonally Transformed by EBV
5. BUTEL, Janet S. Baylor College of Medicine 5 R01 CA 25215-07	Tumor Virus Effects on Mammary Epithelial Cells
6. BUTEL, Janet S. Baylor College of Medicine 5 R01 CA 22555-08	Biological Properties of SV40 Early Proteins
7. CALNEK, Bruce W. Cornell University 5 R01 CA 06709-23	Studies on the Avian Leukosis Complex
8. CHINNADURAI, Govindaswamy St. Louis University 5 R01 CA 31719-04	Genetic Analysis of Adenovirus 2 Early Genes
9. CHINNADURAI, Govindsawamy St. Louis University 5 R01 CA 33616-06	Adenovirus 1p Locus: Role in Oncogenic Transformation
10. CLOUGH, Wendy G. University of Southern California 5 R01 CA 23070-09	EBV DNA Synthesis in Transformed Lymphocytes
11 . CLOUGH, Wendy G. University of Southern California 1 R01 CA 35343-02	DNA Methylation in Lymphocytes
12. CONLEY, Anthony J. St. Louis University 2 R01 CA 33101-04	Regulatory Features of HSV Gene Expression

13. COOK, James L.
National Jewish Hospital
& Research Center
1 R01 CA 38796-01

Tumor Cell Resistance to Destruction by Effector Cells

14. COOPER, Neil R.
Scripps Clinic and Research Foundation
5 R01 CA 14692-13

Humoral Immunity to Viruses and Virus-infected Cells

15. COURTNEY, Richard J.
University of Tennessee (Knoxville)
2 R01 CA 24564-08

Studies of Purified Herpes Simplex Virus Glycoproteins

16. CROCE, Carlo M.
Wistar Institute of Anatomy & Biology
5 R01 CA 16685-11

Mappings of Tumor Virus Genomes in Transformed Cells

17. DE MARCHI, Jeanette M.
Vanderbilt University
5 R01 CA 20806-08

Induction by Cytomegalovirus of Cell DNA Synthesis

18. DESROSIERS, Ronald C.
Harvard University
2 R01 CA 31363-04

Molecular Basis for Herpesvirus Saimiri Oncogenicity

19. GALLOWAY, Denise A.
Fred Hutchinson Cancer Research Center
5 R01 CA 26001-07

Herpesvirus Expression in Transformation and Latency

20. GALLOWAY, Denise A.
Fred Hutchinson Cancer Research Center
5 R01 CA 35568-02

Molecular Studies on Herpesvirus Proteins

21. GALLOWAY, Denise A.
Fred Hutchinson Cancer Research Center
1 R13 CA 38135-01

Ninth International Herpesvirus Workshop

22. GAYNOR, Richard B.
Univ. of Calif. (Los Angeles)
5 R01 CA 30981-05

Transforming Functions of Adenovirus E1A Proteins

23. GLASER, Ronald
Ohio State University
5 R01 CA 29066-05

Epstein-Barr Virus DNA in Transfected Cells

24. GLASER, Ronald
Ohio State University
5 R01 CA 36357-02

Molecular Genetics of Epstein-Barr Virus

25. GREEN, Maurice
St. Louis University
2 R01 CA 29561-28

Biochemistry of Animal Virus Multiplication

26. GREEN, Maurice
St. Louis University
5 R01 CA 21824-09

Transforming Proteins of Three Human Adenovirus Groups

27. HARTER, Marian L.
University of Medicine & Dentistry of New Jersey
5 R01 CA 28414-05

Function of Early Proteins Encoded by Adenovirus Type-2

28. HAYWARD, Gary S.
Johns Hopkins University
5 R01 CA 28473-05

Cellular Transformation by DNA of Human Herpesvirus

29. HAYWARD, Gary S.
Johns Hopkins University
5 R01 CA 22130-08

Structure and Regulation of Human Herpesvirus Genomes

30. HAYWARD, S. Diane
Johns Hopkins University
2 R01 CA 30356-04

EBV Genome Expression: Localization of Specific Functions

31. HENLE, Werner
Children's Hospital of Phila.
5 R01 CA 33324-03

EBV Serology in Human Cancers and Immune Deficiencies

32. HIRSCH, Martin S.
Massachusetts General Hospital
5 R01 CA 12464-15

Immune Reactivity and Oncogenic Virus Infections

33. HORWITZ, Marshall S.
Yeshiva University
5 R01 CA 11512-16

Adenovirus DNA Synthesis and Polypeptide Assembly

34. HOWETT, Mary K.
Pennsylvania State University Medical Center (Hershey)
5 R01 CA 25305-07

Modulation of the Tumorigenicity of Transformed Cells

35. HUANG, Eng-Shang
University of North Carolina (Chapel Hill)
5 R01 CA 21773-07

Cytomegaloviruses and Human Malignancy

36. HYMAN, Richard W.
Pennsylvania State University Medical Center (Hershey)
2 R01 CA 16498-12

Malignancy and DNA Homology among the Herpesviruses

37.	ISOM, Harriet C. Pennsylvania State University Medical Center (Hershey) 2 R01 CA 23931-08	Regulation of Differentiation in Hepatocytes in Vitro
38.	KETNER, Gary W. Johns Hopkins University 5 R01 CA 21309-06	Transformation & Gene Regulation by Adenoviruses
39.	KIEFF, Elliott D. University of Chicago 5 R01 CA 17281-11	EBV Interaction With Lymphoblasts in Vitro and in Vivo
40.	KLEIN, George Karolinska Institutet 2 R01 CA 30264-04	Immune Effector Mechanisms in EBV-Carrying Patients
41.	KLEIN, George Karolinska Institutet 2 R01 CA 28380-04	Epstein-Barr Virus Determined Nuclear Antigen (EBNA)
42.	KNIPE, David M. Harvard University 5 R01 CA 26345-06	Genetics of Herpesvirus Transformation
43.	LEVINE, Myron University of Michigan 1 R13 CA 40850-01	Tenth International Herpesvirus Workshop
44.	LEWIS, James B. Fred Hutchinson Cancer Research Center 5 R01 CA 29600-03	Functions of Adenovirus Proteins in Transformation
45.	MC DOUGALL, James K. Fred Hutchinson Cancer Research Center 5 R01 CA 29350-05	The Biology of Transformation Herpesvirus
46.	MILLER, I. George, Jr. Yale University 5 R01 CA 12055-14	Studies of Epstein-Barr Virus
47.	MILLETTE, Robert L. Portland State University 5 R01 CA 39067-02	In Vitro Transcription of Herpes Simplex Virus Genes
48.	MUNNS, Theodore W. Washington University 5 R01 CA 27801-06	Immunochemical Characterization of Nucleic Acids

49. NAIDU, Yathirajulu M.
Neogen Corporation
1 R43 CA 38459-01
Prevention of Herpesvirus Diseases: I Molecular Biology

50. NEMEROW, Glen R.
Scripps Clinic and Research Foundation
1 R01 CA 36204-01A2
Infection of B Lymphocytes by Epstein-Barr Virus

51. NONOYAMA, Meihan
Showa University Research Institute for Biomedicine in Florida
5 R01 CA 31949-04
Marek's Disease Virus: Transformation and Oncogenesis

52. NONOYAMA, Meihan
Showa University Research Institute for Biomedicine in Florida
2 R01 CA 31950-01A1
Oncogenicity of Epstein-Barr Virus

53. NONOYAMA, Meihan
Showa University Research Institute for Biomedicine in Florida
1 R01 CA 36895-01A1
Latency of Epstein-Barr Virus DNA in Transformed Cells

54. PADMANABHAN, Radha K.
University of Kansas Medical Center
5 R01 CA 33099-02
Structure and Functional Analysis of Adenovirus Genomes

55. PAGANO, Joseph S.
University of North Carolina (Chapel Hill)
5 P01 CA 19014-09
DNA Virus Genomes, Oncogenesis and Latency

56. PEARSON, Gary R.
Georgetown University
5 R01 CA 39617-02
Epstein-Barr Virus-induced Membrane Antigens

57. PEARSON, GARY R.
Georgetown University
1 R13 CA 39551-01
First International Symposium on Epstein-Barr Virus & Associated Malignant Diseases

58. PEARSON, George D.
Oregon State University
5 R01 CA 17699-09
Replication Map of an Oncogenic Virus

59. PRUSOFF, William H.
Yale University
2 R01 CA 05262-25
Iododeoxyuridine, Iodo-DNA, and Biological Activity

60. RAAB-TRAUB, Nancy J.
University of North Carolina (Chapel Hill)
5 R01 CA 32979-03
EBV Transcription in Nasopharyngeal Carcinoma

61. RAPP, Fred
Pennsylvania State University
Medical Center (Hershey)
5 R01 CA 34479-03

Latency and Transformation by Herpesviruses

62. RAPP, Fred
Pennsylvania State University
Medical Center (Hershey)
2 P01 CA 27503-06

Herpesviruses and Neoplasia

63. RASKA, Karel, Jr.
University of Medicine and
Dentistry NJ-Rutgers Medical School
5 R01 CA 21196-08

Adenovirus T, Surface Antigens and Tumorigenicity

64. REKOSH, David M.
State University of New York
(Buffalo)
5 R01 CA 25674-06

Adenovirus Early Gene Function and DNA Replication

65. RICCIARDI, Robert P.
Wistar Institute of Anatomy and
Biology
2 R01 CA 29797-04A1

Organization and Expression of Adenovirus Genes

66. ROBERTS, Bryan E.
Harvard University
5 R01 CA 27447-07

Organization and Expression of Genes in Viral DNAs

67. ROBERTS, Richard J.
Cold Spring Harbor Laboratory
5 P01 CA 13106-14

Cold Spring Harbor Laboratory Cancer Research Center

68. ROBINSON, Robin A.
University of Texas Health
Science Center (Dallas)
5 R23 CA 36143-02

Regulation of Cellular Gene Expression by HSV ICP-4

69. ROEDER, Robert G.
Rockefeller University
5 R01 CA 34223-03

Regulation of Adenovirus Transcription

70. ROIZMAN, Bernard
University of Chicago
5 R01 CA 08494-20

Mechanisms of Viral Infection in Relation to Cancer

71. ROIZMAN, Bernard
University of Chicago
5 P01 CA 19264-10

UCCRC: Viral Oncology Program

72. SCHAFFER, Priscilla A.
Dana-Farber Cancer Institute
2 R01 CA 20260-09

Immediate-Early Genes of HSV

73.	SHARP, Phillip A. Massachusetts Institute of Technology 1 R01 CA 38660-01	Transcription Regulation by Oncogenes
74.	SILVERSTEIN, Saul J. Columbia University (New York) 2 R01 CA 17477-11	Molecular Biology of Herpes Virus
75.	SIXBEY, John W. St. Jude Children's Research Hospital 1 R23 CA 38877-01	Epstein-Barr Virus Expression in Normal Human Epithelium
76.	SPEAR, Patricia G. University of Chicago 5 R01 CA 21776-09	Herpesvirus Gene Expression in Transformed Cells
77.	SPECTOR, David J. Pennsylvania State University Medical Center (Hershey) 1 R01 CA 34381-01	Regulation of Adenovirus E1B Gene Expression
78.	SPECTOR, Deborah H. Univ. of Calif. (San Diego) 5 R01 CA 34729-03	Human CMV, Cell-Related DNA, Oncogenes & Kaposi Sarcoma
79.	SPELSBERG, Thomas C. Mayo Foundation 5 R01 CA 25340-06	A New Class of Epstein-Barr Virus Nuclear Antigen
80.	ST. JEOR, Stephen C. University of Nevada (Reno) 2 R01 CA 28089-05A2	Herpesvirus Induced Malignancy
81.	STROMINGER, Jack L. Dana-Farber Cancer Institute 2 P01 CA 21082-09	Molecular Basis of Viral Oncogenesis
82.	STROMINGER, Jack L. Dana-Farber Cancer Institute 5 R01 CA 24926-05	Study of Epstein-Barr Nuclear Antigen
83.	SUMMERS, William C. Yale University 5 R01 CA 13515-13	Genetic Study of Animal Viruses
84.	THORLEY-LAWSON, David A. Tufts University 5 R01 CA 31893-04	Epstein Barr Virus Membrane Antigen
85.	TIBBETTS, Clark J. Vanderbilt University 5 R01 CA 34126-03	Adenovirus Genome Expression/Assembly/Transfection

86.	WAGNER, Edward K. Univ. of Calif. (Irvine) 5 R01 CA 11861-16	Control of Viral RNA Synthesis in Herpesvirus Infection
87.	WENTZ, William B. Case Western Reserve University 5 R01 CA 31973-02	Sexually Transmitted Disease in Uterine Carcinogenesis
88.	WILLIAMS, James F. Carnegie-Mellon University 5 R01 CA 21375-08	Genetic Analysis of Adenoviruses
89.	WILLIAMS, James F. Carnegie-Mellon University 2 R01 CA 32940-04	Type 12 Adenovirus Transformation-Defective Mutants
90.	WOLD, William S. St. Louis University 2 R01 CA 24710-07	Adenovirus 2 Coded Early Glycoprotein

CONTRACTS ACTIVE DURING FY85

	Investigator/Institution/Contract Number	Title
91.	NEEL, H. Bryan, III Mayo Foundation N01-CP-91006	Application of EBV Markers to Diagnosis & Prognosis of NPC and Occult Tumors of Nasopharynx Area in USA

SUMMARY REPORT

DNA VIRUS STUDIES II

The DNA Virus Studies II component of the Branch involves the investigation of the two major classes of mammalian small DNA tumor viruses: papillomaviruses and polyomaviruses. In this component, there are 82 research grants with an estimated total funding of 13.1 million dollars. These include traditional research grants, program project grants, conference grants and new investigator grants. The major research emphasis of this program component is the elucidation of the molecular events leading to the initiation and maintenance of cellular transformation by the small DNA tumor viruses and the elucidation of the role of these viruses in the etiology of human cancers. In terms of scientific areas, 40% of the grants deal with the structure and expression of viral genes in animal cells, 29% deal with the biochemical properties and mechanisms of action of viral gene products, 18% deal with basic biochemical and physiological studies which use the small DNA tumor viruses as model systems, 9% deal with the expression and function of cellular genes that are involved in the transformation process, and 5% deal with the relationship of small DNA viruses to human cancers. In terms of the viruses being studied 55% of the grants involve the simian polyomavirus, SV40; 18% other DNA viruses and related systems such as viral oncogenes; 15% mouse polyomavirus; 9% human or animal papillomaviruses; and 3% human polyomaviruses. Representative studies for each of these classes of viruses are described below.

Among the more notable advances in the DNA Virus Studies II program this year has been the continued progress in understanding the possible etiological relationship between human papillomaviruses (HPVs) and anogenital cancers including cervical carcinoma. Improved techniques have allowed investigators sponsored by this program to identify and classify HPV DNA from many pre-neoplastic, neoplastic and metastatic lesions in man. HPV subtypes 16 and 18 were usually found associated with severe dysplasias and carcinoma in situ; whereas HPV-6 and 11 were associated with milder lesions. In addition, new in vitro transformation assays and DNA cloning methods have been used to begin an analysis at the molecular level of the transformation process.

Recent work from a number of laboratories has shown the presence of HPV DNA and HPV proteins in many different tumors. In one study supported by this program, HPV DNA was detected in 80% of the anogenital lesions analyzed. In the case of cervical and vaginal intraepithelial neoplasias, 86% were found to contain HPV DNA. These neoplasias included mild to severe cervical dysplasias, carcinoma in situ and verrucous carcinoma. HPV-6 was the most prevalent subtype. In the case of premalignant lesions of the vulva, penis, and perianus, 72% contained HPV DNA and, of these, at least 41% had HPV-16. For microinvasive lesions, HPV genomes were found in 2 of 3 cases in which vulvar microinvasion was associated with carcinoma in situ and in 4 of 5 cases in which microinvasion was associated with condyloma acuminata. In contrast, HPV DNA was detected in only 26% of the invasive cancers of the cervix and vagina and 17% of the invasive squamous cell carcinomas of the vulva. Using similar DNA hybridization techniques, other laboratories have confirmed these findings and have identified two new HPV subtypes, 18 and 31, which appear to be specific for anogenital lesions. The discovery of these new HPV subtypes suggests that the sensitivity of the hybridization methods for the detection of HPV DNA in invasive carcinomas may be

increased in the future by the use of new probes for additional subtypes (24, 43).

Another observation which has further implicated HPV in an etiological relationship to cervical neoplasia was the detection of HPV DNA sequences in lymph nodes containing metastases. Samples of enlarged lymph nodes and the primary cervical carcinoma were obtained from thirteen patients. Those lymph nodes which contained metastases were invariably positive for the same HPV sequences present in the primary cancer. This result clearly showed that HPV genes are associated with malignant cells and that the sequences are maintained in these cells for the several generations required to form a metastatic lesion. Thus, the HPV genes are probably necessary to maintain the malignant phenotype. Similar results had been previously obtained with metastases from epidermodysplasia verruciformis (EV) patients (43,24).

In the past, a major impediment to the molecular and functional study of HPVs was the lack of cultured cells which could be transformed by these viruses. In the last year, several laboratories have reported the development of systems using cloned DNA to transform three mouse cell lines: NIH/3T3, C127 and Pam 212. In one study, the transforming capabilities of HPV-5 DNA (cloned from a tumor) and HPV-1 DNA (obtained from a benign plantar wart) were investigated. Both HPV-5 and HPV-1 DNA were able to transform NIH/3T3 and C127 mouse epithelial cells. These HPV transformed cells were then analyzed for their ability to grow in soft agar and to induce tumors in athymic nude mice. In agreement with the pathology of the tissue from which the viruses were derived, the HPV-1 transformed clones grew poorly in soft agar and failed to induce tumors in athymic nude mice; whereas HPV-5 transformed clones grew well in soft agar and induced tumors efficiently in athymic nude mice. Nude mouse tumors were classified histopathologically as undifferentiated carcinomas and contained HPV-5 specific DNA (24,78,5,14).

Using these transformation assays, the elucidation of the genomic structure and function of HPVs has begun. In one investigation, three deletion mutants and two subgenomic fragments of HPV-5 were shown to be capable of transforming C127 cells. Clones transformed by the deletion mutants contained episomal copies of viral DNA; whereas clones produced by fragments had viral sequences integrated into the cellular genome. The integrated fragments corresponded to the portion of the viral genome representing the E1-E2-E4 early protein coding region. Preliminary results from another laboratory using an animal papillomavirus have indicated that a second separate transforming or transforming/maintaining function(s) may also be encoded by E6 region. These results support the proposed genome organization map of HPV-5 and the hypothesis that only early viral genes are involved in transformation (78).

Simian virus 40 (SV40) has been a major focus of research for many of the projects supported by this branch component. This small DNA tumor virus was isolated from monkeys and has long been used as a model system to study viral transformation of cells in culture and tumorigenesis in susceptible rodents. Previous research has shown that the transformation properties of SV40 reside in the viral gene that encodes the large T-antigen. This protein has been shown to be necessary for both cell immortalization and induction of the transformed phenotype in cultured cells. Its mechanism of action has been and continues to be intensively studied using biochemical, immunological, genetic and transgenic methods.

Transgenic mice have recently added a new dimension to the use of SV40 as a model for tumorigenesis. Using the new technique of microinjection of recombinant DNA plasmids into fertilized mouse eggs, transgenic mice were produced which carry SV40 T-antigen genes integrated into the genome of every cell of the animals. A high percentage of these transgenic mice developed tumors within the choroid plexus. A line of these mice has been established in which the SV40 DNA is genetically transmitted to progeny. In this line, nearly every affected animal succumbed to choroid plexus brain tumors within five months after birth. Thymic hypertrophy and kidney pathology were also observed in some mice. Both SV40 T-antigen mRNA and protein were readily detected in affected tissues; however, SV40 T-antigen gene expression was barely detectable in unaffected tissues or in susceptible tissues prior to overt pathology, suggesting that tumorigenesis depended upon activation of the SV40 genes. Cell lines could be readily established from tissues of transgenic mice; such cultured cells contained T-antigen. Examination of DNA extracted from tumor tissue or from cell lines derived from tumors revealed structural rearrangements and changes in DNA methylation of the SV40 DNA when compared to DNA from unaffected tissues in these same mice. The SV40 genes were frequently amplified in tumor tissue, further indicating that their expression is probably involved in tumorigenesis in transgenic mice. These mice may provide an excellent system to examine both the tissue specific tumorigenesis by SV40 large T-antigen and the selective expression of genes in developing eukaryotic organisms (47).

The SV40 large T-antigen protein has been found primarily in the nucleus of infected cells, but small amounts also appear in the cytoplasm and at the cell surface (as a tumor specific transplantation antigen or TSTA). The portion of the T-antigen protein which is exposed at the cell surface has been identified as the amino terminal segment. Specific antigenic determinants (epitopes) in the exposed amino terminal portion of the T-antigen have recently been identified by examination of cells transformed by plasmids which encode only the amino terminal half of the T-antigen. Cloned cytotoxic T-lymphocytes (CTLs) were used to identify the epitopes. The lymphocytes were derived from animals immunized against T-antigen and were propagated in vitro using T-lymphocyte growth factor. Two clones were identified which could lyse only cells expressing the truncated SV40 T-antigen. One of these clones could also lyse cells expressing BK viral T-antigen. This result indicated the existence of two separate epitopes for SV40 T-antigen, only one of which is shared with BK T-antigen. These results also support the hypothesis that the cellular immune system is involved in a rigorous immunosurveillance which usually prevents tumor formation in vivo by cells transformed by these viruses (71).

The various roles of SV40 T-antigen in the transformation process have been elucidated by studies using well-defined mutants in the T-antigen gene. In one study, a mutation at amino acid position 128 completely prevented transport of newly synthesized large T-antigen to the nucleus, but did not effect T-antigen expression on the cell surface. This mutant was able to fully transform immortalized (partially transformed) cells at efficiencies approaching that of wild-type SV40. In contrast, the mutant was unable to transform primary rat kidney cells. Foci were produced by this mutant in primary mouse embryo fibroblasts, but at about a 10-fold lower efficiency than that of wild-type virus. In the mutant transformed mouse cells small amounts of T-antigen were transported into the nucleus. This low level of T-antigen could account for the low frequency of transformation. Very similar results were also obtained in a study using T-antigen mutants defective in their ability to bind DNA. Taken together,

these results strongly suggest that the immortalization step in transformation is mediated by T-antigen protein molecules which are localized in the nucleus and can bind to DNA. The development of the transformed phenotype, however, appears to require only cytoplasmic or cell surface localized T-antigen (44, 62,57).

One possible effect of the binding of SV40 large T-antigen to DNA is the trans-activation of cellular gene expression. It had previously been demonstrated that T-antigen can bind to cellular DNA. The capability of T-antigen to trans-activate was directly tested on a variety of promotors linked to the CAT gene. The promoters studied were the SV40 late promoter, the adenovirus E3 promoter, the alpha 2(1) collagen promoter, and the Rous sarcoma virus LTR promoter. Large T-antigen activated all of the promoters. These results indicated that transactivation may be a control mechanism used by many genes. The non-selective activation of cellular genes exhibited by large T-antigen may also be one mechanism by which these proteins contribute to the transformation process, i.e. through alterations in the levels of cellular gene expression (2).

A major technical advance in the SV40 area occurred this year: the development of a cell free system which appeared to efficiently mimic the replication of SV40 DNA in infected cells. Procedures were developed for the preparation of soluble extracts from SV40 infected monkey cells which could catalyze the replication of exogenously added DNA molecules containing the SV40 origin of replication. Extracts prepared from uninfected monkey cells also supported origin-dependent replication, but only in the presence of added SV40 large T-antigen. Very little DNA synthesis was observed in the absence of SV40 origin sequences or when the viral origin contained a small deletion known to abolish SV40 DNA replication in vivo. This system will provide a biochemical assay for the replication promoting activity of SV40 large T-antigen, provide insight into the immortalization activity of T-antigen in the transformation of cells, and should also facilitate the purification and functional characterization of cellular proteins involved in viral and cellular DNA replication (41).

SV40 is dependent on many cellular proteins to provide functions necessary for its reproduction. These protein factors may also mediate transformation. One cellular protein which appears to be strongly involved in cell transformation is the host tumor antigen, p53. This protein has been found at elevated levels in naturally occurring tumors and in cells transformed by a variety of agents including SV40, other tumor viruses, and chemical carcinogens. The p53 protein, a phosphoprotein, is tightly complexed to SV40 T-antigen in cells transformed by this virus. Phosphorylation of p53 may be involved in the mechanism of transformation by SV40, as it has previously been shown that T-antigen mutants which cannot transform cells are also affected in the phosphorylation of p53. The recent availability of large amounts of p53 protein has allowed its biochemical characterization. The phosphorylated amino acids and disulfide bonds are being located. Both phosphoserine and phosphothreonine in the ratio of 9:1 have been found but no phosphotyrosine has been detected. The data also suggests that p53 is linked to AMP through a phosphodiester bond on a serine residue. The association of this AMP modification with transformation is currently under investigation (12,5).

The synthesis of p53 protein is stimulated by SV40 T-antigen. As a first step in understanding the mechanism of regulation of the cellular p53 gene by a viral protein, a c-DNA clone containing the entire murine p53 gene was isolated and

its nucleotide sequence determined. The predicted amino acid sequence was confirmed by amino acid sequencing of several p53 tryptic fragments. The murine p53 gene has subsequently been used to isolate a human p53 cDNA clone. The availability of these cDNA clones of p53 should facilitate the detailed analysis of p53 regulation and help in the elucidation of the role of p53 in transformation (48).

A third virus whose study is supported by this branch component is the mouse polyomavirus. This virus is similar to SV40 and its study has helped clarify the common features and possible variations in the transformation process by small DNA viruses. The SV40 and mouse polyoma virions are morphologically identical and they contain nearly identical amounts of DNA. However, their genomes are organized differently, particularly with respect to the genes encoding the early tumor antigen region which is responsible for transformation. Polyoma DNA codes for six proteins including three tumor antigens: large T-antigen, middle T-antigen, and small T-antigen. The relationship among the tumor antigens with regard to cellular transformation is complex. The large T-antigen (which is localized in the nucleus) appears to be able to immortalize primary cells in culture, whereas middle T-antigen is able to induce the transformed phenotype in previously immortalized cell lines. In the past year, investigators supported by this program have focused their studies on characterizing the structure and mechanism of action of these T-antigens. In particular, the intriguing 1983 observation that middle T-antigen forms a tight complex with the cellular protein pp60c-src has been vigorously pursued. pp60c-src, a tyrosine phosphokinase, is the cellular homolog of the transforming protein encoded by the src gene of Rous sarcoma virus (RSV), i.e., pp60c-src is the product of a known cellular proto-oncogene.

It has become clear over the past two years that middle T-antigen does not have intrinsic kinase activity and that the tyrosine kinase activity in the middle T-antigen/pp60c-src complex is due to the associated pp60c-src. The activity of the T-antigen/pp60c-src complex has recently been found to be at least 20-fold greater than that of free pp60c-src. The mechanism of this activation is not yet clear, but there are preliminary suggestions that it may involve a novel tyrosine phosphorylation at a site about 18 kilodaltons from the N-terminus of pp60c-src. Differences in activity have also been found between the cellular and viral homologs of the pp60c-src protein. Mouse cell lines genetically engineered to have elevated levels of unassociated pp60c-src (no middle T-antigen is present) contained normal levels and distributions of phosphorylated proteins. Other cell lines engineered to have elevated levels of the viral homolog of pp60c-src had 7 to 10-fold greater levels of phosphorylated proteins as well as an altered distribution of these proteins. Thus, pp60c-src appeared to be regulated in its extent of phosphorylation activity in normal cells and to have different substrate specificities than its viral homolog. It is therefore possible that middle T-antigen promotes transformation by complexing with pp60c-src and thereby perturbing the normal regulation of pp60c-src tyrosine kinase activity (38,23,69).

An alternative hypothesis for the mechanism of phenotypic transformation by polyoma middle T-antigen/pp60c-src complex has recently been suggested by the discovery of a new enzymatic activity of the complex. Immunoprecipitates of wild-type middle T-antigen pp60c-src complexes were found to have a 20-fold increase over background of a phosphorylation activity for the membrane phospholipid phosphatidylinositol. The level of this kinase activity correlated with

the ability of the virus to transform. Thus, the middle T-antigen from non-transforming mutants lacked this activity; the polyoma mutant Py1178T, which has an intermediate transformation phenotype, had an intermediate kinase activity. This phosphatidylinositol kinase activity apparently arose from pp60c-src when it complexes with middle T-antigen. It has previously been shown that the purified viral protein homolog of pp60c-src also possesses a similar kinase activity. This finding opens a new line of research on polyoma transformation. Phosphatidylinositol and its phosphorylated derivatives are a source of second messengers in the cell (e.g., diacylglycerol and 1,4,5 inositol triphosphate) that have pleiotropic effects on cell phenotype. The observation of this new kinase activity also supports the hypothesis that some DNA and RNA tumor viruses may share common mechanisms for cellular transformation (60).

The association of pp60c-src and polyoma middle T-antigen has also been studied from a structural and regulatory perspective. The N-terminal portion of the middle T-antigen was found to be necessary for formation of the complex. Deletion mutants of middle T-antigen missing the 29 N-terminal amino acids did not possess in vitro phosphokinase activity and were completely defective for polyoma transformation. In addition, the level of middle T-antigen/pp60c-src complex also appeared to be regulated. Only a limited amount of pp60c-src and middle T-antigen were found to be associated in vivo. Increasing the expression of either protein in cells did not increase the amount of complex. The phosphorylation of serine/threonine residues in middle T-antigen also seems to be involved in the regulation of this association. Although only a small fraction of serine/threonine residues in middle T-antigen are found phosphorylated in vivo, all middle T-antigen associated with pp60c-src was modified. A unique phosphorylation site on pp60c-src was also found only on molecules in complexes. Additional regulatory steps may be involved as well, since phosphorylation alone was not sufficient for association of pp60c-src molecules in complex (5,23,60,7, 38).

A workshop on "Oncogenic Human Polyomaviruses" was sponsored by the Biological Carcinogenesis Branch on March 7, 1985. The goals of the workshop were to assess the state-of-the-art in this field and to determine whether particular areas of study within the field of human polyomaviruses should be stimulated since only 3% of the grants in this branch component are focused on human polyomaviruses. The recommendations of the workshop participants are currently being evaluated for possible future program initiatives.

A Request for Applications (RFA) entitled "The Role of Human Papillomaviruses in the Etiology of Cervical Cancer" was issued on February 1, 1985. This RFA was developed from the recommendations of a workshop sponsored by this Branch on "DNA Viruses and Human Cervical Cancer" which was held on June 13, 1984. The primary objectives of the RFA are to stimulate research on the clinical progression of HPV infections of the cervix from initial infection to cervical dysplasias and possibly to carcinomas and to relate this progression to the molecular biology of papillomaviruses. The application due date was June 1, 1985. Grants in response to this RFA are expected to be awarded in FY86.

In summary, during the past year evidence has been steadily accumulating for the role of human papillomaviruses in the etiology of cervical carcinoma and related human cancers. HPV DNA has been shown to transform cells in culture and HPV DNA and protein have been detected in pre-malignant and malignant lesions, and recently, in metastases from HPV positive tumors. In addition, work on the

transforming proteins of SV40 and mouse polyomavirus has continued to elucidate the actual molecular steps in the transformation process. A novel system for the study of transforming proteins and their genes was introduced this year by the development of transgenic mice strains with stably integrated SV40 genes. The tissue specific expression of these genes results in spontaneous brain tumors in these mice.

DNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. ALONI, Yosef Weizmann Institute of Science 5 R01 CA 14995-12	Control of Gene Expression in Tumor Viruses and Cells
2. ALWINE, James C. University of Pennsylvania 5 R01 CA 28379-05	Regulation of DNA Tumor Virus Gene Expression
3. ALWINE, James C. University of Pennsylvania 5 R01 CA 33656-03	Control of Late Gene Expression in DNA Tumor Viruses
4. BASILICO, Claudio New York University 5 R01 CA 11893-16	Cellular and Viral Control of Oncogenic Transformation
5. BASILICO, Claudio New York University 5 P01 CA 16239-11	Biosynthesis in Normal and Virus-Transformed Cells
6. BENJAMIN, Thomas L. Harvard University 5 R01 CA 19567-09	Mechanism of Cell Transformation by Polyoma Virus
7. BENJAMIN, Thomas L. Harvard University 5 R01 CA 25390-07	Effects of HR-T Mutations on Polyoma Gene Expression
8. BERG, Paul Stanford University 5 R01 CA 31928-04	Transduction of Genetic Information Related to Cancer
9. BOTCHAN, Michael R. Univ. of Calif. (Berkeley) 2 R01 CA 30490-05	Transformation of Cells by SV40 Virus
10. BRADLEY, Margaret K. Dana-Farber Cancer Institute 5 R23 CA 38069-02	Nucleotide Binding Properties of SV40 Large T Protein
11. CARMICHAEL, Gordon G. University of Connecticut Health Center 5 R01 CA 32325-03	Regulation of Polyoma Early Gene Expression
12. CARROLL, Robert B. New York University 5 R01 CA 20802-09	Biochemical Properties of the SV40 T Antigens

13. CHEN, Suzie
Columbia University (New York)
5 R23 CA 36319-02
Host Responses to In Vitro Mutated SV40

14. CHOW, Louise T.
University of Rochester
5 R01 CA 36200-02
Human Papillomavirus Gene Expression

15. COLE, Charles N.
Dartmouth College
1 R01 CA 39259-01
The Molecular Biology of SV40 Large T Antigen

16. CONRAD, Susan E.
Michigan State University
5 R01 CA 37144-02
SV40-Induced Changes of Growth Regulation in Host Cells

17. CONSIGLI, Richard A.
Kansas State University
2 R01 CA 07139-21
Studies in Polyoma Transformed Cells Virion Proteins

18. DAWE, Clyde J.
Harvard University
1 R01 CA 38722-01
Molecular Pathology of Polyoma Virus-Host Interactions

19. DE PAMPHILIS, Melvin L.
Harvard University
5 R01 CA 15579-12
Tumor Virus DNA Replication: A Probe Into Oncogenesis

20. DI MAYORCA, Giampiero
University of Medicine & Dentistry of New Jersey
5 R01 CA 25168-05
Transformation Genes of Simian Virus 40

21. DI MAYORCA, Giampiero
University of Medicine & Dentistry of New Jersey
5 R01 CA 25169-06
BK Virus, A Human Papovavirus

22. DIMAIO, Daniel C.
Yale University
5 R01 CA 37157-02
Analysis of Cell Transformation by Bovine Papillomavirus

23. ECKHART, Walter
Salk Institute for Biological Studies
5 R01 CA 13884-13
Viral Gene Functions and Regulation of Cell Growth

24. FARAS, Anthony J.
University of Minnesota
2 R01 CA 25462-07
Human Papillomaviruses and Malignant Disease

25. FLUCK, Michele M.
Michigan State University
2 R01 CA 29270-04A1
Control of Gene Expression on Viral Transformants

	Investigator / Institution / Grant	Title
26.	FOLK, William R. Univ. of Texas (Austin) 5 R01 CA 38538-02	Mammalian Cell Transformation by Oncogenic Viruses
27.	FRIEDMANN, Theodore Agouron Institute 5 R01 CA 37484-02	Cellular and Papovaviral Gene Expression
28.	FRISQUE, Richard J. Pennsylvania State University 1 R01 CA 38789-01	A Molecular Approach to the Unique Biology of JC Virus
29.	FUJIMURA, Frank K. La Jolla Cancer Research Foundation 5 R01 CA 37689-02	Regulatory Functions in Embryonal Carcinoma Cells
30.	GARCEA, Robert L. Dana-Farber Cancer Institute 5 R01 CA 37667-02	Mechanisms in Polyoma Virus Assembly
31.	GHOSH, Prabhat K. Yale University 5 R01 CA 32799-03	Regulation of Simian Virus 40 Transcription
32.	GRALLA, Jay D. Univ. of Calif. (Los Angeles) 5 R01 CA 19941-10	Regulation of Transcription by DNA-Protein Complexes
33.	GREEN, Maurice St. Louis University 5 R01 CA 28689-05	Human Papillomaviruses
34.	GURNEY, Elizabeth T. University of Utah 5 R01 CA 21797-07	Growth Control and Viral Gene Expression
35.	HAGER, Lowell P. Univ. of Illinois (Urbana) 5 R01 CA 17619-09	Biochemical Studies on T-Antigen and Transformed Cells
36.	HALLICK, Lesley M. University of Oregon 5 R01 CA 24799-06	Psoralens as Probes for Viral Nucleoprotein Structure
37.	HANSEN, Ulla M. Dana-Farber Cancer Institute 1 R01 CA 38038-01A1	Mechanism of Initiation at RNA Polymerase II Promoters
38.	HUNTER, Anthony R. Salk Institute for Biological Studies 5 R01 CA 28458-06	Viral Transforming Proteins

	Investigator / Institution / Grant	Title
39.	IMPERIALE, Michael J. University of Michigan (Ann Arbor) 5 R01 CA 19816-10	Role of SV40 Gene A in Cellular Transformation
40.	KELLY, Thomas J. Johns Hopkins University 1 R01 CA 40414-01	Replication of the SV40 Genome
41.	KELLY, Thomas J. Johns Hopkins University 2 P01 CA 16519-11	Program on Molecular Biology of Viral Tumorigenesis
42.	LANCASTER, Wayne D. Georgetown University 5 R01 CA 32603-03	Role of Papillomavirus DNA in Cell Transformation
43.	LANCASTER, Wayne D. Georgetown University 5 R01 CA 32638-04	Papillomavirus DNA and Antigens in Cervical Neoplasia
44.	LANFORD, Robert E. Southwest Foundation for Biomedical Research 5 R01 CA 39390-02	SV40 T-Antigen: Model for Nuclear Transport of Proteins
45.	LEHMAN, John M. University of Colorado Health Sciences Center 5 R01 CA 16030-11	Pathology of Neoplastic Transformation
46.	LEHMAN, John M. Albany Medical College of Union University 7 R01 CA 41608-01	Pathology of Neoplastic Transformation
47.	LEVINE, Arnold J. Princeton University 1 R01 CA 38757-01	Viral Induced Tumorigenesis
48.	LEVINE, Arnold J. Princeton University 2 R01 CA 38964-02	P53 Cellular Tumor Antigen
49.	LIVINGSTON, David M. Dana-Farber Cancer Institute 5 R01 CA 15751-12	Structure and Function of SV40 Non Virion Proteins
50.	LIVINGSTON, David M. Dana-Farber Cancer Institute 2 R01 CA 24715-07	Isolation and Function of Small SV40 T-Antigen

51.	MERTZ, Janet E. Univ. of Wisconsin (Madison) 1 R01 CA 37208-01	Involvement of T-Antigen in SV40 Late Gene Expression
52.	MOUNTS, Phoebe Johns Hopkins University 5 R01 CA 35535-03	Analysis of Papillomavirus in Laryngeal Papillomatosis
53.	OZER, Harvey L. Hunter College 5 R01 CA 23002-09	Host Functions Related to Tumor Virus Infection
54.	PIPAS, James M. University of Pittsburgh 1 R01 CA 40586-01	Genetic Analysis of the SV40 Large Tumor Antigen
55.	POGO, Beatriz G. Mt. Sinai School of Medicine 2 R01 CA 29262-04A2	The Expression of Oncogenicity of Shope Fibroma Virus
56.	POLLACK, Robert E. Columbia University 5 P01 CA 33620-03	Directed SV40 Mutation: Cell and Molecular Consequence
57.	PRIVES, Carol L. Columbia University 2 R01 CA 26905-06	Function/Expression of SV40 & Polyoma Tumor Antigens
58.	ROBERTS, Thomas M. Dana-Farber Cancer Institute 2 R01 CA 30002-04	Isolation of Polyoma T-Antigens Synthesized in E coli
59.	RUNDELL, Mary K. Northwestern University 5 R01 CA 21327-08	Functions of SV40 Small T-Antigen and Cellular Proteins
60.	SCHAFFHAUSEN, Brian S. Tufts University 5 R01 CA 34722-03	Products of the Transforming Genes of Polyoma Virus
61.	SHAH, Keerti V. Johns Hopkins University 5 R01 CA 13478-14	Investigation of SV40-Related Infections of Man
62.	SHENK, Thomas E. Princeton University 5 R01 CA 38965-02	Structure and Function of DNA Tumor Virus Genomes
63.	SHODELL, Michael J. Cold Spring Harbor Laboratory 1 R13 CA 39358-01	Origins of Female Genital Cancer

64. SOMPAYRAC, Lauren M.
Univ. of Colorado (Boulder)
5 R01 CA 34072-02

SV40 Deletion Mutants: Oncogenic Proteins

65. STEINBERG, Mark L.
New York University
5 R01 CA 27869-06

Phenotypic Modulation of Human Infected Keratinocytes

66. TACK, Lois C.
Salk Institute for Biological Studies
5 R01 CA 37081-03

SV40 Chromosome: T-Antigen Complexes and Viral Function

67. TAMM, Igor
Rockefeller University
5 R01 CA 18608-24

Virus Induced Alterations in Animal Cells

68. TEGTMEYER, Peter J.
State University of New York (Stony Brook)
5 R01 CA 18808-11

Mechanisms of Viral Oncogenesis: SV40 Protein Function

69. TEGTMEYER, Peter J.
State University of New York (Stony Brook)
5 P01 CA 28146-05

Tumor Virus-Host Interactions

70. TEVETHIA, Mary J.
Penn State University (Hershey)
5 R01 CA 24694-08

Mutagenesis of Specific Regions of the SV40 Genome

71. TEVETHIA, Satvir S.
Penn State University (Hershey)
5 R01 CA 25000-08

Biology of SV40 Specific Transplantation Antigen

72. TJIAN, Robert T.
Univ. of Calif. (Berkeley)
5 R01 CA 25417-07

The SV40 Tumor Antigen

73. TJIAN, Robert T.
Univ. of Calif. (Berkeley)
5 R01 CA 34724-03

Autoregulation of SV40 Early Gene Expression

74. VARSHAVSKY, Alexander J.
Mass. Institute of Technology
5 R01 CA 30367-05

SV40 Virus Structure and Replication

75. VILLARREAL, Luis P.
University of Colorado Health Sciences Center
5 R01 CA 25819-05

Gene Expression of A Small DNA Tumor Virus: SV40

76. WALTER, Gernot F.
Univ. of Calif. (San Diego)
5 R01 CA 36111-02

SV40 and Polyoma Virus Transforming Proteins

77. WATTS, Susan L.
Cold Spring Harbor Laboratory
1 R23 39775-01

Expression of Human Papillomaviruses in Cell Culture

78. WATTS, Susan L.
University of North Carolina at Chapel Hill
7 R23 CA 41414-01

Expression of Human Papillomaviruses in Cell Culture

79. WEISSMAN, Sherman M.
Yale University
5 P01 CA 16038-12

Program on Molecular Basis of Viral Transformation

80. WETTSTEIN, Felix O.
Univ. of Calif. (Los Angeles)
5 R01 CA 18151-10

Analysis of the Shope Papilloma-Carcinoma System

81. WILSON, John H.
Baylor College of Medicine
5 R01 CA 15743-12

Pathways of Information Exchange in Somatic Cells

82. WOODWARD-GUTAI, Mary
Roswell Park Memorial Institute
5 R01 CA 28250-05

SV40 DNA Replication in Animal Cells

SUMMARY REPORT

RNA VIRUS STUDIES I

The RNA Virus Studies I component of the Branch primarily involves studies of murine and primate tumor viruses, and also lesser numbers of projects on feline, bovine, and hamster tumor viruses. In this program, extramural research is supported by several funding instruments: traditional research grants (R01), program project grants (P01), conference grants (R13), cooperative agreements (U01), outstanding investigator awards (R35), and contracts. The funding level of 93 grants was 15.1 million dollars. The 93 grants involve studies in the murine (73%), primate (14%), feline (9%), bovine (1%), rat (1%), and hamster (1%) model systems in the following areas: gene organization and expression, including studies of oncogenes (50%), virus-cell interactions (30%), and characterization of the biological activity of retroviruses, of the inhibition of viral replication, and of virus-induced cell transformation (20%).

Studies in the RNA Virus Studies I area are concerned with the elucidation of the molecular events associated with the conversion of a normal cell to the malignant phenotype, and utilize retroviruses (including those in animal model systems) to reach this goal. The guiding principle in these efforts is that the malignant phenotype is a stably inherited trait: tumor cells give rise to offspring which are tumor cells. This suggests that oncogenic transformation may be the consequence of genetic alterations. This is clearly the case for cells transformed by oncogenic viruses where specific viral genes are responsible for the maintenance of the neoplastic state. The question naturally arises as to the nature of the genes responsible for naturally occurring tumors and the nature of the genetic rearrangements thought to result in the aberrant activation of these genes. Progress has been enhanced by the important observation that cellular genes homologous to viral oncogenes, in many instances, appear to be responsible for the transfer of the transformed phenotype to fibroblasts in culture. This important observation has spurred a search for the mechanisms by which these endogenous cellular genes may become altered to produce products with a potential for causing malignant transformation. The mechanisms responsible for the activation of cellular oncogenes may involve: (1) local changes or mutations in genes involving base changes or small deletions which alter the functional properties of the gene product; (2) gross changes in the relative position of genes which may either involve translocations of structural gene information from one chromosomal location to another or the introduction of activators (such as viral long terminal repeats adjacent to genes) such that the frequency of gene expression is enhanced; (3) gene amplification mechanisms which may increase the abundance of specific gene products; and (4) changes in the activity of oncogene promotors either by changing the base sequence itself or by altering genome structure in the vicinity of regulatory information (e.g., through changes in the pattern of methylation, the degree of supercoiling, or other aspects of chromatin structure).

Retroviruses first attracted widespread attention as oncogenic agents that replicate via DNA intermediates and involve integration of DNA copies of their genomes in the host chromosomes. Previous studies of these agents have demonstrated that they function: as agents with varied pathological potential, dispersed through many species and transmitted by vertical as well as horizontal

routes; as parasites well-adapted to host functions, thereby facilitating the orderly integration and expression of viral genomes; as intermediates themselves in the relocation of DNA proviruses, which are structural homologs of the transposable elements of other organisms; as mutagens equipped to interrupt or activate cellular genes; and as vectors able to transduce cellular genes and potentially act as agents of evolutionary change. No other class of animal viruses exhibits such profound intimacy with the genomes of their hosts; information gathered concerning this relationship should increase our understanding of the transformation process.

Retroviruses can be 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), which lack oncogenes, induce neoplastic disease in animals only after a long latent period (4-12 months), and do not cause transformation of tissue culture cells at a detectable frequency. A third class of retroviruses, which does not fit clearly into either of these groups, consists of viruses such as spleen focus-forming virus (SFFV) and mink cell focus forming virus (MCF), which appear to be envelope gene recombinants. Although these viruses, in some cases, rapidly induce lesions in infected animals, they do not appear to carry an oncogene of the classic type (i.e., a cell-derived oncogene). Rather, sequences coding for viral envelope located within the env region appear to be responsible for their pathogenic properties by some unknown mechanism. Finally, within the past year, a fourth group of lymphocyte transforming retroviruses without oncogene sequences (T-cell lymphotropic viruses, HTLV) have been recognized which apparently bring about cellular transformation through a novel trans-activation mechanism via a trans-acting protein encoded by a "pX" region (LOR) in the viral genome. Extramural research involving all four types of viruses are being funded in the RNA Virus Studies I component.

As a group, retroviruses often infect lymphoid tissues and many cause leukemias. Some, such as the feline leukemia virus (FeLV), cause a severe immunosuppressive disorder and are responsible for many more deaths by predisposing the host to acute opportunistic infections by other pathogens than by inducing leukemia. A human T-cell lymphotropic virus (HTLV), designated HTLV-III, also appears to have a similar immunosuppressive effect in man; this immunosuppression is known to be the result of a cytopathic effect on a subset of T-lymphocytes involved in conferring immunity (T4 cells). FeLV and other retroviruses, especially T-cell tropic retroviruses, are thus known to be both leukemogenic and immunosuppressive. With the recent recognition that Acquired Immunodeficiency Syndrome (AIDS), an immunosuppressive disorder of man, usually complicated by overwhelming opportunistic microbial infections, may be due primarily to a retrovirus, HTLV-III, there is now an increased awareness of the need to better understand the mechanisms by which RNA tumor viruses may cause immunosuppression. Interest in studies of the lentiviruses (visna, maedi, equine infectious anemia virus), a subfamily of nononcogenic retroviruses, has been aroused with the recent observation that HTLV-III, the causative agent of human AIDS, may be more closely related to this group of cytopathic retroviruses than to the oncogenic HTLV-I.

The genomes of highly oncogenic retroviruses, the sarcoma and acute leukemia viruses, contain host cell derived specific genes responsible for oncogenicity, which in many cases have partially replaced viral sequences necessary for normal viral replication, and thus these viruses are replication defective. For example, the genome of Moloney sarcoma virus includes a single gene (mos) which is responsible for cellular transformation, but is not involved in virus replication. Other sarcoma and acute leukemia viruses contain different transforming genes. These transforming genes of sarcoma and acute leukemia viruses are homologous to DNA sequences present in normal uninfected cells. These normal cell homologs of viral transforming genes are highly conserved in vertebrate evolution and appear to represent normal cell genes. Transcription of several of these genes has been detected in normal and neoplastic cells and, in some cases, normal cell proteins have been identified which are closely related to proteins encoded by the homologous viral transforming genes.

The highly oncogenic sarcoma and acute leukemia viruses thus appear to represent recombinants in which a transforming gene, derived from a homologous gene of normal cells, has been inserted into a retrovirus genome. The transforming genes of these viruses are expressed at high levels in virus-infected cells as a consequence of their association with viral transcriptional regulatory sequences. It is thus possible that transformation by these viruses is a consequence of abnormal expression of normal cell genes. Alternately, transformation might result from structural differences between the viral and cellular proteins.

The retrovirus responsible for the human AIDS has been variously termed human T-cell lymphotropic virus III (HTLV-III), lymphadenopathy associated virus (LAV), and AIDS associated retrovirus (ARV), and was originally thought to be closely related to the oncogenic HTLV-I which is responsible for a small proportion of human adult T-cell leukemia. One AIDS retrovirus isolate has recently been found, by molecular hybridization and heteroduplex analysis, to be closely related to the visna virus of sheep, a member of the lentivirus subfamily. (The lentivirus subfamily of retroviruses are those associated with visna, maedi, and equine infectious anemia.) On the basis of the same molecular techniques, the AIDS retrovirus appeared to be much less related to HTLV-I or any of a variety of other retroviruses. The ability of the AIDS retrovirus to infect and to persist in the brains of AIDS patients, its cytopathic effect on T4 lymphocytes in vitro, and its lack of overt oncogenic potential, thus places this retrovirus in the same class as the cytopathic lentiviruses, some of which are neurotropic (visna, caprine arthritis-encephalitis). The AIDS retrovirus, HTLV-III, is lymphotropic for T4 cells in a manner similar to HTLV-I, and therefore its designation as human T-cell lymphotropic virus-III (HTLV-III) does reflect a biological relationship with the HTLV family of retroviruses. The evolutionary origin of HTLV-III is unknown and is a subject of active research.

Studies were conducted (22,23) to determine if a novel protein product, similar to oncogene products of acutely transforming retroviruses, mediates the unique cell transforming properties of the HTLV family of retroviruses. Antibodies in the sera from patients with adult T-cell malignancies or from healthy carriers of HTLV-I recognized a 42 kilodalton antigen in cells infected with HTLV-I, leading to the conclusion that this antigen is encoded in part by LOR, a conserved portion of the "pX" region which is flanked by the envelope gene and the 3' LTR of HTLV-I. This 42 kilodalton protein apparently mediates cellular

transformation via a trans-activation mechanism recently proposed for this family of viruses by Haseltine (42).

Retroviruses, including the HTLVs, contain LTRs at the ends of their genomes. The LTRs carry regulatory segments, known as promoters and enhancers, which activate transcription of viral genes into messenger RNA, the first step in protein synthesis. Haseltine and associates (42) found that T-cells infected with HTLV-I or HTLV-II produced a factor, presumably a protein (23), encoded by the viral genome which increased transcription from the viral promoter in the LTR, wherever it was located in the host genome. This type of regulation, (trans-regulation) helps to explain the ability of HTLV to rapidly transform lymphocytes in vitro, despite the fact that the viral genome lacks oncogene sequences similar to those acquired from the host genome by acutely transforming retroviruses. The trans-acting factor promotes replication of virus, and may turn on cellular genes that stimulate growth leading to cell transformation. As discussed above, studies of Lee and Essex (23) have identified this 42 kilodalton protein in cells infected with HTLV-I.

The 42 kilodalton protein of HTLV-I was sufficient in itself to mediate the effect of trans-acting transcriptional regulation (42), since stable cell lines that expressed only this protein displayed a high level of trans-acting transcriptional regulation. The LTR of HTLV-II has been found to be responsive to HTLV-I encoded trans-acting factors. The transcriptional unit of the HTLV-II LTR will only work in certain cell types. Thus, cellular factors are required, in addition to virus trans-acting factors, for the trans-acting phenomenon. These studies of Haseltine (42), Lee, and Essex (23) are thus most important and relevant in elucidating a novel mechanism of cancer induction.

Two glycoproteins (gp61 and gp45) of HTLV-I were encoded, at least in part, by the env gene of HTLV, as evidenced by amino acid sequence analysis (23). These major cell associated antigens appeared to be the most immunogenic in persons exposed to HTLV-I. Comparison with amino acid sequence deduced from the primary nucleotide sequence of HTLV-II virus revealed that a gp67 moiety, found in cells infected with this virus was also encoded, at least in part, by the env gene of HTLV-II. The major env gene products of these two types of HTLV were serologically cross-reactive, suggesting that they were conserved to a degree, despite the divergence between the two strains of HTLV.

Simian Acquired Immune Deficiency Syndrome (SAIDS) is a spontaneous disease of macaque monkeys that resembles the human AIDS. It has been reported in several species of macaques. Recent studies at primate centers in California, Washington, and Oregon have implicated a type D retrovirus similar to Mason Pfizer monkey virus (MPMV) as the causative agent. On the other hand, studies at the New England Primate Center have assigned only a passenger role to a similar type D retrovirus isolated at this center. Hunter (47) compared the type D retrovirus isolated from these centers by nucleic acid hybridization techniques. The California and New England isolates were more closely related to one another than they were to MPMV. A type D retrovirus isolate from a case of SAIDS with retroperitoneal fibromatosis observed in Oregon could be distinguished from the other SAIDS retrovirus isolates and the MPMV. This observation conceivably accounts for the ability of the Oregon isolate to cause retroperitoneal fibromatosis in some of these SAIDS affected monkeys.

A counterpart of the human HTLV-I exists in macaque monkeys and may be responsible for lymphomas observed in this species (23). A retrovirus closely related to HTLV-I has been found in varying proportions of healthy individuals of several species of Old World monkeys. HTLV specific viral envelope antibodies were found in 11 of 13 macaques with malignant lymphoma or lymphoproliferative disease, but only in 7 of 95 healthy macaques, suggesting an etiological role for this HTLV-like virus in the development of naturally occurring lymphoid neoplasms in at least one species of nonhuman primates. It appears probable that the human and primate counterparts of HTLVs have, in the past, shared a common origin and natural history.

Different strains of non-acute retroviruses induce dramatically different types of tumors upon inoculation. The Gross passage A, SL3-3, Moloney murine leukemia virus (M-MuLV) and FeLV induce T-cell lymphomas. The avian leukosis virus induces B-cell tumors and the Friend helper virus induces erythroleukemia. What accounts for such tissue specific differences? Recent evidence from two different laboratories (41,45) clearly points to the viral long terminal repeat (LTR) as a major determinant of tissue tropism and pathogenicity. Within the LTR, the tandemly duplicated sequences may be the most important variable. These sequences contained functional enhancer elements (defined by their ability to increase transcription from a heterologous promoter from a distal location). The enhancers of Akv and SL3-3 LTRs displayed remarkable tissue differences in their ability to enhance transcription of heterologous genes. It was suggested that differences in enhancer function were the major determinants of tissue tropism and pathogenicity for this class of viruses.

The studies of Hopkins (45) were aimed at understanding the molecular basis for pathogenicity of nondefective mouse retroviruses. It was first shown that the viral host range property mapped in the transcriptional signals within the LTR sequences. Additional studies sought to ascertain other viral sequences that determine leukemogenicity. Most interestingly, it was found that sequences at the extreme C-terminus of the transmembrane protein, Prp15E, can affect the ability of viruses to replicate in the thymus. In collaboration with Famulari (27) and using recombinant viruses, this observation was extended to make the important finding that these sequences determined the processing of the env glycoprotein in a tissue-specific manner.

Famulari (27) found that MCF specific sequences in the U_3 region of the viral LTR have a strong controlling effect on virus replication in the target organ, the thymus, leading to transformation. A role for the MuLV U_3 region in conferring tissue specificity has been reported in several other virus systems and has been shown to influence the type of leukemia induced by a particular strain of MuLV.

While acutely transforming viruses acquire cellular proto-oncogenes, and as a result become rapidly transforming, many of the slow-acting leukemia viruses may ultimately cause cancer by integrating their genome adjacent to a normally quiescent cellular proto-oncogene. The strong promoter signals arising from this newly-integrated provirus are believed to result in increased transcription of the cellular onc genes, resulting in cell transformation through increased production of products of the oncogene. Recent evidence suggests that this model, termed the "insertional mutagenesis model" of oncogenesis and originally discovered in the induction of B-cell lymphoma by avian leukosis virus, can now be extended to other slowly transforming viruses of the feline leukemia group

(15) and mouse mammary tumor virus (MMTV) group (18,25,80) and some strains of murine leukemia virus (3,45,77,93).

In the murine retrovirus system, studies of Steffen (77) were the first to demonstrate that MuLV proviruses integrate in rat cells adjacent to the proto-oncogene, c-myc. The overwhelming preference for both Moloney MuLV and AKR-derived MuLV was for the provirus to integrate upstream of c-myc and in the opposite transcriptional orientation.

Hopkins and co-workers (3,45) discovered that murine MCF viruses integrate near the c-myc proto-oncogene in 10-20% of the T-cell lymphomas they induce. Results similar to these were obtained independently by Corcoran et al. for spontaneous AKR leukemias, and by Steffen (77) for MuLV, as noted above. Other investigators (Tsichlis, Jolicoeur and colleagues) have found evidence of two or three other preferred sites of integration of MuLV induced rat tumors. These results suggest that in contrast to avian leukosis virus, which integrates adjacent to c-myc and induces B-cell lymphomas, MuLV may have more flexibility in which other proto-oncogenes can be activated to induce tumors of the T-cell type.

Studies of Yoshimura (93) suggest that MuLV involved in the development of thymic lymphoma prefer to integrate in the A-T rich regions of the mouse genome. These preferred regions of integration may be important in lymphoma development.

Similar original studies of Varmus and co-workers (80) have clearly shown that the majority of MMTV-induced mammary carcinomas in C3H mice were accompanied by insertions of MMTV proviral DNA in the vicinity of a heretofore unknown cellular gene which they named lnt-1. This gene is highly conserved in metazoans, present on mouse chromosome 15, unexpressed in all tissues and cells thus far examined, but transcribed to produce about 10 copies of lnt-1 mRNA per cell as a result of proviral insertions. Almost all of these insertions are oriented away from the lnt-1 gene, on either the 5' or 3' side of the gene, implying that an enhancer mechanism activates the gene.

Similarly, in studies with the mouse mammary tumor virus, Etkind (25) has also found integration of MMTV-specific proviral DNA at identical locations in different mouse mammary tumors, suggesting that mammary tumorigenesis in C3H/f mice could result from the activation of a cellular oncogene by an MMTV proviral DNA promoter, as in the promoter insertion model (insertional mutagenesis model) proposed for avian leukosis virus-induced leukemogenesis.

Using Southern blotting and somatic cell hybridization to study the mechanism and possible function of amplified endogenous MMTV DNA in many murine T-cell lymphomas, Dudley (18) found that MMTV DNA acquired during the amplification event mapped to mouse chromosomes 3 and 11. Current work on characterization of elements adjacent to MMTV proviruses and characterization of their transcription will provide insight into the mechanism and function of MMTV DNA amplification in T-cell tumors.

Evidence was obtained by Besmer (6) for the occurrence of multiple cellular proto-oncogenes in the cat genome. The v-kit oncogene of a feline sarcoma virus HZ4-FeSV appeared to be a new oncogene, whereas the oncogene transduced from the cat genome by another FeSV, HZ5-FeSV, was similar to the fms oncogene of Sarma-McDonough FeSV.

Cellular homologs of several known v-onc genes have been detected in human normal and tumor tissues. Further investigation is needed to elucidate their role in a variety of human cancers. For example, oncogenes have been detected by transfection of DNA prepared from different human tumors into immortalized cell lines such as NIH 3T3 cells. DNAs of colon, bladder, lung and pancreatic carcinomas, fibrosarcomas, leukemias, and even neuroblastomas have yielded potent transforming sequences (3,12,84). In those few cases which have been well studied, it is clear that the oncogenes derive from closely related normal cellular sequences, the proto-oncogenes. The weight of present evidence suggests that these oncogenes arise as a consequence of a somatic mutational event which occurs in a target tissue and affects the structure of the proto-oncogene. In fact, the nature of this activating lesion has been well documented in two cases: human bladder carcinoma oncogene and human lung carcinoma oncogene (12, 40). In these instances, a point mutation occurring in the proto-oncogene caused conversion of the proto-oncogene into an oncogene.

The oncogenes detected in many human tumors by transfection into NIH 3T3 mouse fibroblasts are members of the ras oncogene family originally detected in the Harvey (H) and Kirsten (Ki) strains of murine sarcoma virus. The protein product of this oncogene is 21 kilodaltons in molecular weight and is designated p21. The activating lesions cause the replacement of amino acid residues at positions 12 and 61 of the ras polypeptide with other amino acids; this activation appears to occur in a proportion of neoplasms of many cell types and to be unrelated to the histopathology of resultant tumors.

To further analyze the differences between normal and transforming ras genes, Cooper (12) and co-workers have used in vitro mutagenesis to cause changes in the nucleotides encoding position 61 of the ras^H polypeptide. They have thus been able to introduce all nineteen possible amino acid substitutions in place of glutamine at position 61 of the human ras^H polypeptide. Substitution of eleven different amino acids (alanine, serine, threonine, valine, isoleucine, leucine, methionine, arginine, lysine, histidine, and cysteine) at this position yielded mutant proteins with strong transforming activity. Substitution of three other amino acids (glycine, tryptophane and asparagine) led to partial activation of transforming potential such that these mutants induced transformation with 10 to 100-fold lower efficiencies than the fully transforming variants. Mutants having phenylalanine, tyrosine, aspartate, glutamate and proline at position 61 lacked transforming activity.

As originally demonstrated by Hunter, transforming proteins of the retroviruses encoding a tyrosine protein kinase, bring about cell transformation through phosphorylation of certain cellular proteins. More recent studies of Hunter (46) focused on an 81 kilodalton protein (p81) which is a substrate for ST-FeSV p85gag-fes tyrosine kinase and the EGF receptor tyrosine kinase. In most respects, p81 was similar to p36, another tyrosine kinase previously characterized by these workers. A survey of rat tissues showed high levels of p81 in gut and lung, intermediate levels in spleen and kidney, and low levels in liver and brain. Phosphorylation of p36 and p81 may play a part in marked changes in membrane morphology and function characteristic of both virally transformed and growth factor treated cells.

The Philadelphia chromosome, observed in more than 90% of chronic myelogenous leukemia (CML) patients, results from a specific 9 to 22 chromosomal translocation. This cancer may have an oncogene activation mechanism of cancer

induction, similar to that proposed for Burkitt's lymphoma in which there is an 8 to 14 chromosomal translocation involving the c-myc oncogene. Witte (89) obtained evidence to suggest that in the case of CML, the breakpoint on chromosome 9 occurred near the proto-oncogene c-abl and correlated with the production of an altered mRNA and a structurally altered c-abl protein (p210c-abl) which had a tyrosine kinase activity not detectable in the normal c-abl protein (p145c-abl). Based on these results, it was proposed that translocation of c-abl in Philadelphia chromosome positive CML caused the creation of a chimeric gene leading to the production of a structurally altered c-abl protein with an activated tyrosine kinase activity. This altered p210c-abl protein was implicated in the pathogenesis of CML.

Studies of Baltimore (3,4) have focused on Abelson murine leukemia virus (A-MuLV) as a model system to study oncogenesis. A-MuLV can efficiently transform lymphoid and fibroblast cells into tumor cells in vitro. The virus synthesized a single protein, which is the product of a genetic fusion between two genes: a remnant of the parent retrovirus encoded the first 250 amino acids of the fusion protein chain, and a mouse cellular gene (called c-abl) encoded the rest of the protein. The amino-terminal third (the first 400 of 1300 amino acids) of the abl-encoded portion of the A-MuLV protein was capable of phosphorylating the tyrosine amino acid residues of target proteins. Recent results have shown that the protein kinase activity began a chain of phosphorylation reactions that led to phosphorylation of a ribosomal protein called S6, which plays an important role in translating messenger RNA into proteins.

Genetically engineered deletion mutants of A-MuLV were used to define the regions of the A-MuLV protein that are responsible for oncogenesis. A deletion of the carboxy terminal two thirds of the protein only partially diminished its transforming ability; thus it appears that the amino terminal amino acids act as a protein kinase and exhibit the transforming activity. Removal of all but the first 34 of the 250 amino acids from the parental retrovirus contribution to the A-MuLV protein, abolished the transformation of lymphoid cells without affecting fibroblast cell transformation. Reduced stability of the mutated protein in lymphoid cells appeared to be the cause of this behavior. To further characterize the function of the A-MuLV protein, genetic techniques were used to replace portions of the protein with segments derived from the avian Rous sarcoma virus. When the Rous kinase-encoding region replaced the M-MuLV, the resultant virus transformed fibroblasts, but not lymphoid cells. When the N-terminal regions of Rous kinase-encoding region replaced the N-terminal region of A-MuLV, both types of transformation remained intact. Thus, the specificity for lymphoid transformation resided with the domain of the A-MuLV protein that encoded its kinase.

Rosenberg (67) is performing collaborative studies with Baltimore to define the portions of the Abelson MuLV transforming protein necessary for transformation; she has prepared rabbit antibodies specific for various regions of the A-MuLV transforming protein by using, as antigens, either synthetic peptides or fragments of the A-MuLV protein fused to TrpE protein of E. coli. The antibodies are being used to localize A-MuLV transforming proteins in transformed cells using techniques such as immunoprecipitation, western blotting, and immunofluorescence.

Sherr (73) has found that a tyrosine specific protein kinase activity could be detected in immunoprecipitates containing glycoprotein encoded by the cat oncogene, v-fms. The carboxy terminal domain of this protein was found to be

essential for enzyme activity. Two distinct functional domains were required for cell transformation. The v-fms polypeptides were integral transmembrane glycoproteins, oriented with their glycosylated amino termini outside the cell and the carboxy terminal kinase domain within the cytoplasm. The critical properties of the v-fms gene product were similar to those of receptors for growth factors, suggesting that v-fms transformed cells through receptor-mediated signals.

In similar studies with the mos oncogene product, p37mos, Donogue (17) found that the C-terminal portion of p37mos coding region was essential for the transforming activity of this oncogene. A considerable portion of the N-terminal coding region could be deleted without loss of its biological activity, focus formation, in NIH 3T3 cells. On the other hand, this investigator found that the N-terminal coding region of another oncogene, the sis oncogene, derived from the envelope gene of the parental retroviruses, was required for biological activity. This region of the sis gene product encoded a signal sequence, which was presumably required to permit transport of the sis gene product through the endoplasmic reticulum. This is consistent with an autocrine model for transformation by sis, whereby the sis gene product must be secreted in order to be biologically active.

Understanding the events which lead to transformation requires an understanding of the replication of the virus, the determinants of tissue tropism, the stages of the life cycle at which host functions act to block infection and the process of recombination with endogenous viruses. Although the nucleotide sequences of the genome and, therefore, the amino acid sequence of each gene product may be known, we have yet to understand the role of each of these gene products in the virus and host life cycle. Analysis of mutants and their gene products can directly determine the function of each protein. Goff carried out extensive studies (37) on the functions of retroviral genes using mutagenesis as a tool. He generated a number of mutations in the P15 and P12 regions of the gag gene of the Moloney MuLV and found that these mutants were unaffected in virion assembly and late events, but were blocked in early stages of the viral life cycle. Mutations in the P30 domain were much more lethal to the virus and blocked assembly. These mutations, including point mutations, also had important effects on pol function. Mutations in the 3' third of the gene did not affect reverse transcriptase activity, but rather blocked the establishment of the proviral DNA in the host genome. This was the first report conclusively showing that this gene product was required for integration of the viral DNA. The central portion of the pol gene encoded reverse transcriptase; and the 5' portion encoded a function which was required for the processing of the gag and gag-pol precursors. These results showed that the pol gene encoded a protease for nearly all of the processing of the viral gene products. Goff (37) also found that deletion mutants of the virus reverted by recombination with endogenous host sequences. This is a novel finding with important implications for the evolution of these murine retroviruses.

If cellular proto-oncogenes are ultimately responsible for most cancers, what alterations, other than those provided by the intervention of a retrovirus or a mutagen, are necessary for converting a nontransforming proto-oncogene into a cell transforming oncogene? Verma (81,82) found that the c-fos proto-oncogene can transform fibroblasts if two manipulations are carried out: 1) addition of transcriptional enhancer sequences and 2) interruption of 3' sequences. He obtained evidence to suggest that the proto-oncogene c-fos product may be

required in normal cell growth and differentiation. The c-fos gene transcripts can be induced in certain cell lines with a phorbol ester and upon addition of platelet derived growth factor (PDGF). These results indicated that c-fos is an early response gene and that its product may be involved in cell differentiation or aspects of differentiation.

Studies of Wong (90), Kabat (49), and Elder (21) highlight the importance of the env gene and env gene coded glycoproteins in viral pathogenesis. In addition to causing cancers and/or immunosuppressive disorders, certain retroviruses have a neurotropic propensity as well, as in the case of certain feral mouse retroviruses from Lake Casitas. Wong (90) studied a group of temperature-sensitive and paralyotogenic mutants of murine MO-MuLV-TB. This group of mutants, which induce hind leg paralysis in mice, were shown to be inefficient in the intracellular processing of the envelope precursor protein pr80env at the restrictive temperature. Using recombinant DNA technology, it was found that pol-gp70 (Hind III-Bam H1) DNA sequence of the mutant was responsible for this defective processing of env gene precursor and for hind leg paralysis in mice. The mutant virus, but not a mutant-derived virus in which the defect was corrected with the corresponding sequences from wild-type Mo-MuLV-TB, was capable of replicating in CNS tissue at 100 times the titer observed in other tissues of the mouse.

Working with spleen focus forming virus (SFFV), SFFV associated virus, and mutants derived from these viruses, Kabat (49) has been able to show that the membrane glycoprotein encoding env genes are oncogenes required for initiating the process of progressive erythroleukemogenesis in mice. The glycoprotein structure and subcellular localization appeared to be essential for leukemogenic activity.

Elder (21) also obtained evidence to suggest that env genes encoding membrane glycoproteins may be involved in oncogenesis in mice. Thus, a gp70 viral envelope moiety of a mouse type C endogenous retrovirus, termed 81-TAg, was found to be expressed on thymocytes of most strains of mice. The selective occurrence of this antigen in thymus tissues and preferentially in blast cells suggested that the 81-TAg antigen may be the oncogene product involved in mouse thymic leukemia.

The Fv-1 gene controls the host range in mouse cell cultures of the N-tropic and B-tropic murine type C viruses. Yang and co-workers (92) have found another host range restriction of N-tropic viruses in certain Fv-1^{n} genotype mouse cells, such as C57L and 129, based on differences in two different regions of the p30 protein. One change is an alteration of two amino acid residues in the middle portion of p30 and the second is the deletion of an eleven amino acid residue sequence in the carboxy terminal region of Gross virus p30 protein. These differences were distinct from the N- and B-tropic p30 determinants associated with Fv-1 restriction.

Bedigian (5) isolated a myeloid leukemia-inducing B-tropic virus from one of 12 recombinant inbred mouse strains obtained by crossing C57Bl/6J and C3H/HeJ mice. Unlike other ecotropic MuLV strains, this virus is transmitted through the milk in a manner similar to mouse mammary tumor virus. In more recent studies, he has biologically cloned this B-ecotropic MuLV and has been able to transform bone marrow cells in vitro after prolonged latency periods. The transformed cells, which form colonies in soft agar, are currently being characterized for cell type, virus expression, and tumorgenicity in mice.

Duran-Reynals (19) found that mice of the RF strain were highly resistant to endogenous ecotropic virus expression and lymphoma development as a result of the fact that antibodies against the virus were transmitted via milk from resistant female mice to their offspring. These protective antibodies suppressed replication of endogenous ecotropic virus in infant mice. Young adult mice devoid of these antibodies were, however, immunocompetent and, thus, were apparently able to overcome viral infection and in turn transmit antibodies to their young through the milk. These studies highlight the importance of natural host defense mechanisms in protection against virus-induced cancer in mice.

Searching for underlying common mechanism(s) of cancer induction, Haas (39) has compared virus-induced T-lymphoma cells and X-ray induced T-lymphoma cells of mice for oncogene expression, chromosomal characteristics and cellular growth characteristics. Both types of cells expressed five proto-oncogenes c-myc, $c\text{-}ras^{Ha}$, $c\text{-}ras^{Ki}$, c-abl, and c-myb. In contrast to virus-induced T-lymphoma cells, the X-ray induced lymphoma cells did not have newly integrated proviral genomes, did not possess a rearranged myc gene and did not express the new 69 kilobase viral message. Similarly, in contrast to the virus-induced T-lymphoma cell lines (which did not secrete growth factors and were growth factor independent), the X-ray induced T-lymphoma cells secreted a cell growth factor and were dependent on this growth factor (autocrine factor). The virus-induced lymphoma cells often possessed a distinct, highly stable chromosome rearrangement, namely trisomy of chromosome 15. This characteristic was absent in X-ray induced T-lymphoma cells.

Acquired Immunodeficiency Syndrome was first discovered in cats over 15 years ago. With the recognition of AIDS in man, this disease is now called FAIDS (feline AIDS). Using a newly isolated FeLV variant derived from a case of feline thymic lymphosarcoma, Hoover (44) now can experimentally transmit FAIDS to 100% of the cats inoculated with this variant. In collaborative studies with Mullins, it was established that the proviral DNA of the variant virus occurred in linear, unintegrated form in the bone marrow of the cats with FAIDS, presumably causing cytopathic changes in lymphocytes which are responsible for cell-mediated immunity.

In collaborative studies, Rojko (66) and Hoover (44) demonstrated that FeLV infection can exist in a latent state with no demonstrable free virus in the plasma or other tissues of the animal. However, FeLV was found in bone marrow progenitor cells and could be reactivated by in vitro culture of marrow cells or by in vivo treatment of cats with adrenal corticosteroids. This previously unrecognized and important consequence of regressive FeLV infection of cats is apparently due to an effective host immune mechanism, and has important implications in viral latency and pathogenesis in feline leukemia.

Certain FeLV isolates from thymic lymphosarcomas have recently been found to be able to transduce the proto-oncogene, c-myc. This finding is unique for a mammalian retrovirus (the avian retrovirus, MC-29, carries a v-myc oncogene). Deninger (15) determined the nucleotide sequence of the feline v-myc gene and FeLV flanking regions. Both the nucleotide and predicted amino acid sequences were very similar to the murine and human c-myc genes (ca. 90% identity). The entire c-myc coding sequence was represented in feline v-myc and was in phase with the gag gene reading frame; v-myc, therefore, appeared to be expressed as a gag-myc fusion protein. Both feline and chicken v-mycs have lost potential phosphorylation sites in the putative DNA binding region of exon 3. If these

sites were phosphorylated in normal c-myc proteins, their loss may alter the DNA binding affinity of v-myc proteins. The viral hexanucleotide, CTCCTC, was found 3' of the v-myc gene in this provirus and 3' of the v-fes gene in the two feline sarcoma virus isolates that have been sequenced. The presence of this hexanucleotide, and the similarity of restriction enzyme site distribution near 3' onc-FeLV junctions, suggested that some sequence specificity may exist for the transduction of cellular genes by FeLV.

In studies of endogenous retroviruses of the cat, Roy-Burman (70) found that endogenous FeLV elements present in cat DNA were bounded by LTRs and contained major deletions in the gag and pol genes. It was hypothesized that these FeLV elements originated as a result of germ line integration of a complete virus followed by deletion of sequences in the gag and pol genes or by integration of a preexisting deleted variant at a single or limited number of chromosomal loci in a relatively recent ancestor of the cat. This was thought to be followed by gene duplication events and subsequent divergence. In contrast to endogenous FeLV elements, a majority of the endogenous RD-114 sequences in the cat contained major deletions in the env region, and there appeared to be a single or, at most, two complete RD-114 elements which probably were responsible for yielding an infectious virus under permissive conditions. All endogenous RD-114 sequences examined were also bounded by LTR elements, confirming their origin from a germ line infection of the ancestral cat. Analysis of expression of two cellular oncogenes (c-myc and c-myb) in feline tissues suggested that their expression was linked to growth and development as it varied with the gestational age of the fetus and from fetal to adult tissues. Enhanced levels of c-myc transcripts were detected in several feline neoplasms, including hematopoietic malignancies. The feline c-myc locus was cloned and characterized. Polymorphisms at the c-myc locus including alleles differing at 3' coding sequences were defined. Genetic rearrangements at the c-myc locus have been detected in four of the ten cat neoplasms examined. In studies of a variety of human lymphoma-leukemia specimens, it was noted that c-erbB oncogene locus was associated with hematopoietic cell proliferation, and the c-myc locus was transcribed at elevated levels in the majority of the chronic lymphocytic leukemia, B-cell type, cells.

The abundance of endogenous retroviral sequences in the mouse genome is further exemplified by the recent identification of two previously unknown retroviral sequences within the TL locus of the major histocompatibility complex (MHC) of uninfected C57/B110 mice (55). The origin of these incomplete murine retrovirus related sequences is unknown. The study of these sequences may prove significant with respect to the interaction of retroviral sequences within the genome, expression of the genes within the TL locus, and polymorphisms within the MHC.

It has been clearly established in several systems that hormones and other small molecules affect the intracellular concentration of specific RNAs. In the case of glucocorticoid stimulated viral gene expression, Yamomoto (91) showed that this particular biological effector can selectively alter the rate of transcription of specific eukaryotic genes. Thus, it may be possible to use this system for an integrated analysis of multiple mechanisms of eukaryotic gene regulation.

Expression of endogenous proviruses varies with inbred mouse strain and age, and appears to be under stringent cellular control. Regulation of provirus expression is thought to be at the transcriptional level, and is controlled by linked cis-acting inhibitory cellular DNA sequences, by DNA methylation, or both. To begin to differentiate between these different control mechanisms, Copeland (13) molecularly cloned three endogenous ecotropic proviruses: Emv-1, Emv-3, and Emv-13. All three proviruses were poorly expressed in vivo, although they appeared non-defective by restriction enzyme analysis. DNA transfection and marker rescue experiments using cloned DNAs showed that all three proviruses carried small mutations in their genome that inhibited their expression in vitro as well as in vivo. Recombination between different defective proviruses also appeared to occur in vivo, leading to the generation of non-defective recombinant viruses that spread throughout the animal leading to viremia late in life.

Two RFAs for cooperative agreements, initiated by the RNA I component in FY84, were considered for funding during this year. One RFA was to encourage research on human T-cell leukemia/lymphoma virus (HTLV) types I and II. Since AIDS research is being funded through a variety of mechanisms, the purpose of this RFA was to stimulate research on HTLV I and II in order to elucidate such fundamental issues as the mode of virus transmission, virus-host cell interactions, and the molecular basis of virus transformation of two members of this important group of human retroviruses. Four new cooperative agreements submitted under this RFA were funded in FY85. The second RFA initiated in FY84 was designed to stimulate research on bovine leukemia virus (BLV). The virus-host interaction in BLV-infected cattle represents an important model of oncogenesis associated with an exogenous retrovirus transmitted horizontally under natural conditions. The mechanism(s) by which BLV induces tumors remains unknown. It may prove to be similar to the mechanism used by the HTLV. The structural and epidemiological similarities between BLV and HTLV provide a compelling reason to study BLV infection and tumorigenesis. Unfortunately, none of the applications submitted in response to the RFA were scientifically meritorious and therefore no grant awards were made.

Three new concepts for research grant initiatives were presented to the DCE Board of Scientific Counselors (BSC) in FY85. The first research grant initiative concerned feline leukemia virus (FeLV) research and was based on input from a BCB workshop held on November 29-30, 1984 at the NIH. Dr. Myron Essex of the DCE Board of Scientific Counselors chaired the meeting. Other Board members attending were Dr. Marcel Baluda and Dr. Charlotte Friend. The consensus of the participants was that feline leukemia virus offered important models of oncogenesis/immunodeficiency syndrome in an outbred species and that NCI should stimulate additional studies on the biology, immunology, and molecular biology of feline leukemia virus. This concept was presented to the BSC at their February 1985 meeting and was approved for implementation as a program announcement. This announcement is currently in preparation for issuance; awards in response to it will be funded in FY86.

A second research grant initiative concerned basic studies on the development and assessment of retroviral vaccines. It was based on a BCB workshop held at NIH on December 10-11, 1984 concerning retroviral vaccines. Dr. Hilary Koprowski of the DCE Board of Scientific Counselors chaired the meeting. Other Board members attending were Dr. Myron Essex, Dr. Marcel Baluda, and Dr. Charlotte Friend. The consensus of the workshop participants was that NCI should stimulate both in vitro and in vivo experimental studies to develop

and/or assess new retroviral vaccines. The concept was presented to the BSC at their February 1985 meeting and was approved for implementation as an RFA for traditional research grants. This RFA is currently being prepared for release. Awards in response to this announcement will be funded in FY86.

A third research grant initiative concerned studies of novel exogenous and endogenous human retroviruses and was based on a discussion group which met on March 11, 1985 at NIH in Bethesda. Dr. Padman Sarma, the Program Director of the RNA Virus Studies I component chaired the meeting. Three members of the BSC, Dr. Charlotte Friend, Dr. Marcel Baluda, and Dr. Myron Essex, attended. The consensus of the participants was that the NCI should stimulate research to isolate and characterize these novel human retroviral entities and determine their significance in human cancer. This RFA is currently being prepared for release. Awards in response to this announcement will be funded in FY86.

RNA VIRUS STUDIES I

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. AXEL, Richard Columbia University (New York) 5 P01 CA 23767-07	Molecular Virology
2. BACHELER, Lee T. Temple University 2 R01 CA 29519-05A1	Organization and Expression of Leukemia Virus Genomes
3. BALTIMORE, David Massachusetts Institute of Technology 5 P01 CA 26717-05	Molecular Analysis of Oncogenic Viruses
4. BALTIMORE, David Whitehead Institute for Biomedical Research 1 P01 CA 38497-01	Interactions of Oncogenes with Developing Systems
5. BEDIGIAN, Hendrick G. Jackson Laboratory 5 R01 CA 31102-05	A New Murine Model for the Study of Myeloid Leukemia
6. BESMER, Peter Sloan-Kettering Institute for Cancer Research 5 R01 CA 32926-03	Oncogenes of New Sarcoma Virus Strains
7. CHEN, Irvin S. Y. University of California (Los Angeles) 1 R01 CA 38597-01	A Molecular Genetic Study of Human T-cell Leukemia Virus
8. COGGIN, Joseph H., Jr. University of South Alabama 5 R01 CA 23491-07	Etiology of a Lymphoma Epizootic in Hamsters
9. COHEN, James C. Louisiana State University Medical Center (New Orleans) 5 R01 CA 34823-04	The Molecular Genetics of Retroviruses
10. COHEN, James C. Louisiana State University Medical Center (New Orleans) 5 R01 CA 35686-02	Retrovirus Sequence Specific Integration in Human Cells

11. COMPANS, Richard W.
University of Alabama
(Birmingham)
5 R01 CA 18611-11

Molecular Studies of Oncorna and Arenaviruses

12. COOPER, Geoffrey M.
Dana-Farber Cancer Institute
5 R01 CA 18689-10

Infectious DNA for Endogenous RNA Tumor Virus Genes

13. COPELAND, Neal G.
University of Cincinnati
5 R01 CA 37283-02

Ecotropic MuLVs of Normal and Mutant Mouse Strains

14. DARNELL, James E., Jr.
Rockefeller University
5 P01 CA 18213-10

Correlated Program in Viral Oncology

15. DEININGER, Prescott L.
Louisiana State University
Medical Center (New Orleans)
5 R01 CA 31702-03

Molecular Mechanisms of Retroviral Induced Leukemia

16. DESROSIERS, Ronald C.
Harvard Medical School
1 U01 CA 40680-01

T-cell Lymphotropic Viruses of Macaques

17. DONOGHUE, Daniel J.
University of California
(San Diego)
5 R01 CA 34456-03

Expression of Retroviral Envelope Gene Fusion Proteins

18. DUDLEY, Jacquelin P.
University of Texas (Austin)
5 R01 CA 34780-02

Amplification of MMTV DNA in T-cell Lymphomas

19. DURAN-REYNALS, Maria L.
Yeshiva University
5 R01 CA 07160-19

Possible Neoplastic Effects of Non-Neoplastic Viruses

20. ECKNER, Robert J.
University of Massachusetts
Medical School
5 R01 CA 39689-02

Biological and Physical Properties of Friend Virus

21. ELDER, John H.
Scripps Clinic and Research
Foundation
5 R01 CA 25533-05

Structural Studies of Recombinant Retrovirus gp70s

22. ESSEX, Myron E.
Harvard University
5 R01 CA 13885-11

Oncornavirus-Associated Cell Membrane Antigens

23. ESSEX, Myron E.
Harvard University
2 R01 CA 18216-09

Seroepidemiology of Retroviruses in Immune Suppression

24. ESSEX, Myron E.
Harvard University
1 R35 CA 39805-01

NCI Outstanding Investigator Grant

25. ETKIND, Polly R.
Montefiore Medical Center (Bronx, NY)
5 R01 CA 39439-02

Molecular Mechanism in C3HR Mouse Mammary Tumorigenesis

26. EVANS, James W.
University of California (Davis)
1 R13 CA 41069-01

Conference On "Genetic Engineering of Animals: An Agricultural Perspective"

27. FAMULARI, Nancy G.
Sloan-Kettering Institute for Cancer Research
5 R01 CA 36162-02

Stage Specific Events in Viral Leukemogenesis

28. FAN, Hung Y.
University of California (Irvine)
5 R01 CA 32454-04

Studies of Murine Leukemia Virus Integration

29. FAN, Hung Y.
University of California (Irvine)
5 R01 CA 32455-05

Expression of C-type Virus Genes

30. FERRER, Jorge F.
University of Pennsylvania
5 R01 CA 34231-02

Studies on a High Incidence Leukemia Herd of Cattle

31. FIRESTONE, Gary L.
University of California (Berkeley)
5 R01 CA 35547-03

Steroid Regulation of Protein Maturation

32. FLEISSNER, Erwin J.
Sloan-Kettering Institute for Cancer Research
5 P01 CA 16599-11

Hematopoietic Cell Transformation by Retroviruses

33. FLYER, David C.
Dana-Farber Cancer Institute
1 R23 CA 40585-01

Specificity of the CTL Response to Murine Leukemia Virus

34. FRIEND, Charlotte
Mount Sinai School of Medicine
2 R01 CA 10000-19A1

Filterable Agents and Tumor Induction in Mice

35. FRY, Kirk E.
Stanford University
5 R01 CA 03352-29

Biological Aspects of Carcinogenesis by Radiation

36. GARDNER, Murray B.
University of California (Davis)
5 R01 CA 30912-03

Mammary Tumorigenesis in Hosts Lacking MuMTV DNA

37. GOFF, Stephen P.
Columbia University (New York)
2 R01 CA 30488-05

Construction and Analysis of Retrovirus Mutants

38. GOLDSCHNEIDER, Irving
University of Connecticut Health Center
1 R01 CA 38762-01

Cellular Targets of Leukemic Transformation

39. HAAS, Martin
University of California (San Diego)
5 R01 CA 34151-04

Viral Malignant Lymphomagenesis in X-irradiated Mice

40. HARFORD, Esther C.
U.S. Uniformed Services University of Health Sciences
5 R01 CA 34582-03

Oncogenes (c-ras) in Human Cancer Induction

41. HASELTINE, William A.
Dana-Farber Cancer Institute
5 R01 CA 19341-08

The Molecular Biology of Replication RNA Tumor Viruses

42. HASELTINE, William A.
Dana-Farber Cancer Institute
5 R01 CA 36974-02

Study of pX Region of Human T Cell Leukemia Virus

43. HAYS, Esther F.
University of California (Los Angeles)
5 R01 CA 12386-13

Development of Lymphoma in the Thymus

44. HOOVER, Edward A.
Colorado State University
5 R01 CA 32552-02

Pathogenesis of Animal Leûkemia

45. HOPKINS, Nancy H.
Massachusetts Institute of Technology
2 R01 CA 19308-10

Studies on Endogenous and Other C-type Viruses of Mice

46. HUNTER, Anthony R.
Salk Institute for Biological Studies
5 R01 CA 17096-11

Macromolecular Synthesis and Growth Control

47. HUNTER, Eric
University of Alabama
(Birmingham)
5 R01 CA 27834-05

Genetics of Primate D Type Retroviruses

48. JENKINS, Nancy A.
University of Cincinnati
1 R01 CA 38039-01

Retroviruses as Insertional Mutagens

49. KABAT, David
Oregon Health Sciences University
5 R01 CA 25810-07

Leukemogenic Membrane Glycoproteins: gp55s of SFFVS

50. LERNER, Richard A.
Scripps Clinic and Research Foundation
2 P01 CA 27489-06

Consequences of Endogenous Retroviral Expression

51. LEVY, Jay A.
University of California
(San Francisco)
5 R01 CA 33137-03

Role of Endogenous Xenotropic Viruses

52. LILLY, Frank
Yeshiva University
5 R01 CA 19931-08

Mechanism of the H-2 Effect on Viral Leukemogenesis

53. LUFTIG, Ronald B.
Louisiana State University
Medical Center (New Orleans)
2 R01 CA 37380-03

Assembly of Murine Leukemia Viruses

54. MERUELO, Daniel
New York University
5 R01 CA 22247-08

Genetics of Resistance to Leukemia

55. MERUELO, Daniel
New York University
5 R01 CA 31346-03

Loci Affecting Radiation/RadLV-Induced Leukemogenesis

56. MULLINS, James I.
Harvard University
1 U01 CA 40646-01

Viral Determinants of HTLV-I Leukemogenesis

57. MURPHY, Edwin C., Jr.
University of Texas System Cancer Center
5 R01 CA 34734-03

MuSV TS110: A Potential ts Transcriptional Mutant of MuSV

58. O'DONNELL, Paul V.
Sloan-Kettering Institute for Cancer Research
2 R01 CA 31491-04

Kinetic Study of Virus-Accelerated Leukemia

59. OLSEN, Richard G.
Ohio State University
5 R01 CA 30338-05
FeLV Leukemogenesis and Preneoplastic Lesions

60. PALKER, Thomas J.
Duke University
1 U01 CA 40660-01
Monoclonal Antibodies to HTLV I and HTLV II

61. PETERSON, David O.
Texas A & M University (College Station)
5 R01 CA 32695-03
Genetic and Molecular Analysis of Steroid Responsiveness

62. PIKO, Lajos
U.S. Veterans Admininistration Medical Center (Sepulveda, CA)
5 R01 CA 24989-05
Gene Expression in Early Mouse Development

63. PINTER, Abraham
Sloan-Kettering Institute for Cancer Research
5 R01 CA 37107-02
Biochemical and Genetic Studies of MuLV Envelope Proteins

64. RACEVSKIS, Janis
Sloan-Kettering Institute for Cancer Research
1 R01 CA 39509-01
MMTV Gene Products and Transformation

65. RASHEED, Suraiya
University of Southern California
5 R01 CA 27246-06
Leukemia & Sarcoma Genes in Cellular Transformation

66. ROJKO, Jennifer L.
Ohio State University
5 R01 CA 35747-02
Pathobiology of Latent Lymphomagenic Feline Retroviruses

67. ROSENBERG, Naomi E.
Tufts University
5 R01 CA 24220-07
Abelson Leukemia Virus Transformation

68. ROSENBERG, Naomi E.
Tufts University
5 R01 CA 33771-03
RNA-Tumor Virus--Hematopoietic Cell Interaction

69. ROSNER, Marsha R.
Massachusetts Institute of Technology
5 R01 CA 32267-03
Isolation and Characterization of Retrovirus Receptors

70.	ROY-BURMAN, Pradip University of Southern California 1 R01 CA 40590-01	Endogenous Retrovirus Related Genes in Feline Leukemia
71.	SARKAR, Nurul H. Sloan-Kettering Institute for Cancer Research 5 R01 CA 17129-11	Components of the Murine Mammary Tumor Virus
72.	SCHWARTZ, Robert S. Tufts University 5 P01 CA 24530-06	Experimental Leukemogenesis
73.	SHERR, Charles J. St. Jude Children's Research Hospital 5 R01 CA 38187-02	The FMS Oncogene
74.	SODROSKI, Joseph G. Dana-Farber Cancer Institute 1 U01 CA 40658-01	Role of HTLV LOR Region in Transcriptional Regulation
75.	SORGE, Joseph A. Scripps Clinic and Research Foundation 5 R01 CA 36448-02	Gene Transfer and Expression Using Retroviruses
76.	SRINIVAS, Ranga V. University of Alabama (Birmingham) 1 R01 CA 40440-01	Site-Specific Modification of SFFV Glycoproteins
77.	STEFFEN, David L. Worcester Foundation for Experimental Biology 2 R01 CA 30674-04A1	Mechanisms of Viral and Nonviral Leukemogenesis
78.	THOMAS, Christopher Y. University of Virginia (Charlottesville) 5 R01 CA 32995-03	Molecular Genetics of Leukemia Viruses of Inbred Mice
79.	VAIDYA, Akhil B. Hahnemann University 5 R01 CA 22413-08	Etiological Studies of Mammary Carcinoma
80.	VARMUS, Harold E. University of California (San Francisco) 5 R01 CA 19287-10	Molecular Biology of Mouse Mammary Tumor Virus

81. VERMA, Inder M.
Salk Institute for Biological Studies
5 R01 CA 16561-11

Viral and Cellular Oncogenes

82. VERMA, Inder M.
Salk Institute for Biological Studies
5 R01 CA 21408-09

Genetic Organization of RNA Tumor Viruses

83. VOGT, Marguerite M.
Salk Institute for Biological Studies
5 R01 CA 13608-13

Viral Gene Functions Involved in Transformation

84. WEINBERG, Robert A.
Whitehead Institute for Biomedical Research
5 R01 CA 39963-02

Construction of Novel Sarcoma Virus Genomes

85. WEINBERG, Robert A.
Whitehead Institute for Biomedical Research
1 R35 CA 39826-01

Molecular Basis of Carcinogenesis

86. WEISSMAN, Bernard E.
Children's Hospital of Los Angeles
1 R01 CA 36936-01A1

Retroviral Interaction with Epidermal Keratinocytes

87. WEISSMAN, Irving L.
Stanford University
5 R01 CA 32031-03

The Receptor-mediated Leukemogenesis Hypothesis

88. WILSON, Michael C.
Scripps Clinic and Research Foundation
5 R01 CA 33730-03

Regulation of Expression of the gp70 Multigene Family

89. WITTE, Owen N.
University of California (Los Angeles)
5 R01 CA 27507-06

Transformation by Abelson Murine Leukemia Virus

90. WONG, Paul K.
University of Illinois (Urbana-Champaign)
5 R01 CA 36293-02

Molecular Basis of Pathogenesis Induced by MLV Mutants

91. YAMAMOTO, Keith R.
University of California (San Francisco)
5 R01 CA 20535-08

Gene Regulation by Steroid Receptor Proteins

92. YANG, Wen K.
Oak Ridge National Laboratory
5 R01 CA 30308-04

Mechanism of Fv-1 Restriction of Murine Leukemia Viruses

93. YOSHIMURA, Fayth K.
Fred Hutchinson Cancer Research Center
5 R01 CA 25461-05

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 avian tumor viruses and hepatitis B virus. This program consists of 90 research grants with an estimated total funding of 14.5 million dollars for FY85. Of these, approximately 80% are involved with studies of avian tumor viruses, and 14% concern hepatitis B virus (HBV) or other hepatitis viruses and their relationship to primary hepatocellular carcinoma (PHC). The remaining 6% deal with a variety of subjects which are more distantly related to human diseases. The majority of studies funded by RNA Virus Studies II are focused on the molecular nature of the transformation process, the definition and discovery of new oncogenes (genes responsible for the transformation of cells from normal to malignant), and development and testing of hypotheses about the mechanism(s) of oncogenesis of viruses lacking oncogenes. In addition to traditional grants (R01) and program project grants (P01), this component now includes one R35 outstanding investigator award as well as three R13 conference grants and two R23 new investigator awards. Selected examples of the types of studies accomplished within the last year follow.

There are two fundamental questions which investigators in avian retrovirology are attempting to resolve. The first of these is mechanism(s) by which viral oncogene products initiate and maintain the transformed state in cells. The second is the relevance, if any, of cellular oncogenes to the transformation process in terms of the initiation of human and/or animal malignancies. Despite intensive investigation, definitive answers are not yet available for either of these two questions.

Several approaches have been utilized in an attempt to answer the question of how viral oncogene products transform cells. Among the simplest, conceptually (and the most labor intensive), are comparative studies of all of the proteins labeled with ^{35}S methionine or ^{32}P orthophosphate in control chick embryo fibroblasts, in cells transformed by the UR2 sarcoma virus, and by a temperature-sensitive mutant (tsL429) of Rous sarcoma virus (RSV) grown at either the restrictive or permissive temperature. Following labeling of the cells, the proteins were separated by two dimensional electrophoresis on very large polyacrylamide gels in order to detect transformation specific changes in protein synthesis and in total phosphorylation of both viral and cellular proteins, since either or both types of proteins might have a role in the initiation and maintenance of the transformed state.

Virus coded proteins appeared in whole cell lysates of all infected cells. The viral structural proteins were identified by comparison with proteins immunoprecipitated with antivirus antibodies. The transforming proteins pp60v-src and p68v-ros, present in cells transformed with RSV and UR2 respectively, were phosphorylated. Eighteen increases and eight decreases in the levels of cellular phosphoproteins were associated with transformation; these levels reverted to normal when cells infected with the temperature sensitive mutants were incubated at the non-permissive temperature. Although these changes in the degree of phosphorylation are more extensive than previously reported, none of them appear to represent novel phosphorylations, since all proteins phosphorylated in transformed cells also appeared to be phosphorylated to a certain extent in uninfected cells.

Fifteen cellular proteins showed increased relative rates of synthesis apparently related either to transformation or growth at the non-permissive temperature. Four other proteins were increased exclusively in cells incubated at the non-permissive temperature, but not in cells incubated at the permissive temperature whether the cells were transformed or not. Eleven additional increases in the synthesis of cellular proteins, many of which were quite large and one seemingly a de novo induction, appeared to be specific for transformation. These changes in synthesis occurred in cells transformed by either the UR2 or RSV virus at the permissive temperature, but reverted to normal levels of synthesis at the non-permissive temperature. It was felt that these eleven changes may represent increases in cellular gene expression that are related to the maintenance of the transformed state. The search for possible regulatory changes, either complementary to or independent from the known tyrosine phosphorylations, which might be involved in transformation by avian viral oncogene products was prompted by recent reservations expressed about the exclusive role of tyrosine phosphorylation in avian sarcoma virus induced transformation. An additional stimulus was the recent observation that pp60v-src and p68v-ros phosphorylate phosphatidylinositol. This finding casts doubt on the exclusive protein specificity of these kinases and raises the possibility that other biochemical activities may play a role in some of the changes associated with transformation.

This investigation complements previous studies which sought to detect potentially important changes in synthesis or phosphorylation of cellular proteins in cells transformed by avian retroviruses. The phosphorylation results largely confirm and extend data of previous studies. It is interesting to note that both UR2 and Rous sarcoma virus phosphorylate an almost identical set of intracellular proteins. This has been observed previously only in the case of tyrosine phosphorylations. In addition, the levels of all phosphorylations tended to revert to normal values when cells transformed by the temperature sensitive mutant of RSV were incubated at the non-permissive temperature. Perhaps the most notable result of this study is that qualitative and quantitative changes in the synthesis of a few cellular proteins appear to be more conspicuous than changes in phosphoproteins. The fifteen proteins showing increases in synthesis either after transformation or upon incubation at the non-permissive temperature are clearly not specific for transformation, but may be related to enhanced growth rate or to other cellular responses common to transformation and to exposure to relatively elevated temperatures. On the other hand, the role of the eleven transformation specific changes in protein synthesis is worthy of further investigation, since one or more of these proteins may be involved in regulatory events that are unique to the transformed state. Two interesting candidates for transformation regulatory proteins are a de novo 31.5 kilodalton basic protein (detected on non-equilibrium gels) and a protein designated T4 which increases 140-fold in transformed cells (2).

Another line of evidence which implicates cellular proteins in the transformation process comes from studies in which avian macrophages are infected with RSV. The normal target cell for these viral infections in the chicken is the fibroblast, in which transformation is induced with high efficiency. However, when macrophages were infected with the virus, no changes in morphology, growth behavior, or expression of macrophage specific proteins were noted. It was determined that the lack of cellular transformation does not result from a block in the synthesis of viral proteins, since infectious virions were released from a majority of cells in the culture. Although the absolute level of pp60v-src was reduced in macrophages as compared with fibroblasts, the protein exhibited the same

phosphorylation pattern and subcellular distribution and was able to phosphorylate immunoglobulin in the immune complex protein kinase assay. These results imply that the failure of RSV to transform macrophages may be due to a restriction in the cellular response to a functional src protein, perhaps because of the absence in the macrophages of cellular proteins which are essential mediators of the transformation process. The alternative explanation, that the failure to transform macrophages is a result of the reduced synthesis of pp60v-src seems unlikely, since the levels of pp60v-src specific kinase activity in macrophages were similar to those observed in several lines of RSV transformed mammalian cells. However, it is possible that the relative amount of pp60v-src necessary to transform fibroblasts may be significantly lower than that necessary to transform macrophages. Thus, it appears more likely that the macrophage cells are refractory to the transforming activity of pp60v-src possibly through the absence of some developmentally regulated molecule which performs an essential function in the pathway leading to src mediated cell transformation.

These observations in macrophages could represent evidence for a linkage restricted action of src. To test this hypothesis, these investigators also infected murine bone marrow cells with a recombinant murine retrovirus which contains the src gene from RSV and observed that during eight months of culturing of these cells there was no evidence of transformation of the murine macrophages by the recombinant virus. There is precedent for the linkage restricted action of oncogenes among the acute leukemia viruses, but this is the only example among the sarcoma viruses. Of the avian retroviruses, AMV (avian myeloblastosis virus) is the most restricted, since it only transforms the morphologically identifiable members of the monocyte-macrophage lineage. Avian erythroblastosis virus is less restricted; it transforms specific stages of erythroid cell differentiation, but is also able to transform fibroblasts. It is noteworthy that other workers have previously shown that avian erythroblastosis virus, like RSV, is capable of replicating in macrophage cells in the absence of transformation. Both avian reticuloendothelosis virus and the murine Abelson virus transform primarily B cells, although they are both also able to transform fibroblasts. Whether this lineage restriction will hold for all oncogenes warrants more extensive investigation (5,6,10).

Another technique directed at resolving the question of how viral oncogene products transform cells is in the use of site directed mutagenesis to construct a variety of deletion, substitution and insertion mutants having missing amino acid residues, single amino acid substitutions, or insertion of additional nucleotides within the DNA coding sequences which results in a change of the reading frame during transcription.

Results of these studies have shown that the deletion of nucleotide sequences within the 5' (amino terminal) half of the src gene yielded mutants which encoded structurally altered forms of the src protein. One deletion mutant, lacking residues between glycine 82 and arginine 169, showed a loss of transforming activity. Two deletion mutants, lacking amino acid residues between 170-226, resulted in virus that was temperature sensitive for morphological transformation. In both instances, the src protein encoded by the mutant virus retained substantial protein kinase activity in vitro. Therefore, mutations in these regions of the src protein map within a functionally important domain of the src protein distinct from the domain specifying the protein kinase activity. Such a recognition domain might specify the interaction of the src protein with a specific target protein or proteins.

The conservation of amino acid sequences between the carboxy terminal half of pp60v-src and other viral tyrosine protein kinases suggests that this part of the protein contains functional catalytic domain(s) necessary for tyrosine protein kinase activity. This suggestion is supported by the findings that viruses containing single point mutations, resulting in single amino acid changes within this highly conserved region, are both transformation defective and have a decreased protein kinase activity (measured both in vitro and in vivo). Thus, the high degree of amino acid sequence conservation among different viruses in this part of the src protein is likely a reflection of the requirement to preserve the structural integrity of this essential domain of the protein as a whole. The alteration of the carboxy terminus of pp60v-src has a deleterious effect on transformation as well as on protein kinase activity, suggesting that the carboxy terminal portion of this protein is intimately involved in maintaining the overall tertiary structure of the enzymatically active src protein. Since these structural changes alter pp60v-src activity, a logical approach now being pursued is to try to define the role of the carboxy terminus of its cellular counterpart, c-src protein, in the specificity of regulation of c-src activity. These experiments should provide information on the mechanism of regulation of v-src, as a possible functional difference has been suggested between the v-src gene product and its cellular counterpart which resides within a carboxy terminal regulatory domain that is present in pp60c-src, but not in pp60v-src. Therefore, cellular transformation mediated by pp60v-src results from the unregulated expression of protein kinase activity. In contrast, the activity of cellular src protein is likely to be regulated in normal cells and may be modulated in response to external growth factors. This hypothesis predicts the presence of one or more proteins capable of interacting with and modulating the activity of pp60c-src. The search for such proteins is currently in progress in a number of laboratories using appropriate hybrid gene constructs (54,55,56).

Other research using mutant virus as a tool involves the construction of a mutant in v-src in which the major phosphotyrosine site (tyrosine 416) has been converted to phenylalanine. Studies with this mutant have enabled investigators to distinguish between the in vitro and in vivo functions of this enzyme. Thus, mouse cells transformed by this mutant gene and designated RSV-SF1, are tumorigenic only if tested in immunodeficient mice, whereas cells transformed by the wild-type parent are tumorigenic in both syngeneic and immunocompetent animals. When examined in vitro, the RSV-SF1 transformed cells are virtually indistinguishable from cells transformed by the wild-type pp60v-src. This behavior of RSV-SF1 suggests that the overall protein kinase activity of pp60v-src can be separated from the ability of cells transformed by this protein to form tumors. It has been recently reported that mutations in the proto-oncogenes of the ras family at position 12, which is normally the amino acid glycine, convert the normal human genes into active oncogenes. Similarly, evidence from this study suggests that the conformation around tyrosine 416 in pp60v-src possesses a controlling function. Either a change in conformation contributed by an amino acid substitution at position 416 in RSV-SF1 has a subtle effect on the kinase activity of pp60v-src, perhaps affecting its ability to phosphorylate a subset of substrates, or pp60v-src possesses activities affected by the nature of residue 416 which are distinct from its known enzymatic function. The tumorigenicity experiments in mice demonstrate that, whereas a change of tyrosine 416 in pp60v-src to phenylalanine has little effect on the properties of cells in tissue culture, it radically alters the tumorigenicity of these cells. Although the RSV-SF1 transformed cells cannot grow in an immunocompetent mouse, they do form tumors in an athymic animal. These tumors cannot, however, be subsequently grown

in a syngeneic host. Pp60v-src isolated from these tumor cells shows very little carboxy terminal phosphorylation, indicating that a reversion to wild type does not occur.

These results appear, at first glance, to be at variance with those of others (31) who removed the tyrosine 416 of pp60v-src by making a deletion mutation. These workers found that the mutant virus was able to form foci in vitro in cultures of chicken fibroblasts at a reduced efficiency and with an increased time of appearance. This mutant virus was also tumorigenic in chicks, although it required a longer time, as compared to wild type, to induce tumor growth. However, this result is not necessarily conflicting with the results found with RSV-SF1 cells in vivo, since the mouse model differs from the chicken in that no recruitment of cells into the tumor mass by viral spread can occur in mice. One factor in successful tumor formation may be that the rate of transformed cell growth is greater than the rate of recognition and killing by the host immune system. If this is true, then a mutation in pp60v-src which rendered cells more susceptible to immune surveillance could go unnoticed in chickens, where virus can spread, and yet be dramatically evident in mice where the initial injected population must serve as the focal point for tumor indication. Thus, these findings indicate that the protein kinase activity of pp60v-src may not be fully responsible for tumorigenesis by v-src and moreover, evasion of the host immune response may be important in tumorigenesis by v-src (5).

Thus, in spite of a great deal of work, the answer to the question on how viral oncogene products transform cells still eludes us. Through the use of the genetic and biochemical techniques described above, some investigators are continuing to study proteins that are changed or altered on the initiation and maintenance of the transformed state, while others are continuing the dissection of viral oncogenes at an ever finer level of resolution. These kinds of studies should provide us with the answer(s) to this intriguing problem. Although this discussion has been focused primarily on the src gene and Rous sarcoma virus, the conclusions apply equally well to the multiplicity of oncogenes found among the avian retroviruses and thus represent a general phenomena among avian oncogenes.

A second major question confronting avian retrovirologists is whether cellular oncogenes have any relevance to the transformation process or to tumorigenesis. Studies of unmodified oncogenes in their natural host cells suggest that the cellular genes do not, themselves, play a role in the initiation of tumorigenesis. However, the genes do appear to be amplified in many tumors. Research leading to these conclusions is described below.

The transforming activity of the cellular src (c-src) gene as well as of hybrid genes between viral and cellular src were tested by replacing all or part of the RSV viral src gene by the corresponding portion of the c-src gene. After these derivatives were introduced into chick embryo fibroblasts by transfection, replication competent virus was recovered. The expression of pp60src induced was equivalent to pp60v-src expression in cells infected with wild-type RSV. Replacement of the portion of the v-src gene either upstream or downstream of the Bgl I site with the homologous portion of c-src gene resulted in fully transforming virus. On the other hand, the virus stock obtained from cells transformed with Rous sarcoma virus DNA containing the entire c-src gene had a very low titer of focus forming virus, while it contained a high titer of infectious virus. It appeared that rare, small foci were formed by mutant viruses generated from the original c-src containing virus. These results indicate that overproduction of

the c-src gene product does not cause cell transformation and that this proto-oncogene is subject to a relatively high rate of mutation when incorporated into a retrovirus genome, which results in the acquisition of transforming capacity. Thus, the RSV variant that encodes c-src may express a large amount of pp60c-src in chick embryo fibroblasts, but fail to cause cell transformation. The differences in biological activities of various constructed DNAs were reproducible in four independent experiments. The results thus clearly indicate that there is some qualitative difference between the v-src and c-src gene products in functions related to cell transformation. This result is in contrast with the reports of some other proto-oncogenes such as c-mos, c-H-ras, and more recently c-fos. These three proto-oncogenes can lead to cell transformation when linked to viral promoter sequences.

The major divergence in amino acid sequence between pp60c-src and pp60v-src which is located in their carboxy terminal region cannot explain the difference of transforming ability, because mutants which have c-src specific carboxy termini can transform chick fibroblasts quite well. The fact that other mutants which encode reciprocal chimeric pp60s can transform chick embryo fibroblasts indicates that the v-src sequence of the Schmidt-Ruppin A strain of Rous sarcoma virus has at least two mutations critical for transformation, one upstream and one downstream of the Bgl I site. Either mutation is sufficient to convert pp60c-src to a transforming protein.

The small foci observed in cultures infected with virus encoding pp60c-src were demonstrated to be generated by mutation of the RSV pp60c-src containing virus to a transformation competent virus. The high divergence in RNA sequences in the retroviral genome derived from a single cloned virus stock seems to be attributable to the low fidelity of reverse transcription. These results show that after transduction of the c-src gene into a retrovirus, this proto-oncogene is subject to a relatively high rate of mutation and that transforming virus can be selected rather easily by virtue of this capacity. This fact may be relevant to the natural history of RSV formation (31).

Similar results were obtained in studies utilizing a rat cell line, designated RC, containing ten times the normal intracellular level of pp60c-src, which was isolated after transfecting rat 2 cells with a chimeric DNA. These cells produced the protein encoded by c-src in quantities comparable to that of v-src when the latter protein is found in transformed cells. The cells remained phenotypically normal, contained actin cables, and did not grow in soft agar. In contrast, both RC cells and parent rat 2 cells transfected with a molecular clone of v-src, exhibited many properties of biologically transformed cells including a round morphology, disruption of actin cables, and the ability to grow in soft agar. The RC cells contained an intracellular level of pp60c-src three times greater than the amount of pp60v-src in a v-src transformed cell line. These investigators concluded that c-src might acquire the capabilities of an oncogene only when altered in a manner that changes the protein the gene encodes. Such a change probably occurred in the generation of v-src from the parental c-src (5).

In contrast to the above findings, when the partially transformed NIH/3T3 cell line was transfected with plasmids containing Moloney murine leukemia virus long terminal repeats and either chicken c-src or v-src genes, focus formation was found after treatment with both types of plasmids, although the c-src containing plasmids induced only about one percent as many foci as v-src. Cleveland digests indicated that authentic chicken pp60c-src was being overexpressed in the

transfected cells, while immunoprecipitation and secondary transfection experiments indicated that focus formation was caused by rare transfection events that resulted in very high levels of pp60c-src expression (about nine times the level of pp60v-src in transformed NIH/3T3 cells) rather than by mutations of transfected c-src genes. The focus-selected c-src overexpressor cells displayed a partially transformed phenotype which included focus formation, limited growth in soft agar, and reduction in serum requirement for survival, but which did not include unlimited growth in soft agar or in vivo tumorigenicty.

Additional experiments showed that the overexpressed c-src protein was biochemically active and phosphorylated both IgG and exogenous substrates when bound in immune complexes, but with significantly lower specific activity than pp60v-src. Quantitative comparison of immunoprecipitates from ^{35}S methionine metabolically labeled cells showed that the synthesis rates of pp60c-src in three focus-selected cell lines was about four times higher than the level in three co-selected c-src overexpressor cell lines or in the v-src transformed cells. These results suggest that the relatively rare c-src induced focus forming events were caused by rare transfection events which resulted in very high levels of pp60c-src expression against the background of many more transfection events which caused more moderate pp60c-src overexpression without focus formation.

This hypothesis was supported by secondary transfection data which showed that the DNAs purified from the focus-selected c-src and co-selected pp60c-src overexpressor cells had low focus forming activities compared with DNAs from both focus selected and co-selected pp60v-src transformed cells. If mutations had been responsible for the appearance of c-src induced foci, it would be expected that the mutant c-src genes which were integrated into the focus-selected cellular DNAs would have had high focus forming activities like those of the v-src transformed cells. DNAs purified from co-selected c-src overexpressor cells had about one percent of the focus forming activity of the DNAs purified from v-src overexpressor cells, which was in agreement with focus forming activities of the plasmid DNAs.

Thus, the partially transformed NIH/3T3 cell line can demonstrate low levels of some of the classic phenotypic manifestations of transformation upon treatment with pp60c-src. The results of the study demonstrate that the different parameters of transformation can respond in non-coordinate fashion to quantitative and qualitative modifications in proto-oncogene expression, and that at least two separable functional activities are required even for transformation of an immortalized cell line. Lastly, it was shown that pp60c-src has at least one and lacks at least one of these activities when expressed at the described levels (69).

Although it seems clear that, at least in the avian system and probably in other systems as well, unmodified cellular oncogenes do not play a role in tumorigenesis, the finding of amplification of cellular oncogenes and their enhanced expression in tumors is intriguing, particularly in some neuroblastoma tumors and lung cell carcinomas, where the levels of amplification ranged from thirty to two hundred times that found in normal cells.

The contribution of cellular oncogenes to tumorigenesis could take either of two forms: (1) inappropriately enhanced expression that overburdens cells with a normal protein encoded by a cellular oncogene or (2) mutational damage that changes the functions of the gene product. It is easy to suggest, but difficult

to prove, that amplification and enhanced expression of cellular oncogenes has contributed to the genesis of certain human tumors. One observation that lends support to this hypothesis is the observation of the presence of double minute (DM) chromosomes in tumor cells. These DMs are unstable elements which are generally lost from growing cells unless the enhanced expression of a gene within the DM confers a selective advantage on the cells. The presence of these DMs in tumors before they were explanted into culture and their continued occurrence in the cells after innumerable generations of cell growth indicates that the gene amplification has helped to sustain the growth of the tumor cell. Although it is not possible to be certain that the identified cellular oncogenes are responsible for the impact of gene amplification, it seems more than coincidence that in every instance studied to date, karyotypic evidence of gene amplification in tumor cells not subjected to chemotherapy has been accompanied by amplification of either a previously identified cellular oncogene, or a gene that can be recognized by its kinship to a known oncogene (e.g., N-myc).

The point in tumorigenesis when gene amplification might come into play is not yet known. There are two observations which suggest different time points for the amplification. First, insertional mutagenesis is an early event (perhaps the first event) in the genesis of tumors by retroviruses without oncogenes of their own; the consequence of insertional mutagenesis is enhanced expression of a cellular oncogene. On the other hand, amplification and enhanced expression of c-myc apparently occurs in concert with the conversion of human carcinoma of the lung cells to a more malignant phenotype at an advanced stage in the evolution of these tumor cells. It is possible that there may be no mandatory sequence of events for the genesis of any particular tumor, other than a requirement for a concerted set of genetic (and perhaps epigenetic) abnormalities before the full malignant phenotype is achieved. In such a scheme, enhanced expression of an oncogene could play its part whenever amplification happens to occur.

The sequence in which double minute chromosomes and homogeneously staining regions of chromosomes might arise is complex and as yet unclear. The presence of amplification and enhanced expression in neuroblastomas, acute promyelocytic leukemias, carcinomas (including colon carcinomas), chronic myelocytic leukemias, and in a transition from small cell lung carcinoma to a more malignant large cell variant provide an indication that amplification of cellular oncogenes may play some role in either the initiation or progression of certain human or animal tumors. It is hoped that further investigations in this area will result in more clear-cut and definitive answers to this intriguing problem (5).

In studies of hepatitis B virus (HBV) and its relation to liver cancer, viral DNA integrations have been cloned from a primary hepatocellular carcinoma and these molecules have been studied in detail. Viral DNA sequences of these integrations show deletions and duplications, and are incomplete viral entities that retain the hepatitis B surface antigen coding region. One of the clones is surrounded by reiterated cellular DNA and the other contains unique cellular sequences adjacent to the HBV integration. Studies on one of these, designated HL70-3 has shown that: it is located on the short arm of chromosome 11, P13-14, that there is no chromosomal translocation, and that by restriction mapping a minimum of a 13.5 kilobase deletion is present at the site of HBV integration. These findings are interesting in view of the fact that deletions in the same region of chromosome 11 are associated with Wilms' tumor, while other abnormalities in this chromosome region are associated with Beckwith-Wydemann syndrome.

Additional studies are assessing the role of delta agent, a defective RNA virus which requires the persistence and helper function of HBV for its replication. To assess the influence of delta agent on viral replication in patients persistently infected with both viruses and showing chronic liver disease (CLD), the level of HBV DNA was measured in the serum and liver of hepatitis B surface antigen positive CLD patients who were either positive or negative for delta antigen in their livers. In individuals who were delta antigen positive and had chronic liver disease, HBV DNA was detected transiently in the serum of four of twenty-one cases where it was present in low levels. In contrast, forty-three percent of the delta antigen negative/hepatitis B surface antigen positive CLD patients were HBV DNA positive and five of these had high serum HBV DNA levels in the range observed during acute HBV infection. Serum hepatitis B surface antigen and anti-hepatitis B core titers were significantly lower in delta antigen positive cases; this correlated with the reduced amount of hepatitis B core antigen in the liver as demonstrated by immunofluorescence or immunoperoxidase staining. Fewer delta antigen positive/hepatitis B surface antigen positive carriers showed virion or replicating HBV DNA forms in their liver as compared with delta antigen negative/hepatitis B surface antigen positive carriers. These findings indicate that in delta antigen positive chronic hepatitis, synthesis of hepatitis B virus genomes and gene products remains suppressed, whereas production of delta components continues at high levels. These observations may be of value in understanding the pathobiology of chronic delta antigen infection and suggest that liver disease activity in delta antigen positive/hepatitis B surface antigen positive carriers results primarily from the superimposed chronic delta infection and is possibly associated with continued delta replication rather than with HBV replication (68).

Thus, a number of studies in the RNA Virus Studies II area continue to provide data on possible mechanisms of oncogenesis by these agents and on possible roles for cellular oncogenes in transformation; studies on hepatitis B virus point out the complex interactions of this agent in human hepatocellular carcinoma.

RNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. ACS, George Mount Sinai School of Medicine 5 R01 CA 34818-02	Studies on the Replication and Oncogenicity of HBV
2. BALDUZZI, Piero C. University of Rochester 5 R01 CA 32310-03	The Transforming Genes of Avian Sarcoma Viruses
3. BALUDA, Marcel A. University of California (Los Angeles) 5 R01 CA 10197-18	Tumor Induction by Avian Myeloblastosis Virus
4. BEEMON, Karen L. Johns Hopkins University 5 R01 CA 33199-03	Location and Function of m6A in Retrovirus RNAs
5. BISHOP, J. Michael University of California (San Francisco) 2 R01 CA 12705-14	Rous Sarcoma Virus: Replication and Cell Transformation
6. BOETTIGER, David E. University of Pennsylvania 5 R01 CA 16502-11	Genetic Analysis of RNA Tumor Viruses
7. BOETTIGER, David E. University of Pennsylvania 5 R01 CA 30383-05	Virus Induced Myeloid Leukemia
8. BOSE, Henry R., Jr. University of Texas (Austin) 5 R01 CA 33192-02	Transformation by Avian Reticuloendotheliosis Virus
9. BOYD, Juanell N. Cornell University (Ithaca) 5 R23 CA 36160-02	Dietary Choline, Aflatoxin, and Carcinogenesis
10. BRUGGE, Joan S. State University of New York (Stony Brook) 5 R01 CA 27951-06	The Association of Two Cellular Proteins with pp60-src
11. BUTEL, Janet S. Baylor College of Medicine 5 R01 CA 37257-02	Hepatitis B Virus and Human Liver Cancer

12. CARBON, John A. University of California (Santa Barbara) 5 R01 CA 11034-17	Studies on Gene Organization and Expression
13. CASPAR, Donald L. Brandeis University 2 R01 CA 15468-12	Assembly of Viruses, Membranes, and Tissue
14. CHISARI, Francis V. Scripps Clinic and Research Foundation 1 R01 CA 40489-01	Pathogenesis of Hepatitis B
15. CHRISTENSEN, James R. University of Rochester 5 R01 CA 36312-02	Oncogenes and Neoplastic Progression
16. COFFIN, John M. Tufts University 5 R01 CA 17659-10	Relationship of Avian Tumor Virus RNA and Host Genome
17. COFFIN, John M. Tufts University 2 R01 CA 27108-06	Mechanisms of Variability of Tumor Virus RNA
18. DUESBERG, Peter H. University of California (Berkeley) 2 R01 CA 11426-17	Retroviral Onc Genes and Cellular Proto-onc Genes
19. EISENMAN, Robert N. Fred Hutchinson Cancer Research Center 2 R01 CA 20525-09	Control Mechanisms in Avian Oncornavirus Replication
20. ERIKSON, Raymond L. Harvard University 5 R01 CA 34943-04	Biosynthesis of Viral RNA
21. FARAS, Anthony J. University of Minnesota (Minneapolis-St. Paul) 5 R01 CA 18303-11	RNA-Directed DNA Polymerase and 70S RNA of Oncornaviruses
22. GOLDBERG, Allan R. Rockefeller University 5 R01 CA 13362-14	RSV Functions Involved in Transformation
23. GORDON, Julius A. University of Colorado Health Sciences Center 5 R01 CA 35378-02	Studies of pp60-src Activity and Substrate Phosphorylation

Investigator	Project Title
24. GOULIAN, Mehran University of California (San Diego) 2 R01 CA 11705-16	DNA Synthesis Studies
25. GRANDGENETT, Duane P. St. Louis University 5 R01 CA 16312-12	Avian Retrovirus DNA Synthesis and Its Regulation
26. GRANOFF, Allan St. Jude Children's Research Hospital 5 R01 CA 07055-23	Studies on Lucke Tumor Associated Viruses
27. GRODZICKER, Terri I. Cold Spring Harbor Laboratory 1 R13 AI/CA/AM 22056-01	Hepatitis B Virus Meeting
28. GUNTAKA, Ramareddy V. University of Missouri (Columbia) 5 R01 CA 36790-03	Synthesis and Structure of Avian Tumor Virus DNA
29. HAGER, Lowell P. University of Illinois (Urbana-Champaign) 1 R13 CA 38747-01	Plasmids in Bacteria Conference
30. HALPERN, Michael S. Wistar Institute of Anatomy and Biology 5 R01 CA 31514-04	Oncornavirus-Induced Immuno-suppression
31. HANAFUSA, Hidesaburo Rockefeller University 5 R01 CA 14935-13	Cellular Alteration Induced by Rous Sarcoma Virus
32. HARRISON, Stephen C. Harvard University 5 R01 CA 13202-14	Structure and Assembly of Viruses and of Coated Vesicles
33. HAYWARD, William S. Sloan-Kettering Institute for Cancer Research 5 R01 CA 34502-04	RNA Tumor Virus Gene Expression
34. HOLTZER, Howard University of Pennsylvania 5 R01 CA 18194-10	Conversion of Embryonic Cells Into Transformed Cells
35. HUMPHRIES, Eric H. University of Texas Health Science Center (Dallas) 5 R01 CA 32295-03	Endogenous Avian Retroviruses in Non-Permissive Cells

36. HUNTER, Eric
University of Alabama in Birmingham
5 R01 CA 29884-05

Site Specific Mutagenesis of the Envelope Gene of RSV

37. JOKLIK, Wolfgang K.
Duke University
5 P01 CA 30246-05

Regulatory Functions of Protein Nucleic Acid Interaction

38. KNOWLES, Barbara B.
Wistar Institute of Anatomy and Biology
5 R01 CA 37225-02

Hepatitis B Virus and Primary Hepatocellular Carcinoma

39. KOPROWSKI, Hilary
Wistar Institute of Anatomy and Biology
5 P01 CA 21124-08

Genetics and Virology of Cancer

40. KUNG, Hsing-Jien
Case Western Reserve University
1 R01 CA 38659-01

Oncogene and Activator: Tumorigenesis by Cloned DNA

41. KUNG, Hsing-Jien
Case Western Reserve University
7 R01 CA 39207-01

Erythroleukemia: Oncogene Activation by Retrovirus

42. LAU, Alan F.
University of Hawaii at Manoa
5 R01 CA 35578-02

Cellular Substrates of pp60-src in ASV-Infected Cells

43. LEIS, Jonathan P.
Case Western Reserve University
5 R01 CA 38046-02

Studies of Retroviral Proteins

44. LINIAL, Maxine L.
Fred Hutchinson Cancer Research Center
5 R01 CA 18282-10

Viral Coded Functions in Rous Sarcoma Virus

45. MACARA, Ian G.
University of Rochester
1 R01 CA 38888-01

Oncogene Phosphoinositide Kinase Activity and Cancer

46. MAJORS, John E.
Washington University
1 R01 CA 38994-01

Analysis of Retroviral Transcriptional Regulation

47. MARCUS, Philip I.
University of Connecticut (Storrs)
5 P01 CA 14733-10

Gene Expression, Virus Replication and Cell Growth

48. MARTIN, G. Steven University of California (Berkeley) 2 R01 CA 17542-10	Genetics of RNA Tumor Viruses
49. MONTELARO, Ronald C. Louisiana State University 2 R01 CA 38851-05	EIAV: Antigenic Variation and Retrovirus Persistence
50. MOSCOVICI, Carlo University of Florida 5 R01 CA 10697-19	Avian Leukemia Viruses and Cell Differentiation
51. NEIMAN, Paul E. Fred Hutchinson Cancer Research Center 5 R01 CA 20068-10	Molecular Mechanisms in Neoplasia
52. NEIMAN, Paul E. Fred Hutchinson Cancer Research Center 5 P01 CA 28151-05	Retroviruses and Cancer
53. OGSTON, Charles W. Rush-Presbyterian-St. Lukes Medical Center 5 R01 CA 37276-02	Molecular Biology of Hepatitis Virus in Vivo
54. PARSONS, John T. University of Virginia (Charlottesville) 5 R01 CA 27578-06	Expression of Avian Retrovirus Transforming Genes
55. PARSONS, John T. University of Virginia (Charlottesville) 5 R01 CA 29243-05	Avian Sarcoma Virus Specific Tumor Antigens
56. PARSONS, Sarah J. University of Virginia (Charlottesville) 1 R01 CA 39438-01	Role of c-src in Retroviral Transformation
57. PRIVALSKY, Martin L. University of California (Davis) 1 R01 CA 38823-01A1	Characterization of the v-erb B Oncogene Protein of AEV
58. RHODE, Solon L., III University of Nebraska 1 R01 CA 37481-01A2	Replicon Control in Normal and Transforming Cells

	Investigator / Institution / Grant	Title
59.	ROBINSON, Harriet L. Worcester Foundation for Experimental Biology 2 R01 CA 23086-08	Retrovirus and Congenital Transmission
60.	ROBINSON, Harriet L. Worcester Foundation for Experimental Biology 5 R01 CA 27223-06	Avian Leukosis Viruses and Cancer
61.	ROBINSON, William S. Stanford University 5 R01 CA 34514-03	Duck Hepatitis B Virus: Infection and Disease
62.	ROGLER, Charles Yeshiva University 5 R01 CA 37232-02	Molecular Aspects of WHV Induced Persistent Infection
63.	ROHRSCHNEIDER, Larry R. Fred Hutchinson Cancer Research Center 5 R01 CA 20551-09	Mechanisms of Oncornavirus-Induced Transformation
64.	RUECKERT, Roland R. University of Wisconsin (Madison) 5 R01 CA 08662-19	Structure and Synthesis of Retro- and Nodaviruses
65.	SCHUBACH, William H. University of Minnesota (Minneapolis-St. Paul) 5 R23 CA 36977-02	Structure and Expression of the Endogenous myc Region
66.	SCOTT, June R. Emory University 5 R01 CA 11673-16	Lysogeny and Bacteriophage P1
67.	SEFTON, Bartholomew M. Salk Institute for Biological Studies 5 R01 CA 17289-10	Viral Membranes and Viral Transformation
68.	SHAFRITZ, David A. Yeshiva University 5 R01 CA 32605-04	Hepatitis B Virus - Chronic Hepatitis - Liver Cancer
69.	SHALLOWAY, David I. Pennsylvania State University 2 R01 CA 32317-04	Role of pp60c-src Homolog of the RSV Oncogenic Protein
70.	SHANK, Peter R. Brown University 2 R01 CA 32980-04	Stability and Disease Tropism of Proviral DNAs

71. SHENK, Thomas E.
Princeton University
1 R01 CA 39606-01
Functional Analysis of the Adeno-Associated Virus Genome

72. SIDDIQUI, Aleem
University of Colorado Health Sciences Center
5 R01 CA 33135-03
Expression of Hepatitis B Virus Genes and Hepatoma

73. SMITH, Ralph E.
Colorado State University
5 R01 CA 35984-03
Biochemistry of RNA Tumor Virus Replication

74. STAVNEZER, Edward
Sloan-Kettering Institute for Cancer Research
5 R01 CA 32817-03
The Origin, Structure, and Biological Activity of SKVS

75. STOLTZFUS, Conrad M.
University of Iowa
5 R01 CA 28051-07
Retrovirus RNA Metabolism

76. SWANSTROM, Ronald I.
University of North Carolina (Chapel Hill)
5 R01 CA 33147-02
Retrovirus Replication: Interaction with Host Genome

77. TATTERSALL, Peter J.
Yale University
5 R01 CA 29303-05
Molecular Basis of Parvovirus Target Cell Specificity

78. TAYLOR, John M.
Institute for Cancer Research
2 R01 CA 22651-08
Reverse Transcription

79. TEMIN, Howard M.
University of Wisconsin (Madison)
5 P01 CA 22443-08
Molecular Biology and Genetics of Tumor Viruses

80. TENNANT, Bud C.
Cornell University (Ithaca)
5 R01 CA 37264-02
Hepatitis, Aflatoxin, and Hepatocarcinogenesis

81. TIOLLAIS, Pierre
Pasteur Institute
5 R01 CA 37300-02
Hepatitis B Virus DNA Oncogenes and Liver Cancer

82. VARMUS, Harold E.
University of California (San Francisco)
5 R01 CA 37281-02
Oncogenic Potential of the Hepatitis B-Type Viruses

83. VARMUS, Harold E.
University of California
(San Francisco)
1 R35 CA 39832-01

Molecular Analysis of Retroviruses and Oncogenes

84. VOGT, Peter K.
University of Southern California
5 R01 CA 13213-14

Interactions Between Avian Tumor Viruses and Their Hosts

85. VOGT, Peter K.
University of Southern California
5 R01 CA 29777-05

Avian Oncoviruses: Transforming Genes and Proteins

86. VOGT, Volker M.
Cornell University (Ithaca)
5 R01 CA 20081-09

Avian Retrovirus Structure and Assembly

87. VYAS, Girish N.
University of California
(San Francisco)
1 R13 AI 19887-01A1

1984 International Symposium on Viral Hepatitis

88. WANDS, Jack R.
Massachusetts General Hospital
5 R01 CA 35711-02

Pathogenesis, Immunodiagnosis, and Therapy of Carcinoma

89. WANG, Lu-Hai
Rockefeller University
5 R01 CA 29339-05

Transforming Genes of Avian Sarcoma Viruses

90. WEBER, Michael J.
University of Virginia
(Charlottesville)
5 R01 CA 39076-02

Early Cellular Changes in Viral Oncogenesis

SUMMARY REPORT

RESEARCH RESOURCES

The Research Resources component of the Biological Carcinogenesis Branch (BCB), in conjunction with the various research units of the Branch, is responsible for developing, allocating and maintaining a coordinated program of research material support to meet the needs of extramural investigators funded by the Branch. The planning, initiating, and oversight necessary to generate and maintain specific research resources is the responsibility of the individual Program Directors who administer each of the research components of the Branch. However, the storage and distribution of research materials, the management of some resource contracts, the development and maintenance of a computerized inventory, and the day-to-day general management and direction of all resources distribution are the responsibility of this component of the Branch.

Laboratory investigations carried out under the sponsorship of the BCB depend on the availability of adequate quantities of viruses, viral reagents, antisera, animals and clinical and laboratory materials of adequate purity, viability and potency, some of which are not available from the commercial sector. The BCB resources component provides some research materials and other supporting activities through contract operations in four general areas. These include activities directed toward production, characterization and distribution of viral and anti-viral reagents; activities concerned with animal resources, including breeding and maintenance of animal colonies; activities directed toward the provision of specialized testing services for the examination of experimental materials; and activities concerned with the storage, inventory and distribution of human specimens.

The viral reagents produced during this period to meet program needs included avian myeloblastosis virus (AMV), reverse transcriptase and monoclonal antibodies to oncogene products of retroviruses. A consistently active supply of AMV reverse transcriptase is vital to biological carcinogenesis studies involving the production of cDNA copies of retrovirus genomes for use as probes to identify viral sequences in normal or malignant tissues, to compare viral and cellular sequences for homology, to permit expression of viral sequences in bacterial systems and for other molecular biological studies. To meet these needs, more than 1,200,000 units of AMV reverse transcriptase were produced and distributed to research laboratories. While the amount of transcriptase shipped has decreased since the payback system was initiated, demand has remained high. Shipments during this period consisted of approximately 235 to domestic laboratories and about 50 to foreign laboratories. The production and distribution of monoclonal antibodies to oncogene products and the synthetic peptides representing determinants of true oncogene products was initiated during this period; however, it has not reached a sufficient level for evaluation (5,7,9).

Animals have an important role in the biological carcinogenesis research program. Studies to determine the biological activity of putative human cancer viruses cannot be carried out in humans; therefore, it is imperative that another system be developed for these determinations and subsequently for

the evaluation of vaccines or other measures of control. The close phylogenetic relationship of the lower primates to man justifies the utilization of these animals for these purposes. Since the marmoset appears to be especially suitable for use as a comparative model system, a colony of 86 cotton-topped marmosets consisting of 41 adults and 45 juveniles is being maintained. To date, at least five and possibly six virus-tumor models, including Epstein-Barr and Herpesvirus saimiri viruses, have been established in marmoset monkeys. In addition, of particular interest is the occurrence of colon cancer in three animals in this colony. Because of its small size, the marmoset is economical to house, yet it is large enough for routine surgical procedures and serological monitoring (4).

A transfer of funds in the amount of $250,000 was made to the National Institute for Allergy and Infectious Diseases to support production of captive-born woodchucks and their utilization as models for studies of human hepatitis and hepatocellular carcinoma. It has been determined that the animals can be infected with woodchuck hepatitis virus during a limited period in their life, and that an on-site facility for research as well as breeding is required. Significantly, it appears that the chronic carrier state can be established in the woodchuck. This animal may furnish a valuable model for studies on the development of hepatocellular carcinoma in humans; moreover, it is apparently superior to the duck model in that histopathologic characteristics of hepatoma have been demonstrated in the woodchuck and not, as yet, in the duck. The breeding effort to produce seronegative woodchucks will continue; these animals will be used in experimental studies (12).

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 virus presence in tissue cells, the ability to grow virus, or the validity of virus isolation systems are all dependent upon the assurance of the identity of the cell cultures used. To meet this need, procedures were carried out for interspecies and intraspecies cell identification on approximately 235 cultures from 45 laboratories. Three assays were utilized in these determinations: immunofluorescent staining for species specific surface antigens, isoenzyme analysis, and cytological analysis by means of chromosome banding (10).

In addition, during this period, more than 2,000 human specimens and over 500 viral reagents were shipped to research laboratories from the inventory of frozen biological reagents. Materials received at the repository for storage included plasma containing AMV, sera obtained from patients with nasopharyngeal carcinoma, and monoclonal antibodies to oncogene products of retroviruses along with the corresponding synthetic peptide antigens used to induce them. Data relevant to the storage and distribution of research resource materials were added to the data base. Computerization of inventory data makes it possible to rapidly obtain information on availability, location and quantity of resources, permitting rapid response to requests by investigators. Inventory and accountability reports were prepared for program administrators as needed (2,8)

In May 1981, the Branch began implementation of the resources "payback" system approved by the DCCP (now DCE) Board of Scientific Counselors. The payback system is one in which the recipients of resource materials or services reimburse the resource or production contractor directly for the services or materials received, based on a price schedule agreed upon in advance between the NCI and its resource contractor. The contractor in turn credits those funds received from recipients against its production costs and these are shown on monthly vouchers which it submits to the Government for payments under the contract. Initiation of this system was the result of a variety of influences: the noticeable shrinking of the budget; an interest in seeing that the resource dollars utilized by grantees, intramural scientists, and contractors were included in a peer-review system; and the perception that free distribution of resources did not always result in the most effective utilization of available funds.

The research resources payback system, now fully implemented, has been phased in over the past three and one-half years. Total costs of production and distribution of research materials are collected on the contracts in which there are a large number of individual users who are receiving small amounts of material at costs reasonable enough for them to continue to acquire them without financial hardship. In other cases, where past utilization patterns have shown that significant problems would be encountered if total costs were charged, only partial operating costs are collected in order that investigators will not have to unduly curtail their research activities. In either case, as a general rule, all grantees, contractors, and intramural scientists pay for the resources which they receive. Charges are not collected for resource distributions to investigators that are participating in the special bilateral agreements between the United States and certain foreign countries. These bilateral agreements usually contain specific language relating to the open exchange of resources for cancer research and as such are not appropriate for payback implementation.

Resource contracts now operating under the payback system include two for the production and distribution of anti-viral reagents; one for specialized testing services; two for animal resources and the contract for operation of the repository for maintaining and distributing frozen biological reagents. The first contract implemented under the payback system was for the production and distribution of avian myeloblastosis virus (AMV) and AMV reverse transcriptase. Charges imposed this year for AMV materials have been seven cents per unit of transcriptase and $1,000 per gram of virus. Recovered funds and the remaining inventory of frozen virus have made it unnecessary to add new funds to this fully implemented contract for the last four years. The contract for testing services is functioning well. Recipients contribute toward operational costs and the number of tests performed has remained at the projected level. Resource contracts that became part of the payback system during the past year have not operated under the system long enough for evaluation at this time; however, the monitoring and evaluation will continue for all resource contracts. (4,10).

The payback system overall seems to be performing as expected. The demand level for high quality biological reagents not readily available from commercial sources has remained fairly constant. Reimbursement for full or

partial costs of services has led to more careful use of costly resource reagents. This has resulted in a reduced level of effort in several resource contracts or termination of unnecessary activities.

RESEARCH RESOURCES

CONTRACTS ACTIVE DURING FY85

Investigator/Institution/Contract Number	Title
1. BOYSE, Edward A. Sloan-Kettering Institute for Cancer Research N01 CP 71003	Influence of Virus-Related Genes on Susceptibility to Cancer
2. BURTON, Robert Information Management Services, Inc. N01 CP 11014	Computer Support for Resources Management
3. CLAPP, Neal K. Oak Ridge Associated Universities N01 CP 21004	Operation of a Marmoset Colony for Cancer Research
4. CLAPP, Neal K. Oak Ridge Associated Universities N01 CP 51006	Marmoset Colony for Cancer Research
5. ELLIS, Carmen Life Sciences, Inc. N01 CP 11013	Production and Distribution of Avian Myeloblastosis Virus and AMV Reverse Transcriptase
6. FERRER, Jorge F. University of Pennsylvania N01 CP 51003	Bovine Leukemia/Virus Herd
7. HOUTS, G. E. Molecular Genetics Resources, Inc. N01 CP 51007	Production, Characterization and Distribution of AMV Reverse Transcriptase
8. MASSAGEE, Pamela D. Microbiological Associates N01 CP 11000	Repository for Storage and Distribution of Viruses, Sera, Reagents and Tissue Specimens
9. NIMAN, Henry L. Scripps Clinic and Research Foundation N01 CP 41009	Preparation of Monoclonal Antibodies on Oncogene Products to Retroviruses

10.	PETERSON, Ward D. Children's Hospital of Michigan N01 CP 21017	Inter- and Intraspecies Identification of Cell Cultures
11.	TENNANT, B. C. Cornell University N01 AI 02651	Breeding Facility for Woodchucks (Marmota Monax)
12.	TENNANT, B. C. Cornell University N01 AI 52585	Breeding Facility for Woodchucks (Marmota Monax)

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