Division of

CANCER ETIOLOGY

Volume I
October 1, 1983-September 30, 1984

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ANNUAL REPORT

DIVISION OF CANCER ETIOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1983 through September 30, 1984

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ANNUAL REPORT

DIVISION OF CANCER ETIOLOGY

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October 1, 1983 through September 30, 1984

OVERVIEW

This year the name of the Division was changed to the Division of Cancer Etiology (DCE), concomitant with changing the name of the Division of Resources, Centers and Community Activities to the Division of Cancer Prevention and Control. As part of a Division-wide reorganization the Biological and the Chemical Carcinogenesis Intramural and Extramural Programs have been restructured. Thus, new Biological Carcinogenesis and Chemical and Physical Carcinogenesis Programs have been created, with Associate Directors soon to be appointed for each Program. Incorporated into the new Biological Carcinogenesis Program will be 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 Carcinogenesis Extramural Program (Biological Carcinogenesis Branch). In addition, a new laboratory (Laboratory of Tumor Virus Biology) will soon be created within the Biological Carcinogenesis Program and will be located in Building 41.

Incorporated into the new Chemical and Physical Carcinogenesis Program will be 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 will be 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. An Associate Director for this Program was appointed two years ago. As part of the realignment of program activities, a large segment of the Biometry Branch was moved to the Division of Cancer Prevention and Control during the past year. The transfer involved those sections dealing with the Surveillance, Epidemiology and End Results (SEER) Program and with clinical trials. The activities and staff remaining with this Division were formed into a new Biostatistics Branch. Moving into the Epidemiology and Biometry 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 this 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 the consolidation of the Laboratory of Cellular and Molecular Biology in Building 37 and the move of the Laboratory of Chemoprevention to Building 41 were completed. Renovation of space to house the Laboratory of Molecular Virology in Building 41 will be completed this year as will renovations for the Laboratory of Experimental Carcinogenesis in Building 37. A multi-user liquid nitrogen facility in Building 37 has recently been installed. All DCE laboratories are now located in Government facilities which will reduce significantly funds needed for resource support laboratories.

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 viruses and viral reagents, three for animal resources and one for specialized testing services. 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 had 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 14 Cooperative Agreements at a level of 2.4 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. Frederick Cancer Research Facility (FCRF)

   Research conducted by the contractor began in the early 1970s as a series of unrelated tasks or projects. Today, the carcinogenesis program at the FCRF has gradually evolved into a coordinated effort on the mechanisms of action of viral and chemical carcinogens and has been transferred to the Office of the Associate Director, NCI (Dr. Peter Fischinger), which coordinates all FCRF activities. 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.

2. The DCE Board of Scientific Counselors

Chartered in 1978, the Board is an advisory body whose members are drawn from the scientific community outside NIH. Chosen for their recognized expertise in chemical carcinogenesis, molecular biology, viral oncology, 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, comprise 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 first cycle of site visits to the Division's entire intramural operation was completed three years ago with the site visits to the Laboratory of Viral Carcinogenesis and to the Experimental Oncology Section of the Laboratory of Molecular and Cellular Biology. The second cycle of site visits 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 Clinical Epidemiology Branch on April 12-13, 1984; and the Laboratory of Biology on March 19, 1984. The site visit to the Laboratory of Cellular and Molecular Biology occurred on July 17-19, 1984 and the Laboratory of Molecular Carcinogenesis will be site visited on September 13-14, 1984. Two additional site visits, to the Laboratory of Chemoprevention and to the Laboratory of Cellular Carcinogenesis and Tumor Promotion, will occur in the fall of 1984.

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 Grant Application (RFA) in the areas of bovine leukemia virus, human T-cell leukemia/lymphoma virus and the development of cancer chemopreventive agents. In addition, in response to the emerging epidemic of AIDS and because of the potential public health problem that it poses, DCE joined with the National Institute of Allergy and Infectious Diseases in issuing a second RFA for Cooperative

Agreement (U01) applications in order to stimulate research on the infectious etiology of AIDS and Kaposi's sarcoma. Another RFA, originally issued in FY83 but funded this year, sought to stimulate traditional research projects (R01s) in the area of hepatitis B virus and primary hepatocellular carcinoma. A workshop on DNA viruses and human cervical carcinoma was held during the past year, was chaired by a member of the DCE Board of Scientific Counselors and the recommendations and discussions from that workshop are being evaluated for new initiatives. The Division also co-sponsored a symposium/ workshop with the Division of Cancer Treatment entitled "Comparison of Mechanisms of Carcinogenesis by Radiation and Chemical Agents." The purpose of the symposium was to identify opportunities for new or renewed emphasis and to identify areas where technology or specialized resources are lacking.

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 advisory group.

FIGURE 1

DIVISION OF CANCER ETIOLOGY

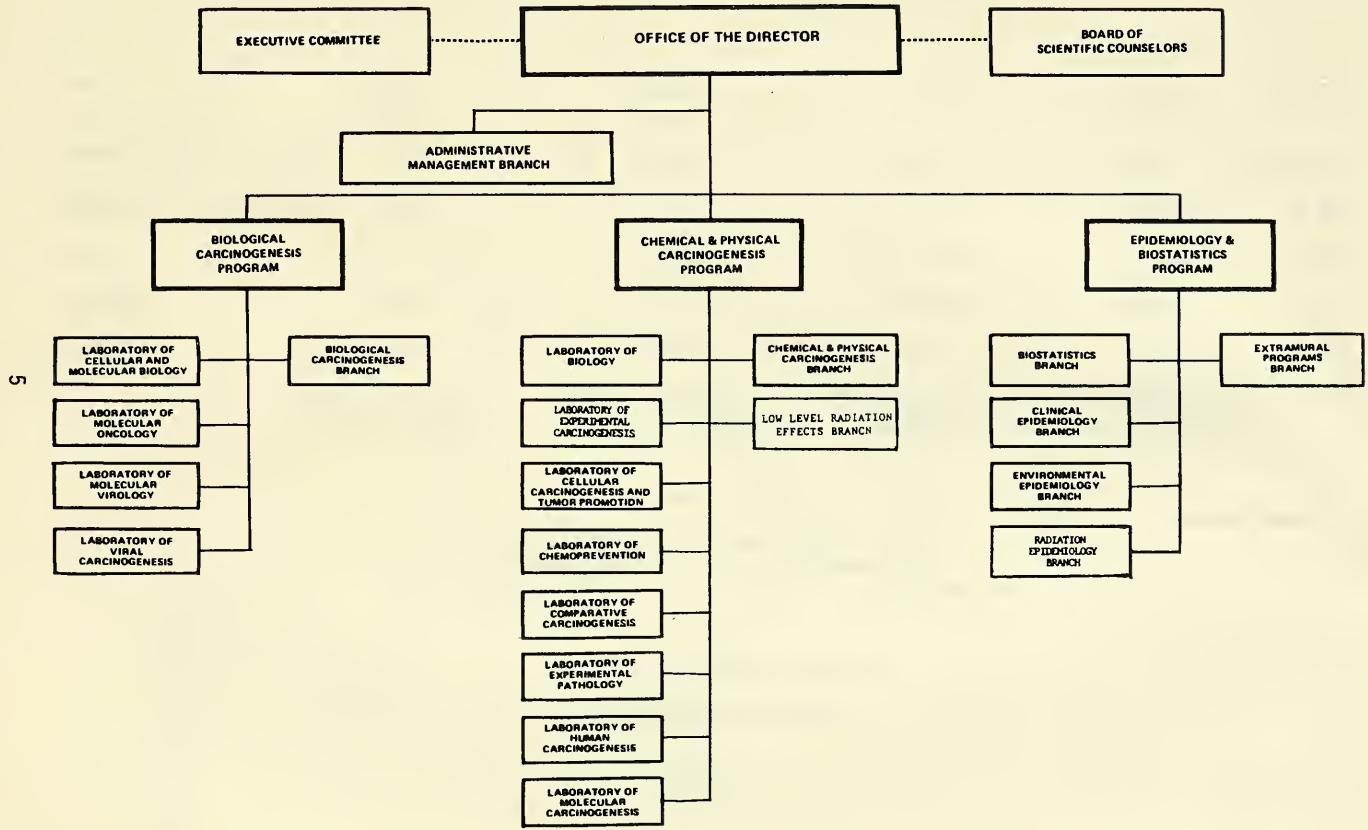


TABLE 1

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER ETIOLOGY

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

FY 1984 Estimate

	Office of the Division Director	Chemical and Physical Carcinogenesis	Biological Carcinogenesis	Epidemiology and Biostatistics	Total
Inhouse	3,304	18,207	14,492	7,622	43,625
Contracts	4,769	7,175	3,128	17,659	32,731
RFA	0	5,471	1,000	1,970	8,441
Cooperative Agreements	550	0	2,372	658	3,580
Research Project Grants	0	41,956	54,018	19,121	115,095
Total	8,623	72,809	75,010	47,030	203,472

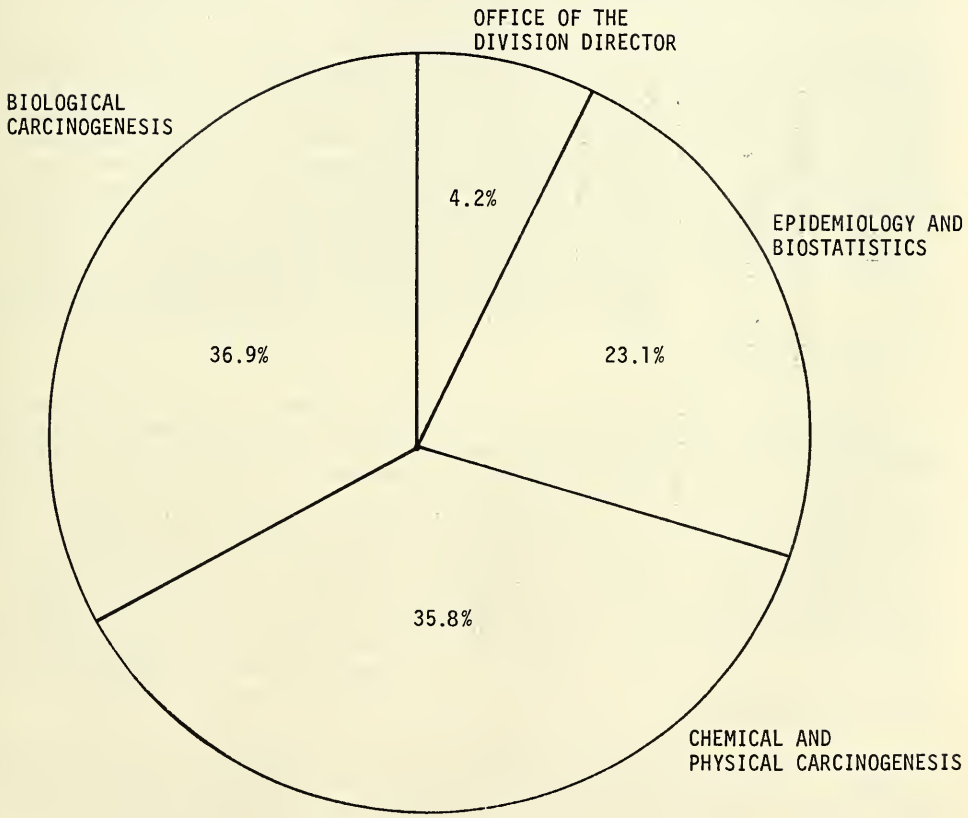
FIGURE 2

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER ETIOLOGY

Current Distribution of Funds

FY 1984 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 biometrics.

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

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, promoters, hormones and growth factors which may be required for the progression of the "initiated" cell to the malignant state.

Finally, studies on the natural history of cancer in humans and on the incidence of cancers in different geographic locations help to identify causal associations of 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, cancer incidence in the workplace, effects of low-level radiation, and environmental pollutants in air, water, and soil are under investigation.

Biological Carcinogenesis:

Oncogenes

Recent studies have shown that oncogenic RNA viruses have derived their ability to cause cancer in animals, and perhaps in humans, from genes found in normal cells. These genetic sequences are unrelated to those necessary for normal viral replication and have been found to produce proteins which are necessary for the initiation of the transformed state. Since the gene products result in transformation, the genes have been termed "oncogenes." Oncogenes have been found to possess the following characteristics: they are essential for certain types of transformation; they are derived from cellular genetic sequences; they have a limited number of specific targets; they act by means of their translational protein products; and they are probably limited in number (approximately 20 have been found). Currently, ongoing research seeks to continue the search for oncogenes in both animals and human tumor systems and to characterize these genes; to explore the emergent area of human oncogenes and their relationship(s) to viral oncogenes in terms of nucleic acid homology and translational (oncogene) products; to purify and characterize the translational gene products of these genes; to use these purified products in delineating the mechanism(s) of transformation; and to define the function(s) and mechanisms of regulation of the cellular homologues (c oncs) of viral transforming genes.

The first direct link between an onc gene and a known biological function has been demonstrated. The simian sarcoma virus (SSV) onc gene, v-sis, has been sequenced and its 28,000 molecular weight (MW) product, p28^{sis}, identified by means of antisera prepared against small peptides derived from sequence analysis of v-sis. Studies on human platelet-derived growth factor (PDGF), a potent mitogen for cells of connective tissue origin, have led to the elucidation of its amino terminal amino acid sequence. Computer comparison of this and additional protein sequence data with the predicted amino acid sequence of p28^{sis} has revealed an extraordinary degree of homology between PDGF and p28^{sis}, implying that the two proteins have arisen from the same or closely related cellular genes. It was further demonstrated that p28^{sis} rapidly undergoes a series of discrete processing steps including dimer formation and proteolytic digestion to yield molecules structurally and immunologically resembling biologically active PDGF.

The human locus related to v-sis was cloned and shown to contain at least 5 exons corresponding to the v-sis coding region. Nucleotide sequence analysis of these exons revealed that the predicted amino acid sequence of human c-sis differed by 6% from that of the woolly monkey derived v-sis. These findings imply that the sis proto-oncogene has been well conserved during primate evolution. By comparison of the known amino acid sequences of PDGF peptides with the predicted human c-sis protein, it was possible to demonstrate that this human

proto-oncogene is the structural gene encoding one of the two major polypeptides of this potent mitogen for connective tissue cells.

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). Efforts were made 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 those observed in cells transformed by SSV DNA. In spite of its transcriptional activation, c-sis clone 8 DNA did not demonstrate focusforming activity.

A putative upstream exon was identified by its ability to detect the 4.2 kilobase (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 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. These findings establish that the normal coding sequence for a human growth factor has transforming activity when provided necessary signals for transcription and initiation of translation.

A series of 22 human hematopoietic tumors and tumor cell lines have been surveyed for sequences capable of transforming NIH/3T3 cells by DNA transfection. A primary human acute myelogenous leukemia, a chronic myelogenous leukemia cell line, and cell lines derived from three independent acute lymphocytic leukemias demonstrated oncogenes capable of conferring the transformed phenotype to NIH/3T3 cells through serial cycles of transfection. One of three transforming genes associated with acute lymphocytic leukemia cells (classified as thymocyte development state II) was identified as the activated cellular homologue of the Kirsten murine sarcoma virus onc gene, kis, a member of the ras family of onc genes. A transforming gene, N-ras, was demonstrated to be common to several human myeloid and lymphoid tumor cells. Thus, the NIH/3T3 transfection assay commonly detects related ras oncogenes in human hematopoietic tumor cells. Moreover, the activation of these oncogenes appears to be independent of the specific stage of cell differentiation or tumor phenotype.

An N-ras-related transforming gene was detected in the human lung carcinoma cell line, SW-1271, and molecularly cloned in biologically active form. 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.

A large proportion of oncogenes so far detected by DNA transfection are related to the H-ras onc gene of Harvey (and BALB) murine sarcoma viruses (MSV), k-ras (the oncogene of Kirsten MSV), and a third member of the ras gene family, N-ras. Individual tumors of many different organs have been associated with the activation of members of the ras gene family. A systematic survey was performed of human urinary tract tumors, processed immediately after surgery, as well as normal

tissues from the same patients, for the presence of such genes. Activation of H-ras as an oncogene was demonstrated in around 10% of unselected urinary tract tumors as well as direct evidence that oncogene activation was the result of a somatic event selected for within the tumor cell population.

Among 21 human mammary tumors analyzed for transforming genes by NIH/3T3 transfection, only DNA of a carcinosarcoma cell line, HS578T, registered as positive. An H-ras oncogene identified in this line was cloned in biologically active form and the activating lesion identified as a single nucleotide substitution of adenine for guanine within the 12 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 alteration at position 12. These findings establish that activation of this oncogene was the result of a somatic event 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.

Two of the six possible single point mutations that can activate the human K-ras locus by altering the coding properties of its 12th codon create restriction enzyme polymorphisms. These polymorphisms have been utilized to develop biochemical assays that discriminate between normal and transforming K-ras genes. Such assays were used to determine the mechanism of activation of K-ras oncogenes in A1698 bladder carcinoma and A2182 lung carcinoma human tumor cell lines as resulting from mutations within the 12th codon. Moreover, a single point mutation was shown to be responsible for the malignant activation of a K-ras oncogene present in tumor but not in normal tissue of a patient with a squamous cell lung carcinoma. These results further demonstrate an association between activation of ras oncogenes by specific mutational mechanisms and the development of certain human cancers.

An active transforming sequence had previously been detected by transfection of DNA isolated from passage 330 of the human teratocarcinoma line PA-1, which was initially derived from a metastatic ovarian germ line tumor. These late passage cells show significant alterations in various malignancy-related phenotypic characteristics, including tumorigenicity in nude mice and the ability to form colonies in agar suspension, in comparison to early passage (<100) PA-1 cells. The transforming activity detected at passage 330 was identified as an N-ras oncogene which had undergone a glycine-aspartic acid transition at position 12. Early passage (36) PA-1 cells are poorly tumorigenic in nude mice and no focus-inducing activity can be detected in DNA transfection assays. The presence of an activated N-ras can thus be correlated with increasing tumorigenicity in PA-1 cells. This system represents an oportunity to study the role of this specific oncogene, as well as other specific genetic changes, in the carcinogenic progression process in PA-1 cells.

The Harvey murine sarcoma virus (Ha-MuSV)p21^{ras} gene was fused to the amino terminal portion of the lambda phage cII gene of pJL6. This ras gene fusion product effected the synthesis of a hybrid protein of 23,000 daltons representing more than 10% of the total cellular protein containing all but four residues

at the amino termini of the $p21^{ras}$ protein. This bacterially produced protein appears to be similar to the Ha-MuSV $p21^{ras}$ protein in that it underwent immunoprecipitation by monoclonal antibodies specifically directed towards that protein, was able to bind guanosine diphosphate and was capable of undergoing autophosphorylation like the authentic viral oncogene protein. It has been shown that avian carcinoma virus MH2 shares a transformationspecific sequence, myc, in common with other acute transforming retroviruses such as MC29, CMII and CKIO. A 5.2 kb MH2 provirus DNA has been 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 has been discovered which appears to be an MH2 transformation-specific onc gene. Hybridizations with normal chicken cellular DNA, as well as 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. 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, CK10 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 with the chicken proto-myc oncogene. The following information was obtained: 1) Δgag-mht forms a hybrid gene with a contiguous 2682 nucleotide reading frame, terminating with a stop codon near the 3' end of mht. 2) The 3' end of 969 nucleotides of the mht region up to the stop codon is 80% homologous to the unique onc-specific raf sequence of the murine sarcoma virus 3611. 3) More 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. 4) The myc sequence is preceded by an RNA splice acceptor site shared in common with the cellular proto-myc gene, beyond which it is colinear up to a 3' termination codon and 40 noncoding nucleotides with the myc sequences of avian MC29 retroviruses and the chicken proto-myc. Thus, myc forms together with a 5' retroviral exon, a second MH2 specific gene. 5) The oncogene myc is followed by a 400 nucleotide 3' terminal c region which is related to the Rous sarcoma virus. The significance of these data, relating to MH2, is that out of 19 known different viral oncogenes, five of them 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 its oncogenic potential and transformation capabilities.

A prokaryotic vector has also been developed which allows the expression of high levels on onc genes in E. coli. This vector (pJL6) has provided a means for studying both the chemistry of the protein and preparing appropriate immunological reagents for studying the expression of the oncogene product in transformed cells and tumor cell lines. This efficient expression vector contains a unique restriction enzyme site 12 codons beyond the lambda phage cII gene initiation codon and was used to fuse the carboxy terminal sequences of the MC29 v-myc gene to the amino terminal sequences of the λcII gene. Transcription of this chimeric plasmid is under the control of the λp_L promoter which, when derepressed,

produced a high level of CII-myc fusion protein amounting to greater than 5% of the total cellular protein. This high level of expression has enabled antibodies to be raised against this protein which is capable of immunoprecipitating the MC29 gene product P110$^{gag\text{-}myc}$. Gardner-Rasheed feline sarcoma virus (GR-FeSV) is an acute transforming retrovirus which encodes a gag-onc polyprotein possessing an associated tyrosine kinase activity. The integrated form of this virus, isolated in the Charon 21A strain of bacteriophage λ, demonstrated an ability to transform NIH/3T3 cells at high efficiency upon transfection. Foci induced by GR-FeSV DNA contained rescuable sarcoma virus and expressed GR-P70, the major GR-FeSV translational product. The localization of long terminal repeats within the DNA clone made it possible to establish the length of the GR-FeSV provirus as 4.6 kb pairs. The analysis of heteroduplexes formed between λ feline leukemia virus (FeLV) and λ GR-FeSV DNAs revealed the presence of a 1,700 base-pair FeLV unrelated segment, designated v-fgr, within the GR-FeSV genome. The size of this region was sufficient to encode a protein of approximately 68,000 daltons and was localized immediately downstream of the FeLV gag gene coding sequences present in GR-FeSV. Thus, it is likely that this 1.7 kb pair stretch encodes the onc moiety of GR-P70. Utilizing probes representing v-fgr, homologous sequences were detected in the DNAs of diverse vertebrate species, implying that v-fgr originated from a wellconserved cellular gene. The number of cellular DNA fragments hybridized by v-fgr-derived probes indicated either that proto-fgr is distributed over a very large region of cellular DNA or represents a family of related genes. By molecular hybridization, v-fgr was not directly related to the onc genes of other known retroviruses having associated tyrosine kinase activity.

The nucleotide sequence of the region of v-fgr encoding its primary translation product, P70$^{gag\text{-}fgr}$, was determined. From the nucleotide sequence, the amino acid sequence of this transforming protein was deduced. Computer analysis indicates that a portion of P70$^{gag\text{-}fgr}$ has extensive amino acid sequence homology with actin, a eukaryotic cytoskeletal protein. A second region of P70$^{gag\text{-}fgr}$ is closely related to the tyrosine-specific kinase gene family. Thus, the v-fgr oncogene appears to have arisen as a result of recombinational events involving two distinct cellular genes, one coding for a structural protein and the other for a protein kinase.

Three types of tumors termed plasmacytomas (ABPCs), lymphosarcomas (ABLSs), and plasmacytoid lymphosarcomas (ABPLs), arise in BALB/c mice treated with pristane and Abelson murine leukemia virus (A-MuLV). While most ABPCs and ABLSs contain integrated A-MuLV proviral genome and synthesize the v-abl RNA, most ABPLs do not. The ABPL tumors were examined for the expression of other oncogenes that may be associated with their transformed state in the absence of transforming virus. These tumors expressed abundant c-myb RNA of unusually large size and showed DNA rearrangements of the c-myb locus. Molecular cloning of the mouse c-myb locus and its rearranged counterpart in the ABPL-2 tumor revealed that the alteration in this locus is due to insertion of a defective MuLV proviral genome containing both LTRs upstream to the v-myb-related sequences. This insertion interrupts the c-myb coding region in a manner similar to that observed in the generation of avian myeloblastosis virus.

Localization of Onc Genes to Chromosomes

Molecular cloning, high resolution cytogenetic procedures and gene mapping panels of somatic cell hybrids between rodent and human cells have been employed in an

extensive series of mapping experiments to genetically locate a variety of human cellular genes which participate in neoplastic transformation. Within the last two years, the human gene map has experienced a large increase in the number of neoplasia loci which have been mapped to specific chromosomal positions. A total of 27 specific human loci have been chromosomally mapped to date. The construction of this extensive human map has played an important role in the resolution of early genetic events in neoplastic transformation in man.

The association between certain human malignancies and unique chromosomal abnormalities has led to the hypothesis that specific cellular oncogenes are involved and consequently activatable by the genetic abnormalities. In particular, it had been observed, using the cloned constructs of the viral oncogene v-myc, that chromosomal translocation involving the human proto-myc gene on chromosome 8 is transferred to the immunoglobulin heavy chain locus on chromosome 14. Although chromosomal breakpoints can be variably located, the recombination sites have not been precisely identified in relation to the functional domains of these loci. Two reciprocal recombination sites between c-myc and the immunoglobulin heavy chain μ in a Burkitt lymphoma have been identified and characterized. Sequencing of the crossover point joining chromosomes 8 and 14 shows that the onc gene is interrupted within its first intron and thereupon joined to the heavy chain μ switch region. This recombination predicts that the translocated onc gene would code for a rearranged mRNA but would express a normal c-myc polypeptide.

The v-raf transducing retrovirus was originally isolated from the murine sarcoma virus 3611-MSV. The raf oncogene is a murine homolog which was independently captured in the avian MH2 transforming virus. Humans have two copies of this proto-oncogene, c-raf-1, a functional locus containing at least eight exons and seven introns, and an intronless pseudogene with chain termination signals in each reading frame. The two raf loci mapped to human chromosomes 3 and 4, respectively, using a human hybrid panel. The 5' and 3' ends of the coding portion of this gene have been identified. Conditions have been determined for its expression as a transforming gene in vitro. The intracellular location of its normal gene products has been analyzed. The c-raf gene products have been purified to homogeneity from mouse cells. Mouse and human raf-specific antisera were obtained by use of raf-sequence-derived synthetic peptides, as well as genetically engineered raf protein. Transformation of cells by the raf oncogene is enhanced in vitro and in vivo in the presence of a second oncogene, v-myc. A combination of both oncogenes was discovered in the genome of the avian carcinoma virus MH2, suggesting a role for this specific pair of genes in the development of natural carcinomas.

A transforming activity associated with a line of human cells, MNNG-HOS, a human osteosarcoma derived cell line which was subsequently transformed to anchorage-independent growth and tumorigenicity, had previously been reported. Analysis of this oncogenic sequence has continued. The transforming sequence, designated met, has been cloned in several overlapping lambda clones totaling 40 kb of human sequence. Met appears to represent a previously unidentified transforming gene, since it shows no sequence homology with known members of the ras oncogene family nor with the oncogenes mos, myc, myb, src, erb, sis, rel or B-lym. Restriction analysis further indicates that it does not represent the human homologues of abl or fes. The activated met sequence shows no detectable gross rearrangement when compared to human placental DNA, several other human tumors, or the parental HOS cell line. Met has been localized to human chromsome 7

using somatic cell hybrids. It appears to represent a new and interesting human sequence with oncogenic potential.

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 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 demonstrate 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 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. Experiments have been designed in an attempt to elucidate the mechanism by which the activator/enhancer sequences function. A principal emphasis for the future will be the definition of biological macromolecules which interact with these regulatory elements.

The retroviral LTR contains transcriptional control elements that affect viral gene expression. By deletion mutagenesis of the genome of the cloned Abelson murine leukemia virus, regulatory signals could be mapped to at least three domains within the LTR. A defective 5' LTR that did not sustain transforming gene function was complemented by an intact LTR positioned at the 3' end of the genome. This versatility of the retroviral genome with respect to its transcriptional control elements appears to provide a strong selective advantage for viral gene expression.

The regulation of the λ int gene has been determined. It can be transcribed from either of two promoters p_I and p_L. P_I requires λ cII protein in addition to RNA polymerase to initiate. It transcribes int and terminates at a site, tI, 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 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.

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. 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 exists 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 class I antigen presentation and surveillance for "aberrant" cells by the immune system.

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, may be a tolerogenic form of the class I antigens, acting as a blocking factor to regulate the function of cytotoxic T-cells in the process of immune surveillance. Studies are in progress to confirm this hypothesis by using this secreted class I antigen to modulate T-lymphocyte recognition of tumor cells.

Studies on DNA Viruses

The oncogenes of small DNA tumor viruses, such as SV40 and 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 mapped. Specific biochemical activities and transformation functions have been assigned to discrete sections of the gene. The immortalization of cells in culture is a function of the amino terminal half of the tumor antigen protein. Induction and maintenance of the transformed phenotype in immortalized cells is a property of the carboxy-terminal half of the protein. It is now clear that these two functions are separable. Although the polyoma virus T antigens are not yet as well characterized, a recent observation involving the middle T antigen of 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 homologue of the viral oncogene from the Rous sarcoma virus (RSV), pp60v-src. Thus, the transformation of cells by polyoma may be mediated by a change in the activity of the pp60c-src phosphokinase in vivo in a way similar to the transformation process of RSV.

In studies of the relationship of Hepatitis B virus (HBV) to primary hepatocellular carcinoma (PHC), a rapid multisite monoclonal radioimmunoassay for measurement of human alpha-fetoprotein (AFP) using two high affinity monoclonal antibodies directed against distinct determinants of the protein has been developed. The test may be useful in the early identification, diagnosis, and monitoring of patients with primary hepatocellular carcinoma and other AFP-producing tumors in high risk populations. Additionally, it had been thought that viral replication is suppressed or inactive in many PHC patients and that HBV potential infectivity is presumably very low or absent in these individuals. However, recent studies indicate that when viral replication is present in hepatitis B surface antigen

carriers ($HBsAg)^+$/antihepatitis B e antigen $(HBe)^+$, active liver disease is often found. In these individuals, active chronic liver disease appears to be related to continued replication and secretion of HBV, and may occur in a much higher proportion of $HBsAg^+$/anti-HBe^+ carriers than was previously suspected.

The ability to detect and diagnose cancer at an early stage of disease increases the probability of successful treatment. Results from a current multi-institution study indicate that certain anti-Epstein-Barr virus (EBV) antibodies are valuable markers for clinicians for the diagnosis of undifferentiated types of human North American nasopharyngeal carcinoma, including occult primary tumors. The IgA anti-virus capsid antigen antibody response is the most specific for this disease and of the greatest diagnostic value when used alone or in combination with an anti-early antigen test. In regard to prognosis, antibody titers to EBV immunofluorescence antigens do not vary greatly from those determined at diagnosis in patients that remain in clinical remission following initial therapy. In contrast, increases in antibody titers measured by immunofluorescence to the D component of the early antigen (EA) complex signal the presence of recurrent or active disease. However, antibodies measured by the antibody-dependent cellular cytotoxicity (ADCC) assay continue to be the most predictive of disease course following therapy. Of patients on study for at least two years, 75% of those with high ADCC titers at diagnosis have remained disease-free as opposed to approximately 35% in the low-titered group. In regard to survival, approximately 80% of the patients in the high-titered group have survived for longer than 3 years as opposed to approximately 55% in the low-titered group. These results strongly indicate that ADCC titers determined at diagnosis are predictive of disease course and can be employed to identify those patients who are candidates for more aggressive therapy.

Studies Related to HTLV and AIDS

Human T-cell leukemia/lymphoma virus (HTLV) is a novel exogenous retrovirus associated with mature T-cell malignancy in man. Extensive seroepidemiological studies indicate that HTLV is present worldwide but is more prevalent in certain areas, including southwest Japan, the Caribbean, parts of South America, and the southeastern United States. There are now about a dozen isolates obtained from patients from different parts of the world. HTLV is distinguishable from all the animal retroviruses by immunological analysis, by analysis of nucleic acid homology, and by amino acid sequencing of structural proteins. The viral structural proteins that have been best characterized are the 19,000 dalton protein, for which a monoclonal antibody is available, and the major core protein, designated p24. Based upon immunological tests of their p24s, there are several subtypes of HTLV. Additionally, hybridization studies show that the DNA sequences of HTLV-II are distinct from those of HTLV-I. In contrast to HTLV-I, HTLV-II is associated only with a relatively benign disease (hairy-cell leukemia). Although HTLV is not closely related to any known animal retrovirus, it appears to be distantly related to bovine leukemia virus (BLV). This has been demonstrated by a weak serologic cross reaction between one of the minor viral proteins of HTLV and BLV. Recent studies have also shown some similarities in amino acid sequences of the HTLV major core protein, p24, with the p24 of BLV, reflecting a common evolutionary background. Evidence suggests that HTLV is not endogenous in humans but rather is acquired by infection. However, HTLV is not readily infectious and may require prolonged intimate contact for transmission. There are as yet no data regarding the presence or absence of a transforming component of HTLV akin to the acute transforming animal leukemia viruses. The HTLV-positive neoplastic

T-cells do not carry an oncogene and it has been suggested that the virus may induce disease by activating cellular genes that are normally repressed.

It has been demonstrated that virtually all HTLV-I-infected T-cell lines express novel, human HLA antigenic determinants which are in addition to and distinct from the HLA phenotype of autologous HTLV-negative B-cell or T-cell lines. A genetic analysis of the novel HLA expression indicated that the antigen was not encoded by the host cell HLA locus, but rather was encoded by the HTLV genome. When the participation of the HLA locus in T-cell function is considered, the homology between a viral protein and an HLA class I protein may be of great biologic significance in the pathogenesis of HTLV-induced neoplasias.

Recently, a newly discovered subgroup or strain of the HTLV family of viruses, designated as HTLV-III, has been suggested as the primary causative agent of the AIDS immunodeficiency disease. The HTLV-III virus has been isolated from a total of 48 subjects including 18 of 21 patients with early AIDS symptoms, 26 of 72 adult and juvenile patients with overt disease, 3 of 4 clinically normal mothers of juveniles with AIDS, and from only 1 of 22 apparently normal homosexuals. The one individual found virus-positive subsequently developed AIDS. No HTLV-III was detected or isolated from 115 normal heterosexual volunteer subjects. The presence of serum antibodies directed against the p41 envelope antigen of the HTLV-III virus in a very high percentage of the AIDS patients (88%) and pre-AIDS subjects (79%) suggest that a reliable serological test for the detection and/or diagnosis of AIDS may soon be available.

Macaque monkeys at four Regional Primate Research Centers have an immune deficiency disease called simian AIDS (SAIDS) characterized by lymphocytopenia, opportunistic infections, and an unusual tumor termed retroperitoneal fibromatosis (RF). A novel, type D retrovirus has been isolated after cocultivation of RF tissues obtained from several species of macaques housed at the University of Washington. This isolate, designated SAIDS-D/Washington, morphologically transforms various rodent cell lines, and can be distinguished antigenically and by hybridization from all known primate retroviruses. There are multiple copies of partially related nucleic acid sequences in Old World monkey cellular DNA; these studies suggest an origin for SAIDS in langur monkey cell DNA. SAIDS-D virus is being biologically and molecularly cloned and the amino acid sequences of the viral polypeptides and the restriction maps of the viral genomes are being compared to those of other type D retroviruses. The finding that SAIDS-D virus seems to be present almost exclusively in animals with RF and SAIDS constitutes preliminary evidence for attributing the etiology of SAIDS to this isolate. In preliminary experiments, the disease was transmitted by inoculation of macaques with cell-free viral filtrates. Vaccination experiments will attempt to elicit a protective antibody response to subsequent challenge with cloned live virus. The viral etiology of simian AIDS has important implications as a model for the etiology, prevention, treatment, and molecular biology of immunosuppressive diseases, and as a marker for the identification, quarantine, and eventual control of AIDS in man.

Chemical and Physical Carcinogenesis:

In Vitro Studies on Human Tissue and Cells

The further refinement of techniques and media for long-term culture of human cells and tissue explants under controlled experimental conditions offers an unparalleled opportunity for the study of many important problems in carcinogenesis. For example, the response of human bronchial epithelial cells (enhanced growth or differentiation) to carcinogens and/or tumor promoters or DNA transfection by oncogenes is being actively investigated. Parallel investigations using epithelial cells and tissues from experimental animals allow investigators to study interspecies differences in response to carcinogens, cocarcinogens and anticarcinogens. A considerable effort has been directed at two important sites of human cancer, bronchus and esophagus. Studies of the factors controlling growth and differentiation of these normal cells and their malignant counterparts are underway. Based on the observation that human bronchial carcinomas and fetal bronchial epithelial (NHBE) cells frequently produce polypeptide hormones such as alpha and beta human chorionic gonadotropin (HCG) and gastrin-releasing peptide (GRP), it has been proposed that such peptides may have normal growth promoting functions during fetal development and that the regulation of these autocrine growth factors is aberrant in carcinoma cells. GRP and its amphibian equivalent, bombesin, has been shown to stimulate clonal growth of NHBE cells. Although neither alpha nor beta HCG alone is a growth factor, the combination is growth stimulatory, which is consistent with the hypothesis that beta HCG binding to its membrane receptor allows access of alpha HCG to its receptor to trigger the subsequent mitogenic stimulus. These observations suggest that studying the regulation of the genes of these polypeptide hormones in fetal versus adult NHBE cells and in bronchial carcinomas may reveal the molecular mechanism for their control and their possible role in carcinogenesis.

NHBE cells have also been used to study the effect of formaldehyde on repair of x-ray-induced single strand breaks in DNA. During metabolic activation, the carcinogenic nitrosamine N-nitrosodimethylamine yields equal molar quantities of methyl carbonium ions and formaldehyde. Both of these metabolites can react with nucleophilic sites in cellular macromolecules, carbonium ions by alkylation, and aldehydes via formation of unstable alkyl-Oℓ derivatives. The monomethylol derivatives of formaldehyde can form intermediary labile products that by secondary reactions can yield stable methylene bridges between macromolecules. Although the alkylating metabolites of N-nitrosamines and their cytotoxic, mutagenic, and carcinogenic effects have been extensively studied, the possible contribution of other metabolites, especially aldehydes, has not received much attention. Human bronchial cells were exposed to x-rays and then incubated with or without formaldehyde, and the repair of DNA single strand breaks was measured. The presence of formaldehyde significantly inhibits the repair of the x-ray-induced single strand breaks correlating with the potentiation of cytotoxicity in human cells and mutation frequency in Chinese hamster V79 cells by combinations of the agents. Formaldehyde also inhibits repair of O^6-methylguanine, decreases O^6-alkylguanine alkyltransferase activity, is mutagenic at high concentrations (> 100 μM), and potentiates the cytotoxicity and mutagenicity of the methylating agent, N-methyl-N-nitrosourea, in normal human cells. Exposure to formaldehyde may lead to the dual genotoxic mechanism by both directly damaging DNA, i.e., formation of DNA-protein crosslinks and single strand DNA breaks, and inhibiting repair of mutagenic and carcinogenic DNA lesions caused by alkylating agents and physical carcinogens.

Epidemiological studies have established that exposure to asbestos fibers is the primary cause of mesothelioma in the industrialized world. Because the latency period for this disease averages 40 years and because there has been a marked increase in the use of asbestos during and since World War II, an epidemic of mesothelioma has been predicted for the latter part of this century. Carcinogenesis studies with animals have shown that mesothelioma can be caused by intrapleural or intraperitoneal injection of asbestos. However, the long-term effects of asbestos fibers on human mesothelial cells in culture have not been reported previously. To study this problem, methods to culture replicative normal mesothelial cells from adult human donors have been developed. The cells contain keratin and hyaluronic acid-mucin, exhibited 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. Mesothelial cells have distinct keratin proteins and have a remarkable ability to regulate their cytoskeletal composition; the content of keratin or vimentin in the cytoskeleton reflects the growth conditions. This uniquely fluid cytoskeleton may be more easily perturbed by penetrating asbestos fibers than are the cytoskeletons of other cell types, possibly leading to an increased risk for chromosomal instability and transformation.

The process of transformation leading to neoplasia can be readily demonstrated in a number of experimental systems, but human cells obtained from normal individuals or those with genetic disorders appear refractory to transformation by known carcinogens. Furthermore, cocarcinogenic experiments utilizing combinations of carcinogens from different categories such as viruses, chemicals, or radiation have also failed to produce transformation in human cells. Because carcinogen treatment of human cells has not resulted in either permanent or tumorigenic lines, the term "transitory" transformation has been suggested to describe the changes produced in human cells. This term is used even though cells subjected to chemicals such as alkylating agents, polycyclic hydrocarbons, lactones, or ultraviolet irradiation will produce progeny that grow in suspension and form small nodules when injected into nude mice. It is apparent, however, that control mechanisms differ between hamster and human cells. Although hamster and human cells have a similar survival with similar doses of the chemical or physical agent, they rely on different repair mechanisms; human cells have been shown to excise DNA damage rapidly relative to their replication rate, while hamster cells continue to divide with unrepaired DNA lesions in the template strand. To demonstrate neoplastic transformation of human cells in vitro, it will be necessary to alter cells to have at least two properties in common with tumor cells: cell immortality and loss of growth control. A cell that has become immortal but exhibits growth control will cease dividing when a signal or contact inhibition is activated. On the other hand, a cell that has lost its growth control will only divide for a finite number of generations if it is not immortal.

A number of "transitory" transformations of human cells have been obtained by first blocking cell multiplication using amino acid deficient media. As a result of treating cells during the S phase of the cycle with aflatoxin B1 or UV light and then immediately culturing in agarose, cell lines were obtained from single colonies, propagated in culture for several passages, and analyzed

for chromosomal alterations. With both carcinogens, cells exhibiting an abnormal chromosome constitution were obtained. Subsequent high resolution banding analysis showed that in the UV light line there was an abnormal chromosome originating from chromosome No. 22 with an extra band present in the long arm; the latter was a tandem duplication at the locus of the c-sis oncogene. The aflatoxin line which also produced nodules in nude mice had chromosomal abnormalities involving the short arms of chromosomes 1, 11, and X. The c-Ha-ras 1 oncogene resides on the short arm of chromosome 11. These observations are important for two reasons: (1) they reflect an attribute of neoplasia, namely aneuploidy; and (2) the rearrangements observed are often associated with oncogenes that become active as a result of new chromosomal configurations.

Other In Vitro Studies

Syrian hamster cells have proved to be useful for studying transformation in vitro and its relevance to cancer. Transformed cells developed in vitro mimic tumor-derived cells resulting from in vivo carcinogen treatment. With hamster cells it has been possible to prove two important primary factors relevant to carcinogenesis. First, the dose response relationships coupled with a Poisson distribution of transformation indicate that an inductive rather than a selective mechanism is responsible for transformation. Secondly, the inability to demonstrate viral activation in this system provides the best evidence to date that the effects of chemical and physical agents that induce transformation must be considered directly responsible for transformation. Thus this system is a valid in vitro approach for investigating carcinogenesis and can now be used to study the steps and/or stages involved in the transition of normal cells to malignant ones in the absence of host modification. Because the model lends itself to quantitation, the modulation of transformation by a variety of carcinogens and noncarcinogens can be investigated. Furthermore, the expression of the initiated and promoted stages of transformation can be separated for study.

Bisulfite, a non-mutagen at neutral pH is being studied in the Syrian hamster system because this compound was reported to enhance UV mutations. Bisulfite alone was able to induce a dose-dependent increase in transformation, and a combination of bisulfite and UV resulted in an additive effect. Because it is considered a ubiquitous pollutant in the form of SO_2 and it induces a dose-dependent transformation frequency up to 3% without cytotoxicity, the biochemical mechanism responsible for transformation is important. The compound does not cause chromosome aberrations but it does minimally increase sister chromatid exchanges at concentrations higher than are necessary for transformation. There is also no indication of induced excision or post-replication repair. However, bisulfite slowed down the rate of DNA synthesis as a result of a reduction in the number of replicons contributing to DNA synthesis. An examination of the neoplastic lines capable of producing progressively growing tumors show that all lines have both structural and numerical chromosome abnormalities. An unusual characteristic of the chromosome picture produced by transformation by bisulfite was an increase polyploidization. Because bisulfite is a non-mutagen having a minimal effect on DNA metabolism, an analysis of protein alterations using 2D gel electrophoresis has begun.

A new serum-free medium has been developed for mouse keratinocytes. It consists of Ca^{2+}-free Eagle's MEM with nonessential amino acids and seven added factors. It supported at least 25 population doublings (PD) with an exponential growth rate of 0.8 PD/day. A clonal growth assay was established and used to define

optimal levels of each growth factor and to investigate the effects of whole serum and serum components on growth and differentiation. Culture methods for enzymatically dissociated epithelial cells from the hamster tracheal epithelium were also developed in analogy with the mouse keratinocyte system. Good growth and subculture were obtained using serum-free F-12 medium with seven factors.

Several epithelial systems are currently in use (i.e., respiratory epithelia, epidermal keratinocytes, urothelia and prostate epithelia) for comparative studies on mechanisms of carcinogenesis. Studies on these systems have indicated that they have comparable patterns of response to specific treatments and culture conditions that can lead, on the one hand, to terminal differentiation, senescence and cell death and, on the other hand, to progressive cell growth, anchorage independence and neoplastic transformation. An important condition for studies on these mechanisms is the ability to grow the target epithelia in serum-free, possibly chemically defined culture media, replacing serum with selected additions of hormones and growth factors at optimal concentrations.

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. Nonhuman primates have been used as an in vivo system to study transplacental carcinogenesis and tumor promotion. In two independent series of experiments, it has been shown that phenobarbital, given in the drinking water after direct or transplacental exposure to N-nitrosodiethylamine (DEN) and after an interval of as long as 4 years, can selectively promote hepatocellular carcinogenesis in the patas monkey. These findings demonstrate that DEN, like the direct-acting alkylating agent nitrosoethylurea (ENU), is a transplacental carcinogen in the nonhuman primate and that the transformed hepatocytes which result from prenatal exposure to DEN may persist, latent, for years after exposure to the carcinogen has ceased. The findings with phenobarbital also provide a positive control compound for further studies of hepatocellular tumor promotion in the patas monkey or other nonhuman primate species and strongly suggest the potential importance of tumor promotion during carcinogenesis in epithelial cells of primates in general, including man.

New primary neoplasms continue to appear in patas and in rhesus monkeys exposed transplacentally to ENU, as long as 10 years postnatally (middle aged adulthood) in patas monkeys and more than 5 years postnatally in rhesus monkeys (young adulthood). It is increasingly evident that in primates as in rodents, neoplastic development can occur late in life as a consequence of transplacental exposure to a chemical carcinogen. Recent identification of primary epithelial tumors of the peripheral lung in patas monkeys prenatally exposed to ENU and surviving to adulthood without further treatment is further evidence that neoplasms of lining epithelia-characteristically the predominant forms of human cancer--can result from transient prenatal initiation by chemical carcinogens.

The pathogenesis and promotion of tumors was also studied using liver initiation-promotion systems in mice and rats; a nitrosomethylurea (NMU) induced thyroid tumor system in rats; skin painting studies in mice; and an aged F344 rat liver model system. These studies indicate 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, effective skin tumor promotion was seen after only two exposures to 12-O-tetradecanoyl-phorbol-13 acetate (TPA). 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 the thyroid gland, removal of the goitrogenic iodine-deficient diet at various time periods after exposure to NMU allowed some of the promoted proliferative lesions to progress to large tumors. 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 effective only after continuous exposure.

In vitro studies using normal human bronchial epithelial (NHBE) cells have demonstrated that tumor promoters can induce terminal differentiation in one cell type and stimulate another subpopulation to proliferate. The effects of a variety 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, 10 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.

Biochemical and molecular mechanisms related to tumor promotion are being investigated in the mouse epidermal JB-6 cell line with clonal variants which, when exposed to tumor promoting agents such as TPA, show a positive or negative transformation (P^+ or P^-) or mitogenic (M^+ or M^-) response. Studies on the 2-deoxyglucose uptake response in these cell lines showed that uptake is required for the mitogenic response to TPA, but not for promotion of transformation. Studies on the phospholipid- and calcium-dependent protein kinase activity (PK-C) associated with the TPA receptor showed that it is present in equal amounts in derivatives of JB-6 of all phenotypes as well as in related mouse epidermal cell lines. By a sensitive technique over 16 substrates were found for PK-C in JB-6 cell lines, i.e., more than all previously described substrates for PK-C in whole cell lysates of several different cell types regardless of phenotype. The preneoplastic phenotype appears not to be determined at the level of substrates for PK-C.

A phosphoprotein species (pp80), which changes in response to TPA, was identified in JB-6 clonal sublines. pp80 is a heat shock protein similar or identical to hsp80. This phosphoprotein was characterized and found to represent approximately 2% of total phosphoprotein in whole JB-6 cell lysates. Transformed JB-6 cell lines were found to lack pp80 and they no longer produce it in response to TPA. The role of this phosphoprotein and the heat shock response mechanism in maintenance of the non-transformed state is being investigated.

Distinct roles for different species of reactive oxygen-free radicals were found in the induction phase of promotion by TPA and expression of the transformed phenotype. Superoxide dismutase effectively blocked induction of transformation if added to cells within 4 hours of exposure to TPA. Of seven different types of eliminators of free radicals tested, those which enzymatically catabolized superoxide anion inhibited TPA promotion most effectively. Inhibitors of the lipoxygenase pathway of the arachidonic acid cascade also inhibited TPA promotion, while an inhibitor of the cyclooxygenase pathway did not. TPA treatment was

found to reduce levels of endogenous SOD activity by 50% in promotion competent subclones of JB-6 but only by 17% in promotion-incompetent subclones. This difference in suppression of SOD with TPA treatment appears to mark the promotion competent phenotype among JB-6 cell lines. Treatment with benzoyl peroxide effectively promoted transformation of JB-6 cell lines. Concentrations of retinoic acid which inhibited promotion by TPA failed to inhibit promotion by benzoyl peroxide.

Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. As with NHBE cells, studies indicate 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 induction of terminal differentiation by phorbol esters appears to be mediated by the phorbol ester receptor, and this action of phorbol esters is enhanced by Ca^{++}. 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. PK-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. Studies on the progression of benign to malignant tumors in vivo indicate that promoters are incapable of accelerating the conversion process while genotoxic carcinogens have a marked enhancing and accelerating effect on malignant conversion. These results suggest a mechanism of multistage carcinogenesis involving three steps. A genetic change in the program of terminal differentiation characterizes the initiation step; this is a preneoplastic change. Tumor promotion involves cell selection and clonal expansion of initiated cells but does not alter their preneoplastic character. A second genetic change is required in the third step to convert benign to malignant lesions.

There is now considerable evidence that PK-C is the major phorbol ester receptor. An impediment to biochemical and immunological analysis of the receptor is that the published purification protocols are time consuming, afford low yields (0.5-5%) and are difficult to scale up. Taking advantage of advances in column chromatography, purification protocols have now been developed that permit the rapid and efficient isolation of the receptor. Stabilization procedures to preserve receptor activity which is otherwise quite labile once the receptor is in the purified state have also been identified. The purified receptor is currently being used for preparation of monoclonal and polyclonal antibodies as well as for biochemical studies. Previous work demonstrated that the phorbol ester binding, like the kinase, showed an absolute requirement for phospholipids, i.e., the binding protein is actually an apo-receptor and phospholipids are an essential cofactor. The phospholipid requirements for binding, with particular emphasis on the contribution which the phospholipid makes to the properties of the complex as a whole, has now been characterized in detail. Different phospholipids vary markedly in their ability to form an active complex. Among active phospholipids, the dose-response curves depend dramatically on the presence or absence of Ca^{++}. In addition to determining whether or not binding can be reconstituted, the nature of the phospholipids also plays a major role in determining the binding affinity of the complex. As would be predicted from this result, reconstitution into mixtures of liposomes of different compositions yields curved Scatchard plots, indicative of heterogeneity in binding affinity.

The current model is that localization of PK-C into different lipid environments may help account for the evidence from both biological and binding experiments that suggests receptor heterogeneity.

The enzyme phospholipase-C, which is unable to cross the membrane bilayer, can fully inhibit the mannosyltransferase activity responsible for the synthesis of retinyl phosphate mannose, without considerable effect on the biosynthesis of the lipid intermediate dolichyl phosphate mannose. These findings suggested a location for retinyl phosphate mannose at the cytosolic side of the endoplasmic reticulum and a function for this molecule as a carrier of mannose across the membrane, possibly to generate guanosine diphosphate mannose. Consistent with this hypothesis is the recent finding that retinyl phosphate mannose synthesis is a reversible process which regenerates guanosine diphosphate mannose in the presence of excess guanosine diphosphate in microsomal membranes.

Studies on Carcinogen Metabolism and Interaction with DNA

The primary enzyme interface between environmental chemicals and higher organisms is the mixed function oxidase system. The various forms of cytochrome P-450 constitute a major part of this system and are the major receptors for a wide variety of drugs, carcinogens, and other environmental chemicals. Efforts are underway to identify and characterize these isozymes in different tissues, species, populations and individuals. A major approach to this problem is to develop a library of monoclonal antibodies (MAbs) which are highly specific for individual cytochromes P-450. The use of MAbs offers a powerful new dimension to numerous aspects of cytochrome P-450 research and will have a large impact on this area of research. Panels of MAbs have been developed to five different cytochromes P-450: two rabbit forms and three rat forms. The latter MAbs have been prepared to the major cytochrome P-450 in livers of rats treated with 3-methylcholanthrene (3MC), phenobarbital (PB), and pregnenolone 16-α-carbonitrile (PCN).

Inhibition of enzyme activity by the MAb 1-7-1, prepared to MC-induced rat liver cytochrome P-450, has been used to detect the cytochromes P-450 recognized by the MAb. This MAb not only inhibited the aryl hydrocarbon hydroxylase (AHH) activity of MC-induced rat liver microsomes, but also the AHH activity of human placenta. Among placentas from different individuals, the extent of inhibition of AHH by this MAb was virtually complete. Inhibition of 7-ethoxy-coumarin deethylase (ECD), however, exhibited a large degree of individual variation. Placentas from both dizygotic and dichorionic, monozygotic twins were examined for both absolute amounts of AHH and ECD and for their degree of inhibition by MAb 1-7-1; high intra-pair concordances were observed, relative to that found in unrelated individuals. The MAb-sensitive activities were also found in human lymphocytes, but not in liver and monocytes. The cytochromes P-450 responsible for these activities in liver and monocytes are therefore antigenically distinct from the enzymes in placenta and lymphocytes.

These results demonstrate the value of MAbs for defining antigenic site relatedness for different enzymatic functions of P-450s, and for identifying and quantifying the amount of a particular enzyme activity in a tissue that is dependent on specific cytochromes P-450. This study may be a prototype for the use of MAbs in phenotyping and mapping of the P-450s responsible for specific metabolic reactions and thus be useful in determining the relationship of P-450 phenotype to individual differences in drug metabolism and carcinogen susceptibility.

Also being developed are radioimmunoassays (RIAs) with MAbs to specific cytochromes P-450. An RIA based on the MAb 1-7-1 has detected 1) the elevation in level of MC-induced cytochrome P-450 in the livers of MC-treated rats, relative to untreated and PB-treated rats; 2) similar differences for guinea pig and C57BL/6 mice but not for hamster and DBA/2 mice; and 3) tissue-dependent differences in MC-induced rats, with higher levels in liver than in lung or kidney. The RIA data are in general agreement with the enzyme inhibition results. The competitive RIA with MAb 1-7-1 was also applied to placenta from different individuals, and a large degree of individual variation was found. The method was compared to the enzyme inhibition method and was found to be more reliable in detecting cytochromes P-450 that readily denature but still maintain intact MAb-specific epitopes. Development of quantitative and sensitive RIAs based on several MAbs should greatly aid in the detection and phenotyping of cytochromes P-450 in tissues and individuals.

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. The carcinogen N-2-acetylaminofluorene (AAF) binds to primarily the C-8 position of deoxyguanosine in DNA following metabolic activation to esters of N-hydroxy-AAF (N-acetoxy-AAF). The modification of DNA by N-acetoxy-AAF creates a locally denatured region recognizable by repair enzymes. There is also evidence that AAF modification impairs the DNA template functions in replication and transcription processes. In one study the ultimate chemical carcinogen N-acetoxy-AAF was shown to inhibit the enzymatic methylation of newly replicated DNA in cultured mouse P815 cells in a dose-dependent manner. Following removal of the carcinogen, a significant de novo methylation of newly replicated DNA takes place, the level of methylation being higher than in control cultures. The aberrant methylation pattern persists in the absence of carcinogen for at least six cell cycles. Cell cloning experiments showed the isolation of clones belonging to either of two classes, one with hypermethylated DNA and one with hypomethylated DNA. The existence of cells containing aberrantly methylated DNA of these two types indicated that both the inhibition of maintenance methylation and aberrant de novo methylation of DNA occurred during the N-acetoxy-AAF treatment. These results suggest that the induction of aberrant methylation patterns may be related to the initial step in chemical carcinogenesis.

The role of fetal versus maternal metabolic activation of metabolism-dependent transplacental carcinogens has been studied using methylcholanthrene (MC) in mice and procarbazine (PCZ) in rats. In a pharmacogenetic study in mice, genetic backcrosses were made to obtain, in the same litter, fetuses which were either inducible or noninducible for the mixed function oxidases that metabolize MC. Inducible fetuses developed a significantly (2-3 times) higher incidence of lung tumors than did those of noninducible genotype. This is the first direct demonstration of a determining role of enzyme inducibility in fetal susceptibility to a carcinogen. Conversely, administration of the transplacental carcinogen, PCZ, to gravid rats resulted in O-6 and N-7 methylguanine adducts in both fetal and neonatal tissues that could not be detected in the corresponding tissues of newborn rats given PCZ directly by injection. This observation constitutes evidence that maternal metabolic activation of carcinogen is crucial for transplacental carcinogenesis by this agent.

Antibodies specific for carcinogen-DNA adducts have probed the nature, extent and consequences of in vitro and in vivo DNA modification. Biological samples of DNA substituted with AAF, benzo[a]pyrene (BP) or cis-dichlorodiammineplatinum

(II) (cis-DDP) were subjected to immunological localization and quantitative immunoassays able to detect one adduct in one hundred million nucleotides. In hepatic DNA of rats fed a carcinogenic level of AAF for 4 weeks, adduct accumulation reached a plateau at 2-3 weeks. During 4 subsequent weeks on control diet, adduct removal was biphasic with a rapid initial phase followed by a slow second phase. A pharmacokinetic model consistent with this data proposes that adducts are formed in two DNA compartments, one from which adducts are removed rapidly and another from which adducts are removed slowly. Persistent adducts accumulate in the slow-repairing compartment, but constitute less than 7% of the total adducts formed. In contrast to the high levels of AAF adducts in liver DNA, binding of BP to deoxyguanosine in DNA of mouse epidermis and cultured epidermal cells is more than 50-fold lower. Binding levels are similar in epidermis and epidermal cells, subsequent to dosages known to induce papillomas in vivo and differentiation-altered foci in the cultured keratinocytes. The kinetics of repair for BPdG in vivo and in vitro are biphasic (as in liver) but much more rapid, with 50% removal by 1-2 days. Thus adduct accumulation and removal seem to be characteristic of interaction between a particular target tissue and an individual carcinogen and may not be quantitatively related to efficiency of tumorigenesis or transformation. Antisera specific for cis-DDP-DNA (bidentate N^7 dideoxyguanosine intrastrand adduct) have been used to measure adducts in DNA of nucleated blood cells from cancer patients on platinum drug chemotherapy. Adducts appear to accumulate both as a function of total cumulative dose and increasing cycle number in individuals who have not received previous platinum drug therapy. The relationship between ability to form adducts and disease response is being analyzed in a prospective clinical study.

Investigations of carcinogen metabolism in cultured human cells revealed that the major carcinogen-DNA adducts formed are identical to those found in experimental animals in which the chemical is carcinogenic. This finding has stimulated the search for adducts and antibodies to adducts in people exposed to environmental carcinogens. For example, roofers, coke oven workers, and aluminum plant workers as well as cigarette smokers are exposed to high levels of polynuclear aromatic hydrocarbons, including BP. A comparison of two very sensitive methods to detect BP-DNA adducts is in progress. Both of the methods appear to detect about one adduct per 1×10^7 nucleotides. Quantitation with both of the methods is possible and both qualitative and quantitative results from preliminary experiments comparing the methods have been promising. Another type of evidence of former or current existence of BP-DNA adducts comes from the presence of antibodies towards BP-DNA in serum from people exposed to BP. These antibodies have been detected in samples from coke oven workers.

In order to assess the role of chemical carcinogens in human liver carcinogenesis, several projects have been initiated with Chinese researchers. For example, the metabolic activation of aflatoxin B_1 and other carcinogens, such as N-nitrosodimethylamine and BP have been investigated using cultured human fetal liver explants. One major aflatoxin B_1-DNA adduct is formed by addition of aflatoxin B_1-2-3-oxide to the 7-position of guanine. This reaction product is unstable, and the imidazole ring of the guanine opens to stablilize the molecule. The major aflatoxin B_1-DNA adduct is similar to the one formed in fetal human liver explants and in rat liver in vivo, an organ susceptible to the carcinogenic action of aflatoxin B_1.

In a related project, urine samples are being collected in Murang'a district, Kenya, for analysis of aflatoxin B_1-guanine, a nucleic acid repair product. It has previously been shown that food samples collected in this district are

contaminated with aflatoxin B_1, and a positive correlation exists between the dietary intake of aflatoxin B_1 and the incidence of liver cancer. The urine samples collected at the out-patient clinic of Murang'a district hospital were concentrated and aflatoxin B_1-guanine isolated. Eleven of 126 samples had a detectable level of a compound whose synchronous fluorescence spectrum was identical to chemically synthesized aflatoxin B_1-guanine. These results are an indication of interactions between the ultimate carcinogenic form of aflatoxin B_1 and cellular nucleic acids in vivo and further support the hypothesis that aflatoxin B_1 may play an important role in the etiology of human liver cancer.

Studies on Z-DNA

A major advance in the effort to determine the biological relevance of the left-handed Z-DNA helix came when it was found that Z-DNA, unlike B-DNA is immunogenic. Antibodies were elicited by immunization with poly(dGdC).poly(dG-dC) in which the 8-position of guanine and the 5-position of cytosine were brominated. In this study rabbits were immunized with a complex of methylated bovine serum albumin and AAF-modified poly (dG-dC).poly(dG-dC). Several populations of antibodies specific for Z-DNA determinants were isolated, purified and analyzed. It was found that the AAF-modified polymer shared common Z-DNA determinants with poly(dGdC).poly(dG-dC) in 3.0M NaCl, poly(dG-m^5C).poly(dG-m^5dC) in 1.5M NaCl and brominated poly(dG-dC).poly(dG-dC) in 0.2M, 1.5M and 3.0M NaCl. Determinants present on the AAF-modified polymer but not on the other Z-DNAs were recognized by another distinct population of antibodies. The greater part of this population appeared to recognize Z-DNA-associated conformational characteristics that were unique to the AAF-modified polymer. The results of these studies are consistent with the interpretation that a continuum of Z-DNA determinants exists which might be capable of functioning as recognition signals for regulatory DNA-binding proteins. In order to investigate the possible role of Z-DNA in the regulation of gene expression, the (dT-dG)n·(dC-dA)n sequence was inserted into the pSV2-CAT plasmid vector and into the c-Ha-ras 1 clone. pSV2-CAT is a recombinant plasmid containing a chloramphenicol acetyltransferase (CAT) gene. Insertion of the Z-DNA sequence resulted in a significant enhancement of expression of the CAT gene and the transforming potentials of c-Ha-ras-1 gene when they were transfected into mammalian cells.

Continuing studies on the relation between the structure and function of chromatin indicate that the transcribable regions of chromatin are hyperacetylated. The same regions were also found to be 30-fold enriched in another minor chromatin protein, histone $H_1°$. Chromosomal proteins HMG-1 and HMG-2, which bind to DNA, bound preferentially to the Z form of DNA. This finding suggests that these proteins may bind selectively to particular regions of DNA, possibly playing some role in the function of the genome. Eleven monoclonal antibodies have been elicited against histone H-5. The epitopes of each of these was mapped. In other studies, the chromatin structure of the gene coding for a 3-methylcholanthrene-induced P-450 enzyme has been studied. A DNAse I hypersensitive region was found near the 5' terminal of the gene.

Studies on Oncogenes in Chemically Induced Tumors

Molecular studies of genes involved in the control of neoplastic transformation are testing the hypothesis that different genes may be activated by different carcinogens and/or cofactors, (e.g., promoting agents), and that transformation may result from the combined effects of these different genes.

Transforming DNAs have been obtained from 7 out of 16 chemically transformed BALB/3T3 cell lines. At least three different patterns of sensitivity to a battery of restriction endonucleases were demonstrated for the transforming DNA of three lines, all transformed by the same carcinogen, BP. Demonstration of a mutated allele of the Ha-ras oncogene was possible after digestion with MspI but not with other tested restriction enzymes. Genes termed pro were identified from DNA of promoter-sensitive mouse epidermal JB-6 cell lines and found to be responsible for susceptibility to promoting agents when transfected to promoter-resistant cell lines. Two pro genes were cloned and one was completely sequenced. Their characterization is under study. Activation and/or modification of cellular oncogenes is likely to be important in carcinogenesis, especially in the latter phases of this multistage process.

The vast majority of studies on oncogenes in tumors has utilized an interspecies assay, i.e., transfection of human tumor DNA into mouse NIH 3T3 cells; however, 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 v-Ha ras oncogenic complementary DNA results in 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 other oncogenes and with chemical and physical carcinogens. TBE-1 has integrated v-Ha ras into its genome and expresses transcripts that hybridize to Ha-specific structural gene and v-Ha LTR probe DNA. TBE-1 cells express detectable levels of phosphorylated v-Ha ras polypeptide p21.

TBE-1 cultures were tested to determine growth characteristics, response to TPA, the ability to form colonies of anchorage-independent cells in soft agar, and tumorigenicity in athymic nude mice. The results indicate that TBE-1 cells (1) are not induced to squamous terminal differentiation by serum, confluence, or 10^{-7}M TPA, (2) form colonies in soft agar, and (3) produce an autogenous growth factor since population doublings per day increase by a factor of 5 when autogenously conditioned medium is used to supplement cell growth at clonal density. The injection of nude mice with TBE-1 cells before selecting an anchorage-independent population leads to the development of nodules less than 0.1 cm in size at a frequency of 2 per 16 animals tested, but the nodules regress after 14 days.

TBE-1SA cells were isolated by selection of anchorage-independent cells growing in soft agar cultures of TBE-1 cells. The TBE-1SA cells were characterized for tumorigenicity, karyology, isozyme phenotype, and histocytochemical staining for keratin and beta human chorionic growth hormone (HCG). The progression of this subpopulation is indicated by their ability to form tumors that can grow to at least 0.5-0.8 cm in size and persist longer than 90 days in 13 of 14 nude mice. The TBE-1SA tumors have not regressed at 2 months, and this experiment is still in progress. Positive histocytochemical staining of TBE-1SA nodules for keratin confirms the epithelial origin of TBE-1SA cells. Since more than 70% of bronchogenic carcinomas contain detectable levels of HCG, TBE-1SA tumors were stained for beta HCG. The histocytochemical characterization reveals a beta HCG product within TBE-1SA tumor cells. The tumorigenicity of TBE-1SA is in contrast with the pretumorigenic phenotype of unselected TBE-1 cell populations. The pretumorigenic TBE-1 cells produced no anchorage-independent colonies within the limits

of detection (10^{-6}) immediately after focus formation and were expressing v-Ha ras gene transcripts and p21 gene product during the period of growth and progression to tumorigenicity of the population.

The modal distribution of chromosomes is 74-75 for TBE-1SA, with marker chromosomes and extensive chromosomal abnormality. The karyotypic instability of TBE-1 cells was also an early event detectable at the first passage after the isolation of foci, and the karyology of TBE-1SA cells shows the extent of abnormality that the bronchial epithelial v-Ha ras transfectants have sustained during their progression to tumorigenicity in athymic nude mice. The progression of v-Ha ras transfectants to immortality, anchorage independence, and tumorigenicity required expression of the oncogene for approximately 100 population doublings and alteration of many phenotypic properties of the cell, inferring a pleiotropic mechanism for the role of the Ha ras oncogene in human carcinogenesis.

A systematic study of the expression of selected oncogenes in chemically induced animal tumors is underway. This project will initially explore the time-course of expression of mutant ras family oncogenes during the development of chemically induced neoplasms in rats. By means of monoclonal or polyclonal antibodies to oncogene products, demonstrable in tissue sections at the cellular level by immunoperoxidase methods, evidence for or against a role of such genes in the induction of neoplasia will be systematically pursued. Transfecting Ki-ras genes have been detected in serially passaged renal mesenchymal tumors. An effort will be made to distinguish between appearance of oncogene products in tumor cells at the earliest identifiable stages of their evolution (consistent with a role in initial transformation to the neoplastic phenotype) or the gradual appearance of such expression during progression of an established primary neoplasm.

Infection of mouse epidermal cells with oncogenic retroviruses containing an activated ras gene indicates 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. Thus epidermal cells with an activated ras gene differentiate to a late basal cell stage but do not advance further. Analysis of RNA isolated from initiated basal cells or epidermal papillomas or carcinomas using a variety of oncogene cDNA probes has failed to show a significantly increased expression of any of these genes.

The raf oncogene has been further studied. To define the raf receptor, a non-transformed cell line which expresses a high amount of raf-specific mRNA has been identified. The plasma membrane fraction from these cells was prepared, and predetermined conditions were employed to release a biologically active receptor. This material is being analyzed for the presence of (1) raf polypeptide (approximately 90 Kda) by immunological methods and (2) receptor by its ability to compete with responsive cells for the growth factor. The mechanism of activation of the raf sequence has not yet been ascertained. Determination of the raf-specific mRNA size in malignant cells revealed no differences relative to normal cells; gross rearrangements and/or truncation of the raf cistron has not occurred. Activation may be the consequence of cellular reprogramming since these carcinomas express other surface antigens (as well as oncogenes--myb and

myc) found on nonepithelial cells. The role of these sequences in human malignancy is not fully understood. Experiments indicate that cells derived from human small cell lung carcinomas express significantly more raf-related RNA than normal bronchial epithelial cells. Small cell carcinomas are especially metastatic. This may be in part the consequence of the cell's expression of a new growth factor receptor (raf) because metastases contain 10-40 times more raf RNA than any of the small cell carcinoma cell lines investigated.

Studies on Gene Expression and Growth Control of Tumor Cells

The hormonal sensitivity and growth control of tumor cells is being evaluated in the context of a working hypothesis that oncogenic transformation may result from a selective increase in sensitivity to external regulators. Hematopoietic cells of (BALB/C X DBA/-2)F1 mice were infected with two strains of Friend virus that differ in their in vivo action (one inducing anemia [EVA], the other polycythemia [FVP]) and the effects of these increases characterized in vitro. Both variants induce erythroid bursts that proliferated and differentiated without added erythropoietin (EPO). However, while the bursts induced by FVP were well "hemoglobinized" (i.e., most cells contained hemoglobin), the cells of FVA-induced bursts contained little or no hemoglobin. The nonhemoglobin bursts, induced by FVA, were established to be erythroid by cytochemistry, electron microscopy, and hormone sensitivity. FVA-induced cells appeared to be hypersensitive to EPO, since small concentrations of the hormone produced marked increases in hemoglobin production, even when the hormone was added to the cultures 3 days post-infection. Time-lapse photography documented that EPO stimulated hemoglobin synthesis in virally transformed cells rather than uninfected erythroid precursors. This observation of FVA-induced hypersensitivity prompted the re-examination of the hormone requirements of FVP-induced bursts--previously considered to be EPO-independent. Reduction of the serum in the cultures allowed the demonstration that FVP-induced erythroid cells also were hypersensitive to EPO. Thus FVA and FVP can be readily distinguished in vitro by the relative EPO sensitivity of virus-induced bursts. These data are consistent with the hypothesis that oncogenic transformation may result from increased sensitivity of progenitor cells for natural, physiological regulators.

Previous analysis of molecular chimeras between the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) and the v-ras transformation gene from Harvey murine sarcoma virus (HaMuSV), and between the LTR and the chloramphenicol acetyl transferase (CAT) gene localized the steroid hormone regulatory sequences between 100 and 200 nucleotides upstream from the cap site in the LTR. Utilization of a competition assay with specific MMTV LTR fragments and total cellular DNA immobilized on cellulose has shown the preferential binding of glucocorticoid receptor to fragments of LTR DNA containing the sequences identified in gene transfer experiments as important for hormone regulation. The addition of transcriptional activator sequences to the MMTV promoter indicates that the hormone regulatory sequence is capable of regulating the activity of the exogenous enhancer. These observations suggest a model for the mechanism of hormone action in which the regulatory sequence acts as a modulator of another cis-dominant positive element. The role of chromatin organization in hormone action has been addressed utilizing chimeras between the MMTV LTR and the BPV 69% transforming fragment. These chimeras replicate uniquely as episomal elements in murine fibroblasts and maintain stable high copy extrachromosomal copy numbers. It was found that nucleosomes are nonrandomly organized on the sequences immediately upstream from the MMTV cap site, and that this phased structure is independent of hormone induction.

A DNase I hypersensitive site is introduced into the chromatin structure upon hormone induction; the location of this site correlates precisely with the sequences required for transfer of hormone regulation in the biological assay. An S1-nuclease hypersensitive site also appears in the chromatin in a hormone-dependent manner. This site maps to the right side of the DNase I site. The phased organization of nucleosomes in the promoter region indicates that a highly organized nucleoprotein structure serves as a template for hormone regulation in vivo. The specific changes induced in this structure upon hormone stimulation suggest that alteration of the chromatin template by interaction with the steroid receptor may play a critical role in the mechanisms of hormone action.

Gene expression during chemical transformation is also being analyzed using hamster embryo cells. The following results have been obtained to date: (1) Although bisulfite has been shown to be a potent transformation agent in vitro, acute bisulfite treatment at neutral pH had no qualitative effect on gene expression. (2) Similarly gene expression during "aging" (time in culture) was relatively stable from early passages (2nd) to later passages (10th). (3) Computer-assisted analysis of polypeptide differences between normal control cells and various bisulfite transformed clones revealed both qualitative (6 proteins) and quantitative polypeptide differences between untreated control cells and each of the individual transformed clones. Following transformation three polypeptides (pI 5.9 MW/55 kDa; pI 5.4/53 kDa; and pI 5.4/32 kDa) were either not expressed or appeared as a charge-shift variant in each of the transformed lines. In addition to the apparent loss of expression or charge-shift two new polypeptides (pI 6.55/45 kDa) were expressed in all clones and one polypeptide (pI 5.62/45 kDa) was expressed in all clones except clone A following transformation with either bisulfite or BP. One polypeptide (pI 6.36/28 kDa) was expressed only in the BP transformed clone.

The cellular macromolecules responsible for the expression of the neoplastic phenotype of chemically transformed human cells have been investigated. A new polypeptide recognized in a chemically transformed human cell line has been identified as a mutant of beta-actin which has aspartic acid at position 244 instead of glycine. Beta-actin genes were isolated from the transformed human cell line. Determination of the structure and organization of the beta-actin gene confirmed that the alteration of beta-actin in the transformed cells is ascribed to a point mutation (transition) in the structural gene of beta-actin.

The synthesis of the mutated beta-actin was correlated with the expression of the transformed phenotype in variants of the transformed line and its hybrids with normal human fibroblasts. A mutation resulted in several defects in the function of beta-actin, such as increased instability, reduced incorporation into cytoskeletal elements, and decreased ability to polymerize in vitro. These defects in the beta-actin molecule were associated with the disruption and loss of the structural organization of the cytoskeleton, such as the actin cable network. The results suggest that a mutation in beta-actin leads the cells to express transformation by disrupting the cytoskeletal structure and its function.

Because DNA transfection is currently a very important technique in carcinogenesis experiments, an understanding of the fate of the transfected DNA is essential. In studies with specially constructed shuttle vectors, it was found that such transfected DNA was susceptible to homologous recombination, leading to an unusually high frequency of spontaneous mutation. Analysis revealed that the mutations involved insertions and deletions of cellular DNA. The host cell DNA was not affected.

Studies on Lymphokines and Growth Factors

Lymphokine preparations from antigen- or mitogen-stimulated lymphocytes from normal hamsters or guinea pigs contain a potent anticarcinogenic activity. They prevent as well as inhibit the subsequent development of carcinogenesis. The new physiologic state, like many hormone responses, is induced rapidly and persists for a short time. When the lymphokine-induced anticarcinogenic state is present at the time of carcinogen exposure, carcinogenesis is prevented and when present shortly after carcinogen exposure, the further development of initiated or complete carcinogenesis is irreversibly inhibited. In fully tumorigenic cells cellular proliferation is inhibited; cellular destruction may also occur with high lymphokine concentrations. Lymphokine also has the ability to increase the sensitivity of cells to destruction by natural killer cell cytotoxicity.

The isolation, characterization and biological activity of transforming growth factors (TGFs) continues to be a major focus of research. The purification of large quantities of TGF-β from human platelets has made it possible to develop antisera to it and to initiate receptor binding studies. Human platelets have been extracted and platelet-derived TGF-β has been purified from the extract. Purified TGF-β is a protein of 25,000-daltons, and it is comprised of two 12,500-dalton subunits held together by disulfide bonds. The purified factor elicits its biological activity at concentrations of less than 4pM. Comparative studies showed that platelets contain 100 times more TGF-β than do other non-neoplastic tissues. Incubation of TGF-β with normal rat kidney fibroblasts (NRK cells) results in an increased number of cell surface EGF receptors. This is an important new finding, since it provides the first evidence for a biochemical mechanism of action of TGF-β.

In NRK cells, both type α and type β TGFs must be present to effect phenotypic transformation as evidenced by the acquisition of the ability to form large colonies of cells in semi-solid agar medium. It has previously been shown that NRK cells transformed by sarcoma viruses secrete type α TGFs and that this secretion is correlated with expression of the transformed phenotype in cells infected with temperature-sensitive mutants of the virus. It has now been demonstrated that these cells also secrete increased amounts of type β TGFs after sarcoma virus transformation and that the levels of TGFs secreted by the cells into the conditioned medium are consistent with an autocrine mechanism of transformation. It has also been shown for the first time that transformed cells secreting type β TGFs have a reduced number of cell surface receptors for that ligand, suggesting that ligand-induced down-regulation has occurred.

Polyclonal antisera have recently been raised to the TGFs and development of monoclonal antibodies is in progress. The effects of these antibodies on the anchorage-dependent and -independent growth of normal and transformed cells are being investigated. Since the first step in the interaction of TGFs with the cell is binding of the growth factor to the cell surface, initial investigations are concentrating on the characterization of cell surface receptors for TGFs. Development of a radioreceptor assay for TGF-β, a TGF that depends on a second TGF (EGF or TGF-α) for activity, has allowed identification and preliminary characterization of a specific high affinity receptor for TGF-β on NRK cells. A similar receptor has been found on all normal and transformed cell lines studied so far. Receptor expression in normal cell lines (NRK and NIH-3T3) is modulated by infection of the cell with acute transforming retroviruses Harvey sarcoma virus (HSV) and Maloney sarcoma virus (MoSV) and by transformation

with certain oncogenes (myc, H-ras). Human cell lines and tissues are being screened for an abundant source of the receptor for use as a starting material for receptor purification and as an immunogen for the production of anti-receptor monoclonal antibodies. Further characterization of the role of endogenously-produced TGFs and their interaction with the cell surface receptor should help elucidate the role these molecules may play in the process of carcinogeness.

A new cell has been derived from a metastatic, human melanoma. It is producing peptides that can stimulate an untransformed cell line to reversibly express a transformed phenotype. This transformed phenotype is expressed in monolayer culture as a disorganized growth pattern and in an anchorage-independent growth (AIG) assay as colonies forming in soft-agar from single cells. One of the peptides in the mixture contributing to this activity is an epidermal growth factor (EGF)-like growth factor that has a molecular weight of approximately 26,000-daltons as determined by gel permeation chromatography. This EGF-like activity requires a second peptide to efficiently stimulate AIG. The second required peptide in this mixture has a modulator activity in that it appears to require the presence of either EGF or an EGF-like activity to efficiently stimulate large colony formation. This TGF-β activity has an apparent molecular weight of approximately 21,000-daltons. Both of these peptides have physical properties that are distinctly different from those previously reported for the TGFs. These "ectopic" peptides may play a role in the expression of the transformed phenotype of the tumor cells producing them. Molecular clones have been derived to determine which genes are regulated by these growth and modulating factors. This knowledge will increase our understanding of the role these factors play in the expression of the transformed state.

The urinary protein of a patient bearing a highly malignant brain tumor (astrocytoma, grade IV) was adsorbed selectively on trimethylsilyl-controlled pore glass beads to yield a high molecular weight (HMW) human transforming growth factor (hTGF). In its apparent molecular size (28,000 M_r), receptor binding, immunologic behavior, and clonogenic activity, the HMW hTGF was indistinguishable from HMW human epidermal growth factor (hEGF). A form of HMW hEGF was previously reported to be present in low concentrations in normal human urine. Thus, rather than being uniquely of glioblastoma tumor cell origin, the HMW TGF/EGF growth factor may reflect a host response to tumor burden.

In NRK cells, retinoids and TGFs were shown to synergize to induce phenotypic transformation as measured by the growth of the cells under anchorage-independent conditions. In new studies employing Fischer rat 3T3 cells transfected with the myc oncogene, it has been shown that type β TGFs synergize with PDGF in the induction of AIG of the cells, and that this effect of the peptides is completely blocked by concentrations of retinoic acid as low as 1 nanomolar. In contrast, when the AIG of the same cells is induced by epidermal growth factor (a type α TGF), retinoids have no effect. Like the retinoids, type β TGFs can also be shown to have opposite effects on these cells: TGF-β stimulates the AIG of the cells when assayed in the presence of EGF. The molecular basis for the changing effects of both the retinoids and the type β TGFs on these cells is being investigated.

Several new developments on retinoid binding proteins have been described. One is the discovery and purification to apparent homogeneity of a new retinol-binding protein from neonatal rats. The protein is distinct from other retinol-binding proteins by several criteria. These include small but distinct differences

in spectra and considerably different immunoreactivity and tissue distribution. It apparently exists in two forms, retinol binding protein A and B, both of which are single polypeptide chains with molecular weights of about 16,000. Both forms seem to coexist in the tissues examined in equal amounts. Tissue distribution studies showed the presence of the retinol-binding protein in a number of tissues of the neonatal rat. However, intestine and liver had 100-fold higher levels than other positive tissues. Only skin among the tissues examined had no detectable activity by the radioimmunoassay procedure employed. In adult rat tissues high levels of the new binding protein were found in the intestine, particularly in the jejunum mucosa. Adult liver, however, had a much lower level than in perinatal liver. Most other adult tissues had non-detectable levels of the new binding protein (less than 4 pmoles per gram of tissue). The high levels in the intestine suggest that the protein may play a role in the absorption process and/or in the esterification of retinol that occurs prior to its incorporation into chylomicrons. In this regard, the gradient in the level of the protein from jejunum to colon of the adult rat is of interest: jejunum levels are 3-fold greater than ileum and both of these are greater than 100-fold the level in colon. Further, as with some other proteins known to be involved in intestinal absorption processes which show increases in their level after birth, so the new retinol-binding protein triples in level from day 19 in fetal intestine to that in the newborn rat. Fetal liver levels also appear to increase in the liver of the newborn rat.

Studies on Diet and Cancer

The degree of caloric restriction necessary to produce the inhibition of tumor development of C_3H mice was not found to be debilitating and did not inhibit the morphological development of the mammary gland. An examination of the influence of total caloric restriction on the estrous cycle of mice revealed no change in the sex cycle. Moreover, a decrease in the caloric intake did not affect the level of serum growth hormone (GH) or thyroid stimulating hormone (TSH), but did significantly reduce the level of circulating prolactin.

A study has been completed to determine whether vitamin E deficiency can lead to an enhancement in mammary carcinogenesis, and secondly, whether excess vitamin E can abrogate the augmentation due to selenium deficiency. The study demonstrated that a low vitamin E intake (7.5 mg/kg of diet) had minimal effect on mammary carcinoma development in rats fed a 5% corn oil diet, but resulted in a marked enhancement in tumor incidence and yield in those rats fed a 25% corn oil ration. Control animals in this experiment received an adequate supply of vitamin E (30 mg/kg). Thus, the effect of vitamin E deficiency on mammary carcinogenesis was accentuated in rats maintained on a high polyunsaturated fat diet, an observation similar to that of selenium deficiency.

Work continues on the role of protease inhibitors as anticarcinogens. It has been demonstrated that when soybean diets containing two principal protease inhibitors, Kunitz and Bowman-Birk, were fed to mice and to Sprague-Dawley rats, tumor promotion and x-ray-induced breast cancer were blocked.

The hypothesis was tested that hepatocarcinogenesis induced by peroxisome proliferators is mediated either directly by carcinogenic H_2O_2 generated by peroxisomal oxidase(s) or indirectly by free radicals produced from H_2O_2, that antioxidants can retard or inhibit such induced neoplasia by scavenging active oxygen species (superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen).

The synthetic antioxidants butylated hydroxyanisole (BHA) or ethoxyquin (EQ) were fed for 60 weeks to male Fischer 344 rats concurrently with ciprofibrate, a hypotriglyceridemic drug and one of the most potent peroxisome proliferators. EQ markedly inhibited ciprofibrate-induced liver carcinogenesis as seen by a decreased incidence in the percentage of animals with tumor, a decreased number of tumors per animal and a reduced tumor size. A significant decrease in incidence and number of carcinomas per liver larger than 5 mm was also found with BHA administration. Since previous studies had shown that these antioxidants neither inhibit ciprofibrate-induced peroxisomal proliferation nor the induction of increased activities of the H_2O_2-producing peroxisomal fatty acid beta-oxidation system, the observed inhibition of hepatocarcinogenesis by these antioxidants was deemed to be consonant with this hypothesis.

Epidemiology and Biostatistics

Case-Control Studies

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. Based on the leads provided by the U.S. cancer atlases, field studies have implicated shipyard work and asbestos exposures during World War II as the explanation for the high rates of lung cancer in several areas along the Atlantic coast. In southern Louisiana, the high rates of lung cancer appeared to be due partly to smoking habits, particularly the heavy use by Cajuns of local brands and handrolled cigarettes that contain high tar levels. An effect of passive smoking was suggested by an excess risk of lung cancer among non-smokers whose spouses were heavy smokers, and among smokers exposed during childhood to maternal smoking. The use of smokeless tobacco was found to account for the elevated rates of oral cancer among women in sourthern rural areas, and the study also revealed a twofold excess risk associated with lower intake of fruits and vegetables. A cluster of colorectal cancer in rural Nebraska was linked to a concentration of Czech migrants, especially those with high fat diets and familial occurrence of digestive tract cancer. The high rates of renal adenocarcinoma in the north central region appeared to be related to ethnic factors (especially German ancestry), cigarette smoking, and obesity and dietary habits (i.e., meat consumption), particularly in females. A parallel study of renal pelvis cancer in this area indicated a dominant influence of cigarette smoking, plus an association with long-term use of phenacetin-containing analgesic drugs and occupational exposures previously related to bladder cancer. A case-control study of nasal cancer in North Carolina and Virginia pointed to the role of smoking (limited to squamous cell carcinoma), chronic nasal disease, and occupational exposure to wood dusts (limited to adenocarcinoma) and textiles to some extent. The latter finding may explain the high rates of nasal cancer noted on the cancer maps among females in this area. A large case-control study of bladder cancer which previously evaluated the risk of artificial sweeteners was also analyzed with respect to a number of other potential risk factors. Of special interest were the excess risks among certain occupational groups, including truck drivers (especially those using diesel engines), painters, railroad workers, metal machinists, metal workers, construction workers, lumbermen and woodworkers, hairdressers, dry cleaners, and cutting operatives. A special study of bladder cancer in northern New England where the rates are high in both sexes revealed excess risks for leather workers in both sexes and for textile workers among males only.

Tobacco smoking is known to account for a substantial proportion of human cancer, and its effects on lung cancer were evaluated by a large-scale case-control study in several European cities. The size of this study enabled more precise estimates of various parameters of risk associated with smoking. Measures of intensity (number of cigarettes smoked per day) and duration (number of years smoked) showed increased risks behaving in a multiplicative fashion. The use of low-tar or filter cigarettes was associated with one-half the risk seen in lifetime non-filter smokers (after controlling for intensity, duration, and years since cessation of smoking). Smoking affected all cell types, and clear gradients of risk related to increasing amounts smoked were seen for adenocarcinoma as well as for other cell types. The number of cases in this study was sufficiently large to show that cigar and pipe smoking increased the risk of lung cancer independent of cigarette smoking.

Occupational Studies

Occupational studies, a time-tested means of identifying physical and chemical carcinogens, were pursued to assess hazards suspected on the basis of experimental, clinical, and field observations. This year a mortality study of structural pest control workers uncovered an excess of lung cancer that rose by the number of years employed as an applicator to nearly a three-fold excess among those licensed 20 or more years. A within-cohort case-control study has been initiated to obtain information on tobacco usage and more detailed information on exposure to pesticides. Proportionate mortality studies of petrochemical workers suggested an increased mortality from cancers of the brain, stomach, skin and lymphatic and hematopoietic systems, but a survey of work records on these cases revealed no strong association with any particular job. 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 was confirmed in a re-analysis of data from the National Bladder Cancer Survey. A large follow-up study of industrial workers in contact with formaldehyde during manufacturing and usage is underway. 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 groups are being designed. Death certificate surveys in Wisconsin and Nebraska revealed that farmers had an increased risk of leukemia, non-Hodgkin's lymphoma, and multiple myeloma. Case-control interview studies to identify specific risk factors in this occupational group are underway. The systematic evaluation of occupational cancer risk, with adjustment for smoking habits, is being carried out among participants of the Veteran's Follow-up Study initiated many years ago by Harold Dorn.

Radiation Studies

Radiation studies received further impetus with the creation of the Radiation Epidemiology Branch. This program of studies is designed to clarify the effects of low-level exposures and the shape of the dose-response curve. The proceedings of a multidisciplinary conference on radiation carcinogenesis was published this year, which emphasized the mechanisms by which cancer is produced. 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. An excess risk was also evident at breast doses in the range of 8-16 rads. Preliminary analyses of the possible interaction between lung cancer and cigarette smoking among atomic bomb survivors were consistent with an additive model, as opposed to the multiplicative

model seen for uranium miners. A study of childhood cancer in twins indicated a two-fold 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. A further follow-up of children irradiated for ringworm of the scalp in Israel revealed an excess of thyroid cancer 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, the radiation regimens were less effective in inducing leukemia than other radiation exposures that have been studied, perhaps related to the cell-killing potential of high-dose radiation to the pelvis. However, a slight risk was found that may have been associated with low-dose radiation received by marrow outside the pelvis. Ovarian damage caused by radiation may have been responsible for 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. Chromosome aberrations following partial-body irradiation were found to persist in circulating lymphocytes for over 40 years. An international study of childhood cancer found that the actuarial risk of developing a second cancer following treatment reached 12% after 25 years. A new study of women who received multiple chest fluoroscopies during pneumothorax treatment of tuberculosis confirmed that repeated low radiation doses increases the risk of breast cancer. Childhood cancer mortality in Utah was not found to be associated with radioactive fallout from nuclear weapons testing, despite positive reports by another investigator. In collaborative studies with the Radiation Effects Research Foundation in Japan, current emphasis is being placed on the analysis of case-control interview studies of breast and lung cancer in efforts to evaluate interactions of radiation with other risk factors.

Drug Studies

Drug studies were continued to evaluate the effects of estrogenic compounds on the risk of various cancers. Data from the Breast Cancer Detection Demonstration Project were also utilized to evaluate the relationship of estrogen replacement therapy to benign breast disease among postmenopausal women. An increased risk of fibrocystic disease and fibroadenoma was associated with use of these drugs. Emphasis was also given to surveys of patients with cancer and some nonneoplastic diseases treated with cytotoxic agents. A study of patients given methyl-CCNU (semustine), a nitrosourea used in cancer chemotherapy, provided the first quantitative evidence linking this drug with acute leukemia and preleukemia. Alkylating agents to treat childhood cancer were also associated with an increased risk of leukemia. An analysis of data from the Survival, Epidemiology and End Results Program suggested that women with breast cancer who received chemotherapy may be at an increased risk of leukemia, while those who received estrogen therapy are prone to endometrial cancer. Recent concerns about the possible tumor-promoting effects of diazepam (valium) and thyroid supplements on breast cancer risk were not substantiated. Despite some reports suggesting that isoniazid may increase the risk of bladder cancer, a case-control study revealed no association with medications used for tuberculosis treatment or prophylaxis. Patients with Hansen's disease showed no excess risk of cancer that could be linked to the use of dapsone, reported to be carcinogenic in laboratory animals.

Nutritional Studies

Nutritional studies were further intensified this year as evidence mounted to suggest that dietary factors contribute to a large fraction of human cancer. Several studies have utilized geographic areas in the U.S. (e.g., Japanese- and Norwegian-Americans) whose cancer risks may be altered by changing dietary habits. In case-control studies the role of dietary fat was suggested for breast and colorectal cancers, a broad nutritional deficiency for esophageal cancer, and a deficiency of fruits and vegetables for oral cancer. Case-control studies of various cancers are underway to measure the intake of various micronutrients, both by interview about usual dietary patterns and whenever possible by laboratory assays of serum samples. In a recent case-control study of lung cancer in New Jersey, lower risks were associated with higher consumption of fruits and vegetables but with no consistent relationship to retinol intake. The dietary association was limited to squamous cell carcinoma and was most evident among current smokers. A case-control study of invasive and in situ cervical cancer is underway at five cancer centers, and this project includes a collection of blood samples for micronutrient and viral assays.

Family Studies

Family and genetic studies, enhanced by collaborative ties with laboratory investigators, 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. This advance in our understanding of melanoma precursors was highlighted by an NIH Consensus Development Conference in October 1983. Non-tumor 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. Surveys of neurofibromatosis and other hereditary syndromes have helped clarify the risks of various cancers and have explored the role of several genetic markers. 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. A study of a family with ten cases of renal cell carcinoma has revealed an inherited 3;8 translocation, and preliminary data show that individuals with the translocation have three copies of the human oncogene, c-myc: one each on the normal chromosome 8, the rearranged 8, and the rearranged 3. The repository of cancer-prone families in the Program has become of special interest to experimentalists involved in the identification of human oncogenes.

Environmental Studies

Environmental pollutants were evaluated through epidemiologic studies that have utilized relevant environmental measurements. 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). In addition, the role of water pollution from halogenated hydrocarbons was evaluated by 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 this cancer after appriopriate adjustments were made. 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. Some evidence of interaction with cigarette smoking was suggested by a positive association with duration of exposure to surface water among nonsmokers, although a negative trend was noted among smokers.

Studies on Infectious Agents

Infectious agents received increasing attention in relation to the role of a type C retrovirus in the etiology of a specific type of aggressive leukemia/lymphoma of mature T-cell origin. A series of collaborative studies clarified the relation of human T-cell leukemia virus type 1 (HTLV-1) to the T-cell malignancy. The distribution of HTLV-1 infection was plotted, with endemic areas demonstrated in southern Japan, the Caribbean Basin, northern parts of South America, Central America, the southeastern United States, and certain parts of Africa. Relatives of persons with HTLV-1 infection showed a higher prevalence of antibodies to this agent than the general surrounding population. Available evidence suggests that this virus is not highly contagious, but transmission probably occurs in a manner resembling that for hepatitis-B (i.e., through close contact and blood product transfusion). The epidemic outbreaks of acquired immunodeficiency syndrome (AIDS) which predisposes to Kaposi sarcoma and opportunistic infections have also been under investigation. Studies have focused on the epidemiologic, immunologic, and virologic characteristics of certain high-risk groups, including male homosexuals and patients with hemophilia. With the discovery of a likely etiologic agent (HTLV-III), specimens collected from the seroepidemiologic surveys are now being analyzed for antibodies to this agent. Also conducted this year were studies to clarify the role of the Epstein-Barr virus in Burkitt's lymphoma and nasopharyngeal cancer, herpesvirus type 2 and human papillomavirus in cervical cancer, and hepatitis-B infection in primary liver cancer.

Biochemical Epidemiology Studies

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 until 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 make it possible to define past exposures and subclinical or preclinical response to initiators, promoters, and inhibitors of carcinogenesis, and to evaluate host-environmental interactions. The most effective ways to effectively utilize this approach are being assessed 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 particular carcinogens or their metabolites in vivo through chemical analyses, mutagenesis assays, or immunologic detection techniques. It has become possible to measure the interaction of specific 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 are being developed to study these mechanisms in lung cancer, and other studies are underway to clarify the role of fecal mutagens in development of colorectal cancer.

REPORTS ON INTERNATIONAL AGREEMENTS AND INFORMATION EXCHANGE ACTIVITIES

(Fiscal Year 1984)

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 by a contract from the Division of Cancer Etiology, and were initiated after pilot studies demonstrated their feasibility. Plans were also developed for a vitamin intervention trial 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 cancer, and investigating in vitro transformation of human epithelial cells by microbial and chemical agents.

SCIENTIST EXCHANGES

U.S. to China:

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

China to U.S.:

Dr. W. Min Cancer Institute (Beijing)	Dr. C. Harris National Cancer Inst. (Bethesda, MD)	2 months	Risk Factors for Liver Cancer

U.S.-Federal Republic of Germany. This agreement primarily concerns cooperation in environmental carcinogenesis. The working groups have agreed to conduct joint investigations on mechanisms of carcinogenesis, including prevention and modulation of the process. The positive results of this program during the past year have included approval for one American to visit a laboratory in Germany, a visit by the U.S. Coordinator to Munich, Heidelberg, and Bonn, and a U.S.-German administrative meeting at the National Cancer Institute, in Bethesda, Maryland. Members of the Ministry of Health and the German Embassy in Washington, D.C., have expressed the need for guidance from American colleagues as to the best way to increase the German effort in cancer research. The U.S. Coordinator and a representative from the Office of International Affairs, NCI, offered suggestions and reviewed the procedure used to implement programs involving other countries. During this year, Professor Schmahl was replaced by a temporary chairman, Dr. Hans Brieskorn of the University of Virginia, Charlottesville, Virginia. The German side agreed to mail specific topics representative of current needs.

SCIENTIST EXCHANGE

U.S. to Federal Republic of Germany

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. J. Coggin, Jr. Univ. of South Alabama (Mobile, AL)	Dr. H. zur Hausen Cancer Institute (Heidelberg)	6 weeks	Monoclonal Antibodies for Detection of Human Fetal Antigens

U.S.-Italy Agreement. Research pertinent to this Division is included in the Cancer Prevention Program. The program involves a variety of activities related to cancer etiology, with recent emphasis on epidemiology. This year a workshop on the epidemiology of childhood cancer was held in Santa Margherita Ligure, Italy, focusing on areas of mutual interest in etiology research and on late effects of cancer therapy. A follow-up meeting on gastric cancer was also held to describe progress in preparing for a collaborative case-control study of this cancer, the leading cause of death in Italy. Plans for pilot testing the study methods were developed, with anticipation of launching a full-scale investigation early next year in high-risk areas of northern Italy.

SCIENTIST EXCHANGES

U.S. to Italy:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
F. Stern NIOSH (Cincinnati, OH)	Dr. E. Buiatti Center for Study and Prevention of Cancer (Florence)	2 weeks	Assessment of Occupational Cancer

U.S.-Japan Agreement. This year marks the last of the second 5-year program of this agreement, which consists of four broad program areas: Etiology, Cancer Biology and Diagnosis, Cancer Treatment, and Interdisciplinary Research. The cooperation between U.S. and Japanese scientists still remains one of the most active and at the June 1984 meeting of the U.S.-Japan Joint Steering Committee, the agreement was renewed for another 5 years. This binational program 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 "Carcinogenicity, Mutagenicity and Metabolism of Heterocyclic Amines" and "Eukaryotic DNA Replication and Repair."

Several members of this Division 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. S. A. Aaronson National Cancer Institute (Bethesda, MD)	Dr. T. Sugimura Natl. Cancer Ctr. Res. Inst. (Tokyo)	1 week	UICC Course on Carcinogenesis
Dr. J. E. De Larco National Cancer Institute (Bethesda, MD)	Dr. T. Sugimura Natl. Cancer Ctr. Res. Inst. (Tokyo)	2 weeks	Effects of Transforming Growth Factors on the Expression of the Transformed Phenotype
Japan to U.S.:			
Dr. Shaw Watanabe Natl. Cancer Ctr. Res. Inst. (Tokyo)	Dr. R. Miller National Cancer Inst. (Bethesda, MD)	6 weeks	Etiological Methods for Multiple Primary Cancers
Dr. M. Miwa Natl. Cancer Ctr. Inst. (Tokyo)	Dr. F. H. Ruddle Yale University (New Haven, CT)	2 months	Effect of Microinjection of Biological Materials into Mammalian Embryonic Cells
Dr. H. Fujiki Natl. Cancer Ctr. Res. Inst. (Tokyo)	Dr. R. Moore Univ. of Hawaii at Manoa (Honolulu, HI)	3 weeks	Isolation of Tumor Promoter (Aplysiatoxin) from Seaweed

Japan to U.S. (contd):

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. K. Oda The Inst. of Medical Sciences (Tokyo)	Dr. W. Eckhart Salk Institute (San Diego, CA)	1 month	Techniques of Immune Affinity Chromatography for Purifying Adenovirus 12 E1A Gene Products
Dr. T. Yamamoto The Inst. of Medical Science (Tokyo)	Dr. I. Pastan National Cancer Inst. (Bethesda, MD)	1 month	Molecular Biology of Cellular Proteins Involved in Malignant Transformation
Dr. M. Kaneko Natl. Cancer Ctr. Res. Inst.	Dr. P. Hanawalt Stanford University (Stanford, CA)	3 weeks	Correlation Between Oncogenes and Tumor Antigens
Dr. T. Seno Saitama Cancer Ctr. Res. Ctr. (Saitama)	Dr. M. Capecchi Dr. K. Lark Univ. of Utah (Salt Lake City, UT)	1 month	Molecular Mechanisms of the induction of Genetic Recombination of Mammalian Somatic Cells

U.S.-France (NCI-Institut National de la Sante et de la Recherche Medicale-INSERM) Agreement. 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.S. to France:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. D. W. Ross Univ. of North Carolina (Chapel Hill, NC)	Dr. M. Bessis Bicetre Hospital (Bicetre)	6 months	Quantitative Analysis of Maturation in Leukemic Cells

France to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. B. Kerdelhue CNRS (Gif-sur-Yvette)	Dr. E. Peck Univ. of Arkansas Medical Center (Little Rock, AR)	2 months	Induction of Pituitary Tumors by Estrogen
Dr. M. Tovey Institut de Recherches Scientifiques Sur Le Cancer (Villejuif)	Dr. Z. A. Cohn Rockefeller Univ. (New York, NY)	6 weeks	Biochemical Study of the Effect of Interferon on Murine Macrophages

U.S.-U.S.S.R. Agreement. We have continued our attempt to redefine and restructure the American-Soviet Cooperative Program in Carcinogenesis. Since this represents a new field of scientific endeavor in the U.S.-USSR Cooperative Cancer Program, we deem it essential to obtain from the USSR as much information as possible on the ongoing work in research areas that were identified as being potentially beneficial to both nations at our meeting in Bethesda, Maryland on September 14-15, 1981. 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.

No scientist exchanges took place this year.

SUMMARY REPORT OF

OFFICE OF THE SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER

NATIONAL CANCER INSTITUTE

October 1, 1983 through September 30, 1984

A. Mission

This program unit comes under the direction of the Office of the Director, Division of Cancer Etiology (DCE), National Cancer Institute, and serves as a focal point and catalyst for development of program interests and activities and collaboration in the area of environmental cancer. Some of the cooperative projects and collaboration with other Federal agencies come under the collaborative programs with the U.S. Environmental Protection Agency (EPA) and the National Institute for Occupational Safety and and Health (NIOSH). Other interfaces are with state agencies, industrial organizations, academic institutions, trade unions, consumer groups, and professional societies. This unit serves as the primary information resource for the Division and the Institute relevant to the role of environmental pollution and industrial exposures in carcinogenesis as well as information on drugs and cosmetics. These activities fulfill one of the essential functions prescribed in the National Cancer Act of 1971 as well as the Biomedical Research and Training Amendments of 1978.

A holistic approach relevant to the assessment of stress from these exposure sources that impact on man is first developed by identification, classification and ultimately a categorization of these levels of exposure (air, water, food, diet, cosmetics, drugs and workplace). Wherever possible, not only single agent exposures are computed but an estimate of total integrated exposure (multiple exposures) is obtained. To achieve these goals for appraisement of occurrence, frequency and concentration of such environmental carcinogens, close working arrangements must be maintained with EPA, NIOSH, the Food and Drug Administration (FDA), the Occupational Safety and Health Administration (OSHA), the U.S. Department of Agriculture (USDA), the Department of Defense (DOD), the U.S. Department of Interior (USDI) and other relevant agencies. These data are derived from intelligence networks (monitoring systems) operable on a national scale and then are published in reports and journal articles as are results from workshops and scientific meetings. These information resources provide factual data for inquiries (public and the NCI Office of Cancer Communications) and are useful as guidelines in projected experimental studies (rodents and fish bioassays) and in epidemiological studies.

A total of 16 contracts and interagency agreements, representing a total figure of $4,462,836, are employed to support the missions spelled out above.

B. Other Assigned Functions

The activities of the Interagency Collaborative Group on Environmental Carcinogenesis (ICGEC), sponsored by this office and now in its eleventh year, also adds to the information exchange activity, but more significantly fulfills one of the missions encompassed within the earlier referenced legislation. While

the ICGEC was originally constituted to provide a mechanism for interagency contacts to secure access to other data bases, it has provided, indirectly, a stimulus for development of Division projects in the area of environmental and occupational carcinogenesis. In an earlier era NCI lacked such mechanisms for interagency cooperation and the ICGEC, along with other activities, provides such an important resource.

This unit also monitors and manages the NCI/EPA and NCI/NIOSH collaborative programs, previously mentioned. Members of our staff serve as project officers on contract projects relating to experimental studies (in vitro and in vivo studies) and information resource contracts which are designed to enhance our data bases.

Two of our staff members 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 and Congressional interest in this task force is still quite evident.

This office continues to support the NCI Chemical Selection Working Group (CSWG) for NCI nominations of chemicals to the National Toxicology Program (NTP) and the Chemical Evaluation Committee of the NTP; this support is provided by staff and by contractor. This office also maintains a Chemical Selection Planning Group (CSPG) which works with a contractor to plan agendas and make prior decisions on chemicals to be submitted to the CSWG. The CSPG also serves to assist the project officer in providing, along with a Steering Committee, an advisory group for a consensus response for planning and budgeting with relevance to this information resource contract.

A resource contract with a local contractor involves the preparation of the PHS-149 report (Survey of Compounds Which Have Been Tested for Carcinogenic Activity). The old contract related to preparation of Volumes for 1974-75, 1976-77, 1979-80). The present contract provides for the preparation of volumes for 1981-86. Distribution of these reports, as well as the IARC (International Agency for Research on Cancer) Monographs, takes place in this office.

An interagency agreement with the Centers for Disease Control and collaboration with the Michigan State Department of Public Health is supported by NCI and a liaison officer from this office is active in that project relating to population studies on polybrominated biphenyls (PBBs). This positive exploitation by academia will be further encouraged.

C. Accomplishments

Interagency Collaboration

As previously stated, one of the most visible mechanisms for collaboration is the Interagency Collaborative Group on Environmental Carcinogenesis. This group consists of representatives from 28 agencies or subagencies and was set up by this office for NCI to fulfill one of the mandates of the National Cancer Act of 1971. The group meets every 6-8 weeks and by October 1984 there will have been about 76 meetings. Topics of meetings held so far this year are as follows: 1) American Petroleum Institute (API) Toxicological Studies of Gasoline, 2) Indoor Air Pollution, 3) Potential Markers for Human Exposure to Carcinogens/Toxic Substances, 4) Problems in Hazardous Wastes, 5) Role of Oxygen Radicals in Toxicity, and 6) Studies of 1,3-Butadiene.

Previous reference was made to the Task Force on Environmental Cancer and Heart and Lung Disease and our participation in all Working Group Activities. A member of the Task Force Planning Group reviewed and planned the activities of Working Group meetings including preparation of the Seventh Annual Report to Congress. A member of our staff continues to serve as Chairperson and coordinator for the Project Group on Exposure and Metabolic Mechanisms. In addition this staff member serves on a planning committee for an upcoming workshop on the role of environmental factors in lung cancer.

The office has prepared a Congressionally Mandated DHHS report entitled "Research Activities of Relevance to the Clean Air Act: Fourth Biennial Report to Congress, 1982-1983."

Assistance is rendered to the Department of Energy's Health and Environmental Effects Program. One of our staff members served as a principal reviewer of their Health and Environmental Effects Documents (HEED) program and participated at their Annual Contractor's Meeting.

An interagency agreement has been initiated with the Northwest and Alaska Fisheries Service, NOAA, U.S. Department of Commerce, to investigate the "Etiology of Tumors in Bottom-Dwelling Marine Fish" in Puget Sound, Seattle, Washington. 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 will be 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 will be assayed in in vitro and in vivo tests. The project will be focused 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 will utilize the English sole also, as this laboratory has demonstrated the capability to utilize this specie in long-term studies. The study will be funded by the NCI and NOAA, with the NCI funding approximately 60% of first year costs.

Interfacing with Trade Associations, Industries, and Trade Unions

The exchange of data and information with a variety of private sector and industrial components--companies and associations--continues. The major component of the dyes class study--conducted in collaboration with the relevant international trade associations and trade union--has been completed. The last two segments, comprising approximately 40% of all the dyes to be evaluated, had to be delayed because of time constraints of the individuals from the private sector. This activity has been resumed, and will be completed in FY'85.

Our staff continues to provide the private sector with guidance in animal model selection, protocol design, and interpretive access to Federally funded research to minimize unnecessary toxicological research. Municipal and state governments, as well as other agencies, are provided with program-derived and retrieved information to aid in legislative decisions.

This office continues to interface between the Institute's epidemiologists and industrial groups to effect collaborating projects. The formaldehyde study is

on schedule and may well be reported out by the beginning of FY'85. It has proved to be an even larger and therefore more significant study than first envisioned and can potentially make a major contribution to an issue of great importance. A similar study of acrylonitrile has been approved and funded. The cooperation offered by private industry during the feasibility study will be continued and probably expanded in the actual study that has just been initiated. Two other compounds with epidemiological study potential have been the subject of discussion between this office and interested companies.

Some 120 compounds have been tested in the in vitro mutagenicity assays and results utilized by intramural scientists, the chemical selection process, and other Federal agencies.

Exchange of information with U.S. Army medical research scientists continues in two spheres. There is an interchange of mutagenicity assay test data on specific compounds to confirm results and, in some cases, to avoid duplicative efforts. Our efforts in aquatic toxicology are shared with the same organization because of their interest in basic research and environmental impact. They have shared funding of one project with us in the past and this continues to be a possibility as our program develops.

Special Projects on Environmental Carcinogenic Contaminants: Air, Water, Food, Drugs and Cosmetics

Expert Panel Review of Monographs on Drugs and Cosmetic Ingredients

The Federation of American Societies for Experimental Biology (FASEB) has been reviewing the contents 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. FASEB has convened a Panel of outside experts to evaluate the content of these monographs and has grouped the monographs into various categories including 1) those substances which should be evaluated, 2) those substances which have recently been evaluated by other Expert Groups, 3) those substances for which evaluations should not be undertaken at this time, and 4) those substances which require further study and analysis before placing them into categories one to three. In addition the Expert Panel will have completely revised and evaluated four to five monographs based on additional information available to the Committee. The Panel will utilize uniform evaluation criteria developed for the assessment of carcinogenicity of these compounds and prepare summary evaluations for those substances included in category two. NCI will then incorporate these revised monographs and evaluations into their final report on these compounds.

Master Agreement: Natural Toxicants in Foods

One hundred and twenty-five compounds which are naturally occurring, inadvertant contaminants or substances, are introduced into foods by processing. The data are presented as summary tables of data on carcinogenicity and short-term test results as well as qualitative and quantitative (where data are available) information on occurrence. The information on carcinogenicity is evaluated in terms of the quality of the published data according to various criteria established by the contractor and approved by a steering committee.

Master Agreement: Monographs on Organic Air Pollutants

A series of air pollutants have been selected for preparation of individual or group IARC-style monographs depending upon the amount and quality of published data available. This project was a more detailed and expanded study than a previously published report entitled "Organic Air Pollutants: Carcinogens, Mutagens and Promoters." Approximately 70 compounds have been selected for this report from an initial list of 770 on the basis of various prioritization criteria and have more detailed discussions of environmental occurrence/fate data than normally appear in current IARC monographs. It should also be mentioned that compounds do not overlap those discussed at the February 1984 meeting of the IARC Working Group. This report has been reviewed by staff at EPA and NCI and is now available for distribution.

Special Reports on Water Pollutants

An initial report on water pollutants appeared as a DHHS/PHS/NIH report on "Biorefractories in Water: Carcinogens, Mutagens and Promoters" and was also published in the Journal of Environmental Science and Health. This earlier report covered only 767 organic contaminants in drinking water. However, it has now been updated and covers about 1565 contaminants and presents an evaluative study on about 52 carcinogens, 68 mutagens, and 23 tumor promoters. This report was sent to outside reviewers and has been edited and amended. The final report was made available for distribution in June 1984. Similarly, a study done on 15 major inorganic chemicals in drinking water is being revised with additional survey data included.

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 Systems). 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. This resource data can now be accessed online through PROPHET or through CIS (Chemical Information System) which has wide availability (academia, industry and government).

Experimental Data on Tumorigenic Stdies on 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 sized fish develop tumors comparable to rodents when exposed to classical carcinogens such as MAM-acetate. From a spectrum of seven species studied, the Japanese medaka and guppy will be the two species used throughout this study. This study, focusing on aquarium-sized fish, will be extended to a carcinogenic evaluation or bioassay of major contaminants in the nation's water supply using the small fish as the test model. 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.

Highlights

During this year, the PHS-149 report, entitled "Survey of Compounds Which Have Been Tested for Carcinogenic Activity," was prepared for 1976-77 and 1979-80. The 1976-77 volume was printed at Government Printing Office and distributed to approximately 750 scientists in the cancer research field in this country and abroad. The 1979-80 volume is in the process of clearance and should be available for distribution in early fall of 1984.

The online data bases on carcinogens, mutagens, and promoters referred to previously as the CCRIS data base has had wide distribution from the reports obtained on those who access this data base using CIS. The National Library of Medicine is now utilizing this data base.

The updated and final report on organic contaminants in drinking water that were identified and classified as carcinogens, mutagens and promoters in the nation's water supplies was published on 1565 contaminants. This report shows, where known, the concentration of these contaminants or range in concentration, reflecting the daily population exposure. A companion project on the inorganic contaminants in drinking water culminated in a final report which will be reviewed by outside consultants prior to publication and distribution.

Considerable progress was made in a project designed to develop a fish model for bioassay of organic contaminants in drinking water. From several species tested it was found that two species, the Japanese medaka and guppy, were most suitable for these studies. A classical carcinogen such as MAM-acetate was found to produce tumors in these fish at target sites similar to those induced in the rodents.

A new Master Agreement task, entitled "Occupational Carcinogens," was awarded for the preparation of 75 monographs which will attempt to link the exposure of specific industrial workers to certain recognized and suspect carcinogens. This project represents a close collaborative study between staff in our office and staff of the Environmental Epidemiology Branch of DCE. Several draft monographs have been prepared and will facilitate future epidemiological studies of these substances.

Two other projects conducted under the above Master Agreement have been completed recently. One report on "Monographs of Organic Air Pollutants" has been prepared by SRI, Int. and has been reviewed by staff at both EPA and NCI. This report contains a series of 75 individual or group monographs containing summaries of published carcinogenesis as well as environmental fate and occurrence data. This report also contains important information on occurrence of these compounds in media other than air and such data will be useful for future studies of human exposure.

The second report, entitled "Natural Toxicants in Foods," is a compendium of summary tables of in vivo and in vitro carcinogenicity associated with approximately 125 substances which occur naturally, enter the food chain inadvertently, or are introduced by processing (e.g. cooking). In addition the data have been evaluated according to the strength of the evidence for carcinogenicity (animal tests) and tables of qualitative and quantitative occurrence data are included where such information is available. Compounds included in this report include

a wide range of chemical classes and should provide a useful resource to scientists in the regulatory community when comparing the biological activity of these chemicals with those produced synthetically.

The Subcommittee on Metabolic Mechanisms of the Task Force on Environmental Cancer and Heart and Lung Disease has prepared a report entitled "Strategies for Determining the Mechanisms of Toxicity." This report is structured in a manner which will allow various Federal agencies to follow a logical stepwise approach in order to develop their own effective and efficient protocols for toxicity studies. This report will be presented to the Working Group following peer review of the report.

Publications

Kraybill, H. F.: Assessment of human exposure and health risk to environmental contaminants in the atmosphere and water with special reference to cancer. J. Environ. Sci. and Health C1(2): 175-232, 1982.

Kraybill, H. F.: Assessment of human exposure and health risk to environmental contaminants in water with special reference to Cancer. In Clayson, D. B., Krewski, D. R. and Munro, I. E. (Eds.): Toxicological Risk Assessment. Boca Raton, FL, CRC Press. (In Press)

OFFICE OF THE SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER

CONTRACTS ACTIVE DURING FY 83

Institution/Principal Investigator/ Contract Number	Title
Fisher, Kenneth Federation of American Societies for Experimental Biology (FASEB) NO1-CP-31014-72	Expert Panel Review of Drugs and Cosmetics Monographs
Fleming, Roy National Institute for Occupational Safety and Health YO1-CP-60505	Conduct of Research on Occupational Carcinogenesis
Haworth, Steve Microbiological Associates NO1-CP-41030	In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Salmonella Typhimurium Assay
Helmes, C. Tucker SRI International NO1-CP-26004-02	Monographs on Organic Air Pollutants
Helmes, C. Tucker SRI International NO1-CP-95607	Resource to Support the Chemical Economic and Biological Needs of NCI/DCCP and to Provide Support to the International Agency for Research on Cancer (IARC)
Hughes, Thomas Research Triangle Institute NO1-CP-15740	In Vitro Evaluation of Chemical Candidates for In Vivo Testing
Kirby, Paul Microbiological Associates NO1-CP-15739	In Vitro Evaluation of Chemical Candidates for In Vivo Testing
Kirby, Paul Microbiological Associates NO1-CP-41004	In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Mouse Lymphoma Assay
Lee, Anthony Technical Resources, Inc. NO1-CP-15761	Survey of Compounds Which Have Been Tested for Carcinogenic Activity
Malins, Donald National Oceanic and Atmospheric Administration (number not assigned as yet)	Etiology of Tumors in Bottom-Dwelling Marine Fish

Overstreet, Robin Gulf Coast Research Laboratory NO1-CP-26008	Biochemical, Pharmacological, and Tumorigenic Studies on a Composite of Drinking Water Carcinogens and Mutagens Utilizing Aquatic Animals as a Bioassay Model
Santodonato, Joseph Syracuse Research Corporation NO1-CP-26002-03	Occupational Carcinogens
Seifried, Harold Tracor Jitco, Inc. NO1-CP-26003-01	Natural Toxicants in Foods
Seifried, Harold Tracor Jitco, Inc. NO1-CP-31046	Evaluation Support of Monographs on Drugs and Cosmetic Ingredients
Ulvedal, F. and Farland, W. Environmental Protection Agency YO1-CP-80205	Performance of Collaborative Studies in the Area of Environmental Cancer
Zack, Matthew Centers for Disease Control YO1-CP-60215	Human Health Consequences of Polybrominated Biphenyls (PBBs) Contamination of Farms in Michigan

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE03509-21 OD

PERIOD COVERED

October 1, 1983 to September 30, 1984

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:	R. J. Parker	Visiting Associate	OD, DCE	NCI
	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)

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 stenderd 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 myeloblastic 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
R. J. Parker	Visiting Associate	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 evaluate the possibility of preventing or reversing chemical carcinogenesis in nonhuman primates.

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 526 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 18 years. A total of 48 monkeys have survived 6 months or longer after the first dose of drug. Fifteen of the 41 monkeys (31.2%) 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 seven 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 received the last dose of adriamycin 74 months ago. 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. 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 two patients receiving treatment for central nervous system (CNS) neoplasms. Both patients were diagnosed with acute non-lymphocytic leukemia after receiving oral doses of nitrosoureas totalling approximately 1420 and 2700 mg/m^2, respectively. 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 195 months. Thirteen of the 22 monkeys (59%) 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 the 22 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 0.95 gm and the dosing interval averages 95 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 13.22 gm over the course of approximately 73 months. Fourteen animals are receiving azathioprine at 5 mg/kg, and have ingested an average cumulative dose of 21.16 gm over an average dosing interval of 53 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 a recently initiated 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 only 32 months; during this period an average cumulative cyclophosphamide dose of 10.23 gm has been administered. Three of the monkeys died during an outbreak of measles in the colony three 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 17 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 39 monkeys necropsied to date developed a total of 29 malignant neoplasms (the histopathology report is pending for one animal), 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. All of the latter animals possessed adenocarcinoma of the pancreas and/or gallbladder or bile ducts as well as urinary bladder carcinoma, fibrosarcoma and osteosarcoma. 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 13 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 8 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. Although two animals at the 2.0 mg/kg dose have been necropsied, neither animal showed evidence of tumor development; however, severe toxic hepatitis with hyperplastic nodules was found in one of the 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.

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 150 months (range 149-152 months), and the second group of 10 monkeys began saccharin treatment approximately 6 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 164 months. Administration of DDT is discontinued after a dosing interval of 130 months is completed. Although seven 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 18 surviving monkeys appear to be in good health.

Arsenic

The carcinogenic potential of arsenic has been under evaluation for approximately 9 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. One monkey died 107 months after the implant; no lesions other than lung mites were found in this animal. The remaining nine 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 14-17 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 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.

The dose rate may also determine the minimum total DENA dose required for tumor induction, since averages for this figure ranged from 700 mg/kg for the 5 mg/kg dose rate to 1,123 mg/kg for the 40 mg/kg dose rate. DENA is also carcinogenic in the more primative primate, Galago crassicaudatus. Ten of 14 treated animals have developed tumors after bimonthly IP injections of DENA at doses of 10-30 mg/kg. In contrast to the DENA-induced primary hepatocellular carcinomas in Old World monkeys, all 10 of the bushbabies developed mucoepidermoid carcinomas of the nasal cavity. In two of these ten animals, carcinoma of the liver was also present, and in both cases metastases to the lungs or to intestinal lymph nodes was noted. The average total dose of DENA given the bushbabies was 0.747 gm and ranged from 0.295-1.485 gm. It is considerably lower than that required to induce tumors in Old World monkeys and reflects the lower body weight of the bushbabies. The average latent period for tumor induction in this species (23 months) is comparable to that in Old World monkeys at the 20 mg/kg dose. No obvious reason exists for the marked difference in the site of DENA-induced tumors noted between Old World monkeys and the bushbabies. It may be related to differences in the metabolism or distribution of DENA, and this possibility will be investigated.

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 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 21 monkeys has received this compound for periods of up to 9 years; thus far, two animals have died of causes unrelated to MNNG toxicity. The remaining 19 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 22 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. Several studies on tumor promotion are planned. One such study, which has already been initiated, involves the use of DENA as an initiator and DOT as a promoter.

Publications

None

CONTRACT IN SUPPORT OF THIS PROJECT

HAZLETON LABORATORIES AMERICA, INC. (NO1-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: Z01CE04548-12 OD

PERIOD COVERED

October 1, 1983 to September 30, 1984

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	DCE	NCI

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 spece 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 2,396 (177 since the 1983 report) single or group accessions from investigators outside the NCI and approximately 59,602 records have been coded. Thirty-five investigators have come to the Registry for study and consultation on single or multiple visits.

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	IPA 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 much 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 2,396 single or group accessions from investigators outside of NIH have been processed since the 1983 report. 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," and "Adrenal Tumors of Mice." These Study Sets, with descriptive material, are loaned to investigators who request them. Twenty-seven loans have been made this year.

Investigators come to the Registry for study and consultation. There have been single or multiple consultations with 35 individuals since the 1983 report.

Major Findings:

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

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.

The Subcommittee on Rat Liver Tumors, appointed by the Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, carried out its work at the Registry of Experimental Cancers where it met one or two days each month over a period of 18 months. The members of the Subcommittee studied the pathologic material in the Registry's Study Set on Rat Liver Tumors and other pathologic material accessed at the Registry. The publication of the report entitled "Histologic Typing of Liver Tumors of the Rat" appeared in January of 1980 (J. Natl. Cancer Inst. 64: 177206, 1980). During the period from January 1980 until April 30, 1984, the Registry has received 8,602 requests for reprints. 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 26 October 1976 and 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

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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, England, Bergaman (In Press)

Newberry, B. H., Liebelt, A. G. and Boyle, D. A. Variables in behavioral oncology: Overview and assessment of current issues. In Fox, B. H. and Newberry, B. H. (Eds.): Impact of Psychoendocrine Systems in Cancer and Immunity. New York, C. J. Hogrefe, Inc., 1984, pp. 86-146.

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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. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE06134-09 OD

PERIOD COVERED
October 1, 1983 to September 30, 1984

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 spece provided.)*

The role of the lymphatic system in the absorption and biodistribution of antitumor agents and monoclonal antibodies administered by IV and SC routes in normal and tumor-bearing rodents is under investigation. Following subcutaneous injection, monoclonal antibodies are delivered via lymphatic channels to regional lymph nodes. Initial studies in mice with monoclonal antibodies to murine histocompatibility antigens showed that over 50% of the dose absorbed from a subcutaneous injection site bound specifically to cells in regional lymph nodes. Similar findings have now been obtained for monoclonal antibodies to B and T lymphocytes which may offer an alternative to radiocolloids in detecting gross abnormalities in lymphatic drainage and lymph node chains by lymphoscintigraphy. Study of in vitro binding characteristics combined with a variety of in vivo pharmacological parameters affecting lymphatic delivery of antibodies has enabled us to develop a firm quantitative understanding of the delivery process with the aid of computer modeling systems. Using this delivery system for a monoclonal antibody against guinea pig line 10 hepatocarcinoma, we have succeeded in detecting early metastatic tumor in lymph nodes of guinea pigs by gamma camera imaging. Attempts at therapy in the guinea pig model using antibody coupled to ricin toxin are currently in progress. Information obtained from studies in guinea pigs has been applied to detection of clinical stage II malignant melanoma and protocols for similar trials have been developed for breast cancer, non-small cell lung cancer and lymphoma.

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 describe 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 establishing a quantitative basis for lymphatic delivery of monoclonal antibodies to lymph nodes from which principals may be applied to clinical settings for immunodiagnosis and immunotherapy. Therapeutic protocols will be developed for antibody-toxin conjugates using the guinea pig L10 tumor system and other animal models of metastatic tumors.

Major Findings:

Monoclonal antibodies against normal cell types were studied initially in order to define the processes by which antibodies are delivered to regional lymph nodes from a subcutaneous injection site. Studies in mice given subcutaneous injection of I-125 labelled monoclonal antibody (anti-K^k) against a murine histocompatibility antigen suggested that lymphatic delivery may provide higher sensitivity, lower background, lower systemic toxicity and faster localization than intravenous injection. Using anti-K^k, in vivo pharmacologic parameters likely to affect lymphatic uptake of antibodies given SC were studied, including dose of antibody, choice of injection site, injection volume and osmolarity. These studies, along with determination of in vitro binding characteristics, were performed in order to optimize uptake of antibody and provide a quantitative understanding of the delivery system. Dose and antibody binding characteristics appear to be the major factors influencing the amount of antibody ultimately bound to cells in regional lymph nodes. Similar studies using antibodies against murine B and T cell sub-populations demonstrate the capability of targeting to specific cell types in lymph nodes for modulating immune function. Attempts to increase lymphatic uptake of antibodies from a subcutaneous injection site by co-administration of pharmacological agents that effectively increase regional lymph flow by increasing peripheral blood flow or capillary permeability are currently being investigated. Monoclonal antibodies externally labelled with radioiodine are widely used both for in vitro and in vivo studies and in diagnostic immunoscintigraphy. However analysis of blood and tissues from mice receiving I-125 or I-131 labelled antibody indicate extensive dehalogenation of antibody occurring within a few hours of injection. The questionable integrity of antibodies labelled with radioiodine for long-term biodistribution and metabolic studies prompted our development of antibodies labelled internally with 14-C or tritium. In vivo metabolic and biodistribution

studies using these antibodies are currently in progress. In addition to antibodies against normal cell types, we have studied lymphatic delivery following SC injection of antibody to a hepatocarcinoma in guinea pigs. Highly specific antibody localization was demonstrated in lymph nodes by gamma camera imaging where tumor occupied only approximately 3% of the total weight of the node. Antibody against normal lymphoid cells has been studied in tandem with antitumor antibody in these animals, the former indicating gross anatomical changes in the node and the latter the presence of tumor in the node.

Significance to Biomedical Research and the Program of the Institute:

Monoclonal antibody conjugation is a potentially useful delivery system for therapeutic and diagnostic agents; this project represents an attempt to improve the selectivity of therapeutic agents by targeting them, through monoclonal antibodies, to specific sites. 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 in regional and even distant lymph nodes. It would, therefore, 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.

Proposed Course:

(1) To continue to pursue goals listed under "Objectives" above. (2) To further develop systems for in vivo delivery of monoclonal antibodies to B and T cell sub-populations as a method of modulating immune function. (3) To continue studies directed toward better describing the in vivo metabolism of monoclonal antibodies using internally labelled antibody. (4) To attempt to improve uptake of monoclonal antibodies from subcutaneous injection sites by co-injection of pharmacological agents that increase regional lymph flow.

Publications

Khato, J., Chirigos, M. and Sieber, S. M.: Antimetastatic effects of maleic anhydride-divinyl ether in rats with mammary adenocarcinoma. J. Immunopharmacol. 5: 65-77, 1983.

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Weinstein, J. N., Steller, M. A., Covell, D. G., Holton, O. D., III, Keenan, A. M., Sieber, S. M. and Parker, R. J.: Monoclonal antibodies in the lymphatics. Cancer Treat. Rep. 68: 257-264, 1984.

Weinstein, J. N., Steller, M. A., Keenan, A. M., Covell, D. G., Key, M. E., Sieber, S. M., Uldham, R. K., Hwang, K. M. and Parker, R. J.: Monoclonal antibodies in the lymphatics: Selective delivery to lymph node metastases of a solid tumor. Science 222: 423-427, 1983.

BIOLOGICAL CARCINOGENESIS PROGRAM

ANNUAL REPORT OF

THE LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1983 to September 30, 1984

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 with 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 activitation, as well as the mode of action of their translational products.

During the past year, our accomplishements 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. Investigators within the Laboratory also discovered a viral oncogene whose tyrosine kinase coding sequence respresents a new member of the expanding family of oncogenes with tyrosine kinase activity. A striking finding was that cellular sequences captured by the virus also included information for a portion of an actin gene. Investigation of the role of actin sequences in this transforming gene is a subject of current study.

During the past year, our investigations of members of the human ras gene family have led to evidence of the high frequency at which these genes are activtated as oncogenes in diverse human tumors as well as knowledge that the major mechanisms of activation involve point mutations at positions 12 or 61 on their coding sequences. Additionally, we established that ras oncogene activation in certain tumors represents somatic events exquisitely selected

for within the tumor cell population. Finally, we uncovered additional examples of the activation at high frequency of one but not other oncogenes in specific carcinogen-induced tumors. We believe that all of these findings argue convincingly for the involvement of ras oncogenes in the human neoplastic process. The major responsibilities for research on transforming genes of retroviruses and cancer cells are within the Molecular Biology, Gene Structure, and Molecular Genetic Sections of this Laboratory.

We demonstrated the first direct link between an onc gene and a known biological function. The simian sarcoma virus (SSV) onc gene, v-sis, has been sequenced and its 28,000 molecular weight (MW) product, p28sis, identified by means of antisera prepared against small peptides derived from sequence analysis of v-sis. Studies on human platelet-derived growth factor (PDGF), a potent mitogen for cells of connective tissue origin, have led to the elucidation of its amino terminal amino acid sequence. Computer comparison of this and additional protein sequence data with the predicted amino acid sequence of p28sis has revealed an extraordinary degree of homology between PDGF and p28sis, implying that the two proteins have arisen from the same or closely related cellular genes. We further demonstrated that p28sis rapidly undergoes a series of discrete processing steps including dimes formation and proteolytic digestion to yield molecules structurally and immunologically resembling biologically active PDGF.

In an effort to obtain sufficient quantitites of the SSV transforming gene product for the detailed analysis of its structural and biologic properties, we placed the v-sis gene under the control of strong phage transcriptional and translational signals that provide for regulated expression of cloned genes in E. coli. When induced, the bacterial cells synthesized levels of the SSV transforming gene product that constituted at least 10% of their total protein. Differences in the structure and processing of the v-sis gene product in prokaryotic and eukaryotic cells provided important insights concerning post-translational modifications of this PDGF-related transforming protein in eukaryotic cells.

The human locus related to v-sis was cloned and shown to contain at least 5 exons corresponding to the v-sis coding region. Nucleotide sequence analysis of these exons revealed that the predicted amino acid sequence of human c-sis differed by 6% from that of the woolly monkey derived v-sis. These findings imply that the sis proto-oncogene has been well conserved during primate evolution. By comparison of the known amino acid sequences of PDGF peptides with the predicted human c-sis protein, it was possible to demonstrate that this human proto-oncogene is the structural gene encoding one of the two major polypeptides of this potent mitogen for connective tissue 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 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 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. These findings establish that the normal coding sequence for a human growth factor has transforming activity when provided necessary signals for transcription and initiation of translation.

We surveyed 22 human hematopoietic tumors and tumor cell lines for sequences capable of transforming NIH/3T3 cells by DNA transfection. A primary human acute myelogenous leukemia, a chronic myelogenous leukemia cell line, and cell lines derived from three independent acute lymphocytic leukemias demonstrated oncogenes capable of conferring the transformed phenotype to NIH/3T3 cells through serial cycles of transfection. One of three transforming genes associated with acute lymphocytic leukemia cells (classified as thymocyte development stage II) was identified as the activated cellular homologue of the Kirsten murine sarcoma virus onc gene, kis, a member of the ras family of onc genes. A transforming gene, N-ras, was demonstrated to be common to several human myeloid and lymphoid tumor cells. Thus, the NIH/3T3 transfection assay commonly detects related ras oncogenes in human hematopoietic tumor cells. Moreover, the activation of these oncogenes appears to be independent of the specific stage of cell differentiation or tumor phenotype.

An N-ras-related transforming gene was detected in the human lung carcinoma cell line, SW-1271, and molecularly cloned in biologically active form. 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.

Oncogenes capable of transforming NIH/3T3 cells are often present in human tumors and tumor cell lines. Such oncogenes were not detected in normal fibroblast lines derived from patients with several clinical syndromes associated with greatly increased cancer risk. Thus, germ-line transmission of these oncogenes does not appear to be the predisposing factor responsible for these high cancer risk syndromes.

A large proportion of oncogenes so far detected by DNA transfection are related to the H-ras onc gene of Harvey (and BALB) murine sarcoma viruses (MSV), K-ras (the oncogene of Kirsten MSV), and a third member of the ras gene family, N-ras. Individual tumors of many different organs have been associated with the activation of members of the ras gene family. We undertook a systematic survey of human urinary tract tumors, processed immediately after surgery, as well as normal tissues from the same patients, for the presence of such genes. We demonstrated activation of H-ras as an oncogene in around 10% of unselected urinary tract tumors as well as direct evidence that oncogene activation was the result of a somatic event selected for within the tumor cell population.

Among 21 human mammary tumors analyzed for transforming genes by NIH/3T3 transfection, only DNA of a carcinosarcoma cell line, HS578T, registered as positive. An H-ras oncogene identified in this line 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 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 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.

Two of the six possible single point mutations that can activate the human K-ras locus by altering the coding properties of its 12th codon create restriction enzyme polymorphisms. These polymorphisms have been utilized to develop biochemical assays that discriminate between normal and transforming K-ras genes. Our investigators used such assays to determine the mechanism of activation of K-ras oncogenes in A1698 bladder carcinoma and A2182 lung carcinoma human tumor cell lines resulting from mutations within the 12th codons. Moreover, we established that a single point mutation responsible for the malignant activation of a K-ras oncogene is present in tumor but not in normal tissue of a patient with a squamous cell lung carcinoma. These results further demonstrate an association between activation of ras oncogenes by specific mutational mechanisms and the development of certain human cancers.

Gullino and co-workers have shown that exposure of 50-day-old Buf/N female rats to nitroso-methylurea (NMU) results in the induction of mammary carcinomas in 90% of the animals with a latent period of about 60 days. The carcinogenic effect of NMU appears to be hormonally regulated. Castration of the rats before NMU injection decreases incidence of tumors to negligible levels. However, if the rats are ovariectomized after NMU treatment, tumors appear with the same frequency as in non-ovariectomized controls, although the number of carcinomas per animal decreases substantially and a general increase in the latency period is observed. These properties made this system suitable not only to study the reproducible activation of oncogenes but also to investigate its putative modulation by physiological factors.

Each of nine mammary carcinomas induced by a single injection of nitroso-methylurea into 50-day-old Buf/N female rats contained a transforming H-ras gene. Molecular characterization of one of the genes revealed that the 12th codon was GAA instead of GGA of the normal allele, encoding glutamic acid in place of glycine.

DNA of a non-Hodgkin's lymphoma induced morphologic transformation of NIH/3T3 cells. First cycle transfectant DNA contained human repetitive sequences and was able to serially transmit the transformed phenotype in additional cycles of transfection. Southern blot analysis showed that specific human DNA

fragments cosegregated with the transformed phenotype through the series of transfections. The pattern of these fragments indicated that this human gene was likely to be 20 kbp or even larger in size. Moreover, the transforming activity of this human gene was inactivated by several restriction enzymes including Hind III, Eco RI, Bam HI, Bgl II, Pst I, and Sac I. NIH/3T3 cells transformed by this gene exhibited strikingly different morphologic properties from transfectants induced by ras oncogenes, frequently identified in human hematopoietic neoplasms. Moreover, hybridization analysis of representative second and third cycle transfectants with DNA probes of a large number of oncogenes showed that the transforming gene of this human lymphoma was not related to any of the ras genes nor to several other retroviral and cellular onc genes.

To better understand the relationship between ras oncogenes and other human genes, we have determined their chromosomal localization by analyzing human rodent somatic cell hybrids with molecularly cloned human proto-oncogene probes. It was possible to assign N-ras to chromosome 1 and regionally localize c-K-ras-1 and c-K-ras-2 to human chromosomes 6pter-q13 and 12q, respectively. These results along with previous studies demonstrate the highly dispersed nature of ras genes in the human genome. Previous reports indicated that the c-myb gene also resides on chromosome 6. It has been possible to sublocalize c-myb to the long arm of chromosome 6 (q15-q21). The non-random aberrations in chromosomes 1, 6 and 12 that occur in certain human tumors suggest possible etiologic involvement of ras and/or myb oncogenes in such tumors.

Gardner-Rasheed feline sarcoma virus (GR-FeSV) is an acute transforming retrovirus which encodes a gag-onc polyprotein possessing an associated tyrosine kinase activity. The integrated form of this virus, isolated in the Charon 21A strain of bacteriophage λ, demonstrated an ability to transform NIH/3T3 cells at high efficiency upon transfection. Foci induced by GR-FeSV DNA contained rescuable sarcoma virus and expressed GR-P70, the major GR-FeSV translational product. The localization of long terminal repeats within the DNA clone made it possible to establish the length of the GR-FeSV provirus as 4.6 kilobase pairs. The analysis of heteroduplexes formed between λ feline leukemia virus (FeLV) and λ GR-FeSV DNAs revealed the presence of a 1,700-base-pair FeLV unrelated segment, designated v-fgr, within the GR-FeSV genome. The size of this region was sufficient to encode a protein of approximately 68,000 daltons and was localized immediately downstream of the FeLV gag gene coding sequences present in GR-FeSV. Thus, it is likely that this 1.7-kilobase-pair stretch encodes the onc moiety of GR-P70. Utilizing probes representing v-fgr, we detected homologous sequences in the DNAs of diverse vertebrate species, implying that v-fgr originated from a well-conserved cellular gene. The number of cellular DNA fragments hybridized by v-fgr-derived probes indicated either that proto-fgr is distributed over a very large region of cellular DNA or represents a family of related genes. By molecular hybridization, v-fgr was not directly related to the onc genes of other known retroviruses having associated tyrosine kinase activity.

The nucleotide sequence of the region of v-fgr encoding its primary translation product, P70gag-fgr, was determined. From the nucleotide sequence, the amino acid sequence of this transforming protein was deduced. Computer analysis indicates that a portion of P70gag-fgr has extensive amino acid sequence homology with actin, a eukaryotic cytoskeletal protein. A second

region of P70gag-fgr is closely related to the tyrosine-specific kinase gene family. Thus, the v-fgr oncogene appears to have arisen as a result of recombinational events involving two distinct cellular genes, one coding for a structural protein and the other for a protein kinase.

The nucleotide sequence of the proviral genome of Abelson murine leukemia virus (A-MuLV), an acute transforming virus of murine origin, was also determined. Like other transforming viruses, A-MuLV contains sequences derived from its helper virus, Moloney murine leukemia virus (M-MuLV), and a cell-derived proto-oncogene (abl) insertion sequence. By comparison of the A-MuLV sequence with that of M-MuLV, it was possible to precisely localize and define sequences contributed by the host cellular DNA. From the nucleotide sequence, we have predicted the amino acid sequence of P120gag-abl, the product of the A-MuLV gag-abl hybrid gene. The amino acid sequence of the putative abl gene, when compared with the sequences of other tyrosine-specific protein kinases (src, fes, fps, and yes), revealed significant homologies, indicating that all these functionally related transforming genes are derived from divergent members of the same proto-oncogene family. In addition to the gag-abl sequence, the proviral genome was found to contain an additional open reading frame that could code for an 18,000-dalton protein, whose role is at present undetermined.

Three types of tumors termed plasmacytomas (ABPCs), lymphosarcomas (ABLSs), and plasmacytoid lymphosarcomas (ABPLs) arise in BALB/c mice treated with pristane and Abelson murine leukemia virus (A-MuLV). While most ABPCs and ABLSs contain integrated A-MuLV proviral genome and synthesize the v-abl RNA, most ABPLs do not. The ABPL tumors were examined for the expression of other oncogenes that may be associated with their transformed state in the absence of transforming virus. These tumors expressed abundant c-myb RNA of unusually large size and showed DNA rearrangements of the c-myb locus. Molecular cloning of the mouse c-myb locus and its rearranged counterpart in the ABPL-2 tumor revealed that the alteration in this locus is due to insertion of a defective MuLV proviral genome containing both LTRs upstream to the v-myb-related sequences. This insertion interrupts the c-myb coding region in a manner similar to that observed in the generation of avian myeloblastosis virus.

The nucleotide sequence of the integrated proviral genome of avian myelocytomatosis virus (MC29) coding for gag-myc protein was determined. By comparison of this nucleotide sequence with the helper virus as well as the c-myc region, it was possible to localize the junction points between helper viral and v-myc sequences. These studies demonstrate that (1) the large terminal repeat sequence of MC29 is very similar to that of Rous sarcoma virus; (2) the viral genome has suffered extensive deletions in the gag, pol, and env genes; (3) the gag region can code for p19, p10, and part of p27; (4) the recombination between viral and cellular sequences occurred in the coding region of p27 such that the open reading frame extends for an additional stretch of 1,266 base pairs, resulting in a gag-myc hybrid protein; (5) the open reading frame terminated within the v-myc region 300 bases upstream of v-myc-helper viral junction; and (6) the v-myc helper-viral junction at the 3' end occurred in the middle of env gene, rendering it defective.

The genome of the replication-defective avian myeloblastosis virus (AMV) contains an inserted cellular sequence (amv) that is part of the oncogene responsible for acute myeloblastic leukemia in chickens infected with AMV. Three antisera raised against distinct synthetic peptides predicted from the long open reading frame of amv specifically precipitated the same 48-kilodalton protein (p48amv) from leukemic myeloblasts but not from normal hematopoietic tissue, fibroblasts, or from fibroblasts infected with the AMV helper virus, MAV-1 (myeloblastosis-associated virus type 1). p48amv is not glycosylated or phosphorylated and does not appear to act as a protein kinase in vitro. The same three antisera that recognized p48amv also specifically precipitated a common 110-kilodalton protein from normal uninfected hematopoietic tissue. This normal cellular homologue of the AMV leukemogenic protein, P110proto-amv, was not present in normal fibroblasts, MAV-1 infected fibroblasts, or, interestingly, in some leukemic myeloblasts. We conclude that p48amv is the leukemogenic product of an altered, transduced, partial proto-oncogene. Short helper-virus sequences provide its carboxyl terminus and also may provide the amino terminus.

Our efforts to investigate cells within the hematopoietic system which are susceptible targets for transformation by retroviral onc genes have continued. A recombinant murine retrovirus (MRSV) containing the src gene of avian Rous sarcoma virus (RSV) was shown to induce hematopoietic colonies in infected mouse bone marrow. MRSV-induced colony formation followed single-hit kinetics and required mercaptoethanol in the agar medium. Cells from the colonies induced by MRSV could be established as continuous cell lines that demonstrated unrestricted self-renewal in vitro and tumorigenicity in vivo. The transformants, all of which expressed high levels of the Rous sarcoma virus transforming protein, pp60src, appeared to be at an early stage in lymphoid cell differentiation. They lacked Fc receptors and detectable immunoglobulin μ heavy chain synthesis, markers normally associated with committed B-cells. The majority of the MRSV-transformed cell lines contained high levels of terminal deoxynucleotidyl transferase, an enzyme present in lymphoid progenitor cells committed to the T-cell lineage. One cell line expressed Thy-1 antigen, but none expressed Lyt-1 and Lyt-2, markers of more differentiated T-cells. These findings demonstrate that the src gene is capable of transforming cells of hematopoietic origin.

The retroviral long terminal repeat (LTR) contains transcriptional control elements that affect viral gene expression. By deletion mutagenesis of the genome of the cloned Abelson murine leukemia virus, regulatory signals could be mapped to at least three domains within the LTR. A defective 5' LTR that did not sustain transforming gene function was complemented by an intact LTR positioned at the 3' end of the genome. This versatility of the retroviral genome with respect to its transcriptional control elements appears to provide a strong selective advantage for viral gene expression.

The role of LTR sequences in the efficient expression of Moloney murine sarcoma virus (MSV-124) transforming gene function was also 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 1,000-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.

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, arylodictyles, carnivores and 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, including primates, 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.

The closed circular form of the endogenous squirrel monkey type D retrovirus (SMRV) was molecularly cloned in a bacteriophage vector. The restriction map of the biologically active clone was determined and found to be identical to that of the parental SMRV linear DNA except for the deletion of one long terminal repeat. Restriction enzyme analysis and Southern blotting indicated that the SMRV long terminal repeat was approximately 300-base-pairs long. The SMRV restriction map was oriented to the viral RNA by using a gene-specific probe from baboon endogenous virus. Restriction enzyme digests of a variety of vertebrate DNAs were analyzed for DNA sequence homology with SMRV by using the cloned SMRV genome as a probe. Consistent with earlier studies, multiple copies of SMRV were detected in squirrel monkey DNA. Related fragments were also detected in the DNAs from other primate species, including humans.

The genetic relationships among molecularly cloned prototype viruses representing all of the major oncovirus genera were investigated by molecular hybridization and nucleotide sequence analysis. We demonstrate two major progenitors of the pol genes of such viruses, one giving rise to mammalian

type C viruses and the other to type A, B, D and avian type C oncoviruses. Evidence of unusual patterns of homology among the env genes of mammalian type C and D oncoviruses illustrate further that genetic interactions between their progenitors have contributed to the evolution of oncoviruses.

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 of the past year are in two research areas: mechanisms of DNA damage and repair, and environmental influences on proliferation and expression of differentiated function in cultured cells, particularly epithelial cells, for analysis of neoplastic transformation in vitro.

Results from irradiating cells during G_2 phase or within 1.5 hours of metaphase together with use of DNA repair inhibitors have implicated deficiences in DNA repair during G_2 prophase in skin fibroblasts from cancer-prone individuals. Ten lines of skin fibroblasts from individuals with genetic disorders predisposing to a high risk of cancer were compared with nine lines from normal adult donors with respect to chromatid damage after x-irradiation [25, 50, and 100 rad (0.25, 0.05, and 1 gray)] during G_2 phase. The 10 cell lines represented five genetic disorders: Bloom syndrome, familial polyposis, Fanconi anemia, Gardner syndrome, and xeroderma pigmentosum, complementation groups A(XP-A), C(XP-C), E(XP-E), and variant (XP-Va). The incidence of chromatid breaks in all cancer-prone lines except XP-E and XP-A was significantly higher than in the normal lines. The incidence of chromatid gaps in all cancer-prone lines except XP-A and XP-Va was significantly higher than in the normal lines. Because each chromatid apparently contains a single continuous DNA double strand, chromatid breaks and gaps represent unrepaired DNA strand breaks arising directly or indirectly during excision repair of x-ray-induced DNA damage. These cytogenetic data together with results from use of the DNA repair inhibitor arabinofuranosyl cytosine (cytosine arabinoside) suggest that cells from all of these cancer-prone individuals are deficient in some step of DNA repair, predominantly excision repair operative during the G_2-prophase period of the cell cycle. It appears that these DNA repair deficiencies are associated with a genetic predisposition to a high risk of cancer.

Five lines of skin fibroblasts from individuals heterozygous for ataxia-telangiectasia (A-T), compared with six cell 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 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, 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. The discovery of a DNA repair defect(s) in skin fibroblasts from individuals genetically predisposed to a high risk of cancer suggests that the acquisition of defects in DNA repair operative during G_2 prophase is prerequisite, possibly the initiation step, for human carcinogenesis.

Hemicyst formation is considered a manifestation of either transepithelial solute and fluid movement or secretory activity in culture. Hemicyst formation in postconfluent monolayers of rhesus monkey kidney (LLC-MK_2) cells is modulated by the dissolved oxygen concentration (PO_2). Either daily replacement of serum-free medium or displacement of the gas phase with 18% vol/vol O_2 (initial medium PO_2 = 125 to 135 mm Hg) enhances formation of hemicysts. Use of 30% O_2 (medium $PO_2 \cong$ 175 mm Hg) does not further increase the incidence, but neither 10% O_2 (medium PO_2 = 90 to 95 mm Hg) nor 1% O^2 (medium PO^2 = 35 to 50 mm Hg), the approximate range of dissolved oxygen values in blood, supports hemicyst formation unless cultures are gently rocked to disrupt diffusion gradients. Phase photomicrography of living cultures shows that the surface of a turgid hemicyst is furrowed, and cinephotomicrography reveals that the walls vibrate subtly. When hypoxic conditions (0 to 1% O_2) are introduced, this vibration ceases within 2 to 3 h, whereas collapse and disappearance of turgid hemicysts requires 18 to 20 h, seems virtually synchronous, and is reversible. Hemicysts form in a broad osmotic range, and increased electrolyte concentration increases the incidence. Hemicysts persist in locally dense areas when cell-free strips are etched in the postconfluent monolayer; no DNA synthesis is detected under these conditions, but two-dimensional cell spreading into the denuded area is seen along the edge of the wound. We conclude that the dissolved oxygen supply in the cellular microenvironment modulates functional expression by differentiated kidney epithelial cells in culture and that increased electrolyte concentration also enhances expression of this phenotypic marker.

Since a wide variety of carcinogenic agents induce DNA-protein cross-links, these lesions may be important in the complex genetic and/or epigenetic processes leading to malignancy. DNA repair is an essential cell response to carcinogenic agents for maintaining viability, function, and their integrated nonmalignant state. Others have shown that these cross-links are repaired by normal cells but that excision repair-deficient xeroderma pigmentosum (XP) Group A cells, XP12BE, are deficient in repair of these bulky adducts. We compared the DNA-protein cross-link repair competency of another XP Group A strain, XP20S, with its more rapidly proliferating simian virus 40-transformed derivative line and with normal human skin fibroblasts. DNA-protein cross-links were induced with 20 μM trans-platinum(II)diammine-dichloride and assayed by the membrane alklaline elution procedure of Kohn. DNA-protein cross-links retard elution of DNA. The repair competency of XP20S cells for trans-platinum(II)diamminedichloride-induced DNA-protein cross-links was similar to that of XP12BE cells, but the competency of the simian virus 40-transformed XP20S cells was nearly equal to that of normal human skin fibroblasts. These results suggest that either cell cycling compensates for the genetic deficiency present in the nucleotide excision process of XP Group A cells or that a process other than nucleotide excision can repair these lesions; this process requires cell cycling or activation by the virus.

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 throughout the country. The ultimate goal of these multidisciplinary studies of virus-induced and spontaneously occurring cancers is to apply the basic information derived to its most important application, the prevention of cancer in man.

CONTRACT IN SUPPORT OF ALL LABORATORY PROJECTS:

HAZLETON LABORATORIES AMERICA, INC. (NO1-CE-01017)

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

Current Annual Level: $307,000

Man Years: 6

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 at the NIH Bethesda campus in December, 1983. As a result, there has been a considerable reduction in level of effort. Additional reductions are anticipated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04805-14 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

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

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

PI: A. Hellman Assistant Chief LCMB NCI

COOPERATING UNITS *(if any)*

FDA, National Center for Medical Diseases and Public Health, Rockville, MD
(M. Swicord and K. B. Hellman)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK *(Use standard unraduced type. Do not excaed the space providad.)*

Excessive morbidity and mortality for leukemia among male workers in occupations emitting electrical fields have been reported. Beneficial efforts in the acceleration of bone and wound healing also appears in the literature and is being applied in clinical medicine. Although the biological mechanism underlying these phenomena are not well understood, it has been suggested that cell membrane receptor-ligand interactors are either directly or indirectly influenced. Since the cell membrane plays an important role in conducting exogenous messages into the cell, its modification may be a significant factor in subsequent cellular responses. Utilizing two well-recognized membrane responder systems, we are investigating the influence of pulsed electrogenetic fields on macrophage and lymphocyte activities. We have observed measurable and reproducible effects brought about by 5.2 msec bursts of bipolar repeats of 15 Hz energy bursts.

PROJECT DESCRIPTION

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

A. Hellman Assistant Chief LCMB NCI

Objectives:

Bone and wound healing are reported to be accelerated when the affected areas are exposed to certain pulsed electromagnetic fields (PEMF). Similarly, recent epidemiological reports suggest an excess of morbidity and mortality from leukemia among male workers in electrical occupations. We are attempting to gain a better biological understanding underlining both of these observations.

Methods Employed:

Certain cells of the immune system are subjected to PEMF and their biological responses measured.

Major Findings:

Exposure of Ficoll-purified mouse splenic lymphocytes to PEMF influenced the incorporation of ^{3}H-thymidine subsequent to phytohemagglutinin stimulation. The effect was dependent on the duration of exposure in either enhancing at short exposure time or decreasing blast transformation in the absence of reducing cell viability. The unidirectional migration of macrophages along a concentration gradient of chemoattractant (chemotaxis) was significantly decreased by up to 51% of contact cultures shortly after exposure to short bursts of PEMF. Removal of these cultures from the field for 4 hrs resulted in a return to normal chemotatic activity, suggesting only a transitory PEMF effect.

Significance to Biomedical Research and the Program of the Institute:

These data suggest that macrophage and lymphocyte functions are a biological indicator of PEMF effects and are reliable biological dosimetry systems. Additionally, they provide the first reliable in vitro system that permits a detailed investigation of PEMF action both in this environment and as a clinical tool.

Proposed Course:

We are continuing to utilize this model system to evaluate various pulsed width and duration effects in order to develop optimum clinical data, as well as to study PEMF interaction of the cell membrane.

Publications:

Hellman, K. B., Hellman, A. and Forsher, A. K.: Pulsed electromagnetic fields influence functional responses of immunocompetent cells. In Pilla, A.A. (Ed.): Ions, Membranes and the Electrochemical Control of Cell Function. Amsterdam, Verlag Chemie. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE04930-13 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters 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	Microbiologist	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NCI

COOPERATING UNITS *(if any)*

CA Dept. Health Services (R. Emmons); Peralta Cancer Inst. (A. Hackett); Univ. of CA (M. Gardner, J. Levy, H. Rubin and M. Stampfer); Children's Hospital Med. Ctr. (K. Walen)

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 vivo studies of the simian sarcoma transforming gene in susceptible mice suggest unique biologic effects, distinctly different from other known oncogenes and including induction of long delayed, slowly growing sarcomas. These neoplasms are currently under intensive laboratory analysis.

Transformation studies using human epithelioid cells infected with DNA and RNA tumor viruses have shown that at least two significant steps are needed to produce fully malignant transformation of normal epithelium.

Collaborative studies on transplantability of human and animal tumors in athymic nude mice have furnished new data on tumor biology and malignant transformation.

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	Microbiologist	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI
J. Pierce	Sr. Staff Fellow	LCMB	NCI
A. Eva	Visiting Associate	LCMB	NCI
M. H. Kraus	Visiting Fellow	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 morphologic transformation with transplantability and tumor production in athymic nude mice.

3. Document tumor induction (neoplastic graft) in athymic nude mice using spontaneous human cancers as well as selected animal specimens; correlate positive neoplastic graft results with malignancy-related gene sequences (oncogenes) and viruses detected.

Methods Employed:

1. In vitro portions of the viral gene experiments are collaborative studies in Dr. Aaronson's and Dr. Robbins' projects (Z01CE04940-17 LCMB and Z01CE05167-04 LCMB). Candidate inocula are furnished in coded vials as deep-frozen aliquots. 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). 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 tissue 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).

An important oncogene, that of the simian sarcoma virus (SSV), is under intensive study in vivo. 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, Drs. Aaronson and Robbins have

constructed fully mapped viruses containing the SSV oncogene in type C virus "packages" which are infectious for newborn mice and are now on test for tumor production.

2. Studies on neoplastic transformation of normal primate as well as some selected nonprimate cells are principally collaborative with Dr. Rhim (Z01CE05060-06 LCMB). 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 line are tested in parallel in athymic nude mice for abililty to form neoplasms. The tumor tissue is reestablished in culture to confirm primate 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 defined murine type C viruses containing the v-sis oncogene have been inoculated by all routes described (Methods). Euthymic and athymic nude newborn mice have been used in these experiments. In contrast to other acute oncogenes (e.g., v-ras, v-mos) which produce local progressive tumors in 10-30 days, the v-sis inocula have never induced early neoplasia. Solid tumors are detected 4-8 months after inoculation by the subcutaneous and intramuscular routes and progress relatively slow, but ultimately reach large size. They are not as rapidly lethal nor invasive as are v-mos- and v-ras-induced tumors. Histologically they have been classed as fibrosarcomas and to date have affected about 10% of inoculated mice at risk. A few intracerebral sarcomas have also been detected at 4-6 months after intracerebral challenge. The neoplasms are transplantable to adult athymic nude mice. Portions of these tumors have been frozen and will be analyzed biochemically and virologically. Cell lines have been established from most sarcomas and are also under study.

2. Transformation studies. In collaboration with Dr. Rhim, transformed human keratinocytes are tested for tumor production in adult and suckling nude mice. The cells are "immortalized" by infection with the hybrid virus adenovirus 12/simian virus 40 (Ad 12/SV40). At this stage they exhibit some properties of neoplasia (high saturation density in vitro, rapid growth, low serum requirement), but do not usually produce tumors in nude mice. When infected with a second virus, Kirsten sarcoma baboon pseudotype, the cells undergo

a second more dramatic change, lose contact inhibition, become heterogeneous and also become tumorigenic, causing progressive typical squamous cell carcinomas which grow rapidly in suckling athymic nude mice, and more slowly in adult nudes. Using 10^7 xenografted cells, the tumor incidence approaches 100% in adults and sucklings. This system is now being studied for its applicability in chemical and transfection oncogenesis studies.

A similar "two-hit" transformation can be shown with epithelioid cells from human amniotic fluid infected with SV40 and Kirsten sarcoma virus. This phenomenon is under study in collaboration with the amniocentesis team at Children's Hospital Medical Center, Oakland, CA.

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

In other experiments, 3T3 cultures transfected with putative human onc sequences have been characterized for tumor production in athymic nude mice.

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) lymphocytes 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. Using murine and simian oncogenes is a preliminary step to studies of their cloned human counterparts.

The ability (or inability) of cultures to form malignant growth by xenografting to 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., Arnstein, P., Price, F. M., Sanford, K. K. and Aaronson, S. A.: Neoplastic transformation of human epidermal keratinocytes by the combined action of adenovirus 12-simian virus 40 hybrid virus and Kirsten murine sarcoma virus. Nature (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. (In Press)

Rubin, H., Chu, B. M. and Arnstein, P.: Heritable variations in growth potential and morphology within a clone of BALB/3T3 cells and their relation to tumor formation. J. Natl. Cancer Inst. 71: 365-375, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04940-17 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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	Acting Chief, Gene Structure Section	LCMB	NCI
	E. P. Reddy	Visiting Scientist	LCMB	NCI
	M. Barbacid	Visiting Scientist	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NCI
	J. Pierce	Sr. Staff Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

Univ. of CA, San Francisco, CA (L. Williams); Harvard Univ., School of Public Health, Boston, MA (H. Antoniades); CA Institute of Technology, Pasadena, CA (M. Hunkapiller); Harvard Medical School, Boston, MA (J. Greenberger)

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.

Some of the most important insights have been developed in the past year. LCMB's accomplishments include: characterization of oncogenes in a number of human cancers and their homologies with defined oncogenes of transforming retroviruses; identification of genetic lesions responsible for activating proto-oncogenes of onc genes into transforming genes as single base alterations which change single amino acids within the proteins coded by the gene; identification of onc genes activated by carcinogen-induced tumors in model systems; mapping the chromosomal locations of a number of onc genes in the human genome and demonstrating the specific rearrangement of one of these genes in translocations associated with Burkitt's lymphoma; detection of the products of previously unidentified onc genes and characterization of their functions.

PROJECT DESCRIPTION

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

S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Acting Chief, Gene Structure Section	LCMB	NCI
E. P. Reddy	Visiting Scientist	LCMB	NCI
M. Barbacid	Visiting Scientist	LCMB	NCI
K. C. Robbins	Expert	LCMB	NCI
J. Pierce	Sr. Staff Fellow	LCMB	NCI
A. Srinivasan	Visiting Associate	LCMB	NCI
Y. Yuasa	Visiting Fellow	LCMB	NCI
D. C. Swan	Expert	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
R. King	Staff Fellow	LCMB	NCI
M. Kraus	Visiting Fellow	LCMB	NCI
S. Needleman	Medical Staff Fellow	LCMB	NCI
S. Srivastava	Visiting Fellow	LCMB	NCI
R. Gol	Visiting Fellow	LCMB	NCI
W. McBride	Chief, Cellular Regulation Section	DCBD	NCI
R. Callahan	Chief, Oncogenetics Section	DCBD	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:

We demonstrated the first direct link between an onc gene and a known biological function. The simian sarcoma virus (SSV) onc gene, v-sis, has been sequenced and its 28,000 molecular weight (MW) product, p28^{sis}, identified by means of antisera prepared against small peptides derived from sequence analysis of v-sis. Studies on human platelet-derived growth factor (PDGF), a potent mitogen for cells of connective tissue origin, have led to the elucidation of its amino terminal amino acid sequence. Computer comparison of this and additional protein sequence data with the predicted amino acid sequence of p28^{sis} has revealed an extraordinary degree of homology

between PDGF and p28^{sis}, implying that the two proteins have arisen from the same or closely related cellular genes. We further demonstrated that p28^{sis} rapidly undergoes a series of discrete processing steps including dimes formation and proteolytic digestion to yield molecules structurally and immunologically resembling biologically active PDGF.

In an effort to obtain sufficient quantitites of the SSV transforming gene product for the detailed analysis of its structural and biologic properties, we placed the v-sis gene under the control of strong phage transcriptional and translational signals that provide for regulated expression of cloned genes in E. coli. When induced, the bacterial cells synthesized levels of the SSV transforming gene product that constituted at least 10% of their total protein. Differences in the structure and processing of the v-sis gene product in prokaryotic and eukaryotic cells provided important insights concerning post-translational modifications of this PDGF-related transforming protein in eukaryotic cells.

The human locus related to v-sis was cloned and shown to contain at least 5 exons corresponding to the v-sis coding region. Nucleotide sequence analysis of these exons revealed that the predicted amino acid sequence of human c-sis differed by 6% from that of the woolly monkey derived v-sis. These findings imply that the sis proto-oncogene has been well conserved during primate evolution. By comparison of the known amino acid sequences of PDGF peptides with the predicted human c-sis protein, it was possible to demonstrate that this human proto-oncogene is the structural gene encoding one of the two major polypeptides of this potent mitogen for connective tissue cells.

We surveyed 22 human hematopoietic tumors and tumor cell lines for sequences capable of transforming NIH/3T3 cells by DNA transfection. A primary human acute myelogenous leukemia, a chronic myelogenous leukemia cell line, and cell lines derived from three independent acute lymphocytic leukemias demonstrated oncogenes capable of conferring the transformed phenotype to NIH/3T3 cells through serial cycles of transfection. One of three transforming genes associated with acute lymphocytic leukemia cells (classified as thymocyte development state II) was identified as the activated cellular homologue of the Kirsten murine sarcoma virus onc gene, kis, a member of the ras family of onc genes. A transforming gene, N-ras, which was demonstrated to be common to several human myeloid and lymphoid tumor cells, was shown to be a distantly related member of the ras gene family. Thus, the NIH/3T3 transfection assay commonly detects related oncogenes in human hematopoietic tumor cells. Moreover, the activation of these oncogenes appears to be independent of the specific stage of cell differentiation or tumor phenotype.

An N-ras-related transforming gene was detected in the human lung carcinoma cell line, SW-1271 and molecularly cloned in biologically active form. 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.

Oncogenes capable of transforming NIH/3T3 cells are often present in human tumors and tumor cell lines. Such oncogenes were not detected in normal fibroblast lines derived from patients with several clinical syndromes associated with greatly increased cancer risk. Thus, germ-line transmission of these oncogenes does not appear to be the predisposing factor responsible for these high cancer risk syndromes.

A large proportion of oncogenes so far detected by DNA transfection are related to the H-ras onc gene of Harvey (and BALB) murine sarcoma viruses (MSV), K-ras (the oncogene of Kirsten MSV), and a third member of the ras gene family, N-ras. Individual tumors of many different organs have been associated with the activation of members of the ras gene family. We undertook a systematic survey of human urinary tract tumors, processed immediately after surgery, as well as normal tissues from the same patients, for the presence of such genes. We demonstrate activation of H-ras as an oncogene in around 10% of unselected urinary tract tumors as well as direct evidence that oncogene activation is the result of a somatic event which is selected for within the tumor cell population.

Among 21 human mammary tumors analyzed for transforming genes by NIH/3T3 transfection, only DNA of a carcinosarcoma cell line, HS578T, registered as positive. An H-ras oncogene identified in this line 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 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 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.

DNA of a non-Hodgkin's lymphoma induced morphologic transformation of NIH/3T3 cells. First cycle transfectant DNA contained human repetitive sequences and was able to serially transmit the transformed phenotype in additional cycles of transfection. Southern blot analysis showed that specific human DNA fragments cosegregated with the transformed phenotype through the series of transfections. The pattern of these fragments indicated that this human gene was likely to be 20 kbp or even larger in size. Moreover, the transforming activity of this human gene was inactivated by several restriction enzymes including Hind III, Eco RI, Bam HI, Bgl II, Pst I, and Sac I. NIH/3T3 cells transformed by this gene exhibited strikingly different morphologic properties from transfectants induced by ras oncogenes, frequently identified in human hematopoietic neoplasms. Moreover, hybridization analysis of representative

second and third cycle transfectants with DNA probes of a large number of oncogenes showed that the transforming gene of this human lymphoma was not related to any of the ras genes nor to several other retroviral and cellular onc genes.

The identification of transforming genes in human tumor cells has been made possible by DNA mediated gene transfer techniques. To date, it has been possible to show that most of these transforming genes are activated cellular analogues of the ras oncogene family. To better understand the relationship between these oncogenes and other human genes, we have determined their chromosomal localization by analyzing human rodent somatic cell hybrids with molecularly cloned human proto-oncogene probes. It was possible to assign N-ras to chromosome 1 and regionally localize c-K-ras-1 and c-K-ras-2 to human chromosomes 6pter-q13 and 12q, respectively. These results along with previous studies demonstrate the highly dispersed nature of ras genes in the human genome. Previous reports indicated that the c-myb gene also resides on chromosome 6. It has been possible to sublocalize c-myb to the long arm of chromosome 6 (q15-q21). The non-random aberrations in chromosomes 1, 6 and 12 that occur in certain human tumors suggest possible etiologic involvement of ras and/or myb oncogenes in such tumors.

Gardner-Rasheed feline sarcoma virus (GR-FeSV) is an acute transforming retrovirus which encodes a gag-onc polyprotein possessing an associated tyrosine kinase activity. The integrated form of this virus, isolated in the Charon 21A strain of bacteriophage λ, demonstrated an ability to transform NIH/3T3 cells at high efficiency upon transfection. Foci induced by GR-FeSV DNA contained rescuable sarcoma virus and expressed GR-P70, the major GR-FeSV translational product. The localization of long terminal repeats within the DNA clone made it possible to establish the length of the GR-FeSV provirus as 4.6 kilobase pairs. The analysis of heteroduplexes formed between λ feline leukemia virus (FeLV) and λ GR-FeSV DNAs revealed the presence of a 1,700-base-pair FeLV unrelated segment, designated v-fgr, within the GR-FeSV genome. The size of this region was sufficient to encode a protein of approximately 68,000 daltons and was localized immediately downstream of the FeLV gag gene coding sequences present in GR-FeSV. Thus, it is likely that this 1.7-kilobase-pair stretch encodes the onc moiety of GR-P70. Utilizing probes representing v-fgr, we detected homologous sequences in the DNAs of diverse vertebrate species, implying that v-fgr originated from a well-conserved cellular gene. The number of cellular DNA fragments hybridized by v-fgr-derived probes indicated either that proto-fgr is distributed over a very large region of cellular DNA or represents a family of related genes. By molecular hybridization, v-fgr was not directly related to the onc genes of other known retroviruses having associated tyrosine kinase activity.

A recombinant murine retrovirus (MRSV) containing the src gene of avian Rous sarcoma virus (RSV) was shown to induce hematopoietic colonies in infected mouse bone marrow. MRSV-induced colony formation followed single-hit kinetics and required mercaptoethanol in the agar medium. Cells from the colonies induced by MRSV could be established as continuous cell lines that demonstrated unrestricted self-renewal in vitro and tumorigenicity in vivo. The transformants, all of which expressed high levels of the Rous sarcoma virus transforming protein, pp60^{src}, appeared to be at an early stage in

lymphoid cell differentiation. They lacked Fc receptors and detectable immunoglobulin μ heavy chain synthesis, markers normally associated with committed B cells. The majority of the MRSV-transformed cell lines contained high levels of terminal deoxynucleotidyl transferase, an enzyme present in lymphoid progenitor cells committed to the T-cell lineage. One cell line expressed Thy-1 antigen, but none expressed Lyt-1 and Lyt-2, markers of more differentiated T cells. These findings demonstrate that the src gene is capable of transforming cells of hematopoietic origin.

The retroviral long terminal repeat (LTR) contains transcriptional control elements that affect viral gene expression. By deletion mutagenesis of the genome of the cloned Abelson murine leukemia virus, regulatory signals could be mapped to at least three domains within the LTR. A defective 5' LTR that did not sustain transforming gene function was complemented by an intact LTR positioned at the 3' end of the genome. This versatility of the retroviral genome with respect to its transcriptional control elements appears to provide a strong selective advantage for viral gene expression.

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.

The closed circular form of the endogenous squirrel monkey type D retrovirus (SMRV) was molecularly cloned in a bacteriophage vector. The restriction map of the biologically active clone was determined and found to be identical to that of the parental SMRV linear DNA except for the deletion of one long terminal repeat. Restriction enzyme analysis and Southern blotting indicated that the SMRV long terminal repeat was approximately 300 base pairs long. The SMRV restriction map was oriented to the viral RNA by using a gene-specific probe from baboon endogenous virus. Restriction enzyme digests of a variety of vertebrate DNAs were analyzed for DNA sequence homology with SMRV

by using the cloned SMRV genome as a probe. Consistent with earlier studies, multiple copies of SMRV were detected in squirrel monkey DNA. Related fragments were also detected in the DNAs from other primate species, including humans.

The genetic relationships among molecularly cloned prototype viruses representing all of the major oncovirus genera were investigated by molecular hybridization and nucleotide sequence analysis. We demonstrate two major progenitors of the pol genes of such viruses, one giving rise to mammalian type C viruses and the other to type A, B, D and avian type C oncoviruses. Evidence of unusual patterns of homology among the env genes of mammalian type C and D oncoviruses illustrate further that genetic interactions between their progenitors have contributed to the evolution of oncoviruses.

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. Accomplishments in Cancer Research, 1983. (General Motors Cancer Res. Foundation) (In Press)

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

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Aaronson, S. A., Yuasa, Y., Robbins, K. C., Eva, A., Gol, R. and Tronick, S. R.: Oncogenes and the neoplastic process. In Bolis, C. G., Frat, L. and Verna, R. (Eds.): Advances in Cell Growth Regulation. New York, Plenum Press (In Press)

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Chiu, I-M., Reddy, E. P., Givol, D., Robbins, K. C., Tronick, S. R. and Aaronson, S. A.: Nucleotide sequence analysis identifies the human c-sis proto-oncogene as a structural gene for platelet-derived growth factor. Cell 37: 123-129, 1984.

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Eva, A. and Aaronson, S. A.: Identification and preliminary characterization of a new transforming gene from a human lymphoma. UCLA Symposia on Molecular and Cellular Biology. New York, Liss, Inc. (In Press)

Fujita, J., Yoshida, O., Yuasa, Y., Rhim, J. S., Hatanaka, M. and Aaronson, S. A.: H-ras oncogenes are activated by somatic alterations in human urinary tract tumors. Nature 309: 464-466, 1984.

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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. The Cancer Cell. New York, Cold Spring Harbor Laboratory Press (In Press)

Robbins, K. C., Antoniades, H. N., Devare, S. G., Hunkapiller, M. W. and Aaronson, S. A.: Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet-derived growth factor. Nature 305: 605-608, 1983.

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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. The Cancer Cell, Vol. 11. New York, Cold Spring Harbor Laboratory Press (In Press)

Yuasa, Y., Gol, R. A., Chang, A., Reddy, E. P., Tronick, S. R. and Aaronson, S. A.: Mechanism of activation of an N-ras oncogene of SW-1271 human lung carcinoma cells. Proc. Natl. Acad. Sci. USA (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04941-12 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

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. Tronick	Acting Chief, Gene Structure Section	LCMB	NCI
Others:	S. Aaronson	Chief	LCMB	NCI
	K. Robbins	Acting Chief, Oncogene Studies Section	LCMB	NCI
	E. Reddy	Acting Chief, Molecular Genetics Section	LCMB	NCI
	I.-M. Chiu	Visiting Fellow	LCMB	NCI
	A. Yaniv	Visiting Scientist	LCMB	NCI
	A. Gazit	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:
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.)*

Analysis of the genomes of replication competent oncoviruses demonstrated that a major progenitor of the pol genes of these viruses gave rise to mammalian type C viruses and another gave rise to types A, B, D, and avian type C oncoviruses. Unusual patterns of homology among the env genes of mammalian type C and D oncoviruses revealed the occurrence of genetic interactions between their progenitors and suggests that such interaction contributed to their evolution. Studies on human proto-oncogenes provided information on the chromosomal localization of members of the ras gene family and the myb proto-oncogene. The ras genes were found to be widely dispersed in the human genome (H-ras-1, chromosome 11; K-ras-1, chromosome 6; K-ras-2, chromosome 11; N-ras, chromosome 1). C-myb was localized to chromosome 6. These findings are being pursued in regard to the specific chromosomal rearrangements that are known to occur in certain human tumors. Structural analysis of the c-sis (human) proto-oncogene showed that a 45-kbp region of human DNA contains all the coding regions of the v-sis oncogene. These sequences are present as six exons, five of which contain the open reading for the sis gene product, p28. An additional 5' coding sequence was identified. Sequence analysis demonstrated that the c-sis gene codes for platelet-derived growth factor chain 2. It was possible to demonstrate that the c-sis (human) proto-oncogene possesses transforming potential.

PROJECT DESCRIPTION

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

S. Tronick	Acting Chief, Gene Structure Section	LCMB	NCI
S. Aaronson	Chief	LCMB	NCI
K. Robbins	Acting Chief, Oncogene Studies Section	LCMB	NCI
E. Reddy	Acting Chief, Molecular Genetics Section	LCMB	NCI
I.-M. Chiu	Visiting Fellow	LCMB	NCI
A. Yaniv	Visiting Scientist	LCMB	NCI
A. Gazit	Visiting Fellow	LCMB	NCI
D. Swan	Expert	LCMB	NCI
Y. Yuasa	Visiting Fellow	LCMB	NCI
W. McBride	Chief, Cellular Regulation Section	LB	NCI
R. Callahan	Chief, Oncogenetics Section	LTIB	NCI
J. Schlom	Chief	LTIB	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:

1. The genetic relationships among oncovirus genera were examined by Southern blotting and molecular hybridization techniques using molecularly cloned DNAs. The pol gene sequences of representative mammalian type A, B, D, and avian type-C oncoviruses were all shown to be related, whereas no homology between the pol genes of these viruses and those of mammalian type-C viruses could be detected. However, computer analysis revealed a sequence of 16 amino acids that were highly conserved among all retroviral pol genes for which sequence data was available. Analysis of retroviral env genes demonstrated some similarities between mammalian type C and D viruses, and,

in addition, a much more highly conserved region was found to be present in the squirrel monkey retrovirus (SMRV) and baboon endogenous virus (BaEV) genomes.

2. A human recombinant clone (HLM-2) was previously shown to contain sequences related to MMTV gag and pol genes. HLM-2 was studied in more detail in order to determine its relationship to representatives of the major oncovirus genera. The HLM-2 genome was not detectably related to mammalian type C viral sequences when examined by low-stringency hybridization and by nucleotide sequence analysis. The HLM-2 pol gene was found to be homologous to those of mammalian types A, B, D, and avian type C oncoviruses. Its env gene sequences most closely resembled those of the type A virus, whereas its long terminal repeat (LTR) sequences were most similar to the type D virus.

3. In studies aimed at examining human tumor cell lines for the presence of ras-related oncogenes, an N-ras oncogene was detected in the SW1271 lung carcinoma cell line. This gene was molecularly cloned and physically characterized. Recombinants between the SW1271 oncogene and the N-ras proto-oncogene were constructed and tested for transforming activity. This analysis indicated that the SW1271 oncogene contained a genetic lesion in either exon 1 or 2 that led to its malignant activation. Nucleotide sequence analysis of these coding regions showed that the SW1271 gene was mutated in codon number 61 such that an arginine residue replaced a glutamine residue in the predicted coding sequence of the N-ras p21.

4. The molecular cloning of the c-myb (human) proto-oncogene made possible its chromosomal localization. A series of somatic cell hybrids between human and rodent cells containing different human chromosomes were examined for c-myb (human) sequences. Hybrids containing the well-characterized chromosome 2;6 reciprocal translocations were used to sublocalize the c-myb (human) proto-oncogene to the long arm of chromosome 6 (6q) distal to the breakpoint at 6q15. Thus, this proto-oncogene could be localized to human chromosome 6 (q15-q21).

5. Clones containing v-sis-related sequences were isolated from a human library of phage DNA. The four overlapping clones represented about 45-kbp of human DNA. The v-sis-related regions were localized within a 14.5-kbp region. Nucleotide sequence analysis of these regions revealed that the 5'-most v-sis-related exon lacked an ATG codon for initiating protein synthesis. All of the non-helper viral-related sequences of simian sarcoma virus (SSV) were contained within the six v-sis-related exons. Sequence comparison to other proteins demonstrated that the c-sis (human) gene codes for platelet-derived growth factor chain 2. An additional coding sequence unrelated to v-sis was demonstrated to be present upstream of the v-sis-related exons. By coupling this sequence to a clone containing all of the v-sis-related regions and adding to this construct a transcriptional control element, a molecule with transforming activity was created. Other constructs consisting of various combinations of these segments (or lacking some) did not transform NIH/3T3 cells.

6. Sequences homologous to the tyrosine kinase specific region of the transforming gene of the GR strain of feline sarcoma viruses were isolated from a human library. The structure of this proto-oncogene [designated c-fgr (human)] was determined. The viral-related regions, of which at least five have been identified, were localized within a 7.6-kbp region. However, sequences from the 3' end of v-fgr may not be present in this clone. Unlike v-fgr, no 5'-actin-related sequences were present in the human clone. A unique sequence probe was prepared and used to map the fgr oncogene to a single human chromosome.

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.

Recombinant DNA techniques are being applied and further developed to study other mammalian transforming viruses and cellular genes involved in transformation.

Publications:

Aaronson, S. A. and Tronick, S. R.: The role of oncogenes in human neoplasia. In DeVita, V., Hellman, S. and Rosenberg, S. (Eds.): Current Topics in Oncology. Philadelphia, J. B. Lippincott Co. (In Press)

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Chiu, I.-M., Reddy, E. P., Givol, D., Robbins, K. C., Tronick, S. R. and Aaronson, S. A.: Nucleotide sequence analysis identifies the human c-sis proto-oncogene as a structural gene for platelet-derived growth factor. Cell 37: 123-129, 1984.

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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 coding sequence for a human growth factor causes cellular transformation. Cell (In Press)

McBride, O. W., Swan, D. C., Tronick, S. R., Gol, R., Klimanis, D., Moore, D. E. and Aaronson, S. A.: Regional chromosomal localization of N-ras, K-ras-1, K-ras-2 and myb oncogenes in human cells. Nucleic Acids Res. 11: 8221-8236, 1983.

Tronick, S. R. and Aaronson, S. A.: Unique interactions of retroviruses with eukaryotic cells. In Oldstone, M. B. A. and Notkins, L. A. (Eds.): Concepts in Viral Pathogenesis. New York, Springer-Verlag (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. The Cancer Cell, Vol. 11. New York, Cold Spring Harbor Laboratory (In Press)

Yuasa, Y., Gol, R. A., Chang, A., Chiu, I.-M., Reddy, E. P., Tronick, S. R. and Aaronson, S. A.: Mechanism of activation of an N-ras oncogene of SW-1271 human lung carcinoma cells. Proc. Natl. Acad. Sci. USA (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04951-08 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

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

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

PI:	J. E. Dahlberg	Microbiologist	LCMB NCI
Others:	D. V. Ablashi	Microbiologist	LCMB NCI
	A. Yaniv	Visiting Scientist	LCMB NCI
	A. Gazit	Visiting Fellow	LCMB NCI

COOPERATING UNITS *(if any)*
U. California School of Veterinary Medicine, Davis, CA (N. East); Animal Research Center, Hebrew U., Rehovot, Israel (K. Perk); Dept. Pathology, Colorado State U., Fort Collins, CO (J. DeMartini)

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.)*

Lentiviruses, a group of retroviruses that cause slow virus diseases in domestic animals and may be distantly related to newly described human retroviruses, are being intensively analyzed by immunological techniques and by molecular cloning technology. The proviral DNA of caprine arthritis-encephalitis virus (CAEV) has been cloned, and cloning of the DNA of equine infectious anemia virus (EIAV) is currently underway. Radioimmunoassays to the major structural proteins of these viruses are being used to study genetic relatedness of these agents to other retroviruses and as a sensitive means of analysis of virus transmission and sero-conversion under natural and experimental conditions.

Hybridoma technology is being used to study the structural proteins of a transforming primate herpesvirus (Herpesvirus saimiri) and also to produce monoclonal antibodies to the proteins produced by normal and transforming onc genes.

PROJECT DESCRIPTION

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

J. E. Dahlberg	Microbiologist	LCMB NCI
D. V. Ablashi	Microbiologist	LCMB NCI
A. Yaniv	Visiting Scientist	LCMB NCI
A. Gazit	Visiting Fellow	LCMB NCI

Objectives:

1. To understand the molecular biology of lentiviruses and other retroviruses associated with slowly developing disease in domestic animals and man and to use molecular techniques to analyze mechanisms of pathogenesis and to develop vaccines capable of preventing disease.

2. To utilize hybridoma technology to develop monoclonal antibodies to structural proteins of transforming viruses and to proteins coded for by cellular and viral onc genes.

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.

The development of a variety of radioimmunoassays, using purified proteins from CAEV and EIAV, were described earlier. We have been using these assays, in collaboration with investigators at the University of California at Davis, to investigate the transmission of CAEV to newborn goats. Using tissue culture grown virus of known titer, we have shown that a single oral dose is sufficient to lead to seroconversion and development of disease, while contact control animals remained antibody negative. The animals were tested weekly for both antigen levels and the presence of antibody, and at no time could significant levels of virus be detected. In a separate study, the antibody titers in serum and milk samples from individual goats were compared. It was observed that although antibody was first detected in the serum as titers increased, they increased more rapidly in the milk so that where titers of over 1000 were seen, they were consistently higher in the milk than in the serum. Since we and others have consistently been able to recover infectious virus from the milk but not from serum or plasma, it was an unexpected finding that so much antibody would be present in the milk. Recently published data from other laboratories, however, indicate that the antibodies present in the serum of infected goats does not neutralize CAEV, and we are currently determining if antibodies in milk will also fail to neutralize the virus. If so, it seems possible that the higher titers in the milk are a reflection of an immune response to relatively active virus replication in the mammary gland. This question will soon be answerable because we have recently cloned an integrated CAEV provirus and can look at the level and site of virus replication by in situ hybridization. This study will be carried out in collaboration with investigators at Colorado State University.

To facilitate research on the mode of replication and disease induction, we are attempting to molecularly clone several of the lentiviruses. At this time, CAEV has been cloned into pBR322 and shown to be identical, by restriction enzyme mapping, to nonintegrated linear proviral DNA present in acutely infected cells. This DNA will be tested for relatedness to other retroviruses to verify and extend our immunological findings and will be used as a probe to investigate the site and mode of replication in diseased goats.

2. The isolation of a set of 27 monoclonal antibodies which could be sorted into 11 unique groups recognizing nearly all of the structural proteins of the transforming Herpesvirus saimiri was described earlier. Of particular interest was the finding that 15 of the 27 antibodies (6/11 groups) immunoprecipitated more than one viral protein, but did not precipitate nonviral cellular proteins. We investigated several of the possible mechanisms for this finding, because by definition a monoclonal antibody recognizes a single epitope. All of our hybridomas have been cloned, so the trivial explanation that more than one antibody could sometimes be present is not possible. Furthermore, identical reactivities to multiple antigens have usually been observed more than once and from separate fusions.

One set of two antibodies immunoprecipates proteins of 125,000, 75,000, and 47,000 daltons. These antigens were shown to be the principal glycoproteins of HVS by labeling with ^{3}H-glucosamine and by showing that treatment of infected cells with tunicamycin led to their disappearance and to the appearance of a single 25,000 dalton protein. The two monoclonal antibodies fail to immunoprecipitate any protein from tunicamycin-treated extracts, suggesting that the

core 25,000 dalton protein does not have the epitope present on the three glycosylated viral proteins that the antibodies recognize. This suggests that in this case, the monoclonal antibody is recognizing an epitope generated on three proteins by virus-specific post-translational modification.

In another set of experiments, virus-infected cell extracts, metabolically labeled with ^{35}S-methionine, were treated with different concentrations of dithiothreitol (DTT) prior to incubation with different monoclonal antibodies. One group of 6 antibodies, which normally precipitate 4 proteins of 25, 32, 49, and 50 kD, recognized only one of the four proteins at DTT concentrations of 9 millimolar or greater. None of the monoclonal antibodies of other groups demonstrated this increased specificity in the presence of DTT. Different antibodies of this group were able to recognize different components of this disulfide-bond linked complex. Of interest is the ability to detect this complex in cell extracts, indicating that this component of the virion is pre-assembled in the cell.

More recently, the main emphasis in hybridoma technology in this laboratory has turned towards an analysis of onc gene products. Other members of the laboratory have cloned several onc genes of viral and cellular origin, and current efforts include introducing these genes into prokaryotic and eukaryotic expression systems. At present, two onc proteins, the p21 of Kirsten sarcoma virus and the p28 of simian sarcoma virus, are being produced at high levels in bacteria, and procedures are being developed for purifying these proteins. Monoclonal antibodies will be produced to these proteins using standard techniques. In the case of p21, the normal human gene has also been introduced into bacteria, and the major objective will be to produce antibodies which can distinguish between the normal and transforming proteins. For SSV p28, which is closely related to one component of platelet-derived growth factor (PDGF), monoclonals to different portions of the p28 molecule may be crucial to understand which of the multiple molecular forms of PDGF are biologically active and how SSV p28 helps to sustain the transformed phenotype.

Significance to Biomedical Research and the Program of the Institute:

1. Lentiviruses are retroviruses which cause widespread disease in domesticated ungulates and may represent a reservoir from which human viruses, such as human T cell leukemia virus (HTLV), can originate. Our work with CAEV, EIAV and other lentiviruses will lead to increased understanding of how virally-induced slow diseases occur, and may ultimately lead to safe and effective vaccines.

2. Hybridoma technology is rapidly fulfilling earlier promises that it would allow new approaches in immunology, biochemistry, cancer research and other areas that would otherwise be difficult or impossible. We have been using this technique to produce antibodies to a transforming herpesvirus that recognize many of the structural antigens of the virion and several nonstructural virus-specified proteins as well. These antibodies are being used to probe the architecture of the virus and the mechanism of how the virus proteins are synthesized and assembled. They will also be made available to other workers interested in HVS and related viruses. In addition, monoclonal antibodies to virally-derived onc genes are being isolated and tested for their ability to

react with the transforming protein. These antibodies will be of enormous value in helping to understand how and where transforming genes function.

Proposed Course:

1. Work on lentiviruses will involve continued efforts to molecularly clone additional viruses. EIAV is of particular importance because of its apparent relatedness to HTLV-III. These cloned viruses will be used to investigate the mechanisms of pathogenesis in vivo, and sequence analysis will be needed to permit an adequate understanding of genomic organization of these viruses and to allow the development of candidate vaccines. At the same time, the immunological assays currently available will be used to study, in greater detail, the mechanism of virus transmission under controlled and field conditions.

2. Most of our work with hybridoma technology will involve the purification of onc gene proteins expressed in prokaryotic and eukaryotic systems, the generation of monoclonal antibodies to these proteins, and use of the antibodies to study onc gene expression in normal and transformed cells.

Publications:

Faggioni, A., Ablashi, D. V., Armstrong, G., Dahlberg, J., Sundar, S. K., Rice, J. M. and Donovan, P. J.: Enhancing effect of N-methyl-N-nitrosoguanidine (MNNG) on Epstein-Barr virus (EBV) replication and comparison of short term and continuous TPA treatment of nonproducer and producer cells for EBV antigen induction and/or stimulation. In Prasad, U., Ablashi, D. V., Levine, P. M. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya Press, 1984, pp. 333-345.

Faggioni, A., Ablashi, D. V., Dahlberg, J., Armstrong, G. and Sundar, S. K.: Interaction of N-methyl-N-nitrosoguanidine (MNNG) with owl monkey kidney cells in enhancing the yield of Herpesvirus saimiri (HVS) and its antigens. Proc. Soc. Exp. Biol. Med. (In Press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04976-07 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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
	W. G. Taylor	Research Biologist	LCMB	NCI
	R. E. Tarone	Mathematical Statistician	BB	NCI
	J. S. Rhim	Microbiologist	LCMB	NCI
	M. H. Greene	Epidemiologist	EEB	NCI
	M. Potter	Chief	LG	NCI

COOPERATING UNITS *(if any)*

Howard University College of Medicine (R. Parshad); Division of Computer Research and Technology (B. Trus); College of Medicine, Univ. of CA (E. Stanbridge); Johns Hopkins Univ. 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:
5.5	1.5	4.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Cultures of skin fibroblasts from normal individuals, individuals with genetic disorders predisposing to a high risk of cancer, cancer patients, as well as cells transformed in culture, 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 G2 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 suggested from studies with somatic cell hybrids between normal and malignant cells. The chromosomal radiosensitivity appears to result from deficient DNA repair during G2-prophase. 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 and quantify cytomorphic changes diagnostic of neoplastic transformation and determine their functional basis.

PROJECT DESCRIPTION

Names, Title, 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
W. G. Taylor	Research Biologist	LCMB	NCI
R. E. Tarone	Mathematical Statistician	BB	NCI
J. S. Rhim	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, computerized image analysis of living cells is being used to identify quantifiable cytomorphologic markers of neoplastic transformation as a nondestructive diagnostic tool for use in transfection and transformation studies and also to provide insights into mechanisms of carcinogenic change at the cellular level.

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.

In developing a transformation system with normal epidermal keratinocytes, it has been necessary to study in detail the calcium and numerous other requirements for enhancing proliferation and minimizing terminal differentiation of epidermal keratinocytes in culture. Several approaches to induce transformation are being evaluated.

Computerized image analysis is carried out on photomicrographs taken at high power of individual living normal and neoplastic cells growing in Dvorak chambers. Emphasis is on nuclear structure and cytoplasmic spread.

Major Findings:

The pursuit of this project has led to the following major new findings and accomplishments:

1. A genetic basis for enhanced G_2 chromosomal radiosensitivity is suggested from studies with somatic cell hybrids produced by fusing a normal human cell with a malignant cell. The hybrids which are nontumorigenic show normal G_2 chromosomal radiosensitivity. On the other hand, the original tumorigenic cell line and those tumorigenic lines that subsequently segregate show enhanced G_2 radiosensitivity. Thus, the deficiency in the malignant cell is complemented by fusion with the normal cell and enhanced chromosomal radiosensitivity segregates with tumorigenicity. (Manuscript in preparation. Collaboration with Dr. E. Stanbridge, University of California College of Medicine).

2. Five lines of skin fibroblasts from individuals heterozygous for ataxia-telangiectasia (A-T) showed a much higher frequency of chromatid breaks and gaps following x-irradiation during G_2 than did cell lines from age-matched normal controls. 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 about 1% of the human population.

3. Skin fibroblasts from 16 cancer patients compared with those from normal donors also showed a significant increase in incidence of chromatid damage after x-irradiation during G_2. The cancer patients were predominantly young and members of families having a high incidence of cancer. Therefore, they were probably individuals at high cancer risk. These results again suggest that enhanced G_2 chromosomal radiosensitivity may provide the basis for a test for cancer susceptibility. (Manuscript submitted for publication.)

4. The increased incidence of radiation-induced chromatid damage in (1) human cells transformed in culture, (2) cells derived from human tumors, (3) cells from cancer-prone individuals, and (4) skin fibroblasts from cancer patients compared with that of normal cells or cells from normal donors could result from deficiencies in DNA repair during G_2 prophase. This possibility is supported by data comparing cytogenetic responses to DNA repair inhibitors and by kinetic studies on the incidence of chromatid damage in a normal and tumor cell line at 0.5 and 1 hr post-irradiation. In normal cells, chromatid damage decreased during the period following irradiation, presumably due to competent DNA repair. In contrast, chromatid damage in tumor cells increased during the same post-irradiation period, presumably through deficient or incomplete nucleotide excision repair; i.e., initial incision of DNA without completion of the repair process. This deficiency would result in the accumulation of DNA single-strand breaks which could subsequently be converted to double-strand breaks by single-strand nuclease. These DNA breaks would be manifest as chromatid breaks and/or gaps by cytogenetic analysis.

5. Use of exogenous catalase and 0% O_2 in light-shielded cultures was shown to reduce chromosomal aberrations and delay onset of malignant transformation in mouse fibroblasts. Exogenous catalase also decreases the incidence of chromosome aberrations in mouse keratinocytes. Thus, photoproducts, including H_2O_2 formed in the presence of O_2, play an important role, directly or indirectly, in genetic damage and neoplastic transformation of mouse cells.

6. Normal mouse epidermal keratinocytes proliferate more rapidly to higher cell density if calcium concentration is increased above levels for optimal clonal growth. Further, even at low calcium concentration, normal and transformed malignant mouse keratinocytes maintain a capacity for terminal differentiation evidenced by cornified envelope and squame formation. Thus, loss of maturation potential appears to be neither a prerequisite nor a causal factor in their neoplastic transformation. (Manuscript submitted for publication.)

7. Photomicrography with computer-assisted morphometric analysis was used as a quantitative nondestructive approach to differentiate between unstained living neoplastic cells and their normal progenitors in culture. When compared to non-neoplastic counterparts, neoplastic human and mouse cells had a significantly higher nuclear:cytoplasmic ratio, larger area and perimeter of nucleoli and chromocenters and significantly reduced cell area and perimeter. (Manuscript in preparation.)

8. In a collaborative study with Dr. J. Rhim, infection of a primary culture of untreated human keratinocytes with Ad 12-SV40 hybrid virus led to a continuous morphologically-altered cell line which expressed neoplastic properties after superinfection with KiMSV. (Manuscript submitted for publication.)

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 from certain cancer patients. 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-prophase and that it has a genetic basis. The DNA damage produced by carcinogenic agents can so overwhelm DNA repair capabilities of the cell as to result in genetic change. Conceivably, one such genetic change, possibly the initiating step in carcinogenesis, is an impairment of DNA repair mechanisms operative during the G_2-prophase period of the cell cycle and responsible for enhanced G_2 chromosomal radiosensitivity. The G_2 period of the cell cycle just prior to mitosis is a critical time for monitoring of chromosomal DNA and repair of any lesions which otherwise would be transmitted to daughter cells. A deficiency in these repair processes could facilitate chromosomal rearrangements, deletions and translocations that can activate oncogenes and lead to the progression of genetic alterations associated with malignant transformation. This genetic defect could also account for the mutability and

heterogeneity of tumor cells. Furthermore, enhanced G_2 chromosomal radiosensitivity may provide the basis for an assay to detect susceptibility to cancer.

Since most human cancers are carcinomas, it is important to develop a transformation system with epithelial cells. We are concentrating on human epidermal cells because of availability of tissue and because the epidermal basal cell is an important target for environmental carcinogens. A reproducible transformation system with normal human epithelial cells provides a model with which to follow the sequence of events culminating in carcinogenic change and to discriminate between events fundamental to transformation and those secondary changes resulting from the genetic instability of transformed cells. A significant step toward this end was optimization of culture conditions for reproducible extended growth of human basal keratinocytes to high cell density without a feeder layer. Tissue-like densities simulate in vivo conditions and enumeration of isolated cell nuclei facilitates accurate quantification of growth responses. Human cells in culture, in contrast to rodent, are karyotypically stable, rarely undergo spontaneous transformation, and these human epithelial cells which readily metabolize carcinogens resisted our repeated efforts to transform them by chemical carcinogens. These results clearly indicate that other approaches are needed to effect transformation, such as impairment of their DNA repair, known to be far more efficient than that of rodent fibroblasts.

The criteria of nuclear cytopathology as used for Papanicolaou-stained preparations, together with cytomorphic markers in cultured cells that correlate with tumorigenicity, provide an accurate, direct and rapid means for diagnosis of carcinogenic change. Certain of these criteria were shown to be applicable to individual living cells and can be quantified.

Proposed Course:

A number of projects recently initiated will be continued. These include the following:

1. 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 should be possible to identify and isolate the genes responsible for susceptibility or resistance.

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

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

4. 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.

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

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

7. 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.

8. In view of the successful transformation of human epidermal keratinocytes with Ad 12-SV40 hybrid virus and superinfection with KiMSV, we will continue efforts to cotransfect these cells with pSV3 neo (containing the origin of SV40 virus and information for large and small T antigens) and the cloned transforming region from Kirsten murine sarcoma virus. The protoplast fusion method will also be used.

9. 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.

10. Repeated low-level ultraviolet treatment of human epidermal keratinocytes will be used to simulate environmental exposure. In addition, DNA repair inhibitors will be used to enhance the probability of transformation.

11. 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.

12. As an adjunct to these studies, quantitative cytomorphic procedures will be used for rapid and reliable assessment of neoplastic transformation in epithelial cells.

Publications:

Parshad, R., Sanford, K. K. and Jones, G. M.: Chromatid damage after G_2 phase x-irradiation of cells from cancer-prone individuals implicates deficiency in DNA repair. Proc. Natl. Acad. Sci. USA 80: 5612-5616, 1983.

Parshad, R., Sanford, K. K., Jones, G. M. and Tarone, R. E.: G_2 chromosomal radiosensitivity of ataxia-telangiectasia heterozygotes. Cancer Genet. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04977-07 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 chereters 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
	W. G. Taylor	Research Biologist	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:
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 formation, consequences, and mechanism of repair of DNA-protein cross-links are being studied in mouse cells and human fibroblasts, including cells from xeroderma pigmentosum (XP) patients and normal individuals. The mechanism of repair of cross-links induced with 20 μM trans-platinum(II) diamminedichloride is currently emphasized. It is known that DNA-protein cross-links are induced by numerous agents, including x-rays, ultraviolet light, visible fluorescent light, and a wide variety of chemical carcinogens such as benzopyrene, methylmethane sulfonate, and AAF. These lesions induced by trans-platinum have been reported to result in transformation of 3T3 and 10T-1/2 mouse cells, and they are repaired by the nucleotide excision pathway. However, because L1210 mouse cells are relatively deficient in nucleotide excision repair compared to normal human fibroblasts, but are at least as competent at repair of these lesions, the mechanism of repair was investigated further. The approach used was to first compare the DNA-protein cross-link repair rates of two human cell lines deficient in nucleotide excision repair, one slowly proliferating (XP20S, group A) and the other rapidly proliferating (SV40-tranformed XP20S); then, the effect of the metabolic inhibitors, cycloheximide and aphidicolin, on the repair of DNA-protein cross-links was examined. Finally, the rates of DNA repair and DNA replication during logarithmic growth and their relation to the cell cycle were studied. From the results of these experiments, we conclude the following: (1) A pathway in addition to the nucleotide excision mechanism can repair DNA-protein cross-links induced by trans-platinum. (2) Cell cycling is necessary for activation of this pathway. (3) DNA synthesis per se is not essential for this repair. (4) The pathway seems to be activated in late G1 or early S phase of the cell cycle.

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
W. G. Taylor	Research Biologist	LCMB	NCI

Objectives:

To identify primary changes in cellular and viral nucleic acids during photochemical, chemical, and viral 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, 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.

Major Findings:

Repair of DNA-protein cross-links. The reagent trans-platinum (trans-platinum (II) diamminedichloride) induces DNA-protein cross-links. We are investigating the mechanism(s) by which mammalian cells repair these cross-links. 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). 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 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. Previous investigations in our laboratory using mouse L1210 cells, normal human skin fibroblasts, and metabolic inhibitors, including cycloheximide, α amanitin and aphidicolin, showed that extent of DNA replication correlated with extent of DNA protein cross-link repair, but that replication per se was not essential. Recent experiments with XP group A cells that eliminate the complications of simple nucleotide excision repair in normal cells indicate, again, that replication per se is not required but that a pathway other than nucleotide excision is available

to repair the DNA-protein cross-links at rates equivalent to those in normal cells. When DNA protein cross-links are induced with 20 μM trans-platinum in rapidly proliferating SV40-transformed, group A, XP20S cells, the cross-links are repaired at essentially the same rate as in normal human skin fibroblasts. DNA-protein cross-links induced in the slowly proliferating untransformed parental line, XP20S, are repaired very slowly, similar to repair rates in XP12BE cells.

If a relationship between cell cycling and DNA-protein cross-link repair exists, it should be observed by changes in the rate of repair during the cell cycle. Several methods to synchronize the L1210 cells (0.5 to 10 mM hydroxyurea w/wo nucleosides; serum depletion w/wo FU; serum depletion with removal of isoleucine or arginine) proved to be unsatisfactory because the DNA in the blocked cells accumulated single-strand breaks to an extent that interfered with adequate DNA-protein cross-link measurements. To circumvent this problem, one hour pulse-label experiments were carried out, and the cells were treated with trans-platinum during the period DNA is labelled (S phase) or six hours later (G2, M, or G1 phase); 5-bromodeoxyuridine is added at the time of trans-platinum treatment to monitor DNA replication. The results of these experiments indicate that there is a clear cell-cycle dependence for repair in these cells and that late G1 or early S phase is the period at which the repair pathway is activated.

These results are additional evidence to support the idea that a second pathway exists which can repair trans-platinum induced DNA-protein cross-links. This pathway is activated sometime in late G1 or early S phase, probably by induction of a new protein, in the absence of DNA damage from trans-platinum treatment. Though cell cycling (as measured by DNA replication) is necessary for activation, DNA replication per se is not essential.

Significance to Biomedical Research and the Program of the Institute:

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

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

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

2. Isolate and characterize the crosslinked protein. Possible approaches:

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

b. Use of antibody techniques.

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

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

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

Publications:

Gantt, R., Taylor, W. G., Camalier, R. F., and Stephens, E. V.: Repair of DNA-protein cross-links in an excision-deficient human cell line and its SV40-transformed derivative. Cancer Res. 44: 1809-1812, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE04978-07 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Growth, Nutrition, and Neoplastic Transformation of Mammalian Cells In Vitro

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:	K. K. Sanford	Chief, In Vitro Carinogenesis Section	LCMB	NCI
	R. R. Gantt	Research Chemist	LCMB	NCI
	G. H. Smith	Research Biologist	LMB	NCI

COOPERATING UNITS *(if any)*

National Center for Drugs and Biologics, Division of Biochemistry and Biophysics (P. C. Noguchi); Division of Computer Research and Technology, Computer Systems Laboratory, NIH (B. L. Trus)

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:
1.0	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 long-term objective of this program is to understand the mechanism(s) of fundamental cellular changes which occur during neoplastic transformation. Studies include: (a) growth, function or transformation of human, primate or mouse epithelial cells; (b) quantification of focal contact formation and association with cell shape changes and neoplastic transformation; and (c) studies on DNA-protein cross-link repair mechanisms(s). The critical role of oxygen for sustained transepithelial transport by high density kidney epithelial cells was completed. Direct quantitative measure of oxygen consumption by kidney epithelial cells and nonneoplastic as well as tumorigenic keratinocytes revealed similar, rapid consumption rates irrespective of initial oxygen concentration, suggesting the cell population modifies the environment to minimize oxidation injury. In related studies, a vigorously proliferating, stable SV40 transformant of XP20S cells repairs DNA-protein cross-links almost as competently as normal skin fibroblasts, whereas untransformed XP20S cells retained most of the DNA-protein cross-link after an identical repair period. The data support the concept that continuous cell cycling compensates for the genetic deficiency in nucleotide excision repair by cells from xeroderma pigmentosum patients or is linked to an alternative repair pathway. Reflection-interference microscopy and computer-assisted morphometric analysis were used to quantify the area of focal contacts on the undersurface of normal and tumorigenic cells; the data show reduced spreading and cell shape change after transformation do not result from an impaired capacity to form focal contacts.

PROJECT DESCRIPTION

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

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

Objectives:

Numerous environmental agents are implicated in human carcinogenesis. The objective of this program is to understand the mechanism(s) of fundamental cellular changes which occur during neoplastic transformation through study of cellular preventive and repair responses to deliberate insults or adverse environmental conditions. Morphologic and biochemical characteristics of normal or genetically abnormal cells are of particular importance for discrimination of fundamental and secondary events.

Methods Employed:

In this laboratory, methods for propagation of primary or low-passage human epidermal keratinocytes have been developed, including formulation of medium NCTC 168 for use without a feeder layer and methods for quantifying proliferation. A similar approach has been used for mouse epidermal keratinocytes whose tumorigenicity was assayed in nude mice and in an in vitro organ culture. Formation of cornified envelopes is assayed by solubilization of keratinocytes in detergent under reducing conditions (1% SDS and 20mM DTT) followed by heating at 90°C to achieve maximum detergent-action, after which cornified envelopes are enumerated. Morphology of keratinocytes and appearance of hemicysts, a marker of secretory or transport activity by epithelium, is followed by light microscopy and staining procedures. Utilization of dissolved oxygen is determined with a Clark electrode calibrated against standard humidified gas mixtures; for dynamic measurement T-60 pyrex flasks have been modified to allow continuous monitoring of dissolved oxygen by the adherent monolayer. DNA-protein cross-links in normal and SV40 transformed human fibroblasts are assayed by the membrane alkaline elution procedure. To study the association between cell shape change and neoplastic transformation, the focal contact formed on the undersurface of fibroblasts of known tumorigenicity were recorded and photographed by reflection-interference photomiocrography. The photographic image was digitized by microdensitometry and the digital image analyzed by computerized morphometric analysis in order to quantify the area of cell undersurface devoted to focal contacts and to determine if transformation-associated differences in area (or distribution) could be detected.

Major Findings:

During the last year, several new findings have been made in three areas: (a) studies regarding growth, function or transformation of human, primate or mouse epithelial cells (see also K. Sanford, Z01CE04976-07); (b) quantification

of focal contact formation and association with cell shape changes and neoplastic transformation; and (c) studies on the mechanism of DNA-protein cross-link repair (see also R. Gantt, Z01CE04977-07).

Earlier studies in this laboratory with human epidermal keratinocytes optimized conditions for growth of human and mouse keratinocytes to dense, tissue-like cell densities. In subsequent studies with C3H mouse keratinocytes, terminal differentiation as reflected in cornified envelope and squame formation occurred at low (0.02 mM) and high (1.20 mM) calcium. A tumorigenic population obtained by implantation assays was morphologically altered, formed areas of keratinization in vivo and cornified envelopes and squames in culture. Results of in vivo tumorigenicity assays were confirmed with an in vitro organ culture assay which circumvents systemic immune responses and is a rapid, useful alternative to the nude mouse. Keratinocytes which are nontumorigenic in nude mice remained confined and well differentiated in organ culture and not extensively invasive. In contrast, the tumorigenic population is transplantable in vivo and highly invasive but poorly differentiated in the organ culture assay. The findings show that expression of differentiated properties and the potential for invasive malignant growth need not be mutually exclusive properties in proliferating neoplastic keratinocytes.

Although extensive work has been done with tissue homogenates and dispersed cells, virtually no information regarding oxygen consumption by adherent epithelial cell sheets was available earlier when studies in this laboratory showed that a 10% CO_2:air gas phase (18% v/v O_2, initial PO_2 ~ 125-130 mm Hg) supports vigorous proliferation of kidney epithelial cells in serum-free medium. As the cell sheet forms, the culture medium PO_2 drops rapidly to ≅ 70-75 mm Hg and approaches proliferation-limiting range for epithelial cells; however, proliferation is retarded with a 1% O_2 gas phase (PO_2 ~ 40 mm Hg).

Studies completed in the past year reveal that continued functional activity by mature, differentiated epithelial cells can be modulated by dissolved oxygen tension. Once tissue-like population densities are attained, cell-mediated transepithelial movement of solutes and fluid to the space between the semipermeable epithelial sheet and the impermeable growth surface causes formation of pulsatile, three-dimensional "domes" or hemicysts. In serum-free medium and without compounds or drugs which induce maturation in other model systems, both formation and maintenance of hemicysts are markedly reduced by use of a 1% O_2 gas phase or elimination of a medium replacement. Conversely, hemicyst incidence is increased by daily replacement of spent medium with fresh medium equilibrated with 18% O_2, reequilibration of spent medium with 18% O_2, or continuous rocking to prevent diffusion gradient formation. In cultures sparged with 10% O_2 (~ 95 mm Hg), virtually no hemicysts appear in stationary cultures, whereas > 500 hemicysts/cm^2 appear in gently rocked cultures. Hemicyst formation is inhibited by ouabain, DPN, and KCN; this may result from drug interference with specific metabolic pathways and/or toxicity leading to disruption of cohesive junctions and loss of semipermeability by the cell sheet.

In subsequent studies we found that monkey kidney epithelial cells and mouse epidermal keratinocytes rapidly (within 30-60 min) utilize dissolved oxygen,

lowering the PO_2 of the cellular environment to a physiologic range. Utilization occurs at a constant rate and is independent of initial gas phase oxygen concentration. In used medium a stable plateau of 45-55 mm Hg is reproducibly attained, whereas replacement of used or conditioned medium with fresh fluid (medium, saline, etc.) consistently results in PO_2 values falling below 10 mm Hg. These data suggest a preventive cellular defense mechanism which functions in the absence of organized homeostatic mechanisms and tissue architecture to maintain an environmental PO_2 comparable to that of intercellular fluids in vivo and thus mitigates extensive cell injury. Such an endogenous mechanism could mitigate extensive cell injury and contribute to the widely observed difficulty in transforming human epithelial cells in culture.

One manifestation of viral-, chemical-, or radiation-induced neoplastic transformation is a cell shape change. To test if the "retracted" shape (morphologically transformed phenotype) resulted from external changes in the undersurface of neoplastic cells, the distribution and frequency of focal contacts (focal attachment sites) were measured. Examination of normal human KD cells by reflection-interference microscopy revealed slender, streak-like focal contacts distributed in parallel arrays and particularly prominent at the leading edge of the lamellar cytoplasm. Because of the difference in cell shape, the focal contacts of transformed HUT 14 cells, though at the periphery, appeared circumferential, a pattern essentially similar to that seen in SV40 transformed fibroblasts. Computer-assisted morphometric analysis revealed that the average area of cell undersurface occupied by focal contacts was significantly greater for nonneoplastic KD cells than for their transformed derivatives; this data quantitatively support the concept that reduced cytoplasmic spreading is associated with neoplastic transformation. Additional studies with paired neoplastic and nontumorigenic mouse and rat cells revealed the distribution of focal contacts on the undersurface of tumorigenic fibroblasts was consistently random and distinct from that in nonneoplastic mouse, rat or human fibroblasts. Thus, undersurface morphology can be used as a rapid, nondestructive diagnostic tool for single cells. However, if the area occupied by focal contacts was expressed as a percent of total cell undersurface, the values were similar for both normal and neoplastic cells of mouse and human origin. We conclude, therefore, that reduced cytoplasmic spreading associated with the onset of tumorigenicity does not result from an impaired capacity to form external focal contacts.

Another manifestation of potentially carcinogenic cell injury is persisting DNA damage. Removal of DNA-protein crosslinks induced with trans-platinum (II) diamminedichloride (DDP) serves as a model for study of DNA repair proficiency. Nucleotide excision repair-deficient xeroderma pigmentosum cells remove DNA-protein crosslinks far more slowly than fibroblasts obtained from normal donors. To test if repair of DDP-induced DNA-protein cross-links is associated with cell cycling, disappearance of crosslinks was compared in slowly dividing Group A XP20S cells and their rapidly growing, stable SV40 transformant by membrane alkaline elution. The results implicate continuous cycling in repair of genomic damage; the SV40-transformant repaired this genetic lesion as competently as normal skin fibroblasts, whereas the untransformed XP cell strain retained ~ 70% of the crosslinks after a 48 hr repair period. (See also R. Gantt, Z01CE04977-07).

Significance to Biomedical Research and the Program of the Institute:

The emerging importance of oxygen-derived free radicals in cell injury and tumor promotion makes study of their formation, mechanism of action, and means of cellular defense of particular importance. Normal epithelium in vivo obtains necessary dissolved gases by diffusion from nearby vascularized stromal tissue and intercellular PO_2 values are low, but the seemingly universal distribution of defense enzymes such as catalase and glutathione peroxidase implies that oxygen-dependent cells are continuously at risk to injury mediated by oxygen metabolites or oxygen-derived free radicals. In vivo, uncommitted basal epidermal keratinocytes, mitotically active and presumably most vulnerable to carcinogen-mediated or oxidative injury, are protected by the superficial epithelium which is relatively impermeable to air. In culture, dissolved oxygen is both an essential metabolite and a substrate for oxygen-derived free radical formation. The rapid consumption of oxygen by epidermal keratinocytes and kidney epithelium conceivably represents an endogenous cellular defense mechanism which acts even in the absence of organized homeostatic mechansisms and tissue architecture to maintain an environmental PO_2 comparable to that of intercellular fluids in vivo and, thus, mitigates extensive cell injury. Such an endogenous mechanism could also contribute to the widely observed difficulty in transforming human epithelial cells in culture. Because heritable changes leading to transformation are most likely to generate misreplication of damaged DNA, study of DNA-repair processes is an important complementary approach.

A hallmark of viral, chemical, or radiation-induced neoplastic transformation is reduced two-dimensional cell spreading resulting in a cell shape change. Our studies show that the capacity to form focal contacts is not impaired as the result of neoplastic transformation and imply that this cell shape change results from cytoskeletal alterations.

Proposed Course:

Because epithelial cells in culture are difficult to transform and epithelium in vivo usually has a low tissue PO_2, the preceding studies suggest additional experiments of fundamental importance and are pertinent to an understanding of human carcinogenesis.

1. Oxygen utilization. Respiratory and cyclo-oxygenase inhibitors will be used to determine if additional normal metabolic "sinks" for oxygen can be uncovered. Reducing agents and appropriate drugs will be used to test if the exaggerated oxygen consumption in fresh medium is a cellular response induced by nonphysiologic conditions in the cell environment.

2. Cell injury and genetic damage. To test the concept that rapid oxygen utilization is a defense mechanism against chronic, nonlethal genomic damage, chemicals (e.g., H_2O_2) or conditions (low-intensity nonionizing radiation) will be used with or without DNA-repair inhibitors. The degree of DNA damage in stationary and gently rocked cultures will be compared with fibroblasts which use significantly less oxygen by measurement of base damage and DNA single-strand breaks. (Methods include

alkaline membrane elution and unscheduled DNA synthesis.) Based on previous studies with fibroblasts, catalase, selenium and mannitol will be used to protect against induced genetic injury. Studies with α-tocopherol and dietary antioxidants (e.g., BHT) will also be done under conditions of optimal viability.

Paired nontumorigenic and tumorigenic mouse epidermal keratinocytes which have been characterized with respect to oxygen consumption will be used in conjunction with monkey kidney epithelial cells in completely defined, serum-free medium and primary or low passage human keratinocytes. Conversely, fibroblasts from patients with a genetic predisposition to DNA damage/cell death will be used to determine how highly susceptible cells can be protected from environmental insults.

3. Cytomorphic analyses. These will be used as diagnostic tools, where appropriate.

Publications

Gantt, R., Taylor, W. G., Camalier, R. F. and Stevens, E. V.: Repair of DNA-protein crosslinks in an excision deficient human cell line and its SV40-transformed derivative. Cancer Res. 44: 1809-1812, 1984.

Taylor, W. G.: Serum-independent modulation of hemicyst formation by dissolved oxygen in postconfluent epithelial monolayers. In Vitro 19: 782-796, 1983.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05056-06 LCMB

PERIOD COVERED
October 1, 1983 to June 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Molecular Studies of Human and Viral Oncogenes

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigetor.) (Name, title, leboretory, end institute affiliation)*

PI:	E. P. Reddy	Acting Chief, Molecular Genetics Section	LCMB	NCI
Others:	S. Lavu	Visiting Fellow	LCMB	NCI
	N. Sacchi	Visiting Fellow	LCMB	NCI
	J. Mushinski	Medical Director	LG	NCI
	M. Potter	Chief	LG	NCI

COOPERATING UNITS *(if any)*

Litton Bionetics, Inc., FCRF, Frederick, MD (M. Barbacid and E. Santos)

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:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

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

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

A single genetic alteration, a guanine-to-cytosine transversion, is responsible for the acquisition of malignant properties by K-ras genes of two human tumor cell lines established from carcinomas of the bladder (A1698) and lung (A2182). As a consequence, arginine instead of the normal glycine is incorporated into the K-ras-coded p21 proteins at amino acid position 12. This mutation creates a restriction enzyme polymorphism that can be used to screen human cells for transforming K-ras genes. This approach was used to identify the mutational event responsible for the malignant activation of a K-ras oncogene in a squamous cell lung carcinoma of a 66-year-old man; this point mutation was not present in either the normal bronchial or parenchymal tissue or in the blood lymphocytes. Hence, malignant activation of a ras oncogene appears to be specifically associated with the development of a human neoplasm.

Molecular cloning of the mouse c-myb locus and its rearranged counterpart in the ABPL-2 tumor reveals that the alteration in this locus is due to insertion of a defective murine leukemia virus proviral genome containing both long terminal repeats upstream to the v-myb-related sequences. This insertion interrupts the c-myb coding region, which is similar to that observed in the generation of avian myeloblastosis virus.

PROJECT DESCRIPTION

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

E. P. Reddy	Acting Chief, Molecular Genetics Section	LCMB	NCI
S. Lavu	Visiting Fellow	LCMB	NCI
N. Sacchi	Visiting Fellow	LCMB	NCI
J. Mushinski	Medical Director	LG	NCI
M. Potter	Chief	LG	NCI

OBJECTIVES:

1. To study the mechanism of activation of a human K-ras oncogene derived from a lung carcinoma and compare its status with that of the normal counterpart derived from the same patient.

2. To study the mechanism of DNA rearrangements in mouse lymphosarcomas.

Methods Employed:

Molecular cloning, DNA sequence analysis, DNA transfection.

Major Findings:

Mechanism of activation of K-ras oncogene. Oncogenes capable of inducing malignant transformation on transfection of NIH/3T3 cells have been found in various human tumor cell lines, as well as in unmanipulated solid tumors. Most of these transforming genes belong to the ras gene family, which contains three members, termed H-ras, K-ras, and N-ras. Although the ras genes have different genetic structures, all of them code for proteins of 189 amino acid residues, generically designated p21.

Molecular characterization of the human H-ras and K-ras loci indicates that these genes acquire malignant properties by single point mutations that affect the incorporation of the 12th or 61st amino acid residue of their respective p21 proteins. Mutations within codon 12 of H-ras alter the sequence CCGG (C, cytosine; G, guanine), which is specifically recognized by certain restriction endonucleases, thus providing a simple biochemical assay for detection of transforming H-ras genes. However, the potential use of these findings is hampered by the infrequent activation of the H-ras locus in human neoplasias in much higher frequencies.

We therefore undertook studies to characterize the K-ras locus that has undergone alterations leading to its malignant activation and studies to determine whether these changes create restriction enzyme polymorphisms which can be utilized to develop simple in vitro assays to detect the point mutations.

Because of the complexity of the human K-ras locus, we limited our cloning efforts to DNA segments containing exon sequences. We subcloned domains of v-kis, the onc gene of the Kirsten strain of murine sarcoma virus, which

contained sequences homologous to each of the K-ras exons. A 124-base pair (bp) Sst II-Sau 3AI DNA fragment of v-kis, consisting of nucleotides located at positions -24 to +120 from the initiator codon ATG (A, adenine; T, thymine), was used as a probe to identify the first exon of the human K-ras gene. This retroviral probe was used to screen a library of human fetal liver DNA partially digested with Eco RI and amplified in λ Charon 4A phages. A recombinant phage, containing 18 kbp of human DNA was isolated, and an internal 6.6-kbp Eco RI DNA fragment was subcloned in pBR322. Sequences hybridizing to the 5' end of v-kis were located within a 1.0-kbp Hinc II DNA fragment, which was subsequently characterized by nucleotide sequence analysis.

Single point mutations responsible for the malignant activation of the H-ras gene present in T24 bladder carcinoma cells altered the coding properties of its 12th codon and also eliminated the tetranucleotide CCGG specifically recognized by the restriction endonucleases Hpa II and Msp I. These results provided the experimental basis for developing a biochemical assay capable of distinguishing between normal and transforming H-ras genes. In the case of the human K-ras proto-oncogene, neither of the two Gs that determine the coding properties of its 12th codon, GGT, were part of any sequence specifically recognized by known restriction endonucleases. Howevever, replacing the first G with a C creates the sequence GAGCTC (+29 to +34), which is recognized by Sac I. Similarly, a mutation replacing the second G with a C generates the sequence of GCTGC (+31 to +35). This sequence is specifically cleaved by Fnu 4 HI. None of the four other point mutations that would change the coding properties of this triplet lead to the generation of restriction endonuclease polymorphism. Hence, K-ras oncogenes activated by certain single point mutations within its 12th codon could be biochemically identified by the generation of new Fnu 4 HI or Sac I cleavage sites.

The first exon of the human K-ras locus is contained within a 14-kbp Sac I DNA fragment. A polymorphic Sac I cleavage site within this exon resulting from a G→C mutation in codon 12 will cause the appearance of two Sac I DNA fragments of sizes 5.8 and 8.2 kbp. These polymorphic DNA fragments can be specifically identified with probes that are specific to the first exon.

DNAs isolated from five human tumor cell lines and three human tumor specimens in which we had identified K-ras oncogenes were submitted to Southern blot analysis after digestion with Sac I. Six of these DNAs showed the wild-type 14-kbp Sac I DNA fragment, and the other two, which had been isolated from A1698 bladder carcinoma and A2182 lung carcinoma cell lines (independently established cell lines with at least four different isoenzyme markers), showed altered Sac I DNA fragments. Instead of the normal 14-kbp Sac I DNA fragment, two new DNA fragments of 8.2 and 5.8 kbp were seen. These are the expected sizes if a polymorphic Sac I cleavage site is generated within the first exon of the K-ras locus in these two human tumor cell lines.

Demonstration that the above results were the direct result of a GGT→CGT mutation in codon 12 of A1698 and A2182 K-ras oncogenes was provided by molecular cloning of a 6.6-kbp Eco RI fragment of human DNA that contained the first exon of the K-ras oncogene present in A2182 lung carcinoma DNA. Partial sequence analysis of this DNA fragment revealed that the first exon of the A2182 K-ras oncogene was identical to that of its allele present in normal

human cells with the exception of codon 12, in which the first base, a G, was replaced with a C. As a result of this point mutation, arginine instead of the normal glycine is the 12th amino acid residue of the transforming p21 protein coded for by the A2182 K-ras oncogene.

Creation of restriction enzyme polymorphisms by the mutational events responsible for the malignant activation of ras genes has made it possible to screen human tumors for the presence of critical mutations and to investigate their role in the development of carcinogenesis. We selected lung carcinomas because they represent the most common form of human cancer and because K-ras oncogenes appeared to be preferentially activated in this type of tumor. Genomic DNAs were prepared from 1- to 3-g portions of surgically removed lung carcinomas, digested with Fnu 4 HI or Sac I and submitted to hybridization analysis. A Sac I polymorphism was observed in one of eight tumors initially screened. Tumor LC-10, a squamous cell carcinoma removed from a 66-year-old man had the same 8.2- and 5.8-kbp Sac I DNA fragments present in the K-ras oncogenes of A1698 and A2182 human tumor cell lines. These results indicate the existence of G→C mutation at position +34 of the first exon of an LC-10 K-ras gene.

In a parallel study, the same human tumor DNAs were tested in NIH/3T3 transfection assays. Only LC-10 DNA exhibited detectable transforming activity (0.01 focus-forming units per microgram of DNA). Transformed cells were cloned in agar and their DNAs were submitted to hybridization analysis. All tested NIH/3T3 transformants contained the 8.2- and 5.8-kbp Sac I DNA fragments, thus demonstrating that the mutated LC-10 K-ras gene had transforming activity.

LC-10 DNA also includes a K-ras allele whose first exon is within a 14-kbp Sac I DNA fragment. The absence of this DNA fragment in NIH/3T3 transformants suggests that this gene does not have transforming activity. These results could be considered as evidence that the lung carcinoma cells are heterozygous at the K-ras locus. However, because solid tumors contain more than one type of cell, the possibility that nonmalignant cells might be responsible for the apparent heterozygosity cannot be excluded.

We also examined normal tissue, including bronchial, and parenchymal cells and blood lymphocytes, from the patient with the LC-10 tumor. None of the normal cells showed the activating G→C mutation detected in tumor tissue. Moreover, DNA isolated from these normal tissues did not show transforming activity in NIH/3T3 transfection assays. These results indicate that a mutational event responsible for the malignant activation of a ras transforming gene is specifically associated with the development of a human cancer.

DNA rearrangements in onc gene loci in mouse lymphosarcomas. Abelson murine leukemia virus (A-MuLV) is a replication-defective transforming retrovirus that arose by recombination of nondefective helper virus Moloney murine leukemia virus (M-MuLV) and cellular sequences present within the normal mouse genome. The latter sequences, termed abl, appear to code for the transforming properties of the virus. This virus induces in adult BALB/c mice a variety of lymphoid neoplasms predominantly of the pre-B cell series (ABLS tumors). However, when the mice are previously injected with pristane, which induces intraperitoneal granulomatous tissue, this virus also rapidly induces plasmacytomas

(ABPC tumors) and occasionally, a morphological subset of lymphosarcomas characterized by plasmacytoid cytoplasm but with very little immunoglobulin production (ABPL tumors). Our preliminary experiments indicated that ABPC and ABLS tumors produced abundant amounts of infectious A-MuLV particles while most ABPL tumors, in striking contrast, did not. In an effort to understand the molecular mechanisms involved in the genesis of these tumors, a detailed study of the expression of abl, myc, and myb oncogenes was undertaken. These studies demonstrated that: (1) ABLS and ABPC tumors contain integrated A-MuLV proviral genome in the cellular DNAs and express abundant quantities of A-MuLV RNAs. In contrast, ABPL tumors, with the exception of ABPL-3, do not contain the A-MuLV proviral genome and do not express detectable levels of A-MuLV RNA. (2) All three tumor types expressed a 2.4-kb myc RNA. The band intensities of myc RNA in the three classes of tumors varied considerably with the ABLS, generally containing the lowest amount of myc RNA. None of the ABLS or ABPL tumors were found to contain rearrangements in myc locus. (3) The ABLS and ABPC tumors do not exhibit any rearrangements in myb locus and do not express any abnormal sized mRNAs. On the other hand, all ABPL tumors show rearrangements in the c-myb locus and express elevated levels of myb RNA. In addition, a majority of them contain an abnormal sized mRNA which readily hybridized with the v-myb probe. These experiments appeared to indicate that DNA rearrangements in the c-myb locus are responsible for the enhanced expression and appearance of abnormal sized c-myb-encoded mRNAs in these cells. In order to understand the molecular mechanisms by which these DNA rearrangements occur, we undertook molecular cloning of normal c-myb sequences from mouse embryonic DNA and the rearranged c-myb sequences from the ABPL-2 tumor line.

Following these studies, a c-myb clone was isolated from a partial Eco RI library of mouse embryonic DNA cloned in Charon 4A vector. This clone, which had a 17-kbp insert, contained three Eco RI fragments (4.2, 1.7, and 0.5 kbp long) which cross-hybridized with the v-myb probe. To orient the mouse sequences with respect to the v-myb gene, the myb-related Eco RI fragments of 4.2, 1.7, and 0.5 kbp were used as probes and hybridized with various restriction digests of the v-myb clone. These results indicated that 4.2-kbp Eco RI fragment hybridized to the 5' end of the v-myb clone, while the 0.5-kbp Eco RI fragment hybridized to the 3' end of the v-myb sequences. The 1.6-kbp Eco RI fragment hybridized to the middle region of v-myb sequences, thus orienting the molecule. This orientation was further confirmed by partial nucleotide sequence analysis of this clone.

Structure of myb locus in ABPL-2 cells. In order to study the altered myb locus in ABPL-2 tumor, the DNA from the tumor was digested with Eco RI and the 7.5-kbp DNA band was cloned in λgt Wes B vector. An Eco RI insert from one such clone was isolated and compared with 4.2-kbp c-myb Eco RI fragment by heteroduplex analysis. These results clearly indicated that the two DNA fragments shared a homology of 0.9 kbp and 3.3 kbp at either end and the rearranged myb fragment from ABPL-2 tumor contained a large insertion of approximately 3.0 kbp towards one end of the fragment.

In order to characterize the nature of the inserted DNA fragment, the DNA was digested with various restriction enzymes and hybridized with a Moloney murine leukemia virus (M-MuLV) long terminal repeat (LTR)-specific probe.

These results indicate that the inserted piece of DNA had at least two LTRs derived from Mo-MuLV. Restriction mapping and nucleotide sequence analysis have indicated that the inserted DNA has a similar nucleotide sequence to that of Mo-MuLV with a large deletion in the gag, pol and envelope regions. This insertion of the proviral genome appears to have occurred towards the 5' end of the v-myb-related sequence.

The enhanced transcription of the c-myb locus therefore appears to be due to the enhancer effect of LTR sequences on the c-myb locus, while the altered size of c-myb DNA fragment in ABPL-2 tumor is due to the insertion of a defective Mo-MuLV proviral genome upstream to the myb-oncogene-related sequences in these tumors. This situation is analogous to that found in avian leukosis virus-induced bursal lymhomas.

The MuLV proviral genome inserted has suffered large deletions in pol and env regions and, hence, is only 3.0 kbp long.

Significance to Biomdical Research and the Program of the Institute.

The experiments performed under this project demonstrate that the mutational event responsible for the malignant activation of a K-ras transforming gene is specifically associated with the development of a human cancer and is not associated with the normal tissues of the same patient. These experiments have also provided a molecular approach for the detection of some of these mutations using the restriction enzyme polymorphism generated as a result of this event. This approach provides a simple and rapid method to screen large numbers of normal and tumor tissues for the presence of mutations that activate ras genes.

The results presented above demonstrate that the rearrangement of the c-myb locus observed earlier by us in ABPL-2 tumor cells is due to the insertion of a proviral genome upstream to the myb-oncogene-related sequences. The retroviral LTRs are known to contain enhancer elements and presumably the insertion of these LTRs leads to the altered rates of expression of myb-encoded mRNAs in these cells. ABPL tumors also seem to produce structurally altered mRNAs from the rearranged c-myb locus. Whether these altered myb mRNAs synthesize myb proteins that are structurally different leading to an altered biochemical function, which in turn is responsible for the oncogenic activity, requires further experimentation.

Proposed Course:

A detailed sequence analysis of mouse c-myb locus and the rearranged c-myb locus has been undertaken. Structural changes similar to those that occurred in c-myb oncogenes are responsible for the activation of this oncogene in mouse plasmacytoid lymphosarcoma. Studies are also being conducted to understand the mode of activation of c-myb locus in several human lymphoid tumors. The project will be continued by other members of the LCMB.

Publications:

Aaronson, S. A., Reddy, E. P., Robbins, K., Devare, S. G., Swan, D. C., Pierce, H. J. and Tronick, S. R.: Retroviruses, onc genes, and human cancer. In Harris, C. C. and Autrup, H. N. (Eds.): Human Carcinogenesis. Academic Press, 1983, pp. 609-630.

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Lavu, S., Mushinski, J. F., Shen-Ong, G. L. C., Potter, M. and Reddy, E. P.: Structural organization of mouse c-myb locus and the mechanism of its rearrangement in ABPL 2 tumor line induced by pristane and Abelson murine leukemia virus. Current Topics in Microbiology and Immunology, Vol. 113 (In Press)

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Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D., Baluda, M. A. and Reddy, E. P.: Complete nucleotide sequence and biochemical organization of the transforming gene and large terminal redundancies of avian myeloblastosis virus. In Scolnick, E. M. and Levine, A. J. (Eds.): UCLA Symposium on Tumor Viruses and Differentiation. New York, A. R. Liss, 1983, pp. 291-296.

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Shen-Ong, G. L. C., Reddy, E. P., Potter, M. and Mushinski, J. F.: Disruption of mouse c-myb locus by M-MuLV insertion within a common region in independently derived plasmacytoid lymphosarcomas. Current Topics in Microbiology and Immunology, Vol. 113 (In Press)

Srinivasan, A., Reddy, E. P., Dunn, C. Y. and Aaronson, S. A.: Molecular dissection of transcriptional control elements within the long terminal repeat of the retrovirus. Science 223: 286-289, 1984.

Yuasa, Y., Eva, A., Kraus, M. H., Srivastava, S. K., Needleman, S. W., Pierce, J. H., Rhim, J. H., Gol, R., Reddy, E. P., Tronick, S. R. and Aaronson, S. A.: Ras-releated oncogenes of human tumors. Cold Spring Habor Conference on Cell Proliferation, Vol II, The Cancer Cell (In Press)

Yuasa, Y., Gol, R. A., Chang, A., Reddy, E. P., Tronick, S. R. and Aaronson, S. A.: Mechanism of activation of an N-ras oncogene of SW-1271 human lung carcinoma cells. Proc. Natl. Acad. Sci. USA (In Press)

Yuasa, Y., Srivastava, S. K., Dunn, C. Y., Rhim, J. S., Reddy, E. P. and Aaronson, S. A.: Acquisition of transforming properties by alternative point mutations within c-bas/has human proto-oncogene. Nature 303: 775-779, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05060-06 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Studies of Oncogenic Expressions 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	Microbiologist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. Fujita	Visiting Fellow	LCMB	NCI
	Y. Yuasa	Visiting Fellow	LCMB	NCI
	P. Arnstein	Veterinary Medical Officer	LCMB	NCI
	K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI

COOPERATING UNITS *(If any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

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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 individuals predisposed to cancer genetically or by virtue of exposure to environmental carcinogens; (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 application in primates, including humans.

PROJECT DESCRIPTION

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

J. S. Rhim	Microbiologist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. Fujita	Visiting Fellow	LCMB	NCI
Y. Yuasa	Visiting Fellow	LCMB	NCI
P. Arnstein	Veterinary Medical Officer	LCMB	NCI
K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
S. Sieber	Deputy Director	DCE	NCI
R. S. Day	Microbiologist	LMC	NCI

Objectives:

1. To develop a cell culture transformation system for identification of individuals at high risk for early cancer.

2. To develop and study human cell transformation systems, particularly epithelial cells, and the factors regulating cell transformation, to elucidate mechanism of cellular 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:

Neoplastic transformation of primary human epidermal keratinocytes by the combined action of DNA SV40 and RNA Ki-MSV ras transforming genes. Most viral carcinogenesis studies of human cells have used fibroblasts. Since the majority of human tumors are of epithelial origin, it is important to study the epithelial cell system. However, in vitro viral carcinogenesis studies involving human epithelial cells have been limited by the difficulty in culturing such cells. There have been a few reports describing DNA tumor virus-induced altered growth of human epithelial cells, but the tumorigenicity of the altered cells has not been demonstrated. In addition, no in vitro neoplastic transformation of human epithelial cells by RNA tumor viruses has been described. Recently, efforts to improve the ability to culture human epithelial cells have led to the development of defined media for growth of a variety of types. For example, it is now possible to obtain primary cultures of human skin epithelial cells in the

absence of feeder cells. Such cells can be successfully subcultured without a feeder layer for at least a few additional passages. Using this system, we have studied alterations in human epithelial cell growth and differentiation resulting from the interaction with transforming genes of DNA and RNA tumor viruses. Primary cultures of human epidermal keratinocytes grown in NCTC 168, supplemented with horse serum infected with adenovirus 12-simian virus 40 (Ad12-SV40) hybrid virus, grew, underwent productive and nonproductive infection, and became established, but nontumorigenic cell lines. The altered epithelial cells contained both SV40 large and small tumor antigens, but did not contain adenovirus early region (E1A and E1B) messages. Subsequent infection of these nonproducer cells with Kirsten murine sarcoma virus (K-MSV) produced striking morphological alteration and led to the acquisition of neoplastic properties. Thus, neoplastic transformation of human epidermal keratinocytes was demonstrated by the combined action of SV40 and K-MSV ras transforming genes. These findings provide the first demonstration of neoplastic conversion of human primary epithelial cells in culture, establishing further a defined set of transforming genes which complement each other in the induction of the malignant phenotype. These results also establish a multiple stage process in the progression of human epithelial cells toward malignancy.

Inherited enhanced susceptibility to retrovirus-induced transformation of Gardner syndrome cells. A number of in vitro studies had previously indicated that skin fibroblasts can be used to distinguish individuals with Gardner syndrome (GS) and familial polyposis coli (FPC) from others in the general population. In order to establish further the accuracy of the test, coded skin samples received from Eldon Gardner and Randy Moon, University of Utah, were tested for various growth properties, including susceptibility to transformation by viral or chemical agents. Parallel specimens were also tested by Suraiya Rasheed, University of Southern California. The results indicated that, based on the higher susceptibility to retrovirus-induced transformation and chromosomal aneuploidy, the GS and FPC cells could be distinguished from those of the general population with more than 70% accuracy. However, much work is in order before any biological assay can be used for clinical diagnosis of GS or FPC patients.

Glucocorticoids enhance retrovirus transformation of mammalian cells. 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). However, the effect of these hormones on the replication of MSV in other mammalian cells has not been examined. Therefore, the effect of glucocorticoids on the replication of MSV in mammalian cells were examined. In contrast to the reported lack of effect of steroid hormones on MSV-induced transformation in mouse cells, we found that the glucocorticoids, hydrocortisone (1 to 10 μg/ml), and dexamethasone (5 μg/ml), enhanced transformation induced by K-MSV in normal rat kidney (NRK) and human cells in vitro. Indeed, hydrocortisone enhances the K-MSV-induced transformation in NRK and human skin fibroblasts 10- to 30-fold. The enhancing effect was much more pronounced in normal human colonic mucosal epithelial-like cells. On the other hand, the hormones estradiol, testosterone, and progesterone (5 μg/ml) had no effect. Individual foci appeared earlier and were larger in hydrocortisone-treated cells compared with untreated cells. This enhancing effect is further evidenced by

the increased virus yield and murine leukemia virus complement fixation antigen production in the test system. Hydrocortisone did not promote the production of K-MSV in mouse embryo cells, as previously reported. However, individual foci in hormone-treated cells appeared earlier and the size of the foci were larger than those appearing in untreated cells. Thus, the hydrocortisone treatment offers a simple, rapid, sensitive transformation assay method for MSV in mammalian cells and may be of practical use in achieving greater production of type C virus from certain retrovirus-producing mammalian cell lines.

Hybrids between human tumor cell strains differing in repair of N-methyl-N-nitrosoguanidine (MNNG)-produced DNA damage. Three groups of human cell strains having differing capacity for DNA repair have been recognized among human tumor strains by comparing their responses to alkylation-damaged DNA with those of normal human fibroblasts. One approach to understanding the genetic basis of the Mer (MNNG repair) and REM (cellular resistance to MNNG) phenotypes has been to study repair in systems which have combined elements from both groups.

Human tumor cell strains with differing responses to MNNG damage in their DNA were treated with precipitates of the plasmids pSV2gpt or pSV2neo. Transfected clones were selected on the basis of the drug resistance which each plasmid confers. Cells with different drug resistances were fused and hybrids were selected in medium requiring the expression of both markers. Hybrids produced by fusion of two different strains hypersensitive to MNNG-produced cytotoxicity and which lack the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (O6MT) failed to show complementation, suggesting that these strains share a common genetic defect. Hybrids from fusions of each of three strains containing O6MT activity with the same strain lacking O6MT activity were of surprising character. In one case the hybrid had resistance to MNNG-produced cell killing and O6MT activity similar to the parent strain possessing O6MT activity. In a second case, the hybrid had greater resistance to MNNG produced cytotoxicity than either parent strain, although the level of O6MT activity was not higher. In a third case, the hybrid had little or no O6MT and as much hypersensitivity to MNNG-produced cytotoxicity as the parent strain lacking O6MT activity. We conclude that the survival of human tumor cell strains after MNNG-produced DNA damage is controlled by several genes. Even individual repair enzymes, like O6MT, are likely to be regulated by the interaction of these genes.

Induction of tumors in chimpanzees with a chemical carcinogen. In collaboration with Dr. Sieber, liver tumors were induced in adolescent chimpanzees following continuous treatment with a chemical carcinogen, DENA. This is the first documentation of experimentally induced chimpanzee tumors. The tumors were hepatocellular carcinomas. Attempts were made to establish the tumor cell lines from tumors, but the tumor cells did not survive beyond a few passages. Attempts were also made to isolate oncogenes in the NIH/3T3 cells by the DNA-mediated transfection method using the hepatoma tissue DNA, but no positive results were obtained.

Isolation of oncogenes from human lung carcinoma-derived cell lines. Several oncogenes were isolated from 20 human lung carcinoma-derived cell lines in the LCMB using the DNA-mediated transfection assay. One of the isolates has been

identified from a carcinoma-derived cell line, designated Hs242. This transforming gene, related to c-bas/has (human), was cloned in biologically-active form and demonstrated a specific transforming activity of $>10^4$ ffu/μg DNA insert. Restriction endonuclease mapping revealed no detectable differences from its normal allele, indicating that relatively subtle changes were responsible for its activation as a tranforming gene. By restriction enzyme and nucleotide sequence analysis, the Hs242 oncogene was shown to be unaltered in the first exon, the region responsible for activation of the T24 and EJ human oncogenes. Thus, genetic changes at more than one site in the same proto-oncogene can be responsible for its acquisition of transforming properties under natural conditions. Studies were aimed at localizing the number and nature of the genetic alterations responsible for activation of the Hs242 oncogene.

H-ras oncogenes are activated by somatic alterations in human urinary tract tumors. DNA-mediated gene transfer studies using NIH/3T3 cells as recipients have demonstrated the presence of transforming genes (oncogenes) in diverse human tumors. A large proportion of oncogenes so far detected by DNA transfection are related to the H-ras onc gene of Harvey (and BALB) murine sarcoma viruses (MSV), K-ras, the oncogene of Kirsten MSV and a third member of the ras gene family, N-ras. Individual tumors of many different organs have been associated with the activation of members of the ras gene family. In this study we present the first systematic survey of human urinary tract tumors, processed immediately after surgery as well as normal tissues from the same patients, for the presence of such genes. We demonstrate activation of H-ras as an oncogene in around 10% of unselected urinary tract tumors as well as direct evidence that oncogene activation is the result of a somatic event which is selected for within the tumor cell population.

Transforming genes were detected by transfection analysis in 2 of 23 unselected primary human tumors of the urothelium. In both cases, H-ras oncogenes were identified as the source of tranforming activity. Neither oncogene was activated by a lesion at position 12 in the coding sequence consistent with other studies, indicating that position 12 lesions in H-ras are not commonly detected in primary human tumors. We demonstrated a genetic alteration(s) within codon(s) 60, 61, or 62 of the H-ras coding sequence of the JPT26 tumor by a Bst NI restriction site polymorphism. Since position 61 is a major "hot spot" for activation of ras oncogene in human tumors, it is likely that the genetic alteration detected at this position of the JPT26 oncogene coding sequence is responsible for its activation as a transforming gene. Neither normal cell DNA from two patients whose tumors contained H-ras oncogenes possessed a transforming gene detectable by transfection analysis. By restriction enzyme analysis, it was possible to obtain direct biochemical evidence that the genetic lesion in the JPT26 oncogene was not present in normal cellular DNA of this same patient. These findings establish that this genetic alteration is the result of a somatic event which must be powerfully selected for within the tumor and, thus, likely to contribute to its development.

Our results also suggest that H-ras oncogenes are more commonly activated in urinary tract tumors than N- or K-ras genes. This is in contrast to hematopoietic tumors, where N-ras appears to be the most frequently activated ras oncogene, or lung and colon carcinoma where K-ras oncogene appears to predominate. The frequency at which oncogenes were detected is likely to be

an underestimate due to the inherent insensitivity of the transfection assay. Single base changes at either position 12 or 61 in the H-ras coding sequence will lead to loss of Msp I or Bst NI sites for 6/6 and 7/8 possible amino acid substitutions, respectively. Thus, a systematic survey of urinary tract carcinomas for these point mutations as well as for amplification or other rearrangements using biochemical approaches may provide a more accurate estimation of the frequency at which H-ras is activated as an oncogene in these naturally occurring human malignancies.

Significance to Biomedical Research and the Program of the Institute:

1. The development of testing systems for identification of genetic susceptibilities to cancer could prove very useful in cancer diagnosis.

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 virus and chemicals in order to elucidate human epithelial cell carcinogenesis.

3. Continued characterization of adeno 12-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 cervical, ovarian, and brain carcinomas and hepatomas, using the DNA transfection 3T3 cell assay.

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

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05061-06 LCMB

PERIOD COVERED: October 1, 1983 to June 1, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Molecular Study of Abelson Murine Leukemia Virus Genome

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

PI:	A. Srinivasan	Visiting Associate	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	S. G. Devare	Visiting Associate	LCMB	NCI
	E. P. Reddy	Visiting Scientist	LCMB	NCI
	Y. Yuasa	Visiting Fellow	LCMB	NCI
	J. Pierce	Senior Staff Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 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 spece provided.)*

The integrated Abelson murine leukemia virus (A-MuLV) genome cloned in bacteriophage vector was used to analyze the functional nature of the different segments of the viral genome. Construction of mutants having sequential deletions of the A-MuLV-specific abl gene revealed that only the proximal 40% of abl with its associated 5' helper viral sequences is required for fibroblast transformation. To define regions of long terminal repeat (LTR) required for A-MuLV transforming gene function, deletion mutants encompassing different domains were construced and assayed following transfection of NIH/3T3 cells. Deletions involving only TATA and CAAT sequences showed reduced transforming activity, whereas deletions extending to the end of the LTR abolished transforming activity. Removal of both 72-bp repeats completely abolished the transforming activity. Interestingly, ligation of wild-type 3' LTR to the defective 5' LTR subgenomic clones restored the transforming activity. Nucleotide sequence analysis of the complete proviral genome of A-MuLV had been determined. The amino acid sequence of the abl gene, derived from the primary DNA sequence, has significant homologies to other tyrosine-specific protein kinases encoded by src, fes, fps and yes onc genes.

PROJECT DESCRIPTION

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

A. Srinivasan	Visiting Associate	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. G. Devare	Visiting Associate	LCMB	NCI
E. P. Reddy	Visiting Scientist	LCMB	NCI
Y. Yuasa	Visiting Fellow	LCMB	NCI
J. Pierce	Senior Staff Fellow	LCMB	NCI
A. Eva	Visiting Associate	LCMB	NCI
S. R. Tronick	Acting Chief, Gene Structure Section	LCMB	NCI
R. Narayanana	Visiting Fellow	LCMB	NCI
A. Gazit	Visiting Fellow	LCMB	NCI

Objectives:

1. Structural and functional analyses of the transformng gene of Abelson murine leukemia virus (A-MuLV).

2. Dissection of A-MuLV long terminal repeat (LTR) by in vitro mutagenesis and its role in viral gene expression.

Methods Employed:

1. Molecular cloning techniques, transfection assay, immunoprecipitation and SDS-PAGE.

2. Molecular cloning, DNA transfection and sequencing.

Major Findings:

1. Molecular cloning of A-MuLV. The integrated proviral genome of A-MuLV was molecularly cloned in E. coli using bacteriophage vector λgtWES·λB. A 7.8-kbp DNA fragment containing the integrated viral genome was obtained following Eco RI digestion of high molecular weight DNA of A-MuLV transformed nonproducer cells and enrichment by RPC-5 chromatography and preparative gel electrophoresis. Recombinant DNA clones containing a 7.8-kbp Eco RI fragment were shown to contain the integrated A-MuLV genome with 5' and 3' ends flanked by 1.8-kbp and 0.2-kbp mink cellular DNA sequences, respectively. The relationship of A-MuLV to its parental genome Moloney murine leukemia virus (M-MuLV) was studied by heteroduplex analysis. Such studies showed that A-MuLV contains regions of homology with M-MuLV at either end of its genome and the central portion is unique to A-MuLV. The extent of homology is 1.7 kbp and 0.7 kbp at 5' and 3' ends, respectively.

   Transformation by cloned whole and subgenomic A-MuLV fragments. In order to identify the minimal A-MuLV DNA sequences required for transformation, we minitored the biologic activity of λ AM-1 DNA after exposure to different

restriction enzymes. Restriction enzymes Ava I, Sma I, Pst I, Sac I, Bgl I and Bgl II, which cleave at multiple sites within the viral genome, completely abolished transforming activity. Xba I and Kpn I each cleave the A-MuLV genome only within the LTR. Xba I significantly reduced and Kpn I completely abolished transforming activity. Pvu I, which cleaves at a unique site 300 bp downstream from the 5' LTR, also abolished A-MuLV biologic activity.

Cleavage with enzymes that cut at unique sites towards the 3' end of the viral genome made it possible to localize the region of abl required for transforming activity. Thus, Bam HI, Hind III, and Cla I, which cut at unique sites towards the 3' end of the genome, did not appreciably alter the biologic activity of λ AM-1 DNA. Even cleavage at a unique Sal I site within abl sequences resulted in transforming activity comparable to that of the parental A-MulV DNA. In contrast, Acc I, which cleaves approximately 700 bp upstream from the Sal I site, completely abolished A-MuLV biologic activity. These results suggested that not more than 56% of abl was required for fibroblast transformation.

Comparison of transforming activities of A-MuLV subgenomic DNA clones. In an effort to provide more quantitative comparisons of their transforming activities, several A-MuLV DNA fragments were subcloned in plasmid vector. In most cases it was possible to utilize the same restriction sites in pBR322 for construction of the subclones. However, for Xba I, Acc I and Kpn I subclones, it was necessary to utilize Eco RI linkers. A-MuLV DNA subcloned in pBR322 demonstrated 1.7×10^2 ffu/μg DNA when utilized to transfect NIH/3T3 cells. Cla I, Bam HI, Hind III, and Sal I DNA subclones each showed comparable transforming efficiencies, while the Acc I subclone was completely inactive under the same assay conditions. These findings directly established that the distal 44% of abl was not essential for efficient transformation of fibroblasts.

Analysis of A-MuLV DNA subclones constructed from the Pvu I, Xba I, or Kpn I restricted wild-type genome provided information concerning the requirements for transformation of sequences at the 5' terminal portion of the A-MuLV genome. Both Pvu I and Kpn I subclones lacked detectable biologic activity. The transforming efficiency of the Xba I subclone was around 3 to 10% that of wild-type A-MuLV DNA. These latter findings demonstrate that while the 5' viral LTR is critical to transformation by A-MuLV, the entire LTR is not absolutely essential.

Identification of gene products coded by A-MuLV subgenomic DNAs. A-MuLV is known to code for expression of a 120,000 molecular weight protein, which contains gag gene products p15, p12, and a small portion of p30, as well as a region unrelated to known MuLV proteins. Since transformation by A-MuLV is thought to be mediated by P120, it was of interest to analyze A-MuLV-specific proteins present in cells transformed by subgenomic A-MuLV DNAs. Cells were metabolically labelled with [^{35}S]-methionine and viral proteins analyzed by immunoprecipitation with anti-M-MuLV sera followed by SDS-PAGE.

Transformants induced by A-MuLV genomic DNA, as well as by Cla I, Bam HI and Hind III subgenomic clones were found to express proteins whose sizes were indistinguishable from P120 coded by wild-type A-MuLV. These results indicate that the A-MuLV sequence coding for P120 terminates prior to the Hind III site. Transformants obtained with the Sal I DNA subclone demonstrated anti-M-MuLV precipitable proteins, whose sizes varied from 82,000 to 95,000 daltons among individual transformants tested.

Previous studies have indicated that wild-type A-MuLV coded P120 possesses an associated protein kinase activity. Analysis of transformants induced by different A-MuLV DNA subclones in each case revealed A-MuLV-specific proteins with this activity. Moreover, the sizes of the proteins corresponded to those of the A-MuLV-specific proteins precipitable with anti-MuLV serum in each transformant. Protein kinase activity was even preserved in the Sal I subclone coded 82,000 daltons protein. These results help to localize the coding region for the A-MuLV transforming protein responsible for its kinase activity to sequences within the proximal 40% of abl.

Nucleotide sequence of A-MuLV. To understand the mechanism of action of the v-abl gene and its relationship with other transforming genes known to code for tyrosine-specific kinase activity, we undertook primary DNA sequence analysis of the proviral genome. The sequence was determined according to the partial chemical degradation method of Maxam and Gilbert. Because the complete nucleotide sequence of M-MuLV, the natural helper virus of A-MuLV, is known, we compared the sequence with that of M-MuLV. The mink cellular sequences immediately flanking the A-MuLV LTRs were found to contain a four-nucleotide direct repeat sequence, T-G-G-G, confirming previous findings of duplication of a short stretch of sequences at the site of retrovirus integration.

The comparison also revealed the occurrence of a sequence homology of 1,776 bp at the 5' end and 793 bp at the 3' end. The region of homology at the 5' end included the noncoding sequence and the amino terminal region of the gag gene.

The noncoding sequence at the 5' terminus included the LTR, the primer tRNA binding site, and a stretch of sequences that are present before the start of the gag sequences. This region of 1,776 bp exhibited 17 differences between the two genomes. Eight of these changes occurred in the LTR region, three in the 5' noncoding sequences, and six within the gag gene. The open reading frame, starting at position 1,067, contained the entire sequence of p15 and p12 and the first 21 codons of p30. Beyond position 1,776, no sequence homology was observed between the two viral genes, thus localizing the point of recombination at the 5' end.

The coding sequences terminated within the cell-derived abl sequences 855 bp upstream from the v-abl helper viral junction at the 3' end. Examination of this additional stretch of 800 bp revealed the presence of a second open reading frame starting with an ATG at position 4,154-4,157 and terminating at position 4,642-4,644 with a TGA codon. This stretch of 492 bases could code for a protein of 18,000 daltons. It is interesting to note that a promoter-like sequence T-A-T-A-A-A occurs at position

4,067-4,072 upstream to this open reading frame. A sequence resembling C-C-A-A-T box is also seen at position 4,019-4,023.

The point of recombination between the M-MuLV and c-abl sequences at the 3' end occurred at the same point within the viral genome as it did with c-mos during the generation of the Moloney MuSV genome. These findings suggest that there may exist preferential sites for recombination within the helper viral genome, and these sites could have played a crucial role in their evolution.

Amino acid sequence homology with other viral oncogenes coding for tyrosine-specific kinase activity. The translational product of the A-MuLV genome, P120, has been shown to be associated with tyrosine kinase activity. Of about 15 described v-onc genes, at least six appear to encode enzymes with analogous function (src, yes, fes, fps, ros, and abl). It was, therefore, interesting to examine the structural relationships among these onc proteins because the sequence for five of them is available. The results of this analysis demonstrated that all these five proteins have extensive sequence homology. Thus, v-abl protein shared 176, 166, 140, and 138 amino acids with yes-, src-, fes-, and fps-encoded transforming proteins. The homology is more pronounced with regions that are implicated with the active site for tyrosine phosphorylation. Less conserved regions of homology are observed between v-abl and v-mos encoded gene products; one of the v-mos products also has been shown to be a protein kinase.

Expression of an A-MuLV abl gene homologue in human cells. In collaboration with A. Eva, expression of the cellular homologue of the abl gene in human cells was analyzed. Normal fibroblasts revealed only two abl-related transcripts, whereas a complex pattern of transcripts was observed in human tumor cell lines derived from sarcoma, carcinoma, melanoma and astrocytoma. The widespread presence in tumors and even normal fibroblasts of multiple transcripts related to the onc gene of A-MuLV suggests that expression of related human genes may be associated with an important cellular function(s). Also, whether the multiple RNAs observed with the abl probe reflect RNA processing or transcription of more than one member of a family of related onc genes remains to be determined.

2. Deletion mutagenesis of long terminal repeat. The proviral genome of a retrovirus contains chracteristic directly repeated sequences, designated LTRs. The LTR is synthesized from viral genomic RNA components specific to the 5' (U5), 3' (U3), and repeated terminus (R) regions of the molecule during reverse transcription into a U3-R-U5 configuration. Because of their location within the viral genome, as well as their resemblance to transposable elements, retroviral LTRs have been said to function in processes including virus transcription, integration, replication, and genome rearrangement.

With respect to viral RNA transcription, certain LTR functions have been tentatively assigned. Nucleotide sequence analysis of LTRs derived from a number of avian and mammalian retroviruses has revealed the existence of consensus promoter sequences in the U3 region located at similar distances

from the RNA initiation site in the R region. Moreover, evidence from eukaryotic cell-free transcription systems has indicated that viral RNA transcripts initiate within the LTR. Evidence is also accumulating for the existence of sequences with an "enhancer"-like function within the U3 region of the LTR. In other systems, enhancer elements, which can act at a distance from the RNA start site and independent of orientation, appear to play an essential role in efficient gene function.

Abelson murine leukemia virus, a prototypical acute transforming retrovirus, arose by recombination of Moloney murine leukemia virus (Moloney-MuLV) with a cellular onc sequence designated abl. Moreover, abl is translated from a message initiated within the 5' LTR. Molecularly cloned A-MuLV demonstrates high-titered transforming activity as determined by transfection analysis on NIH/3T3 cells. Since A-MuLV transforming activity is dependent on efficient abl gene expression, we undertook deletion mutagenesis of the A-MuLV 5' LTR in an effort to precisely locate its transcriptional control elements. We have also explored the possibility that the 5' and 3' LTRs can act in a cooperative manner to allow transforming gene function.

We used an A-MuLV deletion mutant, pHind, which lacks the distal 1,019 kilobases (kb) of the viral genome, including the 3' LTR. This mutant transforms NIH/3T3 cells at a titer comparable to that of the wild-type A-MuLV genome. By use of a pHind, any influence of the 3' LTR on abl expression could be eliminated. In order to construct deletion mutants in TATA, CAAT (A, adenine; C, cytosine; and T, thymine), and the 5' cap site, we cleaved pHind with the restriction enzyme Xba I and treated the linerized plasmid with Bal31 exonuclease. After preliminary screening with appropriate restriction enzymes, we established the precise extent of each deletion by DNA sequencing.

An A-MuLV mutant (pAva), in which the TATA box and the 5' cap site were specifically deleted, showed no impairment in transforming activity. Deletion of the CAAT box as well (pHX25) reduced A-MuLV focus formation by a factor of about 10. Finally, mutants pHX37 and pHX90, in which larger portions were deleted, including the polyadenylation (poly A) signal and other downstream sequences, lacked any detectable biologic activity. These findings indicate that the TATA box and the 5' cap site are not essential for the expression of the abl gene. In the absence of the TATA box and the cap site, adjacent sequences seem to function as alternative promoter or intiation sites for transcription. Such sequences appear to be farther downstream since mutants with large deletions encompassing this downstream region (for example, pHX37 and pHX90) lacked transforming activity.

Nucleotide sequence analysis has revealed a 72- to 74-base pair (bp) repeat sequence in the U3 region of the LTRs of a number of retroviruses, including A-MuLV. A 72-bp unit has been identified within the genomes of papovaviruses. Deletion of this element in SV40 has been reported to reduce expression of the T antigen by a factor of at least 100 and to abolish virus viability. Evidence that the retroviral 72-bp repeat can replace the SV40

repeat and restore SV40 viability has suggested that the retroviral 71-bp repeat may similarly play a role in efficient retroviral gene expression.

To explore the effect of this region of the LTR on A-MuLV transforming gene function, we constructed another series of LTR deletion mutants lacking one or both 74-bp repeats. Loss of one 74-bp repeat did not impair A-MuLV transforming activity; however, loss of both repeats completely abolished this function.

Because the retrovirus genome is organized with an LTR at each terminus, we explored the possible cooperative effects of an intact 3' LTR on A-MuLV deletion mutants lacking transforming activity as a result of a defective 5' LTR. Thus, pHX37 and pPX deletion mutants were cleaved with Hind III, and a wild-type 3' LTR was inserted at this site. The resulting recombinant plasmids, pHX37-3' and pPX-3', respectively, were analyzed for transforming activity in the NIH/3T3 transfection assay. The addition of a 3' LTR failed to complement the defective 5' LTR in pHX37-3'. In contrast, the transforming activity of pPX-3' was restored almost to wild-type levels.

To study the potentiating effect of the LTR positioned 3' to the mos gene, a fragment containing v-mos was linked to an LTR derived from the Abelson MuLV proviral genome. As shown in Figure 2, pSCE and pSHE constructions both demonstrated high titered transforming activity. These results confirmed the ability of the 3' LTR to confer efficient transforming function to the v-mos and indicated, as well, that v-mos sequences to the right of the Hind III site were not essential for transforming activity. Because pSHE provided more sites for construction of LTR and other deletions, we utilized this plasmid in subsequent studies.

We next constructed a series of mutants of pSHE which deleted progressively larger regions from the 3' end of the LTR 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.

Retroviruses and transposable elements. On the structural level, retroviruses have considerable resemblance to transposable elements which have been identified in a number of prokaryotic and eukaryotic organisms since its discovery in maize. The transposable elements of Drosophila are strikingly similar to those of the integrated forms of retroviral proviruses and the properties shared by both are the following: (1) transposable

elements and proviruses are flanked by short, direct repeats of host DNA, presumably resulting from duplication of the target site upon integration; (2) presence of long terminal direct repeats (LTRs); and (3) the LTRs are bounded by small inverted repeats with similar terminal dinucleotides (5' TG---CA3'). These features have led to the speculations that retroviruses have evolved from transposable elements or vice versa. Recently, nucleotide sequences of the LTRs of transposable elements 17.6 and 297 of Drosophila were found to be remarkably similar to the LTR of avian leukosis-sarcoma virus. These structural and sequence similarities have prompted us to investigate whether LTRs of transposable elements of Drosophila are also functionally equivalent to retroviral LTR. In an attempt to answer this question, we have constructed a series of plasmids containing v-mos linked to the LTRs derived from the transposable elements of Drosophila. Transforming activity on NIH/3T3 cells following transfection of DNA was scored as an enhancer function provided by LTR. We show that there is a variation of two orders of magnitudes in the efficiency of various retroviral LTRs in enhancing v-mos transforming activity and that the LTR of copia enhances at least ten-fold the transformation by v-mos. In addition, we have also compared the efficiencies of LTRs derived from retroviruses of different species in relation to the transposable element LTR. Our findings provide evidence for the functional similarity of the long terminal repeat of copia element and retroviruses.

Significance to Biomedical Research and the Program of the Institute:

1. Human cellular sequences, designated proto-oncogenes, have been identified using retroviral transformation-specific sequences as molecular probes. Evidence for the potential involvement of such oncogenes in human cancer is accumulating rapidly. Reports implicating the involvement of the v-abl homologue, c-abl, in chronic myelogenous leukemia is an example in that regard. A c-abl probe would also be useful for analyzing other neoplastic diseases of lymphoid origin. Further, the molecularly cloned proviral DNA is useful for developing viral expression vectors.

2. Several investigators have analyzed the transcriptional control elements present in the viral genome using molecularly cloned DNAs. These studies have brought in new information as to the control signals and helped partly to understand the eukaryotic gene control in general. The regulatory control sequences identified in different viral genomes will be useful in the construction of viral expression vectors.

Proposed Course:

Using the primary DNA sequence data available for the A-MuLV transforming gene abl, attempts will be made to construct mutants to gain more information about the functional domain of abl protein.

The 5' termini of A-MuLV-specific mRNA produced in cells transformed by LTR deletion mutants will be determined. An attempt will be made to compare the enhancer sequence present in transposable element and viral LTR.

These projects will be continued by other investigators in the Laboratory of Cellular and Molecular Biology.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05062-06 LCMB

PERIOD COVERED: October 1, 1983 to September 30, 1984

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
	M. Wong	Visiting Fellow	LCMB NCI
	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC NCI

COOPERATING UNITS *(if any)*

CIIT Research, Triangle Park, NC (J. Swenberg)

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.)*

We surveyed human hemotopoietic tumors and tumor cell lines for sequences capable of transforming NIH/3T3 cells by DNA transfection. We found that the transforming gene associated with one acute lymphocytic leukemia was the activated cellular homolog of K-ras, while N-ras was shown to be the activated form of several myeloid and lymphoid tumor cells. We have also detected an apparently new transforming gene in the DNA of a non-Hodgkin's lymphoma, characterized histopathologically as a diffuse undifferentiated B-cell lymphoma. It was demonstrated that the biologic activity of this transforming DNA was serially transmissible through several cycles of transfections, and that it can induce a series of phenotypic changes in the same cell. Moreover, this transforming gene is not related to any of the known ras genes, nor to several other retroviral and cellular oncogenes. By using the same NIH/3T3 cell transfection assay, we analyzed fibrosarcomas induced in C57Bl/6 and NIH Swiss mice by subcutaneous single dose treatment of MCA. We used high molecular weight DNAs of the tumor cell lines derived from four of these fibrosarcomas for transfection on NIH/3T3 cells. We demonstrated that two of these tumor cell line DNAs are capable of transforming NIH/3T3 fibroblasts, that the transforming gene associated with these tumors is the same and that it is the cellular analog, K-ras, of the transforming gene of Kirsten murine sarcoma virus. We extended our studies to DNAs extracted from primary tumors developed in 75% of BALB/c and NIH Swiss mice treated SQ with a single dose of 100 μg methylcholanthrene. High molecular weight was extracted from each of the tissue specimens and tested on NIH/3T3 by transfection assay. Fifty percent of the tumor DNA analyzed was able to transform the NIH/3T3 cells. We demonstrated that exogenous K-ras sequences were associated with the transformed phenotype of the transfectants and established that K-ras is the transforming gene activated at high frequency in MCA-induced fibrosarcomas.

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
M. Wong	Visiting Fellow	LCMB	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI

Objectives:

To investigate which transforming genes are associated with specific human lymphomas, studies are directed to identify, isolate and characterize these genes in order to determine by what mechanism they are able to induce the transformed phenotype.

To study which genes are involved in transformation induced by chemical carcinogens, utilizing 3-methylcholanthrene (MCA)-induced mouse tumors.

Methods Employed:

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

Major Findings:

We have detected in both acute and chronic myelogenous leukemia cells, as well as in acute lymphoid leukemia cells, transforming genes capable of being serially transmitted to NIH/3T3 cells. The oncogene of one intermediate T-cell leukemia was demonstrated to be the activated human allele of K-ras, whereas two additional intermediate T-cell leukemias contained a different transforming gene, N-ras, related to but distinct from K-ras. This same gene was also found to be the transforming gene of one acute and one chronic myelogenous leukemia.

In efforts to detect additional transforming DNA sequences in human hematopoietic tumor cells, we found a transforming gene in a poorly differentiated diffused primary human B-cell lymphoma. We found that the biologic activity of this gene was serially transmissible through several cycles of transfection. Moreover, the unusual morphology observed in transfectants induced by this gene does not resemble the transformed phenotype induced by a large number of known retroviral onc genes. Our present studies identify that a transforming gene of a human non-Hodgkin's lymphoma is not related to any of the known ras genes nor to several other retroviral and cellular onc genes.

We have shown that DNAs of two of four cell lines established from methylcholanthrene-induced mouse fibrosarcomas contain a common transforming gene which was identified as the activated cellular oncogene, K-ras. We also found that the activation of K-ras occurs in 50% of primary tumors induced in BALB/c and NIH Swiss mice injected SQ with a single dose of methylcholanthrene. The

number of tumor DNAs positive in transfection assay was not equally distributed among the groups of tumors harvested at different times from MCA treatment. The percentage of tumor DNAs containing a transforming gene was inversely related to the time frame between MCA treatment and tumor harvest. Moreover, there was no evidence of relationship between the transforming activity of the tumor DNAs and the size of the tumors from which the DNAs were extracted.

Significance to Biomedical Research and the Program of the Institute:

Oncogenes of the ras family have been detected in activated genes in many different human tumors. We showed in our experiments that N-ras gene is the more frequently activated cellular oncogene in human hematopoietic tumor cells, and that its activation is independent of the stage of cell differentiation or tumor phenotype. Another ras oncogene, K-ras, is less frequently detectable in human hematopoietic tumor cells. The discovery of a new transforming gene, which is unrelated to many viral and cellular genes and is associated with a human lymphoma, and the characterization of its mechanisms of activation and function may be critical to the development of a better understanding of the pathways by which normal human cells become neoplastic.

It will be important to establish when in the course of oncogenesis these genes are activated. In this respect, the detection of a common transforming gene, K-ras, in 50% of MCA-induced fibrosarcomas has the relevance of providing a defined experimental system in which it may be possible to define its precise involvement in the progressive development of tumors.

We showed a relationship between the number of methylcholanthrene-induced tumor DNAs which have an activated K-ras and the length of time frame between MCA treatment and tumor harvest. These observations may indicate that K-ras gene is activated early during the development and growth of the MCA-induced fibrosarcoma and that its presence as an activated gene is not required for the maintenance of the transformed state in a fully developed tumor. The lack of transforming activity of the K-ras gene of older tumors may be attributed to DNA repair mechanisms which would occur or become effective late during tumor growth.

Proposed Course:

Efforts are underway to molecularly clone the 31-23-306 human transforming gene in biologically active form as well as to clone its normal human counterpart. Such studies should make it possible to precisely define its mechanism of activation. Efforts will also be undertaken to sequence the coding region of the gene in order to prepare appropriate reagents necessary to detect and characterize its product. Finally, using the approaches that have been successfully used in studies of the ras transforming gene of human origin, efforts will be made to define the role of the 31-23-306 transforming gene in neoplastic conversion of other diffuse lymphocytic lymphomas as well as tumors of different phenotype.

Further studies on the MCA-induced fibrosarcoma system will be attempted to correlate the presence of an activated K-ras allele with a particular time course of tumor development or tumor phenotype. Since MCA is known to induce

other types of tumors, such as lung carcinomas and lymphomas, we will attempt to analyze oncogene activation in other target tissues to assess if the activation of K-ras in MCA-induced tumors is related to target tissue or to the specific carcinogen used. Furthermore, in collaborative studies, we are currently assessing the role of oncogenes detectable by transfection in a number of other carcinogen-induced animal tumors.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05063-06 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

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
	P. Levine	Senior Investigator	CEB	NCI

COOPERATING UNITS *(if any)*
U. Malaya, Kuala, Lumpur, Malaysia (U. Prasad); U. Montreal, St. Justin Hospital, Canada (J. Menzes and S. Sundar); U. Erlangen-Nurnberg, West Germany (B. Fleckenstein and S. Schirm)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

Epstein Barr virus (EBV) immunovirology was investigated as a marker for diagnosis/prognosis in nasopharyngeal carcinoma (NPC) patients, their families, and individuals from ethnic populations in Malaysia known to be at higher risk of developing NPC by comparing IgA antibody to EBV virus capsid antigen (VCA) and lymphocyte stimulation by EBV antigens. All 75 VCA-IgA antibody-positive sera from NPC patients, regardless of age, sex and race (IF titers 10-640), blocked lymphocyte stimulation from NPC patients (20). Those sera which did not contain VCA-IgA antibody (<1:5) did not block lymphocyte stimulation and these patients were found either to have been in remission for at least a year or had no evidence of tumor. Of 25 family members, two had VCA-IgA titers of 5 and 10 and their sera contained lymphocyte stimulation inhibition (LSI). Even though VCA-IgA in one remained between 10-20 over 1.5 years, LSI titers increased after a year and NPC was diagnosed. The peak incidence of NPC in Malaysian Cantonese Chinese was 40-49 years of age for VCA-IgA and was found in all three histological types. In Malay Kadazans, higher incidences were found in young as well as old age groups and were different than those of the Chinese. Human cord blood mononuclear cells were shown to possess receptors for Herpesvirus saimiri (HVS). The infected cells were identified as T cells, IL2 dependent and HVS virus- and antigen-positive. Three different human fibroblast cell lines were tested for HVS replication/transformation. Only one was infectable with HVS regardless of the strain used. Infected cells were virus positive. A rabbit HVS-T cell line (7710), even after 50 passages over a period of 2.5 years, remained totally IL2-dependent and molecular studies revealed L-DNA deletion of 42.5 kbp. The DNA in the 7710 cell was of covalently closed circular form and contained 15 copies of HVS genome. This is the only HVS line which is highly oncogenic and bears DNA deletions which are different than those of T cell lines of monkey origin.

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	Research Microbiologist	LCMB	NCI
P. Levine	Senior Investigator	CEB	NCI

Objectives:

1. To study the EBV immunovirology associated with nasopharyngeal carcinoma (NPC), with particular emphasis on the rise and fall of IgA antibody to EBV virus capsid antigen (VCA) in identification of individuals at risk for NPC, identification of occult primary tumors of the head and neck as NPC, clinical evaluation of IgA antibody and EBNA as aids in NPC diagnosis and prognosis, and the mechanisms involved in specific EBV-induced LSI by IgA antibody recovered from NPC patients in active stages of the disease.

2. Previous reports indicate that human peripheral blood mononuclear cells do not absorb Herpesvirus saimiri (HVS), suggesting lack of HVS receptors on human T cells. In this study, we infected human cord blood lymphocytes and other cell types with HVS to immunologically and histologically characterize the cell type and to look for evidence of antigen/virus production and transformation in the presence and absence of T cell growth factor and/or glucocorticoid hormones.

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 patients with cancers under study, healthy donors, and individuals working with the oncogenic herpesviruses in the laboratory. Monoclonal antibodies, as well as sera from nonhuman primates with or without HVS-induced tumors were used. Three strains of HVS (prototype-S29SC, OMI, and 11) were used for infection of human cells. Human IL_2 was used in the various experiments. Hydrocortisone was used in growth medium for cultivation of human cells after HVS infection.

Major Findings:

1. In a collaborative study with Prof. Prasad (University of Malaya), Dr. Levine and Drs. Sundar and Menzes (St. Justin Hospital, Canada), sera from 75 undifferentiated NPC patients in various stages of the disease (I-IV) were tested by indirect immunofluorescence (IF) for antibody to EBV-VCA/IgA and for blocking human lymphocyte stimulation by UV-treated EBV or soluble antigen prepared from EBV nonproducer Raji cells. The VCA/IgA titers in these sera varied from 10-320 and lymphocyte stimulation inhibition (LSI) activity ranged between 10->320. The IgA antibody titers generally correlated with the

disease stage by not fluctuating as rapidly as LSI titers. Furthermore, follow-up of LSI and EBV antibody titers of three patients for 40 weeks post-diagnosis showed that LSI titers are independent of EBV-VCA/IgA titers, possibly reflecting different target sites. Our data on 12 NPC patients showed that EBV-VCA/IgA titers do not drop suddenly with the patient's response to therapy. The patient's VCA/IgA titer did not increase or decrease significantly until 16-18 months of therapy; however, LSI titers started to reflect between 2-4 months post-therapy, thus implicating that LSI is a much more reliable prognostic test and is a better marker than ADCC which is currently used as a prognostic test in treatment of NPC patients.

Twenty NPC patients, who received treatment (radiotherapy, chemotherapy, surgery, or a combination of radio-chemotherapy), were screened for LSI activity in their sera. Since they remained in remission at least 2 years, no LSI activity was detected at 1:5 titer and biopsies taken at various times also revealed no tumor. Thus, lack of LSI activity can be considered a good marker of response to treatment and arrest of tumor.

Since family members of Cantonese Chinese NPC patients have been found to be at higher risk of developing NPC, we followed 20 family members of NPC patients who were of Chinese and Malay origin. The EBV-VCA/IgA and LSI titers in sera of 18 patients remained <5 for a period of 2 years. Two individuals (one Chinese and one Malay) showed VCA/IgA titers of 20 and 10, respectively, 8 months after the first sample was tested (previously <5). The LSI titer in Chinese patients increased significantly (20-120) for the next 10 months; however, the VCA/IgA titers remained at 1:20. The biopsy of one patient taken first at 8 months was negative for tumor, but at 18 months it was diagnosed as undifferentiated NPC. The results on a second individual are unknown as yet. Thus it can be concluded that LSI could also be useful in restricted epidemiological studies.

One hundred Chinese NPC patients from various age groups and all histological types (WHO I, II and III) were analyzed for VCA-IgA antibody. The peak incidence was 40-49 years of age regardless of histologic types. However, the antibody titers reflected with the tumor, i.e., higher titers in stages III and IV. Thirty-five NPC Kadazan patients (who are originally Malay and are somewhat isolated) revealed two age peaks, 15-21 years and 37-46 years. The earlier age peak in Kadazans, based on VCA/IgA titers, suggested similarity to that we previously reported in Algerians. This peak was not found in Chinese patients in Malaysia even though isolated cases of NPC in a younger age group have been reported. Thus, it is evident that exposure to EBV in Kadazans may be different and may relect the etiological cofactors specifically related to this population.

2. It has been reported that HVS can be adsorbed by two human acute leukemia-derived cell lines, but human peripheral blood did not adsorb the virus before or after fractionation, suggesting lack of receptors for the virus. To our knowledge, the infection of human cord blood mononuclear (HCMC) cells with HVS has not been reported. Thus, the possibility of infecting HCMC with HVS was investigated to study (a) virus adsorption, (b) virus expression, and (c) the

possibility of transformation. HCMC infected with HVS (strains S295C, 11 and OMI) showed only 4-5% of cells were infectible, and infected cells upon cocultivation with owl monkey kidney (OMK) cells released HVS. However, these cells degenerated and no HVS-positive cells could be recovered. Since HVS is T tropic and induces malignant polyclonal lymphoproliferative T cell lymphoma in owl monkeys, we infected OMK monolayer cells with HVS strains 11 and OMI (which are less lytic in cell culture), added HCMC stimulated with PHA, and maintained them for 2-3 days in IL_2. About 10-20% of the HCMC showed HVS antigen as detected by IF and HVS was also detected (<1.0 log) in the culture fluid when HCMC was allowed to grow independent of OMK cells. The HVS-expressing cells were identified by human anti-T monoclonal antibody (Leu 2) as T cells. The cells clumped considerably; however, there was persistent cell killing by the virus. The cells in the presence of IL^2 have survived for more than 6 months, but thus far there is no evidence of transformation.

In 1970, it was reported that certain human and monkey cells can replicate HVS, but infected cells induce an abortive infection. We infected three human fibroblast cell lines (established by Dr. Rhim) with three strains of HVS. The prototype HVS (titer $>10^7$) killed 95% of cell line PC109 but considerable HVS could be recovered in the supernatant. The same cell line infected with strains 11 and OMI and maintained in the presence of 5 µg/ml hydrocortisone produced cytopathic effects in 10-12 days, 85% of the cells degenerated, and HVS antigen could be detected. The surviving cells, grown in isolated areas, ultimately formed monolayers. The first and second passages of these cells revealed HVS antigens, however, passages three and four lacked HVS antigen. These cells showed altered morphology. Whether these cells are transformed and contain HVS genome is not yet known. The other two human fibroblast lines could not be infected with HVS.

Previously we had reported that inbred rabbits develop generalized T cell lymphoma upon HVS inoculation and cell line 7710 could be established from infected spleen. The 7710 cells are T lymphocytes, HVS nonproducers, and highly oncogenic in inbred rabbits (strain III/J). This cell line was characterized for HVS molecular aspects and its dependency on IL2. Over a period of 2-1/2 years, 7710 cells have undergone 50 passages, but are totally dependent on IL_2. In the absence of IL_2, these cells degenerate, but when 7710 cells are inoculated in rabbits they induce tumors. The possibility that 7710 cells produce their own IL_2 in vivo is not known.

Molecular studies in collaboration with Drs. Fleckenstein and Schirm (University of Erlangen-Nurnberg, West Germany) revealed that 7710 cells contain 15 copies of genome equivalent as detected by liquid hybridization and also bear L-DNA deletion of 42.5 kbp, which is different from that observed in HVS nonproducer marmoset and OMK cell lines. The HVS DNA in 7710 cells is a covalently closed circular form. No virus could be rescued from 7710 cells by in vitro cocultivation with OMK cells or by other means or upon in vivo inoculation in rabbits. Like the other HVS nonproducer nonhuman primate cell lines, the persisting viral DNA in 7710 cells was highly methylated. To our knowledge, this is the first HVS nonproducer line which is oncogenic. The other monkey lines either established in vitro or from tumors are not tumorigenic.

Significance to Biomedical Research and the Program of the Institute:

Our findings indicate that LSI is a powerful tool for use in follow-up of NPC patients. The change in LSI titers is detected with relapse in a few weeks, reflecting the patient's response to therapy. This change is not apparent by use of VCA/IgA or ADCC titers. The LSI test is a good indicator for early intervention to arrest tumor activity. Our studies on NPC families also indicate that LSI could be used as a marker for follow-up of individuals who are at risk of developing NPC. However, the LSI test is not applicable on a large scale because of the cost of reagents and complexity of the test. The LSI test, being EBV specific and its activity manifested solely in IgA fractions, strengthens the role of EBV in NPC as the major contributing factor in the multifactoral etiology. LSI is also a more sensitive and better clinical marker than ADCC because ADCC failed to distinguish between NPC and non-NPC patients. In conjunction with VCA/IgA titers, the first test to screen for NPC patients and other risk groups, LSI may be used for follow-up of patients and individuals at risk. Since NPC age incidence peaks in Chinese and Malays are different, it is apparent that other cofactors need to be investigated in both populations. Genetic factors, such as specific HLA types (HLA A2/BW46 is consistently reported in NPC patients of Cantonese Chinese origin), in Kadazans should be investigated.

Infection of HCMC clearly shows that certain T cell populations possess receptors for HVS. Whether such T cells will ultimately undergo transformation and establish a cell line is not known yet. It is evident that in the absence of IL_2, such cells fail to survive. Like HTLV-I and II, the T cells which get infected and transform remain dependent on IL_2 for their survival, and a similar process may prevail with HVS infection of human T lymphocytes.

The infectivity of only one fibroblast line with three strains of HVS clearly suggests that all fibroblast cells are not infectible with HVS. Whether survival cells which are virus/antigen positive would undergo transformation remains to be determined. It is evident that hydrocortisone may be an important cofactor in further growth and ultimately in establishment and transformation of these human cells. The infection of human fibroblast cells is significant in assessing the role of HVS in humans.

To our knowledge, 7710 is the first well-characterized HVS cell line which is tumorigenic and may provide the basis for studying the role of HVS in oncogenesis in the absence of detectable virus. Moreover, in view of its reliability, low cost and reproducibility, the 7710 tumor system could also prove to be a useful model for studying the effects of chemotherapy on lymphoblastic lymphoma in humans. Since viral DNA is present in transformed cells in multiple copies, it is feasible to isolate the viral genomes from these cells and to study their molecular mechanisms, replication, persistence, transformation, immunopathogenesis, and maintenance of cells in the transformed state.

Proposed Course:

These projects will be pursued to take advantage of new findings and developments. The mechanism of impaired cell-mediated immunity (CMI) is not understood at present, so the precise nature, specificity and mechanism of action of LSI, particularly with regard to CMI, will be determined.

Since serum IgA values are considerably increased in NPC of the squamous cell type, whereas patients' sera contain no detectable EBV-VCA/IgA antibody, it would be important to determine whether LSI is also present in this histologic type of NPC and, secondly, whether it is EBV specific.

To determine whether the total IgA assayed in NPC patients prior to treatment accurately reflects the levels of IgA during remission and relapse and thus could be developed as a prognostic marker, and to determine if removal of serum IgA from NPC patients with high LSI titers (>320) would be associated with response to therapy or improvement in general CMI response.

To determine whether increases or decreases in IgA-producing plasma cells within tumors reflect LSI titers.

The study of Herpesvirus saimiri infection of human cells will be continued in order to understand the precise steps involved in HVS infection, replication, and ultimately transformation. Specifically, we intend to investigate whether line 7710 expresses viral proteins, particularly transforming proteins, and to determine the role of such proteins in transformation and maintenance of the transformed state. Moreover, since HVS DNA is oncogenic, it will be important to investigate the oncogenicity of DNA from 7710 in III/J and other inbred rabbits and to transfect III/J and other inbred rabbit cells, as well as monkey and mouse 3T3 cells with high molecular weight 7710 cell DNA, and to study the biologic and molecular events in such transfected cells. As most frequent chromosome abnormalities are found on chromosomes 6 and 15, it will be important to investigate whether any known viral oncogenes can be found on these chromosomes.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05164-04 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

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. Pierce	Sr. Staff Fellow	LCMB	NCI
Others:	S. Aaronson	Chief	LCMB	NCI
	S. K. Srivastava	Visiting Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*
Merk, Sharp and Dohme Research Laboratories, Westpoint, PA (S. Anderson); Litton Bionetics, FCRF, Frederick, MD (J. 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:
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.)*

A recombinant murine retrovirus (MRSV) containing the src gene of avian Rous sarcoma virus was shown to induce hematopoietic colonies in a bone marrow colony-forming assay. Cells from the colonies induced by MRSV could be established as continuous transformed lines, all of which expressed high levels of pp60src, and appeared to be at an early stage in lymphoid differentiation. These findings provide the first demonstration that the src gene is capable of transforming cells of hematopoietic origin.

BALB- and Harvey-murine sarcoma viruses (MSVs) were shown to alter the growth and differentiation of cells within the myeloid series in a manner that commonly leads to self-limited proliferation but infrequently induces neoplastic conversion, as well.

Abelson-MuLV was shown to induce mast cell transformation in vitro when interleukin-3 was initially utilized to enhance the proliferation of the mast cell phenotype. Abelson-MuLV was the only retrovirus analyzed that conferred factor independence and leukemogenicity to the mast cell cultures.

PROJECT DESCRIPTION

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

J. Pierce	Sr. Staff Fellow	LCMB	NCI
S. Aaronson	Chief	LCMB	NCI
S. K. Srivastava	Visiting Fellow	LCMB	NCI

Objectives:

1. Investigate the ability of replication-defective retroviruses, other than Abelson murine leukemia virus, to induce in vitro soft agar colony-forming assay.

2. To determine whether ras-containing viruses are capable of altering the growth and differentiation properties of myeloid cells in a factor-independent manner.

3. 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.

Methods Employed:

Standard hematopoietic culture techniques included an in vitro hematopoietic colony-forming assay developed to detect transformation of murine lymphoid cells by retrovirus, 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:

1. Efforts to demonstrate transformation of avian hematopoietic cells by Rous sarcoma virus (RSV) have not been successful. For example, although RSV replicates efficiently in avian macrophages, it is unable to transform them in culture. Recently, the RSV src gene was introduced into an amphotropic murine helper virus, yielding a recombinant virus, designated murine Rous sarcoma virus (MRSV). Since this virus was capable of inducing erythroleukemia and sarcomas in newborn nice, we examined its ability to alter the growth properties of mouse hematopoietic cells in culture. MRSV induced compact colonies that

followed single-hit kinetics and required mercaptoethanol in the agar medium. Cells from the colonies induced by MRSV could be established as continuous cell lines. The initial use of normal adherent bone marrow feeder layers was required to establish permanent lines. Once MRSV-induced cell lines became independent of the feeder layer requirements, they demonstrated unrestricted self-renewal and were tumorigenic. The seven transformants isolated from each expressed high levels of pp60^{src} that was active in the protein kinase assay. Cells from these lines lacked Fc receptors and detectable immunoglobulin μ chain synthesis. The majority contained high levels of TdT. One cell line expressed Thy-1 antigen but none expressed Lyt-1 or Lyt-2 antigens. These findings provide the first evidence that the src gene of RSV is capable of inducing malignant transformation of lymphoid progenitor cells.

2. It has previously been demonstrated that ras-containing viruses are capable of inducing proliferation and differentiation of cells of the erythroid series in vitro and erythroleukemia in vivo. We have also shown that these viruses are able to transform a lymphoid progenitor cell both in culture and in vivo. In our present report, a hematopoietic colony-forming assay was utilized to demonstrate that BALB- and Harvey-murine sarcoma viruses (MSVs) were capable of inducing the growth of myeloid colonies. Murine bone marrow cells were infected in vitro with BALB- or Harvey-MSV and then cultured in soft agar without the addition of exogenous growth factors. Under these conditions, large diffuse colonies formed in the semi-solid agar medium 5 to 8 days after infection. Analysis of bone marrow from BALB- or Harvey-MSV infected animals also revealed the presence of diffuse colony-forming cells.

The single-hit titration pattern of colony formation and release of ras-containing sarcoma virus from diffuse colony cells provided evidence that sarcoma virus infection was required for diffuse colony formation. Moreover, the presence of the p21 transforming protein could be detected in the cells from these colonies by radioimmunoprecipitation. Diffuse colonies were not induced by infection of bone marrow cells with the murine helper viruses used to pseudotype the ras-containing sarcoma viruses or by Abelson murine leukemia virus (A-MuLV) or MRSV infection. The presence of myeloid colony stimulating factors was also excluded as being the cause of diffuse colony formation.

Phenotypic analysis of hematopoietic cells from colonies whose growth properties were altered by these viruses revealed that the majority expressed markers associated with mature macrophages, including nonspecific esterase, phagocytosis, lysozyme production and the presence of Fc receptors and Mac-1 cell surface antigen. The doubling time, saturation density and replating efficiency of diffuse colony cells was increased in comparison to normal mouse macrophages. Cells from these colonies could be propagated for several passages but only one permanently growing line could be established. Cells from the BALB-MSV-induced continuous line grew in soft agar and were tumorigenic. They expressed high levels of the p21 ras-specific transforming protein. They displayed phenotypic markers similar to those of diffuse colony cells, although at relatively low levels. These cells could be induced to differentiate with phorbol myristate acetate to a mature macrophage phenotype. The effects of BALB- and Harvey-MSV on the myeloid lineage appear to more closely resemble the growth stimulating and differentiating actions of these viruses on erythroid precursor rather than their direct transforming effects on lymphoid precursor cells.

3. It has been reported that A-MuLV can occasionally induce mastocytomas in vivo. Interleukin-3 (IL-3) is known to promote the growth of normal basophil/mast cells in vitro. We generated IL-3 dependent fetal liver cultures in order to determine the effects of A-MuLV infection on an enriched population of mast cells. A-MuLV infection led to the conversion of mast cells from factor dependence to factor independence. The A-MuLV-induced independent mast cell lines were tumorigenic in nude mice and had a high cloning efficiency in soft agar containing no exogenous growth factor. A-MuLV appears to be unique in its ability to induce factor independence to these cells since a variety of other retroviruses including Harvey-MSV, Kirsten-MSV, Moloney-MSV, and Snyder-Theilen feline sarcoma virus, although capable of productively infecting the mast cell cultures, were unable to release these cells from dependence on IL-3. We are presently analyzing the A-MuLV-induced mast cell transformants to determine if they produce their own IL-3 and if they still possess IL-3 receptors.

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 differentiated 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 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.

Proposed Course:

1. Recombinant retroviruses generated in our laboratory, which have activated human onc genes have been or presently are being generated. We are currently analyzing the ability of these viruses to induce murine hematopoietic cell transformation in the bone marrow assay system. We are also analyzing the ability of mammalian retroviruses to transform human bone marrow cultures utilizing amphotrophic pseudotypes of replication-defective retroviruses.

2. We are interested in determining whether BALB- or Harvey-MSV lymphoid cell transformants display immunoglobulin or T cell receptor gene rearrangement in order to more precisely define their stage in the lymphoid developmental pathway.

   We intend to determine whether lymphoid progenitor cell transformants have the capacity to differentiate along either the T or B cell developmental pathways following transformation by temperature-sensitive mutants or exposure to agents which promote differentiation in other systems. We have currently isolated two cell lines transformed by a temperature-sensitive mutant of Kirsten-MSV, another member of the ras-containing retroviruses,

and are investigating the ability of these lines to differentiate upon shift to the nonpermissive temperature. These lines should also help to confirm whether transformation of hematopoietic cells results from a block in differentiation caused by the action of a transforming protein.

3. Efforts are underway to determine whether 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.

4. We have developed techniques to allow for the growth of normal murine B and pre-B lymphoid cells on feeder layers. The electroporation transfection technique will be employed to determine whether these normal lymphoid cells can be transformed by gene transfer of A-MuLV DNA.

Publications:

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Yuasa, Y., Eva, A., 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. The Cancer Cell, Vol. 11. Cold Spring Harbor Laboratory Press (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05167-04 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Analysis of the Transforming Gene of Simian Sarcoma Virus

PRINCIPAL INVESTIGATOR *(List other professionel 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	Acting Chief, Gene Structure Section	LCMB	NCI
	A. Gazit	Visiting Fellow	LCMB	NCI
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COOPERATING UNITS *(if any)*

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

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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.)*

Recent studies have identified for the first time the normal function of a proto-oncogene. The v-sis gene of simian sarcoma virus (SSV) is responsible for the transforming function of this virus. The primary amino acid sequence predicted from nucleotide sequence analysis served as a basis for obtaining synthetic peptide antibodies which allowed identification of the v-sis coded protein, p28sis. Independently, studies on human platelet-derived growth factor (PDGF) have led to the elucidation of much of its amino acid sequence. Computer comparisons of the PDGF sequence with the predicted amino acid sequence of p28sis has revealed an extraordinary degree of homology. By analysis of the p28sis molecule itself, it has been possible to demonstrate a very close structural similarity between these proteins. Moreover, p28sis is processed in SSV-transformed cells to forms which closely approximate PDGF conformationally and functionally.

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 v-sis-related exons as well as the upstream c-sis exon leads to the acquisition of high titered transforming activity.

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	Acting Chief, Gene Structure Section	LCMB	NCI
D. Swan	Expert	LCMB	NCI
A. Srinivasan	Visiting Associate	LCMB	NCI
P. Reddy	Visiting Scientist	LCMB	NCI
A. Gazit	Visiting Fellow	LCMB	NCI
H. Igarashi	Visiting Fellow	LCMB	NCI
I.-M. Chiu	Visiting Fellow	LCMB	NCI
D. Givol	Fogarty Scholar-in-Residence	LCMB	NCI
A. Yaniv	Visiting Scientist	LCMB	NCI
F. Leal	Guest Researcher	LCMB	NCI

Objectives:

1. To examine the interaction of the simian sarcoma virus (SSV)-transforming protein (p28^{sis}) with other cellular components.

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 and to understand the etiology of cancers in humans.

Methods Employed:

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

Major Findings:

The transforming protein of a primate sarcoma virus and a platelet-derived growth factor (PDGF) are derived from the same or closely related cellular genes. This conclusion is based on the demonstration of extensive sequence similarity between the transforming protein derived from the simian sarcoma virus *onc*

gene, v-sis, and a human PDGF. The mechanism by which v-sis transforms cells could involve the constitutive expression of a protein with functions similar or identical to those of a factor active transiently during normal cell growth.

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 been implicated as important targets for genetic alterations that may lead normal cells to become malignant. Despite great strides in identifying cellular genes with transforming potential, little is yet known about proto-oncogene function or how the altered counterparts of these genes disrupt normal growth regulation.

Very recent findings have provided the first direct link between an onc gene and a known biological function. The SSV onc gene, v-sis, has been sequenced and its 28,000 molecular weight (MW) product, $p28^{sis}$, identified by means of antisera prepared against small peptides derived from sequence analysis of v-sis. Studies on human PDGF, a potent mitogen for cells of connective tissue origin, have led to the elucidation of its amino terminal amino acid sequence. Computer comparison of this and additional protein sequence data with the predicted amino acid sequence of $p28^{sis}$ had revealed an extraordinary degree of homology between PDGF and $p28^{sis}$, implying that the two proteins have arisen from the same or closely related cellular genes. The present studies directly established structural and immunological relationships between the SSV transforming gene product and human PDGF.

The transforming gene of SSV, an acute transforming retrovirus, and human PDGF, a potent mitogen for connective tissue cells, appear to have arisen from the same or very closely related cellular genes. In an effort to obtain sufficient quantities of the SSV transforming gene product for the detailed analysis of its structural and biologic properties, we placed the v-sis gene under the control of strong phage transcriptional and translational signals that provide for regulated expression of cloned genes in E. coli. When induced, the bacterial cells synthesized levels of the SSV transforming gene product that constituted at least 10% of their total protein. Differences in the structure and processing of the v-sis gene product in prokaryotic and eukaryotic cells provided important insights concerning post-translational modifications of this PDGF-related transforming protein in eukaryotic cells.

The SSV transforming gene, v-sis, encodes a protein, $p28^{sis}$, which is closely related to human PDGF. The human locus related to v-sis was cloned and shown to contain at least 5 exons corresponding to the v-sis coding region. Nucleotide sequence analysis of these exons revealed that the predicted amino acid sequence of human c-sis differed by 6% from that of the woolly monkey derived v-sis. These findings imply that the sis proto-oncogene has been well conserved during primate evolution. By comparison of the known amino acid sequences of PDGF peptides with the predicted human c-sis protein, it was possible to demonstrate that this human proto-oncogene is the structural gene encoding one of the two major polypeptides of this potent mitogen for connective tissue cells.

GR-FeSV is an acute transforming retrovirus which encodes a gag-onc polyprotein possessing an associated tyrosine kinase activity. The integrated form of this virus, isolated in the Charon 21A strain of bacteriophage λ, demonstrated an ability to transform NIH/3T3 cells at high efficiency upon transfection. Foci induced by GR-FeSV DNA contained rescuable sarcoma virus and expressed GR-P70, the major GR-FeSV translational product. The localization of long-terminal repeats within the DNA clone made it possible to establish the length of the GR-FeSV provirus as 4.6 kilobase pairs. The analysis of heteroduplexes formed between λ feline leukemia virus (FeLV) and λ GR-FeSV DNAs revealed the presence of a 1,700-base-pair FeLV unrelated segment, designated v-fgr, within the GRFeSV genome. The size of this region was sufficient to encode a protein of approximately 68,000 daltons and was localized immediately downstream of the FeLV gag gene coding sequences present in GR-FeSV. Thus, it is likely that this 1.7-kilobase-pair stretch encodes the onc moiety of GR-P70. Utilizing probes representing v-fgr, we detected homologous sequences in the DNAs of diverse vertebrate species, implying that v-fgr originated from a well-conserved cellular gene. The number of cellular DNA fragments hybridized by v-fgr-derived probes indicated either that proto-fgr is distributed over a very large region of cellular DNA or represents a family of related genes. By molecular hybridization, v-fgr was not directly related to the onc genes of other known retroviruses having associated tyrosine kinase activity.

The nucleotide sequence of the region of GR-FeSV encoding its primary translational product, p70gag-fgr, has been determined. From the nucleotide sequence, the amino acid sequence of this transforming protein was deduced. Computer analysis indicates that a portion of p70gag-fgr has extensive amino acid sequence homology with actin, a eukaryotic cytoskeletal protein. A second region of p70gag-fgr is closely related to the tyrosine-specific kinase gene family. Thus, the v-fgr oncogene appears to have arisen as a result of recombinational events involving two distinct cellular genes, one coding for a structural protein and the other for a protein kinase.

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.

Proposed Course:

1. Examine the interaction between sis gene products and cellular components in order to identify their substrate(s).

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

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

Publications:

Aaronson, S. A., Reddy, E. P., Robbins, K. C., Devare, S. G., Swan, D. C., Pierce, J. H. and Tronick, S. R.: Retroviruses, onc genes, and human cancer. In Harris, C. C., and Autrup, H. W. (Eds.). Human Carcinogenesis. New York, Academic Press, 1983, pp. 609-630.

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Robbins, K. C., Antoniades, H. N., Devare, S. G., Hunkapiller, M. W. and Aaronson, S. A.: Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet-derived growth factor. Nature 305: 605-608, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05168-04 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

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

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

PI:	D. Swan	Expert	LCMB	NCI
Others:	S. Tronick	Microbiologist	LCMB	NCI
	R. Balachandran	Visiting Associate	DCBD	NCI
	W. McBride	Chief, Cellular Regulation Section	DCBD	NCI

COOPERATING UNITS *(if any)*
None

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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.)*

1. Chromosomal mapping of human immunoglobulin genes and several human onc gene analogues has been carried out using somatic cell hybrids. The precise localization of certain proto-oncogenes has been established by direct in situ hybridization to human chromosomes in both normal and neoplastic cells.

2. Continuous murine B-cell lines have been established from Rauscher leukemia virus-infected cells. These cell lines fall into five categories, each representing a different developmental stage.

PROJECT DESCRIPTION

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

D. Swan	Expert	LCMB	NCI
S. Tronick	Microbiologist	LCMB	NCI
R. Balachandran	Visiting Associate	DCBD	NCI
W. McBride	Chief, Cellular Regulation Section	DCBD	NCI

Objectives:

1. Chromosomal localization of human proto-oncogenes.

2. Correlation of proto-oncogene loci with chromosomal anomalies in neoplasia.

3. Immunoglobulin gene rearrangements in B-cells transformed by Rauscher murine leukemia virus.

Methods Employed:

1. Hybridization of cloned human immunoglobulin genes and human proto-oncogenes to an array of human/rodent somatic cell hybrids has allowed us to establish the human chromosomal location of each of the genes in question. Hybrid cell lines were formed between human and either mouse or Chinese hamster cells. The resultant hybrids were known to segregate human chromosomes and in each instance were tested for human chromosome content by isozyme analysis at the same time as DNA was prepared. Hybrid cell DNA was cut with a restriction endonuclease which was known to give a size difference between hybridizing fragments in human and rodent DNAs. Restricted DNAs were electrophoresed in agarose gels, transferred to cellulose mitrate filters and hybridized to each of the probes. The human fragment hybridizing to each of the probes was seen only in those cell lines which had retained the human chromosome carrying the corresponding gene. Since each hybrid line usually contained more than one human chromosome, it was necessary to test many hybrids before an unequivocal assignment could be made. In most cases it was necessary to subclone some of the positive cell lines in order to allow further segregation of human chromosomes.

More precise localization of human proto-oncogenes has been achieved by direct in situ hybridization of cloned human genes to metaphase spreads of human lymphocyte chromosomes.

2. Mouse tumor cell lines were established from tumors induced by Rauscher murine leukemia virus. Each of the cell lines was tested for the presence of B-cell markers, Fc surface receptors and intracellular and surface IgM. They were shown to lack antigenic markers exhibited by other hematopoietic cells.

DNA was extracted from different cell lines, digested with restriction endonucleases, separated by agarose gel electrophoresis transferred to nitrocellulose filters and hybridized to the appropriate immunoglobulin or oncogene probes.

Major Findings:

1. The following human proto-oncogenes have been mapped:

N-ras	human chromosome	1p36	(in situ)
Ki-ras-1	" "	6p23	(in situ)
Ki-ras-2,3	" "	12	(SCH)
mos	" "	8	(SCH)
myc	" "	8q24	(in situ)
abl	" "	9	(SCH)
Ha-ras	" "	11	(SCH)
sis	" "	22	(SCH)

2. Cells transformed by Rauscher murine leukemia virus were shown, by virtue of their immunoglobulin gene rearrangements and expression, to belong to five different B-cell developmental stages. The earliest stage showed only embryonic gene patterns for both immunoglobulin heavy and light chains, while the most terminally differentiated cells possessed rearranged and expressed heavy and light chain genes. Intermediate stages were shown, in certain instances, to have rearranged immunoglobulin genes without concomitant expression, thus indicating either aberrant rearrangement or control at the transcriptional or translational level.

The involvement of the c-myc gene in potentially aberrant immunoglobulin rearrangements was tested. Several lines which contained rearranged but unexpressed heavy chain genes were shown to have rearranged c-myc loci. In contrast to the situation in mouse plasmacytomas and human Burkitt's lymphomas, however, the c-myc gene was not found to be in close proximity to the immunoglobulin locus.

Significance to Biomedical Research and the Program of the Institute:

1. It is necessary to know the location of genes within the genome in order to understand the interaction between different genes. An example to illustrate this point is seen in Burkitt's lymphoma cells. Well-documented translocations involving chromosomes 8, 14, 2, and 22 had been shown in BL cells. Only after we showed that chromosome 8 carried the c-myc gene at the precise region where the break occurs and that chromosomes 14, 2, and 22 carried the immunoglobulin genes, was it possible to understand how these translocations could lead to activation of the c-myc locus and consequent cell transformation. As a result of any of the three potential translocations (8:14, 2:8, or 8:22) the c-myc gene becomes translocated to either the heavy chain or the light chain immunoglobulin loci.

Many additional chromosomal anomalies are present in other human neoplasias and the role of other oncogenes, mapped by us, to loci involved is currently under investigation.

2. Studies involving Rauscher murine leukemia virus have allowed us to establish B-cell lines at different developmental stages. Further studies on these lines will allow us to determine the precise sequence of genetic events involved in the progression from immature pre-B-cell to immunoglobulin producing B-cell.

Proposed Course:

1. Further human proto-oncogenes will be localized by in situ hybridization as they become available.

2. Involvement of human oncogenes in various human neoplasias will be investigated.

3. The role of Rauscher murine leukemia virus in the transformation of mouse B-cell will be investigated.

Publications:

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05234-03 LCMB

PERIOD COVERED: October 1, 1983 to September 30, 1984

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
	S. Sukumar	Visiting Associate	LCC	NCI

COOPERATING UNITS *(if any)*

Litton Bionetics, Inc., FCRF, Frederick, MD (M. Barbacid and D. Martin-Zanca)

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) N-ras activated oncogenes have been detected in human colon and liver carcinomas, as well as in one liver carcinoma-derived cell line (HepG2). The molecular structure and their transcriptional and translational products have been characterized. (2) Mammary carcinomas have been reproducibly induced in Buf/N female rats by injecting them with a single dose of nitroso-N-methylurea. All tumors obtained in that way contained a transforming H-ras-1 gene. Molecular cloning and characterization of one of these genes revealed that the 12th codon was GAA instead of GGA found in the normal allele, also cloned from untreated rats for comparison. The transforming gene encodes glutamic acid in place of glycine at position 12 of its p21 protein product. These findings demonstrated that chemical carcinogens can act on normal ras genes in a specific way and thus provides a useful model for studies aimed at determining the role of ras genes in the development of neoplasia. (3) A 27-kbp Eco RI DNA fragment, which seems to encompass the whole c-sis locus of a human glioblastoma (A172), has been molecularly cloned and partially characterized. (4) DNA sequences showing about 80% homology to retroviral v-fgr, v-src and v-sis oncogenes have been detected in the genome of the yeast Saccharomyces cerevisiae under relatively relaxed hybridization conditions. A 9 kbp Eco RI fragment enclosing the v-sis-related sequences has been cloned into the phage vector lambda L47.1, and its structure and function are being characterized.

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
S. Sukumar	Visiting Associate	LCC	NCI

Objectives:

To isolate and characterize human 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, are currently being set up for this project.

Major Findings:

1. By means of gene transfer techniques, the presence of activated oncogenes in human colon and liver carcinoma has been established. Both transforming ability and presence of human repetitive Alu sequences cosegregated in the course of several transfection cycles. The transforming activity was encompassed within a 8.6 kbp Nde I DNA fragment which was partially purified by sucrose gradient centrifugation and electrophoresis on low-melting agarose gels. The use of Alu-free, N-ras-specific fragments as probes identified the oncogenes present in solid colon and liver carcinomas and liver carcinoma cell lines as N-ras oncogenes structurally similar to that of the HT-1080 cell line (described in previous reports). In all cases, a 2.2-kbp message was found to be the main transcriptional product in the transformants. The electrophoretic mobility of their p21 product suggests that all have been activated by single point mutations at the 61st codon.

2. Single-shot injections of nitroso-methylurea induced reproducibly, and almost exclusively, the development of mammary carcinomas in Buf/N female rats after variable latency periods (60-90 days). The presence of an activated H-ras-1 gene in 9/9 of the tumors was established by gene transfer techniques using NIH/3T3 cells as recipient. The tranforming activity was ascribed to a 12-kbp Bam HI DNA fragment present in all the transformants. One of these fragments was molecularly cloned into the bacteriophage λ-1059, and structurally characterized by restriction endonuclease mapping and nucleotide sequence analysis. The normal counterpart of the NMU-activated oncogene was also molecularly cloned from breast tissues of untreated rats into the same λ-1059 vector and characterized. Comparison of both genes revealed that in the NMU-induced oncogene the 12th codon was GAA, encoding

for glutamic acid, instead of the GGA, encoding for glycine, found in the normal allele. Studies of restriction enzyme polymorphisms around the 12th codon allowed us to establish that precisely the same mutation could be found in at least 7/9 of the NMU-induced mammary tumors.

3. A single Eco RI DNA fragment of approximately 27 kbp, which hybridized with v-sis-derived probes, was partially purified by sucrose gradient centrifugation from total cell DNA of the human glioblastoma (A172), where the c-sis gene is expressed. The 27 kbp Eco RI DNA fragment was molecularly cloned into the cosmid pHC79. Restriction endonuclease mapping and Southern blot analysis revealed a molecular structure similar to that of the c-sis locus in normal cells. The cosmid c-sis clone did not have transforming activity in the NIH/3T3 standard transfection assay.

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of transforming and normal alleles from human tumors and tumor cell lines may enable us to understand the origin of certain human neoplasias. In this same respect our findings on the induction of tumors in rats by nitroso-methylurea strongly support the important role that chemical carcinogenesis can play as a model system for studying the mechanism by which ras transforming genes promote tumor development.

The finding of oncogene-related sequences in yeasts has two main implications: (a) the genes carry out essential functions for the eukaryotic cells, since they have not been lost in the course of evolution; and (b) yeast cells, among the simplest eukaryotic organisms, may be used to determine the basic function for oncogenes by simple genetic analysis, since yeasts are very well known genetically.

Proposed Course:

Current work is focused on the characterization of the human sis proto-oncogene sequences which determine its transcriptional control.

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 E. coli and purification of their encoded p21 proteins. Proc. Natl. Acad. Sci. USA (In Press)

Notario, V., Sukumar, S., Santos, E. and Barbacid, M.: A common mechanism for the malignant activation of ras oncogenes in human neoplasia and in chemically-induced animal tumors. In Vande Woude, G. F., Levine, A. J., Topp, W. C. and Watson, J. D. (Eds.): Cancer Cell: Oncogenes and Viral Genes. New York, Cold Spring Harbor Laboratory, 1984, Vol. 2, pp. 425-432.

Sukumar, S., Notario, V., Martin-Zanca, D. and Barbacid, M.: Induction of mammary carcinomas in rats by nitroso-methylurea involves the malignant activation of the H-ras-1 locus by single point mutation. Nature 306: 658-661, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05235-03 LCMB

PERIOD COVERED: October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Evolutionary Relationships among Types A, B, C and D Retroviruses

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

PI:	I.-M. Chiu	Visiting Fellow	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Acting Chief, Gene Structure Section	LCMB	NCI
	A. Yaniv	Visiting Scientist	LCMB	NCI
	A. Gazit	Visiting Fellow	LCMB	NCI
	K. C. Robbins	Expert	LCMB	NCI

COOPERATING UNITS *(if any)*

Johns Hopkins University, Baltimore, MD (R. C. Huang)

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:
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.)*

The genetic relationships among molecularly cloned prototype viruses representing all of the major oncovirus genera were investigated by molecular hybridization and nucleotide sequence analysis. One of the major progenitors of the pol genes of such viruses gives rise to mammalian type C viruses and another gives rise to type A, B, D, and avian type C oncoviruses. The evolutionary studies were also extended to slow viruses such as caprine arthritis encephalitis virus (CAEV) and equine infectious anemia virus (EIAV). It was found that CAEV is homologous to intracisternal A particles and M432 viruses, while EIAV is homologous to human T-cell leukemia virus. Evidence of unusual patterns of homology among the env genes of mammalian type C and D oncoviruses illustrates that genetic interactions between their progenitors contributed to the evolution of oncoviruses.

The human locus related to v-sis was cloned and shown to contain at least 5 exons corresponding to the v-sis coding region. Nucleotide sequence analysis of these exons revealed that the predicted amino acid sequence of human c-sis differed by 6% from that of the woolly monkey-derived v-sis. These findings imply that the sis proto-oncogene has been well conserved during primate evolution. By comparison parison of the known amino acid sequence of protein-derived growth factor peptides with the predicted human c-sis protein, it was possible to demonstrate that this human proto-oncogene is the structural gene encoding one of the two major polypeptides of this potent mitogen for connective tissue cells. The v-sis-related c-sis does not contain an initiation codon ATG. A putative exon upstream from the v-sis-related sequence was identified by Northern hybridization. Its sequence possesses an initiator codon ATG and splicing signal in phase with the downstream exon. This fragment, when ligated to the v-sis related sequences, enabled c-sis proto-oncogene to transform.

PROJECT DESCRIPTION

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

I.-M. Chiu	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Acting Chief, Gene Structure Section	LCMB	NCI
A. Yaniv	Visiting Scientist	LCMB	NCI
A. Gazit	Visiting Fellow	LCMB	NCI
K. R. Robbins	Expert	LCMB	NCI
R. Callahan	Chief, Oncogenetics Section	DCBD	NCI

Objectives:

To study evolutionary relationships among retroviruses. Studies are directed to give insight into human endogenous retroviral-related sequences and their potential functions in human malignancies and normal states. Efforts are also directed to study the structural organization of the human c-sis proto-oncogene.

Methods Employed:

A relaxed hybridization technique has been developed which enables us to discover the relationships among all major genera of a retroviral family for the first time. This technique is now extended to screen the human library, using v-sis as a probe, for the platelet-derived growth factor (PDGF) related genes. Other methods used include standard gene cloning methods, restriction enzyme analysis, gel electrophoresis, library screening, DNA sequencing, and cDNA cloning.

Major Findings:

1. The squirrel monkey retrovirus (SMRV) long terminal repeat (LTR) is 421-bp long. It has four 43 bp direct repeats.

2. The tRNA binding site sequence of SMRV is identical to that of $tRNA^{Lys}_{1,2}$ suggesting $tRNA^{Lys}_{1,2}$ is the primer for SMRV replication.

3. Localization of the homology region between intracisternal A particles and infectious oncoviruses in the pol gene.

4. Sequence analysis of genes coding for mouse mammary tumor virus (MMTV) pp32 and SMRV counterparts showed that their predicted amino acid sequences are homologous to the Rous sarcoma virus (RSV) counterpart.

5. A consensus peptide sequence of 16 amino acids is identified which is highly conserved among MMTV, RSV, Moloney murine leukemia virus (Mo-MuLV), human T-cell leukemia virus (HTLV), and SMRV.

6. The predicted amino acid sequence of cDNA clones for IgE-binding factor showed homology with the consensus sequence and their flanking sequences.

7. The nucleotide sequence homology between CAEV and IAP/M432, as well as between EIAV and HTLV.

8. Human retroviral-related sequences obtained using MMTV pol gene sequences as a probe were found to be homologous to MMTV, HTLV, RSV, and SMRV by using nucleotide sequence analysis.

9. Molecular cloning and characterization of human c-sis proto-oncogene.

10. Nucleotide sequence analysis identifies the human c-sis proto-oncogene as a structural gene for PDGF.

11. Nucleotide sequence analysis of a putative c-sis upstream identifies three open-reading frames with initiation codons and splicing signal in phase with the downstream exons. The v-sis-related human c-sis, when ligated with the upstream exon and provided with transcription initiation elements, is capable of transforming NIH/3T3 cells upon transfection.

Significance to Biomedical Research and the Program of the Institute:

The identification of a consensus sequence in the diverged genera of oncoviral subfamilies makes it possible to characterize the retroviral-related sequences isolated from human genomic libraries. The establishment of relationship between HTLV I and EIAV makes possible a detailed analysis of human malignancies in animal models. The cloning, sequence analysis, and activation of human c-sis proto-oncogene can help shed light on the role of PDGF.

Proposed Course:

1. To make antibodies against synthetic peptide made from the retroviral consensus sequence and to screen for endogenous DNA polymerase activities or endonucleolytic activity and to study its relationship with IgE receptor factor.

2. To make c-sis cDNA from human poly(A+) mRNA and to further characterize the human genomic c-sis locus.

3. To study the derepression of c-sis transcription in glioblastoma cells.

4. To search for other human PDGF-related genes by relaxed hybridization and nucleotide sequencing.

5. To study the transcriptional enhancing activity of SMRV-LTR.

Publications:

Chiu, I.-M., Andersen, P. R., Aaronson, S. A. and Tronick, S. R.: Molecular cloning of the unintegrated squirrel monkey retrovirus genome: Organization and distribution of related sequences in primate DNAs. J. Virol. 47: 434-441, 1983.

Chiu, I.-M., Callahan, R., Tronick, S. R., Schlom, J. and Aaronson, S. A.: Major pol gene progenitors in the evolution of oncoviruses. Science 223: 364-370, 1984.

Chiu, I.-M., Reddy, E. P., Givol, D., Robbins, K. C., Tronick, S. R. and Aaronson, S. A.: Nucleotide sequence analysis identifies the human c-sis proto-oncogene as a structural gene for platelet-derived growth factor. Cell 37: 123-129, 1984.

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 coding sequence for a human growth factor causes cellular transformation. Cell (In Press)

Yuasa, Y., Gol, R. A., Chang, A., Chiu, I.-M., Reddy, E. P., Tronick, S. R. and Aaronson, S. A.: Mechanism of activation of an N-ras oncogene of SW-1271 human lung carcinoma cells. Proc. Natl. Acad. Sci. USA. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05305-02 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analysis of Human Tumors and Normal Tissues for Oncogene Activation

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

PI:	S. Needleman	Medical Staff Fellow	LCMB NCI
Others:	M. Kraus	Visiting Fellow	LCMB NCI
	S. A. Aaronson	Chief	LCMB NCI

COOPERATING UNITS *(if any)*

Fels Institute, Philadelphia, PA (S. K. Shore and P. N. Magee)

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.)*

Genomic DNAs from a variety of neoplastic and normal tissues were analyzed by transfectional analysis for the presence of activated cellular oncogenes. Despite reports to the contrary, normal tissues from patients with many primary tumors and/or strikingly positive family histories did not exhibit oncogene activation. A variety of human tumors were found where a small minority were positive (e.g., 1/7 sarcomas, 1/14 Hodgkin's lymphomas, and 1/22 chronic myelogenous leukemias (CML). However, two tumors, human acute myelogenous leukemia (AML), and dimethyl-nitrosamine (DMN)-induced renal sarcoma were positive in a much higher percentage of tumors (3/6 AML and 5/5 renal sarcomas). Efforts were thus directed at these two tumors. In the case of AML, transforming capacity is associated with acquisition of human c-N-ras, and the gene product, p21, appears to have the same aberrant mobility in each patient. Patients whose tumor DNA is positive in transfection appear to be younger and have larger, more massive tumor burdens. Further studies are needed to confirm these preliminary observations.

In DMN-induced renal sarcomas, transformation of 3T3 cells is associated with acquisition of rat c-ras-K. This is of interest in that nitrosomethylurea, which is metabolized to the same active metabolite as DMN, causes breast cancer when given IV to rats in association with c-ras-H activation.

PROJECT DESCRIPTION

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

S. Needleman	Medical Staff Fellow	LCMB NCI
M. Kraus	Visiting Fellow	LCMB NCI
S. A. Aaronson	Chief	LCMB NCI

Objectives:

Having screened approximately 100 tumors and cell lines for oncogene activation in the transfection assay, it became apparent that in most human tumors and tumor models only a small minority of specimens tested will exhibit demonstrated oncogene activation. This small proportion of tumors positive in the transfection assay has proved invaluable for the further isolation and characterization of genetic elements intimately associated with transforming capacity and tumorigenesis in model systems. The question remains whether tumor genes that can transform in vitro and cause rodent tumors in vivo acquire these activities as an event primary to spontaneous neoplasia, or merely as a secondary reflection of carcinogenesis, which is known to cause many varied phenotypic changes in most systems.

In view of this dilemma, the study of tumors, where the majority of tumors score positive, seemed attractive in that, if cellular proto-oncogene activation is a primary event, it should be in these tumors where this might be demonstrated. Thus, we undertook to study AML where 3 of 6 were positive, and DMN-induced rat kidney sarcoma where 5 of 5 were positive.

Methods Employed:

The laboratory has utilized DNA transfection by the coprecipitation method, modified from previous reports (Wigler et al., Cell 14: 725, 1978). Briefly, total genomic DNA, extracted by standard procedures, is coprecipitated with calcium phosphate and layered onto 1.3 x 10^5 NIH/3T3 cells in log phase. Cells are maintained according to standard techniques and scanned morphologically for transformed foci at 14-21 days after transfection. Foci which do not appear spontaneously are cloned by the glass cylinder method and grown for DNA extraction. These transfectant DNA specimens are hybridized by the method of Southern (J. Mol. Biol. 98: 503, 1975) with a probe specific for human repetitive sequences. If the hybridization shows no human DNA present superimposed on the mouse background, the focus must be spontaneous. If there is human genetic information present, the DNA is transfected in subsequent cycles, as well as analyzed by hybridization with known oncogene probes. Monoclonal antibodies to the ras gene product are incubated for 30 min at 4°C with lysates of cells labeled with ^{35}S-methionine. Goat anti-rat IgG bound to staphylococcal protein A complexes with sepharose is added for 30 min at 4°C to bind antibody and any protein it has bound. These complexes are washed, electrophoresed in 14% polyacrylamide in the presence of SDS and mercaptoethanol. The gel is then autoradiogrammed for 24 hr at -80°C and p21 migration compared to controls without ras activation as well as those with activation where the mutation is fully characterized.

Major Findings:

In the study of a AML transfectants for p21 expression, increased levels of p21 compared with controls was noted. Moreover, the aberrant mobility was identical for each patient, suggesting the identical mutation. Genomic DNA from the AML was probed for N-ras, c-mos, and c-myc. In no case was amplification or rearrangement noted. When the clinical data from the three AML patients was compared with the three negative patients or published AML data as a whole, it suggested that the transfection positive patients are younger (median 40 yr) and have more aggressive disease than negative patients.

In the DMN-induced rat kidney sarcoma, positivity in transfection was associated with acquisition of rat c-ras^K sequences. This is of interest when compared to another rodent model (Sukumar et al., Nature 306: 658, 1983) in which NMU, given by a different route, produces mammary carcinoma. The tumors are positive in high frequency in transfection in association with acquisition of c-ras^H. Since NMU and DMN are metabolized to the same active intermediate and cause the same biochemical lesion (methylation of quanine residues at the O-6 position), it appears that the target tissue has specificity for activation of a particular oncogene in association with carcinogenesis.

Significance to Biomedical Research and the Program of the Institute:

There has been much excitement about the role of activated oncogenes in human neoplasia. Studies of the kind described above could further our understanding of human cancer and eventually lead to therapeutically exploitable manipulations, if it is indeed the case that the transfection model represents events which are germane to the genesis of clinical neoplasia. The finding that only a minority of human tumors tested score as positive might lead one to conclude that this is not the case. Alternatively, since human cancer is really a very heterogeneous group of diseases, these studies might sort out a subgroup of neoplasms where oncogene activation, which is detectable in this assay, is an important mechanism. Another possibility is that the system at this point is a crude and insensitive one and only detects relatively high levels of an event which is, in fact, more universal than the data suggest. There appears to be at least one human tumor and several rodent tumors where the frequency of detection of activated oncogenes affords the opportunity to study oncogene activity predictably associated with tumor formation. In AML, it affords the possibility of a new parameter of utility in difficult diagnostic situations, or perhaps a prognostic marker.

The study of rodent tumor models such as ours and that of Sukumar affords two unique opportunities. First is the ability to make temporal correlation between the activation of c-ras^K and tumor formation. If c-ras^K activation is a primary event, then it should be detectable before or very early after appearance of the tumor. Second is the pursuit of tissue specificity for oncogene activation with agents that cause O-6 methylation of guanine residues. Is this apparent specificity due to higher transcriptional activity of c-ras^H in breast than kidney, or conversely, higher activity of c-ras^K in kidney than breast? Does estrogen, necessary for breast tumor induction, set up the c-ras^H gene for activation?

Proposed Course:

These projects will be continued.

Publications:

Needleman, S. W., Yuasa, Y., Srivastava, A. and Aaronson, S. A.: Normal cells of patients with high cancer risk syndromes lack transforming activity in the NIH/3T3 transfection assay. Science 222: 173-175, 1983.

Yuasa, Y., Eva, A., Kraus, M. H., Srivastava, S., Needleman, S. W., Pierce, J. H., Rhim, J. S., Reddy, E. P., Tronick, S. R. and Aaronson, S. A.: Ras-related oncogenes of human tumors. The Cancer Cell, Vol II. New York Cold Spring Harbor Laboratory Press. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05306-02 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

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

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 spece provided.)*

Human ras oncogene (H-ras, K-ras and N-ras) encoded proteins (p21) have been characterized using monoclonal antibodies against p21 or antioligopeptide antibodies. p21 proteins with 12th amino acid substitutions were grouped into slow moving species as compared to fast moving species of p21 proteins having amino acid substitutions at the 61st position. Analysis of p21 proteins together with diagnostic restriction enzyme analysis of the oncogenes provided a useful method for the detection of site of activation in human ras oncogenes. Although p21 proteins exhibited a heterogeneous pattern on SDS-polyacrylamide gels, there appeared to be a conserved mode of post-translational processing. The altered electrophoretic mobilities of p21 proteins as a result of amino acid substitutions probably reflect the confirmational change in the protein which may be responsible for the oncogenic potential of p21 proteins.

Transforming efficiency of DNA from a lung carcinoma-derived cell line Hs242 was 10-25-fold lower compared to other tumor cell lines and the Hs242 line was comprised predominantly of stromal cells of normal appearance. To exclude the possibility that the Hs242 transforming gene arose from minor undetectable fraction of tumor cells, a transforming gene (c-H-ras) was cloned and characterized directly from Hs242 cells. Three positive clones were obtained in λL47 cloning vector and all the clones were positive in NIH/3T3 transfection assay. These results suggest the presence of activated c-H-ras oncogenes in Hs242 cells, although the cells exhibit normal phenotype.

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

Objectives:

1. To understand the molecular basis for the heterogeneity in electrophoretic patterns of p21 proteins from various tumor cell lines.

2. Studies on the biochemical properties of p21 proteins to deduce a common denominator which could be used for understanding the mechanism of action.

3. Analysis of tranforming gene in Hs242 lung carcinoma cell line.

Methods Employed:

1. Immunoprecipitation of transforming proteins using anti-synthetic peptide and monoclonal antibodies to p21, and their analysis on SDS-polyacrylamide gels.

2. In vitro assays for GDP binding and autophosphorylation functions of p21.

3. Purification of p21 proteins using various purification methods.

4. Molecular cloning of transforming DNA sequences using the λ phage system and characterization of gene by restriction enzyme and Southern blot techniques.

5. DNA transfection techniques to assess biological activity of transforming genes.

Major Findings:

1. Characteristic altered mobilities of p21 proteins have been found to be associated with position 12 and 61 substitutions. p21 electrophoretic mobilities may be used as diagnostic marker for the tentative assignment of site of mutation resulting in the activation of ras proto-oncogenes.

2. Three transforming gene clones, obtained directly from Hs242 lung carcinoma cell line, were positive in NIH/3T3 transfection assays, suggesting the presence of activated ras gene in Hs242 cells of normal phenotype.

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:

No intracellular substrate for p21 has been so far identified. However, this does not rule out the possibility that p21 proteins have kinase functions. In the absence of any known substrate, the peptide sequence susceptible to phosphorylation by v-ras and v-kis encoded p21 will be used to test kinase functions of human ras gene encoded p21. If kinase function is common to the p21s, a search for phosphorylated substrates in eukaryotic cells will be undertaken in an effort to understand the mechanism of action of p21 proteins.

Since p21 is localized in the membrane, cross-linking with homo- and heterobifunctional reagents will be performed in an effort to detect topographically associated proteins with which p21 may interact to create its transforming potential. Finally, structure and functional knowledge gained by study of p21 proteins synthesized by eukaryotic cells will be applied to the characterization of bacterially expressed p21 proteins.

Publications:

Needleman, S. W., Yuasa, Y., Srivastava, S. and Aaronson, S. A.: Normal cells of patients with high cancer risk syndromes lack transforming activity in the NIH/3T3 transfection assay. Science 222: 173-175, 1983.

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. Cold Spring Harbor Conference on Cell Proliferation and Cancer Vol II. The Cancer Cell (In Press)

Yuasa, Y., Srivastava, S. K., Dunn, C. Y., Rhim, J. S., Reddy, E. P. and Aaronson, S. A.: Acquisition of transforming properties by alternative point mutations within c-bas/has human proto-oncogene. Nature 303: 775-779, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05307-02 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Activation of Normal Human Proto-oncogenes as Transforming Genes

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
	A. Srinivasan	Visiting Associate	LCMB NCI
	Y. Yuasa	Visiting Fellow	LCMB NCI
	S. Tronick	Acting Chief, Gene Structure Section	LCMB NCI
	E. Reddy	Visiting Scientist	LCMB NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 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 c-H-ras was activated as a transforming gene in NIH/3T3 cells by its linkage to LTR sequences. The transformation, which was associated with high level of expression, could be transferred through several cycles of transfection. By molecularly cloning the transforming gene, we showed, unequivocally, that over-expression of the normal proto-oncogene was responsible for its transformation activity.

2. The normal protein-derived growth factor-2 (PDGF-2)/c-sis gene was activated as a transforming gene in NIH/3T3 cells. We constructed a series of recombinants in which a retroviral long terminal repeat (LTR) was linked to various regions of the human c-sis locus. The first construct, where LTR was linked to a region containing all the v-sis-related exons, lacked transforming activity, although sis-related transcripts were expressed. Insertion of a putative non-v-sis-related upstream exon between the LTR and the first v-sis-related exon resulted in a high-titered transforming activity equivalent to that of simian sarcoma virus (SSV) DNA.

3. To determine the biological activity of the human c-ras oncogene in mammalian cells, it was transferred within the genome of a retrovirus to cultured cells or inoculated into mice. Thus, the activated c-H-ras gene was inserted into the genome of Abelson murine leukemia virus (A-MuLV) or Moloney-MuLV. The defective viral particles were rescuable by superinfection with a helper amphotropic MuLV.

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
A. Srinivasan	Visiting Associate	LCMB	NCI
Y. Yuasa	Visiting Fellow	LCMB	NCI
S. Tronick	Acting Chief, Gene Structure Section	LCMB	NCI
E. Reddy	Visiting Scientist	LCMB	NCI

Objectives:

1. To determine the mechanisms responsible for the activation of the normal human c-ras as a transforming gene.

2. To activate the transforming potential of the normal human protein-derived growth factor-2 (PDGF-2)/c-sis gene.

3. To determine the transforming capacity of the activated human c-ras gene in mammalian cells in vitro as well as in vivo by using retroviruses as vectors.

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:

1. The normal human c-ras was activated as a transforming gene by linkage to Abelson long terminal repeat (LTR). The chimeric molecules exerted transforming activity in NIH/3T3 cells with an efficiency 10 to 100 times lower than that of the mutated c-H-ras oncogene. The transformants showed a very high level of H-ras transcripts as well as elevated levels of p21. Moreover, the transformed phenotype could be transferred through several cycles of transfection. Analyses of restriction enzyme polymorphisms did not detect any alterations at position 12 or 61.

To exclude the possibility of some other activating lesion, we molecularly cloned the transforming gene from genomic DNA. The cloned gene was not more active than the parental LTR-H-ras constructs. Moreover, substitution of its

coding exons into the H-ras proto-oncogene did not confer transforming properties to the normal gene. Thus, over-expression of c-H-ras-1 results in the transformation of NIH/3T3 cells.

2. A human c-sis clone, which contains the entire PDGF-2 coding sequence, was neither transformed nor transcribed when transfected onto NIH/3T3 cells. This clone could be transcriptionally activated by positioning the 5' LTR derived from Abelson-MuLV upstream from the first v-sis-related exon of this clone. Nevertheless, no transforming activity was detected. When a putative c-sis upstream coding exon containing potential N-terminal coding sequences and appropriate splicing signals was inserted in the proper orientation between the LTR and the first v-sis-related exon in this construct, high-titered transforming activity was elicited. The transformants contained high levels of v-sis-related transcripts, as well as PDGF-like protein. These findings establish that the normal human sis proto-oncogene possesses transforming potential which can be activated upon its expression in an appropriate assay cell.

Significance to Biomedical Research and the Program of the Institute:

1. A major question in cancer research is the mechanism responsible for oncogene activation. Likely possibilities are qualitative changes in the oncogene encoded protein, quantitative changes in expression of the normal oncogene products, and/or expression at the wrong time or in the wrong tissue. The present study shows conclusively that the unaltered human proto-H-ras gene can function as a cancer gene in NIH/3T3 cells through elevation of its expression.

2. Recent studies have identified for the first time the normal function of a proto-oncogene; the human sis proto-oncogene was identified as the structural gene for PDGF-2. The present study establishes that the normal human PDGF-2 coding sequence has transforming potential which can be activated by provision of appropriate transcription and translation initiation signals. These findings have potentially important implications concerning the role of normal genes in the neoplastic process. Thus, derepression of the coding sequence for a normal human growth factor may be involved in the neoplastic process, leading to tumors of cells responsive to its growth stimulatory properties.

3. Although various methods for transferring DNA sequences into animal cells have been developed, one of the best methods for DNA transfer is the use of animal viruses as vectors. Retroviruses in particular appear to provide a potential vehicle for high efficiency and stable integration into the cell genome without lysing or morphologically transforming the eukaryotic cells. We used retroviral vectors to insert cellular oncogenes into mammalian cells. This approach will permit the study of the activity of human oncogenes in various mammalian cells both in culture and in vivo.

Proposed Course:

1. Overproduction of the normal c-H-ras product induced morphological transformation of NIH/3T3 cells. The transformed phenotype will be compared to that induced by the mutated c-H-ras by soft agar as well as nude mice assays. The correlation between the level of expression and the efficiency of transformation will be studied in order to determine the minimal level of the normal p21 required for eliciting transforming activity.

2. The high level of c-sis product in the transformants will enable comparison of the structure and biologic properties of the processed forms of human sis/PDGF-2 transforming gene product with that of dimers found in native PDGF preparations. Additional LTR recombinants containing other regions of c-sis locus will be constructed in order to elucidate their possible role in regulating PDGF-2/c-sis expression.

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 coding sequence for a human growth factor causes cellular transformation. Cell. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05308-02 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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. Robbins	Expert	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	A. Srinivasan	Visiting Associate	LCMB	NCI
	P. Reddy	Visiting Scientist	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 v-sis transforming gene and its human homologue, c-sis, contain coding sequences for protein-derived growth factor-2 (PDGF-2), a major polypeptide chain of human PDGF. The primary v-sis gene product, p28sis, 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, p28sis undergoes dimer formation and subsequent 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.

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 play an important role in the experimental induction of 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, the transcriptional activation of this gene may be involved in the neoplastic 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. Robbins	Expert	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Strucutre Section	LCMB	NCI
A. Srinivasan	Visiting Associate	LCMB	NCI
P. Reddy	Visiting Scientist	LCMB	NCI
A. Gazit	Visiting Fellow	LCMB	NCI
A. Yaniv	Visiting Scientist	LCMB	NCI

Objectives:

1. To determine the status of c-sis expression in normal tumor cells and tissues.

2. To assess the transforming potential of c-sis (human).

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

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 simian sarcoma virus (SSV) 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 protein-derived growth factor (PDGF), demonstrates that the expression of this onc gene is associated with the continuing proliferation of these cells. Moreover, demonstration that the normal human c-sis gene is capable of causing morphologic transformation of cultured fibroblasts upon transcriptional activation provides further evidence that expression of this locus is involved in the process leading normal cells toward malignancy.

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 simian sarcoma virus (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.

Significance to Biomedical Research 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. Identify the c-sis (human) gene product to compare its physical and biological properties 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 coding sequence for a human growth factor causes cellular transformation. Cell (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05312-02 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structural and Functional Analysis of MC29 Proviral Genome

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

PI:	S. Lavu	Visiting Fellow	LCMB	NCI
Others:	E. P. Reddy	Visiting Scientist	LCMB	NCI
	N. Sacchi	Visiting Fellow	LCMB	NCI
	A. Srinivasan	Visiting Associate	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

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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.)*

1. In an effort to understand the molecular mechanisms involved in the transformation process mediated by myelocytomatosis virus (MC29), molecular cloning of the complete proviral genome was undertaken. We have successfully cloned the proviral genome in lambda Charon 21A phage. Studies are now being performed to test the transforming activity of the viral genome using various cell lines derived from mouse, rat, and avian species. In vitro mutagenesis of the MC29 proviral genome is currently being carried out in an effort to understand the molecular mechanisms involved in the transformation process mediated by this genome. A recombinant virus containing the v-myc oncogene with Abelson long terminal repeats (LTRs) was constructed and live virus rescued upon cotransfection. Studies of the in vitro and in vivo effects of this virus in mouse cell lines are currently being carried out.

2. In an effort to delineate the molecular mechanisms involved in the rearrangement of c-myb locus in mouse plasmacytoid lymphosarcomas induced by Abelson murine leukemia virus, molecular cloning of mouse c-myb locus was undertaken and the comparison of it with the molecular clone of its counterpart in ABPL-2 tumors reveals that the rearrangement observed in the c-myb locus of the tumor line is due to insertion of a defective Moloney murine leukemia proviral genome upstream to v-myb-related sequences.

PROJECT DESCRIPTION

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

S. Lavu	Visiting Fellow	LCMB	NCI
E. P. Reddy	Visiting Scientist	LCMB	NCI
N. Sacchi	Visiting Fellow	LCMB	NCI
A. Srinivasan	Visiting Associate	LCMB	NCI

Objectives:

1. To test the transforming activity of the proviral genome of MC29 in various cell lines and to delineate the role of v-myc sequences in the transforming process. In vitro mutagenesis of the proviral genome in an effort to understand the molecular mechanisms involved in the transformation process mediated by the viral genome is currently being studied.

2. To test the molecular mechanisms involved in the rearrangement of c-myb locus in mouse plasmacytoid lymphosarcomas by cloning and comparing the mouse c-myb locus.

Methods Employed:

1. Restriction enzyme analysis of DNA; gel electrophoresis and Southern blotting; enrichment of the inserted fragment by electroelution and sucrose gradient separation; and molecular cloning of the genome in λ phages.

2. Restriction enzyme analysis of DNA; gel electrophoresis and Southern blotting; partial restriction of the genomic DNA and preparation of genomic libraries in Charon 4A phage; screening of the library for positive clones; and subcloning the restriction fragments of the clone in pBR 322.

Major Findings:

1. Total cellular DNA from two quail nonproducer cell lines, Q5 and Q8, transformed by myelocytomatosis virus (MC29), were analyzed for the presence of proviral genome. The DNA was cleaved with several different restriction enzymes and analyzed by Southern blot analysis and hybridized with a radiolabelled v-myc probe. Two bands, 20 kbp and 7 kbp in length, were identified in Q5 cell DNA cleaved with Hind III. Q8 cell DNA, on the other hand, yielded multiple bands under the same conditions, ranging in size from 2 to 20 kbp. Normal quail cell DNA yielded a single band of 20 kbp hybridizable with v-myc probe. Since Hind III does not cleave within the proviral genome, it was assumed that the 7 kbp present in Q5 cell DNA contained the entire proviral genome with flanking quail cellular sequences, while the 20-kbp band contained the quail c-myc sequences. These experiments also indicated that Q8 cell DNA contained multiple proviral genes, some of which represent deletions within the viral genome. Q5 cell DNA was therefore used for purification and molecular cloning of the MC29 proviral genome.

Total Q5 cell DNA was cleaved with Hind III and was enriched for MC29 proviral sequences by sucrose density gradient fractionation. The enriched DNA was ligated to the arms of bacteriophage λ Charon 21A and packaged in vitro into phage particles and plated onto E. coli BNN45. Approximately 200,000 plaques were screened with a v-myc probe using the plaque filter hybridization technique. Two positive clones were identified and plaque purified. Recombinant phage DNA was prepared from the two clones and a restriction map was developed.

These results indicated that both of the clones obtained had undergone a deletion at the 3' end in the envelope region. These proviral clones were subcloned in pBR322 and tested for their transforming potential of NIH/3T3 cells using transfection assays. The results were negative.

Using recombinant DNA techniques, two recombinants of MC29 viral genome with Abelson LTRs were constructed. One of them has a 5' long terminal repeat (LTR) of MC29, the v-myc oncogene, and the 3' LTR of Abelson-murine leukemia virus (A-MuLV); the other has the 5' and 3' LTRs of A-MuLV and the v-myc oncogene. Both of the subclones in pBR322 were tested again for transforming potential of NIH/3T3 cells by transfection procedures. Again, the results were negative.

Using the cotransfection assay, live virus of v-myc was rescued and its transforming activity in vitro and in vivo is currently being tested.

2. Total mouse DNA was subjected to partial digestion with Eco RI and ligated to λ Charon 4A vector, and genomic libraries were constructed. Screening of these libraries with a v-myb-specific probe resulted in obtaining a homologous clone, designated λ-MM1. Further characterization of this clone revealed that it contained a 17-kbp insert, which on digestion with Eco RI, gave rise to fragments of 7.5 kbp, 4.9 kbp, 1.8 kbp, 1.7 kbp, 0.5 kbp and 0.4 kbp in length. Hybridization with the v-myb-specific probe revealed that the 4.2 kbp, 1.7 kbp and 0.5 kbp fragments contained v-myb-specific sequences. Restriction enzyme analysis and hybridization data has indicated that the c-myb locus in plasmacytoid lymphosarcomas has been rearranged. The rearranged c-myb DNA from ABPL-2 tumor was cloned and characterized. Hybridization of the c-myb locus of ABPL-2 tumor DNA with M-MuLV LTR DNA showed that a partial proviral genome of M-MuLV has been inserted 5' to the 4-kbp region of mouse c-myb locus, resulting in the rearrangement of c-myb locus.

The DNAs from the two clones, mouse c-myb locus and mouse c-myb locus of ABPL-2 tumors, are currently being subjected to sequence analysis.

Significance to Biomedical Research and the Program of the Institute:

1. Myelocytomatosis virus (MC29) is an acute transforming retrovirus of Aves. The c-myc oncogene appears to be involved in lymphomagenesis of man and other mammalian species. Delineation of the molecular mechanism of action of this oncogene will lead to a better understanding of its role in human lymphomagenesis.

2. Avian myeloblastosis virus (AMV) is an acute transforming retrovirus of aves. The oncogene of this virus appears to be involved in lymphomagenesis of aves and mammals. Delineation of the molecular mechanisms of the rearrangements of this oncogene in mice will lead to a better understanding of its role in lymphomagenesis.

Proposed Course:

1. Studies with the cloned proviral genome of MC29 to test its transformation potential on various cell lines and to study the molecular mechanisms involved in using in vitro mutagenesis techniques will be continued.

2. Studies with the cloned c-myb locus of mice and its comparison with the c-myb locus of plasmacytoid lymphosarcomas and sequence analysis of the clones will be continued.

Publications:

Lavu, S., Mushinski, J. F., Shen-Ong, G. L. C., Potter, M. and Reddy, E. P.: Structural organization of mouse c-myb locus and the mechanism of its rearrangement in ABPL-2 tumor line induced by pristane and Abelson murine leukemia virus. Current Topics in Microbiology and Immunology, Vol. 113 (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05362-01 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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
	D. Swan	Expert	LCMB	NCI
	A. Yaniv	Visiting Scientist	LCMB	NCI
	H. Igarashi	Visiting Fellow	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.)*

Studies were initiated to look for expression of sis oncogene in primate embryos and normal term placenta. Preliminary studies have failed to demonstrate expression of the sis oncogene during embryogenesis, but sis has been demonstrated to be expressed in most placentas which have thus far been studied using v-sis and c-sis probes hybridized to poly-A RNA extracts of the placentas.

Another line of study is an examination of an epidermal growth factor (EGF)-dependent mouse keratinocyte line. Studies are underway to see whether oncogenes are expressed when the cells are stimulated with EGF following a period of EGF privation. Another effort has been to try to adopt RNA in situ hybridization to the cell-specific study of oncogene expression. This has thus far been unsuccessful, probably due to low copy number. However, the method has been successful in demonstrating viral infection of cultured cells.

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
D. Swan	Expert	LCMB	NCI
A. Yaniv	Visiting Scientist	LCMB	NCI
H. Igarashi	Visiting Fellow	LCMB	NCI

Objectives:

To study the role of oncogenes in normal versus abnormal cellular growth and differentiation via a search for RNA transcripts of cellular oncogenes in (1) embryonal tissue and (2) cultured cells following growth factor stimulation. Another objective is to study the feasibility of in situ RNA hybridization for detecting oncogene expression in tissue sections.

Methods Employed:

1. Human placentas (fresh and frozen) and various samples of primate embryonal tissue were extracted for RNA and oligo-dT selected for poly A RNA. These were run on agarose-formalin gels and blotted to nitrocellulose via the Northern method. Double-stranded and M-13 clones were p32 labeled and hybridized to the RNA samples.

2. A newborn mouse (BALB) keratinocyte cell line has been established. This cell line is epidermal growth factor (EGF)-dependent for growth. These cells are grown in media with EGF, then passed into EGF-free media and allowed to equilibrate. A timed experiment is then performed with cells harvested at various times following EGF stimulation. Harvested cells are then extracted for RNA, and this RNA is dot blotted onto nitrocellulose paper and hybridized to various DNA probes of oncogenes.

3. RNA in situ hybridization is attempted utilizing the following protocol. Cultured cells are cytofuged onto glass slides and fixed via either glutaraldehyde or formaldehyde and various treatments are employed prior to hybridization (HCl, proteinase-K, etc.). M^3-labeled probes are employed for hybridization under glass coverslips and then slides are washed. Autoradiography is performed by dipping slides in photographic emulsion and exposing for 1 to 4 weeks. Various appropriate positive control cells known to express certain oncogenes in high RNA copy numbers are used and probed with the same oncogene probes.

Major Findings:

1. The sis oncogene is expressed in term human placenta but its role in embryogenesis is still unclear.

2. In EGF-dependent keratinocytes, there may be an increased expression of some oncogenes following EGF stimulation. Preliminary results indicate that the Abelson oncogene may be expressed in this situation.

3. Our studies indicate RNA in situ hybridization can demonstrate oncogene transcripts only when they are present in high copy numbers. Thus, it may be unsuitable for searching for low levels of oncogene expression in histologic specimens such as sections of tumors or embryos.

Significance to Biomedical Research and the Program of the Institute:

1. A fundamental question is the role of oncogenes in normal growth and development. An understanding of this could lead to an understanding of the true nature of oncogenes and the way in which their expression is controlled. Recent work has suggested homologies between various oncogenes and either growth factors or growth-factor receptors. Other studies have suggested oncogene expression in embryogenesis. Our work attempts to forward this knowledge. By studying oncogene expression following growth-factor stimulation, we seek to determine whether oncogenes are involved in pathways leading to cellular growth, differentiation, and DNA replication.

2. If in situ RNA hybridization could be adapted to the sensitive detection of c-onc transcripts, it would be of value in pinpointing precisely which cells within an organ, tumor, or embryo are expressing oncogenes.

Proposed Course:

1. A thorough study will be embarked upon using the mouse keratinocyte system. Stimulated cells will be screened for oncogene expression and these results will be compared to the pattern of oncogene expression for sessile and normally growing cells. Oncogene expression in terminally differentiated versus dividing cells may also be studied. Other cell lines may also be studied to see whether oncogene expression following growth-factor stimulation is a general phenomenon.

2. An animal system (possibly rodent) may be set up to study systematically the expression of sis and other oncogenes during embryogenesis.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05363-01 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analysis of the v-sis Oncogene by in vitro Mutagenesis

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:	K. Robbins	Expert	LCMB	NCI
	S. Aaronson	Chief	LCMB	NCI
	C. Dunn	Biologist	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 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 structure and function of the oncogene product of the simian sarcoma virus, v-sis, is being analyzed by means of in vitro mutagenesis. Mutations have been introduced into the v-sis coding sequence by site-directed methods. Resulting variants are tested by their ability to transform NIH/3T3 cells in culture using DNA-mediated gene transfer. The coding sequence of the site of polypeptide glycosylation has been altered without affecting the ability of the viral genome to transform. Mutations have also been generated at sites believed to be involved in polypeptide cleavage. The effect of these mutations on the cellular transforming activity of v-sis and on the polypeptide structure is currently being investigated.

PROJECT DESCRIPTION

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

C. R. King	Staff Fellow	LCMB	NCI
K. Robbins	Expert	LCMB	NCI
S. Aaronson	Chief	LCMB	NCI
C. Dunn	Biologist	LCMB	NCI

Objectives:

Pulse chase polypeptide labelling has revealed that p28*sis* undergoes several post-translational modifications. The polypeptide is glycosylated, forms a dimer, and is cleaved of N-terminal and C-terminal sequences. We are currently using site-directed mutation in an attempt to assay the functional relevance of post-translational changes in the v-*sis* oncogene product.

Methods Employed:

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 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 expression in cells transfected with nontransforming variants.

We have used the directed mutagenesis approach of Wallace and Itakura to introduce single base changes into the v-*sis* coding region. The v-*sis* coding sequence was removed from pSV2 SSV and inserted into the single-stranded bacteriophage M13 mp9. This allowed selection of recombinant phage DNAs containing only the coding strand of v-*sis*. Synthetic oligonucleotides of 15 bases in length and complementary to 14 bases of the v-*sis* coding sequence were annealed to the single-stranded DNA of M13 *sis*.

Using the *E. coli* DNA polymerase large fragment, the M13 *sis* can be made into a double-stranded molecule with one strand containing a single desired nucleotide mismatch. When introduced into *E. coli*, bidirectional DNA synthesis results in two different phage populations, one wild type and the other containing a single nucleotide alteration in the v-*sis* coding information. The mutated variants can be detected by increased stability of hybridization to the oligonucleotide used in the site-directed mutagenesis. Direct DNA sequencing is used to confirm the incorporation of the desired mutation.

Major Findings:

Studies at the protein level indicated that the p28^{sis} polypeptide is rapidly glycosylated upon synthesis in SSV-infected cells. Analysis of the sequence of the sis coding region predicts a polypeptide sequence of Asn-Met-Thr at amino acid position 48 of the viral polypeptide. This sequence fits the required Asn-X-Ser/Thr requirement for N-linked glycosylation. No other such sequence is found in v-sis. Evidence that this site is used in vivo for glycosylation of p28^{sis} has been previously obtained by examination of tunicamycin-treated cells infected with SSV. To test if N-linked glycosylation is a necessary requirement for the biosynthesis of functional v-sis polypeptide, we utilized site-specific mutagenesis to introduce into the v-sis gene a mutation which changes the Asn codon (AAC) to a Leu codon (ATC). The variant sis oncogene was returned to pSV2 SSV and transfected onto NIH/3T3 cells in culture. No difference was observed in the efficiency of focus formation between the wild type and variant. These results indicate that N-linked glycosylation is not likely to play a vital role in the biosynthesis of a biologically active v-sis transforming gene product.

Cleavage of polypeptide hormones often occurs adjacent to pairs of basic amino acids (Arg, Lys). Such pairs occur at the likely N-terminal cleavage site of v-sis (amino acid positions 65, 66) and at five positions in the C-terminal half of the molecule. We have introduced site-specific mutations to disrupt the basic amino acid pair at the putative N-terminal sequence and at three of the five basic amino acid pairs found in the C-terminal region. The variant coding regions have been returned to pSV2 SSV. The ability to transform NIH/3T3 cells in culture is currently under analysis.

Two other types of site-directed mutations have been made to analyze the polypeptide cleavage process. We have deleted regions of DNA sequence corresponding to amino acid 12 to 67 in the v-sis polypeptide. This alteration retains an open reading frame but deletes most of the amino acids from the N-terminus of the protein. The mutant coding region therefore encodes a polypeptide which requires no N-terminal processing. We have also generated a mutation which results in an abbreviated C-terminus. When the Bst EII site at nucleotide position 582 of v-sis is removed by fill-in reaction using E. Coli DNA polymerase I (Klenow fragment), an in-frame translation termination codon is generated at amino acid 194 of v-sis. This C-terminal truncation at the gene level is designed to mimic the C-terminal cleavage observed in v-sis protein maturation.

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 tumor 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 initial finding demonstrates that v-sis protein is biologically active even though it cannot be glycosylated. This finding is of interest as the v-sis protein is not likely to be glycosylated in heterologous systems such as E. coli. It is anticipated that the mutagenesis approach

might make it possible to elucidate functional domains of the transforming protein and may also lead to the development of molecules which could act as competitive antagonists to the transforming protein.

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 in cysteine codons of v-sis. Conserved structural elements responsible for receptor binding will also be investigated. In addition to the transforming activity and polypeptide structure of altered proteins, other aspects of protein biosynthesis will be analyzed. Comparison of nontransforming proteins with wild type SSV protein may shed light on the site of action of the v-sis oncogene product. Moreover, the ability of variant proteins to bind to and cause autophosphorylation of the platelet-derived growth factor receptor will be examined. Other approaches such as nonspecific mutagenesis are also contemplated.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05364-01 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Studies on Retroviral and Human Oncogenes

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

PI:	N. Sacchi	Visiting Fellow	LCMB	NCI
Others:	E. P. Reddy	Visiting Fellow	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

None

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SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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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.)*

1. Several deletion mutants of the T24 human bladder carcinoma oncogene were constructed to study the transcription regulatory signals present in this gene.

2. Partial cDNA clones containing sequences cross-reactive to v-abl oncogene were prepared and their biochemical organization is currently being studied.

PROJECT DESCRIPTION

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

N. Sacchi	Visiting Fellow	LCMB	NCI
E. P. Reddy	Visiting Scientist	LCMB	NCI
A. Srinivasan	Visiting Associate	LCMB	NCI

Objectives:

1. To study the transcriptional regulatory signals present in T24 human bladder carcinoma oncogene.

2. To prepared cDNA clones containing mouse c-abl sequences.

Methods Employed:

mRNA preparation, cDNA cloning, nucleotide seqence analysis, molecular cloning, and site specific mutagenesis.

Major Findings:

1. Regulatory sequences controlling the expression of T24 human bladder carcinoma oncogene have been identified towards the 5' end of the genome. Several deletion mutants of the T24 human bladder carcinoma oncogene were constructed. For this purpose, the DNA clone of the oncogene was subjected to digestion with various restriction enzymes as well as Bal 31 nuclease and the mutants generated were characterized by restriction enzyme mapping and nucleotide sequence analysis. These approaches have led to the isolation of several deletion mutants with deletions toward the 5' end of the genome. These clones are currently being tested for transforming activity to study the effect of these deletions on the expression of the gene product.

2. cDNA clones containing mouse c-abl sequences have been isolated. In an effort to isolate cDNA clones containing the c-abl sequences, RNA was isolated from several mouse tissues and tested for hybridization with a v-abl specific probe. These results revealed that mouse thymus contain abundant amounts of two mRNAs of 5.5-kb and 6.5-kb long which readily cross-hybridize with the v-abl probe. Following these studies, RNA was isolated from pooled mouse thymus tissue and enriched for poly A containing RNA using an oligo-dT cellulose column. Complementary DNA was prepared from the mRNA using reverse transcriptase and the cDNA preparation was rendered double-stranded using DNA polymerase. This double-stranded DNA preparation was inserted into pBR322 and used for transformation of E. coli cells. Hybridization with a v-abl specific probe revealed the presence of a few colonies that reacted with this probe. Currently these colonies are being characterized by nucleotide sequence analysis.

Significance to Biomedical Research and the Program of the Institute:

The studies in the T24 human bladder carcinoma oncogene is expected to provide insights into the molecular mechanism that regulates eukaryotic gene transcription. The experiments on the isolation of cDNA clones containing the c-abl specific sequences is expected to provide further understanding of the biochemical organization of this oncogene which is involved in several human malignancies.

Proposed Course:

This project will be continued to determine the biochemical organization of cDNA clones containing sequences cross-reactive to v-abl oncogene.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05365-01 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Studies on 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	Microbiologist	LCMB	NCI
	S. K. Srivastava	Visiting Fellow	LCMB	NCI
	M. Kraus	Visiting Fellow	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 spece provided.)*

In order to assess the significance of activated oncogenes in the pathogenesis of human urinary tract tumors, fresh urothelial tumors were screened by DNA-mediated gene transfer (DNA transfection) using NIH/3T3 cells as recipients for the presence of such genes. Activation of Ha-ras oncogene was found in 2 out of 23 specimens. By restriction endonuclease analysis, this activation was shown to be the result of a somatic event. The basis for the activation was investigated through gene cloning and found to be due to a point mutation within 61st codon of its coding sequence in both cases. The frequency of Ha-ras activation by point mutation at 61st or 12th codon in human urothelial tumors was further estimated to be around 10% by both DNA transfection and restriction enzyme analysis. The involvement of other mechanisms of activation of oncogenes (gene amplification, gene rearrangement and others) are being explored. Modification of the effect of activated ras oncogenes in transformed cells will also be tried.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professinal Personnel Engaged on this Project:

J. Fujita	Visiting Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. S. Rhim	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 to identify 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 transformatin 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:

The pursuit of this project has led to several findings. Activated Ha-ras was found in 10% of human urothelial tumors by DNA transfection. This activation was a somatic event. An alteration of 61st amino acids of c-Ha-ras encoded protein (p21) glutamine to either arginine or leucine led to an activation of the gene and abnormal electrophoretic mobility of p21 in polyacrylamide gel. By utilizing restriction endonuclease polymorphism, most point mutations at 61st and 12th codons of p21 should be detected but these mutations were found in less than 10% of human urothelial tumors. Due to the small number of activated oncogenes detected, the relationship between activation of oncogenes and the pathological and clinical findings was not apparent.

Significance to Biomedical Research and the Program of the Institute:

The urothelial tumors are considered to be a gross manifestation of a generalized neoplastic change of the urothelium and several chemicals are identified as a bladder carcinogen in humans. Since oncogenes are frequently activated in chemically-induced tumors in rodents, the knowledge of what and how oncogenes

are involved in human urothelial tumors will help elucidate the pathogenesis and prevention of the tumor. The demonstration that Ha-ras proto-oncogene is activated in 10% of human urothelial tumors by point mutation confirms the importance of oncogenes in clinical medicine and encourages the development of new diagnostic and therapeutic modalities based on the studies on oncogenes and their products.

Proposed Course:

Further studies are a logical extension of the present one. Point mutation is not the only mechanism of oncogene activation. The presence of gene amplification, gene rearrangement, and the level of mRNA and proteins encoded by the oncogenes will be investigated. There seems to be a certain limitation on the kind of oncogene detectable by DNA transfection using NIH/3T3 cells as recipient. Other methods, including biochemical as well as biological, will be developed to detect active oncogenes. The correlation between mechanism of activation and the oncogene activated will be investigated using chemically-induced murine tumors and human occupational and endemic bladder tumors. Using revertants from transformed cells, molecular mechanism that suppress the transformed phenotype will be studied and may be applied for development of a new treatment.

Publications:

Fujita, J., Yoshida, O., Yuasa, Y., Rhim, J. S., Hatanaka, M. and Aaronson, S. A.: Ha-ras oncogenes are activated by somatic alterations in human urinary tract tumors. Nature 309: 464-466, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05366-01 LCMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

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
	E. P. Reddy	Visiting Scientist	LCMB	NCI
	S. K. Srivastava	Visiting Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*
None

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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.)*

Studies were aimed at the isolation, characterization, and function of transforming genes potentially associated with the development of human mammary tumors. Analysis of primary mammary tumors and tumor cell lines of human origin for transformation activity following transfection onto NIH/3T3 cells revealed the presence of an H-ras oncogene in a mammary carcinosarcoma cell line, HS578T. The HS578T oncogene was molecularly cloned in its biologically active form. Recombinant gene constructs and nucleotide sequence analysis identified the activating lesion as a single nucleotide change from guanine to adenine at position 35 of the first exon, substituting aspartic acid for glycine at the 12th codon of the p21 gene product. The absence of restriction polymorphism for Hpa II/Msp I, induced by this single point mutation in HS578T tumor cells, from normal cells of the same patient demonstrated the somatic origin of the genetic lesion responsible for the transforming activity of HS578T. Moreover, the presence of the transforming H-ras allele as evidenced by Msp I/Hpa II restriction polymorphism in all single cell-derived clonal lines of HS578T with underrepresentation of the normal H-ras proto-oncogene suggests a selection for the transforming H-ras allele in the HS578T tumor cell population.

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
E. P. Reddy	Visiting Scientist	LCMB	NCI
S. K. Srivastava	Visiting Fellow	LCMB	NCI

Objectives:

1. To identify and isolate transforming DNA sequences in human mammary tumors.

2. To determine the mechanism which led to the activation of the HS578T oncogene.

3. To analyze the functional relationship of the transforming and the normal allele in the HS578T mammary carcinosarcoma cell line.

Methods Employed:

1. DNA transfection onto NIH/3T3 mouse cells using the calcium-phosphate coprecipitation assay.

2. Blot hybridization according to Southern.

3. Molecular cloning using λ phage and bacterial plasmid systems.

4. Nucleotide sequence analysis according to Maxam and Gilbert.

5. Immunoprecipitation and SDS-PAGE of transforming proteins using monoclonal antibodies against p21.

Major Findings:

1. The presence of an H-ras oncogene in the cell line HS578T derived from a human mammary carcinosarcoma.

2. The genetic lesion responsible for the transforming activity of the HS578T oncogene has been localized to a single point mutation in the first exon resulting in the substitution of aspartic acid for glycine at codon 12.

3. The genetic alteration leading to the activation of the HS578T oncogene was not transmitted through the germ line, but was a somatic event. A selective advantage for the transforming allele versus the normal allele has been shown in the HS578T tumor cell population.

Significance to Biomedical Research and the Program of the Institute:

Activated ras genes had been detected in human malignancies of various tissue origin. We demonstrated that ras proto-oncogene activation by a single point mutation can be associated with human mammary tumorigenesis. The precise localization of the activating genetic lesion for the HS578T oncogene supports the evidence that codons 12 and 61 are the major spots where members of the ras gene family become activated by single point mutations. It is of critical importance to establish the direct relevance of an oncogene activated in a human tumor to the neoplastic process leading to tumor development. Our findings of the mutated allele in all tumor cells and its absence from normal cells of the same patient establish that the activating lesion was not inherited but rather is a somatic event. The strong selective pressure for the presence of the mutated allele within the HS578T tumor cell population implies that the somatic activation of this H-ras oncogene must have played a role in the development of the HS578T carcinosarcoma.

Proposed Course:

The relative infrequency of carcinosarcomas among human mammary tumors and the lack of transforming activity of other human mammary tumors as determined in the NIH/3T3 transfection assay imply that mechanisms other than activation of H-ras, K-ras or N-ras by single point mutations at codon 12 or 61 could exert a critical influence on human mammary tumorigenesis. This contrasts with the frequent H-ras activation in carcinogenesis-induced rat tumors of the mammary gland. Biochemical studies will be undertaken to elucidate whether different mechanisms, such as activation of proto-oncogenes by gene rearrangement or transposition, as well as gene amplification of proto-oncogenes, are involved in the etiology of the majority of human mammary neoplasms.

Studies on the functional relationship of mutated and normal H-ras alleles in the HS578T cell line will be continued in order to more precisely address the question of how essential ras gene activation is in tumor initiation. Single cell-derived clonal lines of HS578T with different ratios of normal and mutated H-ras alleles will be assayed for RNA expression of H-ras, N-ras and K-ras. Biochemical analyses of subclones derived from HS578T clonal lines containing both alleles will allow us to more precisely determine the extent of the selective pressure for loss of the normal allele. Comparison of malignant properties of clonal lines possessing both phenotypes should help to correlate the degree of malignancy with the loss of the normal allele.

Publications:

Kraus, M. H., Yuasa, Y. and Aaronson, S. A.: A position 12-activated H-ras oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. Proc. Natl. Acad. Sci. USA (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. The Cancer Cell. New York, Cold Spring Harbor Laboratory Press. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05416-01 LCMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Molecular Analysis of Retroviruses of the Lentivirus Group

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

PI:	A. Yaniv	Visiting Scientist	LCMB	NCI
Others:	J. Dahlberg	Microbiologist	LCMB	NCI
	S. Tronick	Microbiologist	LCMB	NCI
	I-M. Chiu	Visiting Fellow	LCMB	NCI
	S. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*

None

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

The integrated forms of caprine arthritis-encephalitis virus (CAEV) and of equine infectious anemia virus (EIAV) were cloned in the L47.1 strain of bacteriophage λ. The restriction enzyme map of CAEV was determined and oriented relative to the viral RNA. The size of the long terminal repeats of CAEV is approximately 500 base pairs. The determination of the genetic relatedness of CAEV to other retroviruses as well as the molecular characterization of the cloned EIAV are now in progress.

PROJECT DESCRIPTION

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

A. Yaniv	Visiting Scientist	LCMB	NCI
J. Dahlberg	Microbiologist	LCMB	NCI
S. Tronick	Microbiologist	LCMB	NCI
I-M. Chiu	Visiting Fellow	LCMB	NCI
S. Aaronson	Chief	LCMB	NCI

Objectives:

1. To determine the molecular structure of caprine arthritis-encephalitis virus and equine infectious anemia virus.

2. To study the molecular basis of induction of nonmalignant diseases by retroviruses.

Methods Employed:

Standard molecular biological techniques for nucleic acid isolation, gene enrichment, gel electrophoresis, Southern transfer, molecular hybridization, recombinant DNA techniques for cloning and amplification of genes, and restriction enzyme mapping of cloned genes.

Major Findings:

Restriction enzyme analysis of the CAEV unintegrated linear DNA was performed in order to devise a molecular cloning strategy. This analysis revealed the presence of a single Xba I site. The supercoiled CAEV DNA was cleaved with Xba I and ligated to Xba I-cleaved WES B λ phage DNA. Eighteen clones containing CAEV DNA were isolated. The sizes of the CAEV inserts ranged between 1.5 - 8.1 kbp, whereas the full length of CAEV unintegrated DNA is 9.7 kbp. Therefore, additional attempts were made to clone the integrated form of CAEV. The restriction map of the unintegrated linear form of CAEV indicated the absence of a Hind III site. Upon examination of Hind III cleaved high-molecular weight DNA obtained from Himalayan tahr-CAEV-infected cells, we could detect one DNA fragment of 11 kbp using CAEV cDNA probe. This fragment was enriched by sucrose density gradient centrifugation and ligated to purified arms of phage L47.1. One clone was isolated. The λ CAEV clone was shown to contain 9.7 kb of viral information as well as 1.1 kb and 0.25 kb of cellular flanking sequences. The cloned CAEV genome was indistinguishable from that of the unintegrated proviral CAEV DNA with respect to its restriction endonuclease map. The λ CAEV restriction map was oriented relative to the viral RNA by using a cDNA probe representing the 3' end of the viral RNA. Restriction enzyme analysis and Southern blotting demonstrated that the CAEV long terminal repeats are approximately 500 base pairs long. Restriction enzyme digests of a variety of vertebrate DNAs were analyzed for DNA sequence homology with CAEV by using the cloned CAEV genome as a probe. CAEV sequences were not detected in the DNA of its species of isolation, indicating that CAEV is not

an endogenous virus of goat. Of the other species tested, single bands were observed in digests of mouse and horse. CAEV-related bands were not detected in chicken, rat, sheep, pig, cow, rhesus monkey or human DNAs under conditions of non-stringent hybridization and washing.

The EIAV-infected equine dermis cells were found to contain several copies of the viral genome integrated at multiple sites. To obtain clonal cells in which virus integration occurred at a single site, canine cells, also permissive for EIAV, were infected at low multiplicity, and single cells were used to initiate EIAV cell lines. Restriction enzyme analysis of several of these cell lines demonstrated the presence of a single copy of the EIAV genome. When high molecular weight cellular DNA obtained from one of these clones was cleaved with Hind III, two fragments of 11 and 7.2 kbp were detected by using EIAV cDNA as a probe. All other enzymes suitable for molecular cloning cut more frequently. The high molecular weight DNA was partially cleaved with Hind III and enriched for 16-19-kbp fragments by sucrose density gradient centrifugation. The enriched DNA fragments were ligated with purified arms of phage L47.1 and two positive clones were isolated. The molecular characterization of these clones is now in progress.

Significance to Biomedical Research and the Program of the Institute:

Both CAEV and EIAV are nontransforming retroviruses that cause severe diseases in two domestic animals of great economic importance. The isolation and cloning of the viral genomes is extremely important for studying the mechanism of induction of diseases by these retroviruses. The CAEV might also serve as an animal model for studying similar diseases in man, while EIAV has lately gained great significance in view of the finding that its major structural protein cross reacts with HTLV-III, a virus associated with the AIDS syndrome of human.

Proposed Course:

Determination of a detailed restriction enzyme map of EIAV and evaluation of its nucleic acid homology with HTLVs.

Elucidation of the origin and relationship of CAEV and EIAV to other mammalian retroviruses.

Qualitative and quantitative examinations of CAEV and EIAV genomes in tissues of diseased animals.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF MOLECULAR ONCOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1983 to September 30, 1984

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 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 normal to 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. The Cellular Transformation Section investigates the malignant transformation of cells by avian sarcoma viruses, including the function of the virus-coded protein directly responsible for transformation, the primary physiological effects of the functioning protein, 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 viral- and cell-coded proteins. The Molecular Control and Genetics Section conducts studies to control gene expression in the prokaryote, E. coli and its phage, lambda. The molecular basis of gene regulation is determined at the level of transcription initiation, transcription termination, RNA translation, and RNA processing. Mutants are isolated in control sites and in control genes and the effects of these mutations on RNA and protein syntheses are studied in vivo. The Tumor Biochemistry Section investigates the biochemical and genetic elements responsible for cellular transformation. Specific regions of viral and cellular DNA thought to be involved in the expression of cellular transforming phenotypes are isolated by recombinant DNA techniques. Structural properties of the cloned fragments are determined and their transforming activities characterized in biological assays. The characterization and identification of these elements provide a means for examining normal eukaryotic DNA for the occurrence of molecular elements with similar properties.

The major portion of the present and future emphases of this Laboratory concerns the identification, isolation and analysis of genomic sequences by molecular cloning techniques, as well as their onc gene 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 homologues, 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. 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. The following information was obtained: 1) Δgag-mht forms a hybrid gene with a contiguous 2682 nucleotide reading frame, terminating with a stop codon near the 3' end of mht; 2) The 3' end of 969 nucleotides of the mht region up to the stop codon is 80% homologous to the unique onc-specific raf sequence of the murine sarcoma virus 3611; 3) More 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; 4) The myc sequence is preceded by an RNA splice acceptor site shared in common with the cellular proto-myc gene, beyond which it is colinear up to a 3' termination codon and 40 noncoding nucleotides with the myc sequences of avian MC29 retroviruses and the chicken proto-myc. Thus, myc forms together with a 5' retroviral exon, a second MH2-specific gene; 5) The oncogene myc is followed by a 400-nucleotide, 3' terminal c region which is related to the Rous sarcoma virus. The significance of these data, relating to MH2, is that out of 19 known different viral oncogenes, 5 of them 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 its oncogenic potential and transformation capabilities. 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 both 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 a unique restriction enzyme site 12 codons beyond the lambda phage cII gene initiation codon and was used to fuse the carboxy-terminal sequences of the MC29 v-myc gene to the amino-terminal sequences of the lambda cII gene. Transcription of this chimeric plasmid is under the control of the lambda p_L promoter which, when derepressed, produced a high level of cII-myc fusion protein, amounting to greater than 5% of the total cellular protein. This high level of expression has enabled antibodies to be raised against this protein which are capable of immunoprecipitating the MC29 gene product $P110^{gag\text{-}myc}$.

In similar fashion to the previous expression vector construct, we have fused the Harvey murine sarcoma virus (Ha-MuSV) $p21^{ras}$ gene to the amino-terminal portion of the lambda phage cII gene of pJL6. This ras gene fusion product effected the synthesis of a hybrid protein of 23,000 daltons, representing more than 10% of the total cellular protein and containing all but four residues at the amino-terminus of the $p21^{ras}$ protein. This bacterially produced protein appears to be similar to the Ha-MuSV $p21^{ras}$ protein in that it underwent immunoprecipitation by monoclonal antibodies specifically directed towards that protein, was able to bind guanosine diphosphate and was capable of undergoing autophosphorylation like the authentic viral oncogene protein.

We have expanded the versatility of our E. coli expression vector, pJL6, by constructing a number of useful modifications. These include the introduction of a variety of restriction sites by cloning oligonucleotide linker DNA or polylinker-containing regions from segments of other vector DNA. Another modified plasmid, pJLA16, allows the cloning of blunt-ended DNA adjacent to the phage gene, permitting fusions of this gene fragment with the target gene DNA.

Associations between certain human malignancies and unique chromosomal abnormalities have led to the hypothesis that specific cellular oncogenes are involved and consequently activated by the genetic abnormalities. In particular, it had been observed, using the cloned constructs of the viral oncogene v-myc developed in our laboratory, that chromosomal translocation involving the human proto-myc gene on chromosome 8 is transferred to the immunoglobulin heavy chain locus on chromosome 14. Although chromosomal breakpoints can be variably located, the recombination sites have not been precisely identified in relation to the functional domains of these loci. We now have identified and characterized two reciprocal recombination sites between c-myc and the immunoglobulin heavy chain μ in a Burkitt lymphoma. Sequencing of the crossover point joining chromosomes 8 and 14 shows that the onc gene is interrupted within its first intron and thereupon joined to the heavy chain μ switch region. This recombination predicts that the translocated onc gene would code for a rearranged mRNA, but would express a normal c-myc polypeptide.

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 the Ca^{++}-PO^{-}_{4}-dependent transfection and transformation of the NIH 3T3 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 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 investigating the process of 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 homologue 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 investigations, 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 two-fold: 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 DNA's isolated from transformed human cell lines which would induce tumors in nude mice. One of these, derived from a pancreatic carcinoma cell line, was an activated ras^K sequence. Nucleotide sequence analysis has shown that this gene contains an aspartic acid in place of glycine at position 12 in the coding sequence. Position 12 changes have been shown in several isolates of activated ras oncogenes, but this represents the first case in which a gly-asp modification has occurred. The nude mouse assay has also detected active, transfectable oncogenes in a primary gastric adenocarcinoma, a nasopharyngeal carcinoma and a carcinoma of the prostate. We have identified the transferable oncogene as N-ras in the adenocarcinoma, and the others are currently being characterized.

An active transforming sequence had previously been detected by transfection of DNA isolated from passage 330 of the human teratocarcinoma line, PA-1, which was initially derived from a metastatic, ovarian germ line tumor. These late passage cells show significant alterations in various malignancyrelated, phenotypic characteristics, including tumorigenicity in nude mice and the ability to form colonies in agar suspension, in comparison to early passage (<100) PA-1 cells. The transforming activity detected at passage 330 was identified as a N-ras oncogene which had undergone a glycine-aspartic acid transition at position 12. All previous naturally occurring N-ras activations had been shown to involve changes at codon 61. Early passage (36) PA-1 cells are poorly tumorigenic in nude mice and no focus-inducing activity can be detected in DNA

transfection assays. The presence of an activated N-ras can thus be correlated with increasing tumorigenicity in PA-1 cells. This system represents an opportunity to study the role of this specific oncogene, as well as other specific genetic changes, in the carcinogenic progression process in PA-1 cells.

A transforming activity associated with a line of human cells, MNNG-HOS, a human osteosarcoma derived cell line which was subsequently transformed to anchorage-independent growth and tumorigenicity, had previously been reported. Analysis of this oncogenic sequence has continued in collaboration with members of the Molecular Mechanisms of Carcinogenesis Laboratory, Litton Bionetics, and Dr. C. Cooper of the Laboratory of Molecular Carcinogenesis, Dana-Farber Cancer Institute. The transforming sequence, designated met, has been cloned in several overlapping lambda clones totaling 40-kb of human sequence. Met appears to represent a previously unidentified transforming gene, since it shows no sequence homology with known members of the ras oncogene family nor with the oncogenes mos, myc, myb, src, erb, sis, rel or B-lym. Restriction analysis further indicates that it does not represent the human homologues of abl or fes. The activated met sequence shows no detectable gross rearrangement when compared to human placental DNA, several other human tumors, or the parental HOS cell line. Met has been localized to human chromosome 7 using somatic cell hybrids. It appears to represent a new and interesting human sequence with oncogenic potential.

The major efforts to identify and characterize human oncogenic sequences have involved the use of continuous lines of rodent cells for identification and analysis. Therefore, this section has sought to develop a human cell system which can be used to identify human oncogenic sequences and measure their oncogenic potential. Efforts have been concentrated on several non-tumorigenic, immortalized human cell lines which were felt to be analogous to the useful and proven, rodent cell lines. An SV40-transformed human fibroblast line, SV80, and a hybrid of HeLa cells and normal diploid fibroblasts were found to be transfected for cloned, selectable markers and could be shown to possess a cryptic tumor potential. Transfection of SV80 cells with cloned Moloney mos or cloned, activated-human H-ras onc-genes did not induce obvious foci or render the transfected cells tumorigenic in nude mice. Expression of MSV-encoded gag antigens could be detected, and individual cells expressing these antigens were morphologically altered. Preliminary experiments suggest, however, that cotransfection of MSV and adenovirus E1a and E1b sequences can produce cells capable of forming tumors in nude mice. Continued studies of these and other transfected human cells should lead to an increase in the understanding of the role of multiple oncogenes in human malignancy.

It is well established that the transforming genes activated in human tumors represent, in many cases, the human homologues of these viral oncogenes present in acute transforming retroviruses. The oncogene mos was initially identified as the transforming sequence of the Moloney murine sarcoma virus, and so this section has been interested in its mechanism of transformation and its potential role in human oncogenesis. Characterization of the v-mos-encoded protein has been hindered by the fact that it is apparently expressed at extremely low levels in transformed cells. A project is underway to develop a eukaryotic expression system which will express v-mos, and potentially any other gene product, at high levels. Vectors have been constructed in which v-mos is under the control of the murine beta-globin major promoter. This construct has been

introduced into murine erythroleukemia cells. When these cells are treated with inducers of differentiation, the exogenously added, β-globin promoter should be activated and v-mos should be expressed at elevated levels. This should facilitate the characterization of the v-mos protein, its physical properties and its mode of action in a eukaryotic environment.

Additionally, the Microbiology Section is continuing a series of studies on the human homologue of mos and its transforming potential in collaboration with members of the Basic Research Program, Litton Bionetics, Inc., and the Nucleic Acids and Protein Synthesis Laboratory, Program Resources, Inc. The human cellular homologue of mos, c-mos^{Hu}, does not transform NIH 3T3 cells, even when linked to MSV-derived, long terminal repeat (LTR) sequences. However, certain recombinants between c-mos^{Hu} and v-mos, which contain mos sequences from both parents, are active in transfection at reduced efficiencies. Active recombinants containing the 3' portion of c-mos^{Hu} apparently express high levels of the mos gene product detectable by peptide antisera specific for the contrary terminus of c-mos^{Hu}. The pattern of activity in c-mos^{Hu}/v-mos recombinants is consistent with the presence of at least three functional domains, two of which interact in mos^{Hu} to prevent the expression of the transforming function of the protein. Continued study of mos hybrids formed between sequences widely separated on the evolutionary scale offers a novel approach to the study of the structure-function requirements of a transforming protein, as well as the evolutionary conservation of transformation potential in a normal cellular gene.

The Molecular Control and Genetics Section studies the regulation of gene expression in the bacterium E. coli and its phages, as well as during 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.

The regulation of the lambda int gene has been determined. It can be transcribed from either of two promoters, p_I or p_L. p_I requires lambda cII protein in addition to RNA polymerase to initiate transcription. 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 lambda 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 cleavage 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.

In E. coli, two types of terminators exist: those that can terminate with RNA polymerase alone and those that require a protein, Rho, in addition. Although the structure of Rho-independent terminators is well understood, very little is known about the Rho-dependent terminators. The project this section has undertaken, emphasizes experimentation towards a better understanding of the transcription termination/antitermination mechanisms at the Rho-dependent tRI terminator on lambda. Transcription antitermination positively regulates the early

gene expression in phage lambda. The antitermination machinery comprises both phage and host encoded proteins. The phage-encoded N gene product somehow modifies the transcription process at a specific sequence on the phage genome, called "nut" site (for N-utilization), so that the RNA polymerase can read through the transcription terminator signal at the Rho-dependent, tRI terminator site. A host protein, nusA, is also involved in the termination/antitermination process. Mutations generated in vivo and in vitro are being used to direct the Rho-dependent termination site, tRI, and the N-utilization site nearby.

The section also has constructed several expression vectors and is participating in the cloning and expression of mos, myc, and other oncogenes, as well as the env gene of HTLV and several other eukaryotic and prokaryotic genes.

The cellular slime mold, Dictyostelium discoideum, is one of the simplest organisms that undergo true multicellular differentiation. It is being used as a model system to study 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. Cell-cell interaction is a necessary prerequisite for the synthesis and stability of the late messenger RNAs. Following activation, the actual rate of transcription and the subsequent stability of many of the messenger RNAs transcribed off of this portion of the genome are further regulated by a cyclic AMP-mediated process. Two closely integrated approaches are being used to further our understanding of this regulation. First, studies on the physiology of late gene expression have better defined the nature of the requirement for cell-cell interaction and are allowing us to develop conditions under which we can use transformation and in vitro mutagenesis to identify control sites involved in cAMP-mediated regulation of developmental gene expression. Second, since 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 are being used to resolve oligonucleosomes specifically derived from actively transcribed genes in order to determine their protein composition.

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 (p-myc) encoded by the avian MC29 virus. The protein is found in the nucleus in monomeric and dimeric forms, and has a short half-life typical of regulatory proteins. A similar cellular

protein has been identified and localized. This cellular protein disappears after infection and transformation of cells with MC29 and related viruses, suggesting that an autoregulatory process controls the synthesis of the cellular protein. Revertants of transformed cells show a reappearance of the cellular protein, and an investigation of the regulatory process is in progress.

Another virus, MH2, encodes a protein similar to p-myc and another unrelated transforming protein, p-mht. Molecular DNA clones containing deletions in transforming genes have been used to transfect avian and mammalian cells, with the intention of identifying the distinct functions of p-myc and p-mht. The association of the myc gene with a variety of vertebrate neoplasms has suggested a possible role for cellular p-myc in the maintenance of the malignant state.

The studies underway in the Laboratory of Molecular Oncology have enabled us to better understand how eukaryotic and prokaryotic genes are controlled and expressed. We have been using the knowledge gained to express genes from higher organisms (man included) in bacterial systems. Such studies will provide many benefits for the molecular elucidation of the oncogenes and their products, and, additionally, provide an understanding of the basis of gene control and gene expression in higher organisms. These studies could only have been accomplished by having this unique combination of investigators skilled in multidisciplinary areas such as prokaryotic genetics and in oncogene transformation systems of higher eukaryotes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04876-12 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

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)*
Department of Physics, Georgetown University, Washington, D.C. (G. Malinin)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Cellular Transformation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.05	1.05	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.)*

Ts-68 virus-transformed chick embryo fibroblasts (CEF) can uniquely express transformation by a shift in temperature of about 5°. Ts-68-infected and transformed quail cells showed about two times the thymidine kinase (TK) activity of normal quail cells. Ts-68-transformed CEF incubated at 41° showed about two times the TK activity of normal CEF incubated at the same temperature. The ts line incubated at 37° exhibited a higher protein concentration present in the post-microsomal, supernatant fraction than did the cells incubated at 41°. The TK from the ts-68-transformed CEF showed a higher temperature for optimal activity than did normal CEF. The effects of succinylacetone (SA), an inhibitor of the heme biosynthetic pathway, on growth and respiration of L1210 cells in vitro were examined. The cell growth with SA was normal for 2 days, after which time growth ceased. SA treatment of L1210 cells, even when growth ceased, caused less respiration inhibition than inhibition of growth after incubating the cells at high density. Both untreated and SA-treated cells responded similarly to mitochondrial inhibitors and uncoupling agents, suggesting that SA has no specific effect on respiration. Growth inhibition due to SA was not caused by heme depletion or impairment of respiration. Thus far, the only effect of SA which has been observed is the inhibition of the heme pathway.

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 if thymidine kinase (TK) activity is related to transformation in a temperature-sensitive mutant of Rous sarcoma virus (RSV), ts-68. To determine how succinylacetone (SA), a new, potent inhibitor of the heme pathway, affects the metabolism of cancer cells and inhibits growth. SA has been found to inhibit the growth of L1210 cells, but it does so by some mechanism other than inhibition of heme production. To determine if SA causes a block in the electron transport chain to restrict the growth of L1210 cells.

Methods Employed:

Virus-transformed cell lines were generated by infecting secondary chick embryo fibroblasts (CEF) with SR-A, Ta, and ts-68 avian sarcoma viruses, and cultivating the progeny cells. Ts-68-transformed cells express the transformed phenotype at 37° and appear normal when incubated at 41° or higher. Respiration of L1210 cells incubated with and without SA, and in the presence of various mitochondrial inhibitors, was measured in a polarograph equipped with a Clark oxygen electrode.

Major Findings:

1. Relationship of TK activity with transformation. Ts-68-infected and -transformed Japanese quail cells showed 2-2.5-fold TK activity of normal quail cells. Also, ts-68-transformed CEF incubated at 41° showed about two times the TK activity of normal CEF. However, at the temperature expressing the transformed phenotype (37°), the ts-68 line exhibited the same TK activity as the normal CEF, most likely due to the much higher protein concentration present in the post-microsomal supernatant fraction of the transformed phenotype. The TK from the ts-68-transformed CEF grown at 37° showed optimal activity at a temperature of 60°, while optimal TK enzymatic activity for normal CEF was observed at 54°, suggesting that the transformed cells may have a modified or virus-coded enzyme.

2. Effect of SA on the growth and respiration of L1210 cells. SA, a new, irreversible inhibitor of δ-aminolevulinic acid dehydrase of the heme biosynthetic pathway, was studied for its effects on the growth and respiration of L1210 cells in vitro. Cells cultivated in the presence of SA grew normally for 2 days, after which growth ceased. L1210 cells in the stationary phase exhibited 76% less oxygen consumption than cells growing logarithmically. However, SA treatment caused only a 26% decrease in respiration after 3 days, even when growth had stopped. Both untreated and SA-treated cells responded similarly to mitochondrial inhibitors and uncoupling agents, suggesting that SA has no specific effect on respiration. Most ascites cells show an inhibition of respiration when glucose is added to cells depleted of glucose (Crabtree

effect). In contrast to this general observation with ascites cells, L1210 cells were found to be slightly stimulated with added glucose, subsequent to incubation in glucose-free medium. SA was not immediately toxic to L1210 cells, but inhibited further growth after 2 days without lowering heme levels. Thus, growth inhibition due to SA was not a result of heme depletion, as seen in murine erythroleukemia cells, nor of impairment of respiration. This data further demonstrates the absence of respiratory toxicity of SA as seen in normal liver cells and in rats, other than that specifically affecting the heme pathway.

3. Comparative activation of splenocytes by selenium dioxide and periodic acid. Murine splenocytes showed increased thymidine (TdR) incorporation when treated with selenium dioxide (SeO_2). In contrast to splenocyte activation by periodic acid (H_5IO_6), the SeO_2-induced response was not inhibited by neuraminidase degradation of cell membranes prior to oxidation, nor by prior hydroxylamine (NH_2OH) treatment. Reduction of the cell membrane by borohydride ($NaBH_4$) previous to oxidation by SeO_2 or H_5IO_6 inhibited cell activation. Sequential oxidation by H_5IO_6 and SeO_2 increased the TdR uptake to a greater extent than that by SeO_2 or H_5IO_6 alone. The reverse order of the sequential oxidation decreased the TdR uptake to the oxidation level produced by H_5IO_6 alone. It is concluded that the stimulation of splenocytes by SeO_2 is produced primarily by the conversion of carbonyl groups in the cell membrane to dicarbonyls.

Significance to Biomedical Research and the Program of the Institute:

Certain properties of transformed cells can be turned on and off by incubation of ts-68-transformed cells at 37° and 41°, respectively. Transformation events can be examined in detail by observing progressive changes in certain parameters following a shift in incubation temperature from 41° to 37°. SA has shown anti-tumor activity against several malignant cell lines in vitro and in vivo. Although SA has been shown to be active only against the second enzyme of the heme pathway, the drug inhibits the growth of Walker 256 carcinoma and L1210 cells in vitro without reducing the heme levels of these cells. Determination of this secondary mechanism of action may lead to the development of analogs and other compounds which exploit this presently unknown, growth inhibitory property.

Proposed Course:

A goal of the project is to identify new specific proteins and enzymes which appear during the transformation of cells by avian tumor viruses, and which are dependent upon new transcription. The sequence of events leading to changes in transcription will be analyzed during the process of virus-induced transformation. Further attempts will be made to determine if the TK in RSV-transformed cells is a new enzyme distinguishable from the normal CEF TK and if a unique protein(s) is being produced in the ts mutant incubated at the temperature of the transformed phenotype. Other markers of transformation will be studied in the ts mutant system in attempts to better understand the process of transformation.

Publications:

Ebert, P. S., Smith, P., Bonner, R., Hess, R. A., Costa, J. and Tschudy, D. P.: Effect of defined wavelength and succinylacetone on the photoinactivation of leukemia cell growth in vitro by hematoporphyrin. Photobiochem. Photobiophys. 6: 165-175, 1983.

Malinin, G., Hornicek, F. J. and Ebert, P. S.: Comparative activation response of splenocytes oxidized by periodic acid and selenium dioxide. Res. Commun. Chem. Pathol. Pharmacol. 41: 425-439, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04899-12 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

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 Fellow	LMO	NCI
	K. P. Samuel	Visiting Associate	LMO	NCI
	C. Flordellis	Visiting Fellow	LMO	NCI
	U. Rovigatti	Visiting Scientist	LMO	NCI

COOPERATING UNITS *(if any)*
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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:
1.10	1.10	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 research has elucidated the structure of the cloned 5.2-kilobase (kb), integrated DNA provirus of MH2; a major portion (3.5 kb) of the 3' region has been sequenced. The complete genetic structure has been determined and found to be: 5'-Δgag(1.9 kb)-mht(1.2 kb)-myc(1.3 kb)-[non-coding]c-region(0.2 kb)-3'. Aside from the genetic elements shared in common with other nondefective avian retroviruses, the mht gene was found to be a unique oncogene sequence. Hybridizations have revealed that mht is derived from a normal cellular gene distinct from the proto-myc family of oncogenes. The Δgag-mht forms a hybrid gene, containing a 2682 nucleotide contiguous reading frame with a stop codon near the 3' end. The 3' region of mht (containing 969 nucleotides) is 90% sequence-related to the onc-specific raf sequence of the MSV 3611, and 95% homologous at the deduced amino acid sequence level; the closest homology determined so far of the 19 known onc sequences. Using a unique, expression vector, pJL6, we have cloned and expressed the carboxy-terminal portion of the avian MC29, v-myc oncogene as a fusion protein in bacteria produced as more than 10% of the total cellular protein. This expression product has enabled the preparation of antibodies raised against it; these antibodies immunoprecipitate the MC29 oncogene product, p110gag-myc. Employing the same expression system, we prepared a Harvey MSV, p21ras oncogene product as a fusion protein, produced as more than 10% of the total protein; this protein exhibited GDP binding activity and was capable of autophosphorylation, similar to authentic, Ha-MSV p21ras. We have sequenced and characterized two reciprocal recombinant sites between c-myc and the immunoglobulin heavy chain IHC μ region in a Burkitt lymphoma, showing that the onc gene is interrupted within its first intron region and joined to the IHC μ switch region.

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 Fellow	LMO	NCI
K. P. Samuel	Visiting Associate	LMO	NCI
C. Flordellis	Visiting Fellow	LMO	NCI
U. Rovigatti	Visiting Scientist	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 and 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 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 implicate 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 phos phatase. Ligation of each isolated fragment to vector DNA was 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 by the same procedure. Extensive use was made of published DNA sequences in the design 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 trichloracetic 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 diazobenzyl-oxymethyl (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 fragment, 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; (ix) DNA sequence analysis of deletion mutants by the method of Maxam and 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. Tryptic peptide analyses of viral and cellular, onc-related polypeptides by two-dimensional thin layer electrophoresis and chromotography.

11. Microsequence analysis of onc-related polypeptides to define initiation of translation.

Major Findings:

1. The avian carcinoma virus, MH2, has been grouped together with MC29, CMII, and OK10, because all of these viruses share a transformation-specific onc-gene termed myc. A 5.2-kilobase (kb) DNA provirus of MH2 has been molecularly cloned. The complete genetic structure of MH2 is 5'-Δgag(1.9 kb)-mht(1.2 kb)-myc(1.3-kb)-Δenv-[non-coding c-region](0.2-kb)-3'. Δgag, Δenv, and c are genetic elements shared with nondefective retroviruses, whereas mht is a unique, possibly MH2 transformation-specific, sequence. Hybridizations with normal chicken DNA and cloned chicken c-myc DNA indicate that the mht sequence probably derived from a normal cellular gene that is distinct from the c-myc gene. The genetic structure of MH2 suggests that the Δgag and mht sequences function as a hybrid gene that encodes the p100, putative, transforming protein. The myc sequence of MH2 appears to encode a second transforming function. Therefore, it seems that MH2 contains two genes with possible oncogenic functions, whereas MC29, CMII, and OK10 each carries a single hybrid Δgag-myc transforming gene. It is remarkable that, despite these fundamental differences in their primary structures and mechanisms of gene expression, MH2 and MC29 have very similar oncogenic properties.

2. The 5.2-kb RNA genome of the avian carcinoma virus, MH2, has the genetic structure 5'-Δgag(0.2 kb)-mht(1.3 kb)-myc(1.3 kb)-poly(A)(0.2 kb)-3'. Δgag is a partial retroviral core protein gene; mht and myc are cell-derived, MH2-specific sequences; and c is the 3'-terminal, retroviral vector sequence. Here we have determined the nucleotide sequence of 3.5 kb, from the 3' end of Δgag to the 3' end of molecularly cloned proviral MH2 DNA, in order to elucidate the genetic structure of the virus and to compare it with other mht- and myc-containing oncogenic viruses, as well as with the chicken proto-myc gene. The following results were obtained: (i) Δgag-mht forms a hybrid gene with a contiguous reading frame of 2682 nucleotides that terminates with a stop codon near the 3' end of mht. The 3' 969 nucleotides of mht up to the stop codon are 80% sequence-related to the onc-specific, raf sequence of the murine sarcoma virus 3611 (94% at the deduced amino acid level). (ii) The myc sequence is preceded by an RNA splice acceptor site shared with the cellular proto-myc gene, beyond which it is colinear up to a 3'-termination codon and 40 noncoding nucleotides with the myc sequences of the avian retrovirus, MC29, and chicken proto-myc. Thus, myc forms, together with a 5' retroviral exon, a second, MH2-specific gene. (iii) myc is followed by the 3'-terminal c region of about 400 nucleotides, which is related to that of Rous sarcoma virus. It is concluded that MH2 contains two genes with oncogenic potential, the Δgag-mht gene, which is closely related to the Δgag-raf transforming gene of MSV 3611, and the myc gene, which is related to the transforming gene of MC29. Furthermore, it may be concluded that the cellular proto-onc genes, which on sequence ransduction become viral onc genes, are a small group because, among the 19 known onc sequences, five are shared by different taxonomic groups of viruses, of which the mht/raf homology is the closest determined so far.

3. A common cellular sequence was independently transduced by the avian carcinoma virus, MH2, (v-mht) and the murine sarcoma virus MSV 3611 (v-raf). Comparison of the nucleotide sequences of v-mht and v-raf revealed a region of homology that extends over 969 nucleotides. The homology between the corresponding amino acids was about 95%, differing only in 19 of 232 amino-acids. This example, brings 5 out of the 19 known, different, viral onc genes, that share homologous sequences, which have been in viruses derived from different taxonomic groups. These data indicate that (i) the number of cellular proto-onc genes is limited because, like other viruses of different taxonomic groups, MH2 and MSV 3611 have transduced the same onc gene-specific sequences from different cell species; and (ii) that specific deletions and linkages of the same proto-onc sequences to different viral vector elements affect the oncogenic potential of the resulting viruses. The differences in the transformation capabilities of MH2 and MSV 3611 serves as an example.

4. A plasmid, pJL6, was constructed that contains a unique ClaI site twelve codons beyond the bacteriophage λ cII gene initiation codon. This site allowed us to fuse the carboxy-terminal sequences of the avian myelocytomatosis virus (MC29) v-myc gene to the amino-terminal portion of the cII gene. Transcription of the hybrid gene is controlled from the phage λ p_L promoter. When this promoter is derepressed, E. coli cells harboring the chimeric plasmid produce a level of cII-myc fusion protein greater than 5% of the total cellular protein. Antibodies raised by this protein immunoprecipitate the MC29 gag-myc gene product, $p110^{gag\text{-}myc}$.

5. The gene for the Harvey murine sarcoma virus (Ha-MuSV) $p21^{ras}$ protein was fused to the amino-terminal portion of the bacteriophage λ cII gene on the expression vector, pJL6. The fusion was such that transcription was controlled by the well-regulated phage λ p_L promoter, and translation initiated in the cII gene continued in frame into the ras gene sequences that code for p21. When the p_L promoter was derepressed, the E. coli cells harboring the fusion plasmid synthesized a 23,000-dalton protein, which represented more than 10% of the total cellular protein. This protein was chimeric and contained 14 residues, which were specified by the vector; these residues were followed by all of the amino acids that make up Ha-MuSV $p21^{ras}$ protein in that it undergoes immunoprecipitation by monoclonal antibodies directed toward that protein, binds guanosine diphosphate, and is capable of autophosphorylation.

6. Several useful modifications of the E. coli expression plasmid, pJL6, have been constructed. These include the introduction of a wide variety of restriction sites by the cloning of oligonucleotide linker DNA or of a short DNA fragment containing many sites. These plasmids can allow the expression of genes adjacent to one of the restriction sites provided. Another plasmid, pJA16, allows the cloning of blunt-ended DNA adjacent to the fragment of phage gene, and fusions of this gene fragment with target gene DNA. This facilitates the expression of genes on blunt-ended DNA fragments that are either directly the product of restriction enzyme digestion or fragments that are resected with Bal-31 exonuclease.

7. The association between certain human tumors and characteristic chromosomal abnormalities has led to the hypothesis that specific cellular oncogenes may be involved and consequently "activated" in these genetic recombinations. This

hypothesis has been strongly supported by the recent findings that some cellular homologues of retroviral onc genes are located in chromosomal segments which are affected by specific, tumor-related abnormalities. In the cases of human undifferentiated B-cell lymphoma (UBL) and mouse plasmacytomas, cytogenetic and chromosomal mapping data have identified characteristic chromosomal recombinations directly involving different immunoglobulin genes and the c-myc oncogene. In UBLs carrying the t(8:14) translocation, it has been shown that the human c-myc gene is located on the region of chromosome 8(8q24) which is translocated to the immunoglobulin heavy-chain locus (IHC μ) on chromosome 14. Although it is known that the chromosomal break-points can be variably located within or adjacent to the c-myc locus and within the IHC μ or IHC γ locus, the recombination sites have not been exactly identified and mapped in relation to the functional domains of these loci. We report here the identification and characterization of two reciprocal recombination sites between c-myc and IHC μ in a Burkitt lymphoma. Nucleotide sequencing of the cross-over point joining chromosomes 8 and 14 on chromosome 14q- shows that the onc gene is interrupted within its first intron and joined to the heavy-chain μ switch region. This recombination predicts that the translocated onc gene would code for a rearranged mRNA, but a normal c-myc polypeptide.

Significance to Biomedical Research and the Program of the Institute:

It is clear that the formation, the maintenance, and the expression of pro-viruses are the central features of the life cycle of RNA tumor viruses. Many questions related to these aspects are unsettled. To elucidate the process of oncogenesis induced by these viruses, it is important that the structural organization of the transformed genes (oncogene) be defined within the host chromosome and the mechanism by which these genes are expressed and regulated be known.

Proposed Course:

1. Purification of the bacterially expressed human c-sis protein. Various methods will be used to solubilize the c-sis protein and to purify it. One method will include resuspension in guanidinium chloride and renaturing in the presence of an equimolar mixture of reduced and oxidized glutathione. Once solubilized, the protein will be purified by ion exchange or antibody affinity chromatography. The purified protein will be analyzed for mitogenic activity. This will be done by introducing the protein to quiescent BALB/c 3T3 cells in platelet-poor serum. Mitogenic activity will be detected by observing the induction of DNA synthesis as measured by the incorporation of ^{3}H-thymidine into DNA. The bacterial c-sis protein will also be assayed for its ability to compete with platelet-derived growth factor (PDGF) for binding to PDGF receptors and to stimulate the synthesis of myc RNA in BALB/c 3T3 cells. Both of these procedures will elucidate the relationship between the sis gene and PDGF.

Antibodies will be raised against the bacterially expressed c-sis protein eluted from polyacrylamide gels of crude lysates, and against more purified

fractions. These antibodies will be used to determine the level of c-sis protein expression in normal and malignant, animal cell lines. Of speical interest are the glioblastoma line, A173, and the human sarcoma line, 8387. These cell lines have been found to contain high levels of sis RNA. Recent studies have indicated that sis proteins undergo processing through dimerization, specific proteolytic cleavage, and glycosylation. The antibodies raised against the bacterial c-sis protein will be used to detect processing intermediates. Tunicamycin will be used to inhibit glycosylation so that unglycosylated forms can be detected.

2. Development of improved expression vectors and hosts. Presently, the p_L host used by pJL6 and its open reading frame (ORF) derivatives are rather difficult to transform, apparently due to the deletion of host membrane genes that occurred when phage killer genes were deleted. By use of in vitro and in vivo recombination techniques, a host will be made that lacks the killing functions, but retains all host DNA. Together with the p_L-containing ORF vector, this new host will allow a much more rapid means of detecting bacteria that express eukaryotic proteins. This is because recombinant plasmids expressing such proteins could be detected in the same host, into which they were introduced by transformation.

An improvement will be made to the lacZ contained in the ORF vector by placing an amber mutation early in the lacZ sequences. In a host that contains an amber suppressor, this vector will behave as the present lac vector, i.e., colonies that express foreign sequences appear blue on XG plates. This will allow the rapid detection of such strains. When the plasmid is moved into a strain lacking a suppressor, the foreign sequences will continue to be expressed, but will be terminated just after read-through into the lac sequences. This will allow purification of the chimeric protein without a large number of β-galactosidase residues attached.

The vector, pJL6, and its lac derivative will be modified by inserting oligonucleotides into the ClaI or HindIII sites at the end of the cII fragment. For example, SmaI linkers of appropriate lengths will be inserted so that the blunt ends produced by this enzyme will produce ends in all three frames. This will allow the fusion of the cII fragment with any blunt-end site into a protein-coding region without any need for trimming, repairing, or adding linkers to the ends of the target fragment. Of those restriction enzymes that cut DNA frequently due to a four-base recognition sequence, several produce blunt-ended termini. Such enzymes can be used to cleave almost any protein-coding sequence in such a way that it can be inserted into the appropriate blunt-end DNA vector and expressed as protein in bacteria.

3. To elucidate the oncogenic functions of the Δgag, mht, and myc genes in MH2, we are constructing deletion mutants in vitro covering different portions of the MH2 genome. The mutated DNA molecules will be used in the DNA-mediated transfer experiments into primary cultures of chicken embryo fibroblasts or into mammalian tissue culture cells, such as NIH/3T3 cells.

4. Biochemical methods will be employed to detect the integrated MH2 genome in transformed tissue culture cells, including cells transformed by MH2

deletion mutations. These methods include the DNA blot hybridization technique developed by E. Southern (J. Mol. Biol. 98: 503-517, 1975), and a variety of DNA cloning techniques. RNA blot hybridization and immunoprecipitation methods will also be used to investigate the expression of deletion mutants in MH2-transformed cells.

5. We are inserting the viral mht gene into the expression vectors, pJL6 and pJLA16, in order to express the v-mht gene in E. coli. Antibodies raised against the expressed v-mht gene will be used to immunoprecipitate the p100 protein in MH2-transformed cells. This experiment should provide direct proof that the p100 protein is a Δgag-mht hybrid protein which does not contain any myc sequence. Anti-mht and anti-gag antibodies will also be used to investigate the expression of MH2 deletion mutants in tissue cultures.

6. 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.

7. Ongoing and proposed experiments will involve: (i) expression of portions of the envelope gene of HTLV-I in bacteria; (ii) expression of the regions of the pX sequences that may code for a putative transforming protein(s) in HTLV-I; (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 of antisera from HTLV-infected patients and for identifying those at risk in endemic regions; (v) isolation of the rearranged c-myc allele of NC37 cells and its characterization by restriction endonuclease mapping and nucleic acid sequencing; and (vi) comparing sequence data of the first exon and intron of both the normal and rearranged c-myc molecular clones to identify point mutations, deletions, or insertions.

8. Antibodies specific to human c-myc protein will be raised using synthetic peptides derived from the predicted human c-myc protein and/or expressed human c-myc protein as antigen. Using these antibodies as a probe, the myc polypeptide in normal human cells, as well as in various tumor-derived cell lines, will be studied.

9. The translation-initiation site of the human c-myc polypeptide will also be determined by micro-sequencing the amino-terminus.

10. The extent of homology among human, chicken and fish c-myc genes will be assessed by heteroduplex analyses of cloned DNA, and by DNA sequencing analyses. These analyses will also be carried out on fish c-ets, c-mht and other c-onc genes.

11. The sequence of chicken c-myc 5' to the v-myc-specific sequences will be further analyzed. The sequence of chicken myc cDNA will be determined and compared to the previously determined sequence of the chicken genomic clone. This comparison will allow clear identification of the cellular exons and the cellular initiation signal, thus allowing the assignment of the amino acids for the normal cellular gene.

12. Chicken and human genomic ets clones will be further characterized by restriction enzyme and sequence analyses. A cDNA library prepared from CEF (polyA+) RNA will be used to isolate several ets cDNA clones.

13. 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 will also be used as antigens to elicit antibody production.

14. Cellular transformation assays will also be conducted using v-ets DNA in order to define sequences essential for tumorigenesis.

15. DNAs from human cell lines and solid tumors will be analyzed for polymorphism and gene rearrangement of c-ets. Using RNA isolated from these sources, overexpression of the ets gene will also be investigated.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04963-08 LMO

PERIOD COVERED: October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Toward a Molecular Description of Malignant Transformation by the p21 ras Oncogenes

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

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Others:	L. S. Ulsh	Microbiologist	LMO	NCI
	S. Hattori	Visiting Fellow	LMO	NCI
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	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI

COOPERATING UNITS *(if any)* Nucl. Acids Protein Synth. Lab., PRI(S. Showalter, M. Zweig) & LMVC, LBI(S. Oroszlan), Frederick, MD; LCDB, NIADDK(M. Lin) & LB, NHLBI(B. Chuck), NIH, Bethesda, MD; Inst. Biochemistry, National Taiwan Univ., Taipei, Taiwan (J.K. Lin); Dept. Biochemistry, Johns Hopkins University, Baltimore, MD(P.C. Huang)

LAB/BRANCH: Laboratory of Molecular Oncology

SECTION: Tumor Biochemistry Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.80	1.10	0.70

CHECK APPROPRIATE BOX(ES)

☐ (a) 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 ras oncogenes of the highly oncogenic Harvey and Kirsten murine sarcoma viruses encode the 21,000-dalton, p21, proteins. To elucidate the molecular mechanism of oncogenic activation, a metabolic turnover study of the p21 of EJ bladder carcinoma and its viral and normal cellular homologues has been carried out. It reveals the first functional difference between the p21 of the viral ras gene and its cellular homologues. Although these p21s appear to be synthesized by a very similar pathway and to have a similar subcellular distribution, the intracellular half-life of the viral p21 is much longer than that of the cellular p21 due to phosphorylation. The viral p21s of the highly oncogenic Ha-MuSV and Ki-MuSV differ from the cellular p21s by having specific point mutations at the phosphorylation sites, in addition to the mutations found at the 12th codon which activate the proto-oncogenes to the oncogenes found and occurring in many tumors. These studies indicate that there may be several steps in the activation of proto-oncogenes to oncogenes, of varying degrees of potency. Recently, the viral p21 oncogene product has been expressed at high levels in bacteria. As a result, several monoclonal antibodies against the p21 protein have been developed. The enzymatic activities of the bacterially produced p21 have been characterized using the purified protein. In other studies, partial revertants of Ha-MuSV-transformed MDCK cells have been isolated and characterized.

PROJECT DESCRIPTION

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

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S. Hattori	Visiting Fellow	LMO	NCI
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J. A Lautenberger	Senior Staff Fellow	LMO	NCI

Objectives:

Cancer is a collective category of many diseases affecting different organs and tissues of the body. There are many cellular origins for different types of cancers, and perhaps quite diverse causes for different malignant diseases. However, at the cellular level, a common characteristic of different types of malignant diseases is uncontrolled cell growth. Recent advances in cancer research have identified a group of oncogenes, approximately 20 in number, whose inappropriate expression may be important in the development of human cancer. The ras oncogenes were first identified in Harvey and Kirsten murine sarcoma viruses, and cellular homologues were found in most animal species, including man. The ras genes encode a group of highly conserved proteins of 21,000 daltons, designated p21. Studies on the p21 of a temperature-sensitive mutant of Ki-MuSV indicated that the p21 protein was the gene product mediating the virus-induced cell transformation. This protein is required, not only for initiation of transformation, but also for the maintenance of the malignant phenotype. Both cytochemical and biochemical studies have localized the p21 proteins to the inner surface of the plasma membrane. Purification of the p21 from virus-transformed cells indicated that p21 proteins possessed a guanine nucleotide binding activity and an enzymatic activity of autophosphorylation. Expression of the ras gene in bacteria indicated that these biochemical activities were intrinsic to the p21 protein.

Recent studies have identified many activated ras oncogenes in many human tumors. These oncogenes differ from the proto-oncogenes by point mutations in the p21 molecules. The long range objectives of this program are to seek understanding of the molecular mechanisms of cell transformation induced by these genes and their protein products, and to elucidate the biochemical mechanism of oncogenic activation of the p21 proteins. The technologies of genetic engineering and analysis by monoclonal antibodies were employed to study the structure-function relationship of the p21 proteins and to thereby develop novel means of cancer diagnosis, monitoring and treatment.

Methods Employed:

1. 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-gel electrophoresis and visualized by autoradiography.

2. Peptide mapping. Peptides generated by protease digestion were analyzed by a 2° dimensional procedure on thin-layer chromatography (TLC) plates. The first dimension was electrophoresis and the second dimension was chromatography.

3. Phospho-amino acid analysis. The ^{32}P-labeled p21 was hydrolyzed in 6 N HCl and the phospho-amino acids were identified by a 2° dimensional procedure on TLC plates.

4. Purification of the p21 from Ha-MuSV-transformed cells. The p21 was purified from NIH 3T3 cells transformed by Ha-MuSV. The purification procedures involved isolation of plasma membranes in a two-phase solvent system; ammonium sulfate precipitation, DEAE-cellulose chromatography, and phenyl-Sepharose columns. The final purification was approximately 2000-fold and the p21 was about 90% pure.

5. Purification of the p21 overproduced in E. coli. The v-ras^H gene was inserted into a plasmid expression vector developed by Lautenberger and Papas. High level expression of the p21 was achieved in bacteria. The p21 in bacterial cells was isolated by lysing the bacteria, fractionation of the lysates by centrifugation, ammonium sulphate precipitation and column chromatography. The p21 protein was isolated in bulk quantity to a purity of more than 90%.

6. Enzyme activity assays of the purified p21. Autophosphorylation activity of the p21 was assayed by incubating the purified p21 with γ-^{32}P-GTP. The phosphorylated proteins were visualized by SDS-gel electrophoresis. The guanine nucleotide binding activity of the p21 was assayed by mixing p21 with ^{3}H-GDP. The ^{3}H-radioactivity associated with the p21 following immunoprecipitation was determined by liquid scintillation counting.

7. Development of monoclonal antibodies against the p21. The p21 isolated from the bacteria or the synthetic peptides made according to the p21 coding sequences were used to immunize Balb/c mice. The 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 the hybridoma cells were characterized with various cell lines transformed by different ras genes.

Major Findings:

1. Metabolic turnover of the human c-ras p21 of the EJ bladder carcinoma, its viral and normal cellular homologues. The EJ bladder carcinoma oncogene is activated by a point mutation of the c-ras^H proto-oncogene at the 12th amino acid

codon, and the EJ p21 migrates slightly slower than the normal c-ras^H p21 in SDS-PAGE. In an attempt to understand the mechanism of oncogenic activation, a comparative study was performed to examine the metabolic turnover and subcellular localization of the p21 encoded by the EJ oncogene and its normal cellular homologue. Pulse-labeling experiments indicated that both p21s were synthesized by a very similar pathway, as had been observed with the viral p21 of Harvey murine sarcoma virus (Ha-MuSV). The pro-p21s were detected in the free cytosol fraction of cell lysates and the processed products were associated with the plasma membrane fraction. The intracellular half-life of p21 proteins was determined by pulse-labeling with ^{35}S-methionine and chasing in the presence of excess, unlabeled methionine. Both p21s of EJ and its normal cellular homologue have a half-life of 20 hr. When the half-life of the c-ras p21, which is not phosphorylated, was compared to the phosphorylated viral ras p21 of Ha-MuSV, it was found that the viral p21 was much more stable in cells than the c-ras p21s. While the non-phosphorylated form of the v-ras p21 has a similar half-life of 20 hr, the phosphorylated form of the v-ras p21 has a much longer half-life of 56 hr. The half-life of the total v-ras p21 is 42 hr. The v-ras genes of Ha-MuSV and Ki-MuSV differ from their c-ras proto-oncogenes by the 12th codon mutation, and in addition, by a common mutation at the 59th codon of an alanine residue to a threonine residue which generates an acceptor site for the autophosphorylation activity seen associated with the viral p21 proteins. This latter, additional mutation, in the v-ras genes of these highly oncogenic viruses, may further alter the oncogenic potential of these oncogenes. Although the activated, EJ oncogene has a limited capacity to transform the NIH 3T3 cells by itself, the Ha-MuSV and Ki-MuSV have a much wider spectrum of oncogenicity. Therefore, it is tempting to speculate that there may be more than one step involved in the activation of proto-oncogenes to oncogenes, of varying degrees of potency. The prolonged intracellular half-life of the v-ras p21 may be a manifestation of altered interaction with its target substrates in the transformed cells, which is responsible for its observed increased oncogenicity.

2. Monoclonal antibodies to the p21. In order to study the structure-function relationship of the p21 proteins and to develop novel assays for detecting different forms of p21 in human cancer, a new panel of monoclonal antibodies is being developed using the purified p21 protein overproduced in E. coli. A bulk quantity of bacterial p21 was isolated from bacterial cells which was more than 90% pure. These purified proteins were used to immunize Balb/c mice. Many hybridoma cell lines producing antibodies with reactivity to the p21 antigens were isolated. These new monoclonal antibodies are being characterized and should be useful in future studies, particularly involving the subcellular localization of these proteins.

3. Biochemical characterization of the p21. The p21 overproduced in E. coli possesses the enzymatic activity of autophosphorylation using γ-^{32}P-GTP as the phosphoryl donor and also the ^{3}H-GDP binding activity. Procedures have been developed to isolate and purify the p21 proteins in an enzymatically active form. Progress along these lines has been very promising, since the purified, bacterially produced enzyme can now be used to characterize the biochemical enzymatic activities. In other experiments, in order to map the guanine nucleotide binding site of this protein, photoaffinity labeling with many photoactive analogues has

been tried. Although photoaffinity labeling of the p21 has been obtained, it has been at low levels; therefore, no specific labeling of p21 has been too successful, at the present time. It was found, however, that the ^{3}H-GDP binding activity of the p21 was very labile during its purification. Improvements in fractionation procedures are being made to preserve this binding activity.

4. Partial revertants of Ha-MuSV-transformed MDCK cells. Madin-Darby canine kidney cells (MDCK) in tissue culture is an interesting cell line, retaining many of the physiological functions of kidney cells. These functions will be useful markers to assess the transforming function of Ha-MuSV. Four subclones of Ha-MuSV-transformed MDCK cells have been isolated. These subclones fall into two categories. The first transformant class differs from normal, untransformed MDCK cells, in that they have fibroblastic morphology, have lost growth responses to T_3 and PGE_1, grow in soft agar, do not respond to glucagon or vasopressin, have reduced rates of PGE_2 secretion and have high amounts of viral p21. The second class of subclones resembles the normal MDCK cells more closely. They are epithelioid in morphology, are stimulated to grow by PGE_1, however, they grow in soft agar, respond to vasopressin but not to glucagon, have a moderate rate of secretion of PGE_2, and have reduced amounts of viral p21. This second subclass represents partial revertants of the transformed cells. The transforming characteristics can be related to the cellular levels of p21. These partial revertants may, therefore, be reminiscent of the heterogeneity of cell types observed in many human tumors.

Significance to Biomedical Research and the Program of the Institute:

The p21 ras oncogenes have been demonstrated to be associated with many major forms of human cancer, 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 some 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 systems to study the molecular mechanism of malignant transformation. The objective of this program is, first, to seek understanding of the mechanism of transformation and activation of cellular proto-oncogenes. In addition, identification of oncogenes and their protein products will also offer us new insights into developing creative new approaches to cancer diagnosis and treatment. The powerful technologies of genetic engineering and monoclonal antibody development will permit us to devise novel means of cancer detection and monitoring, and therapy through analysis of oncogenes and gene products in cancer patients.

Proposed Course:

Several new members have joined the group recently. With their participation in this program, the research effort will be stepped up and intensified in the following areas, which are to be the projected emphases of our research:

1. Further characterization of the new monoclonal antibodies with the aim of obtaining site-specific antibodies for structure-function studies of the p21 proteins and for eventual development of immunodiagnostic tests for cancer.

2. Further improvement of the purification procedure for the p21 in bacteria to characterize the biochemical properties of this protein. Of special interest will be elucidating the roles of p21 in phospholipid metabolism and growth factor actions which, may be possible candidates for p21 cellular targets in transformation. Obtaining the enzymatically active p21 in the purified form will be the necessary step for crystallization of this protein and eventual X-ray crystallography to reveal the three-dimensional structure of this protein. Determination of the structure of this protein is very critical for understanding the ligand binding and molecular mechanism of oncogenic activation which result from point mutations in the p21 molecules.

3. Site-directed mutagenesis of the ras genes will be employed to derive mutants of the ras genes. These genetic engineering techniques will be used to study the problem of the structure-function relationship of the p21 proteins by expressing the ras genes in bacteria and mammalian cells following gene transfer.

4. Professor Jen-Kun Lin is currently on sabbatical leave in this laboratory. His main interests are aflatoxin B_1 carcinogenesis and environmental carcinogens. He is joining the group to develop a collaborative project on aflatoxin carcinogenesis and oncogenes, especially with the ras genes.

Publications:

Lautenberger, J. A., Ulsh, L., Shih, T. Y. and Papas, T. S.: High-level expression in Escherichia coli of enzymatically active Harvey murine sarcoma virus p21 ras proteins. Science 221: 858-860, 1983.

Shih, T. Y., Ulsh, L. S. and Huang, R. J.: Toward a molecular description of malignant transformation by p21 ras oncogenes. In Chang, E. and Huang, P. C. (Eds.): Proceedings of Symposium on Molecular Biology of Neoplasia. New York, Plenum Press (In Press)

Shih, T. Y. and Weeks, M. O.: Oncogenes and cancer: The p21 ras genes. Cancer Invest. 2: 109-123, 1984.

Ulsh, L. S. and Shih, T. Y.: Metabolic turnover of human c-ras p21 of EJ bladder carcinoma, its viral and normal cellular homologues. Mol. Cell. Biol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04970-08 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

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
	T. S. Papas	Acting Chief	LMO	NCI

COOPERATING UNITS *(if any)*
Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung and Blood Institute, NIH, Bethesda, MD (R. S. Balaban)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Cellular Transformation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.80	0.80	1.00

CHECK APPROPRIATE BOX(ES)

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

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

Malignant transformation of cells by viruses often is induced by a virus-coded protein which plays no role in virus reproduction. Cells infected with the avian MC29 virus produce a protein, p110 gag-myc, containing a viral structural domain (gag) fused to a cellular domain (myc). We have found that the protein migrates to the nucleus soon after synthesis, and has a brief intracellular half-life. The p110 gag-myc exists in transformed cells in both monomeric and dimeric forms, similar to several other eucaryotic and procaryotic regulators of transcription or DNA synthesis. No cellular proteins were found consistently to associate with p110 gag-myc. The cellular homologue p55 myc also was found to exist in dimeric form, as was the avian myeloblastosis virus protein, p50 myb. Possible biochemical activities associated with dimeric forms are under investigation.

In another series of experiments, morphological variants of cells infected with an MC29-related virus, MH2, were isolated. Whereas p55 myc was unable to be found in cells transformed with MC29 or MH2 viruses, the p55 protein reappeared in the cellular variants, suggesting the possibility that in MH2-infected cells the viral myc proteins control the synthesis or modification of cellular p55 myc.

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	Biochemist	LMO	NCI
T. S. Papas	Acting Chief	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 obtain revertants of transformed cells with deficiencies in virus-coded proteins, in order to identify functions important in virus transformation.

Methods Employed:

1. Transformation of cells in culture by avian viruses, morphological resolution of transformed cells by microscopy, resolution of cellular organelles by supravital staining.

2. Isolation of cellular organelles, including microsomes, cell surface membranes, nuclei and nuclear components.

3. Quantitative chemical determinations of protein and a variety of enzymes.

4. Complement fixation, immunoprecipitation, immuno-affinity chromatography.

5. Paper chromatography.

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. Chemical and enzymatic treatment of proteins and nucleic acids.

Major Findings:

Many retroviruses with acute transforming activity contain sequences related to cellular DNA sequences which have no role in virus reproduction. These sequences presumably code for proteins responsible for the malignant transformations induced by these viruses. Two avian viruses, MC29 virus and AMV, contain such sequences. The protein encoded by MC29 virus, p110 gag-myc, contains both viral and cellular domains, and can be immunoselected by antibodies to either domain.

We have confirmed the reported intranuclear location of p110 gag-myc, and kinetic studies showed this to be a protein with an intracellular half-life of about 30 minutes. Such lability suggests that continuous synthesis of p110 gag-myc may be required for the maintenance of transformation. The location and lability of p110 gag-myc also suggested a possible regulatory role in transcription or DNA replication. Several other regulatory proteins in both eucaryotic and procaryotic organisms have been shown to exist and function as oligomers. Our investigation of p110 gag-myc showed that this protein exists predominantly as monomers and dimers in infected, transformed cells. This type of interaction was demonstrated by extracting radioactively labeled cells with nondenaturing detergent, sedimenting proteins through sucrose gradients, immunoprecipitating p110 gag-myc from the fractions, and resolving and identifying p110 gag-myc in polyacrylamide gels.

Some protein bands other than p110 gag-myc were selected in fractions containing dimeric forms. We tested for the possible association of cellular proteins with p110 gag-myc by using antisera directed against both viral and cellular domains of p110 gag-myc. The p110 gag-myc was the only band immunoprecipitated in common by these various antisera, showing that p110 gag-myc was not stably associated with any cellular protein.

Antibody to a cellular domain had been prepared by injecting a rabbit with synthetic peptide derived from the MC29 nucleotide sequence. This antibody was capable of precipitating a cellular protein, p55 myc, homologous to the viral p110 gag-myc (see Project Number Z01CE05238-03 LMO). The fact that no p55 myc was associated with p110 gag-myc in sedimentation experiments demonstrates that the viral p110 gag-myc acts by a mechanism other than via inactivation of cellular p55 myc by heterologous association. When p55 myc from nontransformed chicken cells was examined in sucrose gradients, it, too, was found to exist as a dimer.

The virus-coded protein of AMV, p50 myb, is found in avian myeloblasts infected with AMV. The p50 myb has only cell-related sequences, but is also found in the nucleus and has a short half-life. As in the case of p110 gag-myc, p50 myb is found as a dimer after sedimentation through sucrose gradients. We are presently attempting to define biochemical activities for p110 gag-myc, p55 myc, and p50 myb, which may help us to understand their function(s) in normal and aberrant cell processes.

In the course of the above studies, we noticed the absence of p55 myc, the normal, cellular myc protein, in cells transformed by MC29 virus, and in cells transformed by other independently isolated avian viruses (MH2, OK10) which contain a myc region in the genome. We isolated several morphological variants of cells transformed by MH2 virus, after mutagenizing the cells with ethylmethane sulfonate. Four of these variants exhibited a reappearance of p55 myc. The possibility that the synthesis of normal p55 myc is regulated or modified by the synthesis of viral myc proteins is under investigation.

The increased capability of Rous sarcoma virus-transformed cells for glucose uptake, and the relation of increased glycolysis to transformation has been under investigation for some time. Increased glucose comsumption leads to

higher ATP/ADP levels in transformed cells, and in turn, we have been able to show that the ATP derived from glycolysis is selectively used for Na-K-ATPase activity. These findings are directly correlated with the transforming activity of mutants of Rous sarcoma virus, but do not yet explain the malignant potential of transformed cells.

Significance to Biomedical Research and the Program of the Institute:

The recognition of the formation of dimers by molecules of p110 gag-myc, p55 myc, and p50 myb proteins is consistent with the notion that myc and myb proteins are involved in the regulation of RNA or DNA synthesis. In fact, certain activities of other regulatory proteins have been shown to be limited to oligomeric forms; and it is important, therefore, to test dimeric forms of myc and myb proteins for recognized biochemical activities. The added possibility that synthesis of viral RNA or protein can affect the modification or synthesis of a homologous cellular protein suggests a new avenue for investigation of the virus-induced malignant process.

Proposed Course:

To test the myc and myb proteins for certain biochemical activities in determining the function of these proteins in malignant transformations. To characterize more completely the morphological variants of MH2-transformed cells with respect to possible virus variants produced by these cells, and possible modification of the normal cellular protein, p55 myc. We have terminated our studies on energy relationships in order to concentrate more directly on the characterization and analysis of virus-coded transformation proteins and their normal cellular homologues.

Publications:

Balaban, R. and Bader, J. P.: On the relationship between glycolysis and Na-K-ATPase in cultured cells. Biochim. Biophys. Acta (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05120-05 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
High-level Expression of Oncogene Specific Proteins in Escherichia coli

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:	T. S. Papas	Acting Chief	LMO	NCI
	K. P. Samuel	Visiting Associate	LMO	NCI
	D. K. Watson	Senior Staff Fellow	LMO	NCI
	L. Virgilio	Chemist	LMO	NCI
	W. Sisk	Biologist	LMO	NCI
	D. Court	Biologist	LMO	NCI

COOPERATING UNITS *(if any)*
Basic Research Program, Litton Bionetics, Inc., Frederick, MD (A. Seth)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	0.75	1.0

CHECK APPROPRIATE BOX(ES)

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

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

Proteins derived from the mos, myb, ras, and sis oncogenes have been expressed in Escherichia coli by use of the expression plasmid, pJL6, and its derivatives. This plasmid incorporates the strong, well-regulated, phage λ pL promoter and a small amino-terminal fragment of the λ cII gene. For each of the proteins made, the size is close to that predicted from the DNA sequence. Thus, the E. coli expression system provides an independent confirmation of the presence and length of open reading frames. The c-sis protein was derived from cDNA cloned from human T-cell leukemia virus-transformed cells (HTLV). The expressed bacterial protein was shown to be the expected c-sis product as judged by its reactivity with antibodies raised against purified sis peptides.

The λ sequences in pJL6 were placed adjacent to the E. coli β-galactosidase gene (lacZ) by in vitro recombination, such that the lacZ sequence is out of frame with respect to the lambda cII sequence. A restriction enzyme recognition site (NruI) is located between cII and lacZ, allowing the 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.

PROJECT DESCRIPTION

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

J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
T. S. Papas	Acting Chief	LMO	NCI
K. P. Samuel	Visiting Associate	LMO	NCI
D. K. Watson	Senior Staff Fellow	LMO	NCI
L. Virgilio	Chemist	LMO	NCI
W. Sisk	Biologist	LMO	NCI
D. Court	Biologist	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.

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 the analytic preparation of restriction fragments. Preparative 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 6: 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 by the same procedure. Extensive use in the design of the recombinant was derived from the computer analysis of published DNA sequences.

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 cystine, 0.01% biotin, 0.1% 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]-cystine and incubated for 1.5 min. After labeling, some cultures were chased by adding unlabeled cystine 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 in 1% SDS/0.1% β-mercaptoethanol 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. 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.

6. Computer analysis of DNA sequences. A package of Fortran programs has been written by us to be used with the Laboratory's MINC 11/23 minicomputer. This package includes: an editor to create and modify files that contain DNA sequences; a program to search for restriction sites; a program to translate DNA sequences into amino acid sequences; a program to create hard copy, dot matrix, homology diagrams of the type described by Maizel and Lenk (Proc. Natl. Acad. Sci. USA 78: 7665-7669, 1981), using the laboratory LA34-VA graphics printer. A high-speed communications interface, and the NIH DCRT CLINK program, have allowed the efficient use of programs run on the NIH DEC and IBM 370 mainframes. Frequently used programs on these mainframe machines include: the SEQ program, and Queen and Korn programs for DNA sequence analysis; the DNA and protein sequence alignment program; the DNA and protein data base homology search program of D. Lipman and J. Wylbur (NIADDK); the protein hydrophobicity and secondary structure analysis program of R. Feldmann (DCRT); and the DNA and protein data base search and retrieval programs of J. Owens (NICHHD).

Recent accessions to our computer include: a voice synthesizer to facilitate proofreading of sequences; a digitizer to automatically enter band positions from gels, and a miniature key pad to allow direct reading of sequence gels. New programs using our system's graphics capability include one that is able to plot potential protein initiation and termination codons and another program that charts the Hopp and Wood hydrophilicity values of deduced amino acid sequences.

Major Findings:

1. The human c-sis gene has been expressed as protein in E. coli. A segment of DNA from a cDNA clone of sis-related sequences from human T-cell leukemia virus-transformed cells was placed in the expression vector, pJL6. When E. coli cells harboring this plasmid are induced by heat inactivation of the λ repressor, a 33,000-dalton protein is made which is very likely the expected c-sis protein. This product has the size expected from the DNA sequence and is not seen in uninduced cells or in induced cells containing the vector alone. Four preparations of antisera have been obtained, which were raised against peptides translated from the sis DNA sequence. These antisera all react with the bacterial c-sis protein. By contrast, none of the proteins in control extracts react with all four of the antisera. Most significantly the bacterial c-sis protein is present in bacterial extracts as more than 5% of the total protein. Also, its low solubility makes it possible to remove most of the contaminating E. coli proteins and allows one to achieve a rapid, partial purification.

2. An effective method has been developed by us, for the rapid detection of open reading frames, in DNA placed in an expression vector containing the phage lambda p_L promoter. The region of the expression vector, pJL6, containing λ sequences, was placed adjacent to the E. coli lacZ gene by in vitro recombination. The junction between the amino-terminal, λ cII gene sequences and the carboxy-terminal lacZ (β-galactosidase) sequences was not in frame, so that cells harboring this plasmid were not able to ferment lactose. However, insertion of an open reading frame between these two gene loci can enable the translational read-through from the cII gene through the open reading frame (ORF) and into the lacZ gene. In spite of the fact that the β-galactosidase produced from such a "tribrid" gene has undergone a substitution at its amino-terminal end, it still remains enzymatically active. Thus, cells containing such plasmids possess a lac+ phenotype, and can easily be identified by their ability to form blue colonies on (X-Gal) plates. This vector is similar to the ORF vector of Weinstock et al. (Proc. Natl. Acad. Sci. USA 80: 4432-4436, 1983), but makes use of the stronger and more easily regulated phage λ, p_L promoter.

Significance to Biomedical Research and the Program of the Institute:

To understand more fully how oncogenes are involved in malignant transformation it will be necessary to study the protein products of these genes. The expression of such proteins in bacteria will aid these studies in several ways. Many such proteins have been shown to be enzymatically active. In these instances they can then be analyzed for biological activity. Even when only part of an

oncogene is expressed, the protein product will be useful because antibodies can be raised that may cross-react with the normal or abnormal protein, oncogene products in mammalian cells. Such a study is of special interest for the myc gene, since this gene is a preferred integration target for avian leukosis virus in bursal lymphoma and in rearrangements occurring in murine plasmacytoma and human Burkitt lymphoma.

Studies of bacterially synthesized $p28^{sis}$ are of special interest 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. Another factor indicating the importance of this study is the observation that PDGF stimulates the high-level expression of mRNAs transcribed by the myc gene. This interrelationship suggests that carcinogenesis may involve the interaction of several oncogenes.

Proposed Course:

1. Purification of bacterially expressed human c-sis protein. Various methods will be used to solubilize the c-sis protein and to purify it. These will include resuspension in guanidinium chloride and refolding the protein in the presence of an equimolar mixture of reduced and oxidized glutathione. Once solubilized, the protein will also be purified by ion exchange or antibody affinity chromatography. The purified protein will be analyzed for mitogenic activity by introducing the protein to quiescent BALB/c 3T3 cells in platelet-poor serum. Mitogenic activity will be measured by observing the induction of DNA synthesis as determined by the incorporation of ^{3}H-thymidine into DNA. The bacterial c-sis protein will also be assayed for its ability to compete with PDGF in the binding of PDGF receptors, as well as for its ability to stimulate the synthesis of myc RNA in BALB/c 3T3 cells. Both of these procedures will elucidate the relationship between the sis gene and PDGF.

Antibodies will be raised against the bacterially expressed c-sis protein eluted from the polyacrylamide gels of crude lysates, as well as against more purified protein fractions. These antibodies will be used to determine the levels of c-sis protein expression in normal and malignant, animal cell lines. Of special interest are the glioblastoma line, A173, and the human sarcoma line, 8387. These cell lines have been found to contain high levels of sis RNA. Recent studies have indicated that sis proteins undergo processing through dimerization, specific proteolytic cleavage, and glycosylation. The antibodies raised against the bacterial c-sis protein will be used to detect processing intermediates. Tunicamycin will be employed to inhibit glycosylation so that unglycosylated forms of the protein can be detected.

2. Development of improved expression vectors and hosts. Presently the p_L host used by pJL6 and its ORF derivatives is rather difficult to transform, apparently due to the deletion of host membrane genes that occurred when phage killer genes were deleted. By use of in vitro and in vivo recombination techniques, a host will be made that lacks the killing functions, but retains all host DNA. Together with the p_L-containing ORF vector, this new host will allow a much more rapid method of detecting bacteria expressing eukaryotic proteins.

Recombinant plasmids expressing such proteins should be readily detectable, since it is the same host into which they were introduced by transformation. Another improvement will be made to the lacZ-containing vector by placing an amber mutation early in the lacZ sequences. In a host that contains an amber suppressor, this vector will behave like the present lac vector, i.e., colonies which express foreign sequences appear blue on XG plates. This color change will allow the rapid detection of such strains. However, when the plasmid is moved into a strain lacking a suppressor, the foreign sequences will continue to be expressed, but will be terminated just after read-through into the lac sequences. This modification will allow purification of the chimeric protein without a large number of β-galactosidase residues attached.

The vector, pJL6, and its lac derivative will be modified by inserting oligonucleotides into the ClaI or HindIII sites at the end of the cII fragment. For example, SmaI linkers of appropriate lengths will be inserted so that the blunt ends produced by this enzyme will produce ends in all three frames. This will allow the fusion of the cII fragment with any blunt-end site, into a protein-coding region without any need for trimming, repairing, or adding linkers to the ends of the target fragment. Of those restriction enzymes which cut DNA frequently and recognize a four-base sequence, several produce blunt-ended termini. Such enzymes can be used to cleave almost any protein-coding sequence in such a way that they could be inserted into an appropriate blunt-end DNA vector, and therefore, be expressed as a protein product in bacteria.

Publications:

Kan, N. C., Flordellis, C. S., Mark, G. E., Lautenberger, J. and Papas, T. S.: A unique cellular sequence was independently transduced by avian carcinoma virus MH2 (v-mht) and a murine sarcoma virus (v-raf). In Ahmad, F., Black, S., Schultz, J. J., Scott, W. A. and Whelan, W. J. (Eds.): Advances in Gene Technology: Human Genetic Disorders. Miami, ICSU Press, 1984, pp. 188-189.

Lautenberger, J. A., Court, D. and Papas, T. S.: High level expression of Escherichia coli of the carboxy-terminal sequences of the avian myelocytomatosis virus (MC29) v-myc protein. Gene 23: 75-84, 1983.

Lautenberger, J. A., Kan, N. C., Court, D., Pry, T., Showalter, S. and Papas, T. S.: High-level expression of oncogenes in Escherichia coli. In Papas, T. S., Chirikjian, J. G. and Rosenberg, M. (Eds.): Expression of Cloned Genes in Prokaryotic and Eukaryotic Cells: Gene Amplification and Analysis. New York, Elsevier/North Holland, Inc., 1983, Vol. 3, pp. 147-174.

Lautenberger, J. A., Seth, A., Jorcyk, C. and Papas, T. S.: Useful modifications of the Escherichia coli expression plasmid pJL6. Gene Anal. Tech. (In Press)

Lautenberger, J. A., Ulsh, L., Shih, T. Y. and Papas, T. S.: High level expression in Escherichia coli of enzymatically active Harvey murine sarcoma virus $p21^{ras}$ protein. Science 221: 858-860, 1983.

Papas, T. S., Kan, N. C., Watson, D. K., Flordellis, C. S., Psallidopoulos, M. C., Lautenberger, J., Samuel, K. P. and Duesberg, P.: Myc-related genes in viruses and cells. In Levin, A., Topp, W., Vande Woude, G. F. and Watson, J. D. (Eds.): Cancer Cell. New York, Cold Spring Harbor Press, 1984, pp. 153-163.

Papas, T. S., Watson, D. K., Reddy, E. P., Duesberg, P. H. and Lautenberger, J. A.: Nucleotide sequence analysis of the chicken c-myc focus reveals, homologous regions with the transforming gene of MC29. In Rich, M. A. (Ed.): Leukemia Reviews International. New York/Basel, Marcel Dekker, Inc., 1983, pp. 300-302.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05186-04 LMO

PERIOD COVERED: October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 cherecters 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 Investigetor.) (Name, title, laboratory, and institute affiliation)*

PI:	K. P. Samuel	Visiting Associate	LMO	NCI
Others:	T. S. Papas	Acting Chief	LMO	NCI
	C. Flordellis	Visiting Fellow	LMO	NCI
	D. K. Watson	Senior Staff Fellow	LMO	NCI
	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
	D. D. Blumberg	Senior Staff Fellow	LMO	NCI
	T. T. Chen	Expert	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:
1.15	1.15	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 spece provided.)*

Construction of protein expression plasmids containing the entire envelope gene sequence of the human T-cell leukemia virus type-I (HTLV-I) provirus, as well as DNA fragments representing subregions of this gene, has been accomplished. These plasmids were put into the bacterial expression vector, pJL6, or its derivatives, pJLA16 and pCJX. Work pertaining to the construction of hybrid plasmids containing DNA sequences coding for the putative polypeptides of the HTLV-I genome has also been initiated.

Examination of the c-myc locus of the human lymphoblastoid cell line NC37 has revealed: (1) a rearranged c-myc locus, (2) an overexpression of c-myc mRNA, and (3) that it is tumorigenic in nude mice. A cosmid genomic library of NC37 cell DNA was constructed for the studies relating to the structure and regulation of the human c-myc locus.

Efforts to isolate and characterize the "primordial" c-myc oncogene in slime mold, and other prokaryotic species, are continuing. Preliminary characterization of putative clones from an EcoRI library of the slime mold, Dictyostelium discoideum, is under investigation using specific subclones derived from our cloned myc constructs.

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. Flordellis	Visiting Fellow	LMO	NCI
D. K. Watson	Senior Staff Fellow	LMO	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
D. D. Blumberg	Senior Staff Fellow	LMO	NCI
T. T. Chen	Expert	LMO	NCI

Objectives:

The scope of these investigations is many-fold. In general, they are designed to: (1) express the envelope gene product of the human T-cell leukemia virus (HTLV) in bacterial expression vectors; (2) develop antibodies to the expressed envelope gene product in rabbits; (3) utilize these specific antibodies to HTLV envelope glycoprotein in the development of diagnostic screening techniques for the detection of HTLV antigens in the sera of patients; (4) study the expression of the pX region of HTLV at the protein level, by constructing expression plasmids of pX sequences and by production of its putative proteins; (5) continue to study the c-myc oncogene structure and regulation of expression in normal and neoplastic B-cell neoplasms in humans; and finally (6) to investigate lower eukaryotic and prokaryotic organisms for the presence and expression of cellular oncogene progenitors.

Methods Employed:

1. Cloning - Vector Construction: The expression vector, pJL6, was constructed by Lautenberger in this laboratory (see Project Number Z01CE05120-05 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 cII translation initiation codon of its protein. The ampicillin-resistant gene of pBR322 is present, conferring a marker which selects for an antibiotic for screening of recombinant plasmids. The pJL6 vector contains the unique restriction enzyme sites, HindIII and ClaI, in phase with the cII gene, while pJLA16 contains NruI and HindIII sites, useful for cloning.

The noncoding (first) exon of the normal, human c-myc locus was subcloned as a 600-bp SmaI to PvuII DNA fragment into the HindIII restriction site of the pBR322 plasmid, utilizing an 8-nucleotide HindIII linker fragment. Clones were picked by screening for ampicillin resistance on NZYDT agar plates.

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.

All other subcloning experiments utilized $C600_{r-k}m^{+}_{k}$. Both E. coli strains grow well at 37° or 32°C, in NZYDT media (broth or agar plates).

The E. coli strains, N4830 and N4831, were used to transfect plasmid clones for the expression of proteins. Briefly, N4830 harbors a heavily defective, phage λ prophage, which carries a temperature-sensitive (ts) repressor, C1857, and an active N gene. This strain will grow at 32°C, at which temperature the ts repressor is active, but at 42°, where protein is induced, the repressor becomes inactive. N4831 is isogenic with N4830.

3. 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. 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; (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; and (e) ClaI and XbaI which generate two pX fragments: a 1.35-kb fragment representing the sequences of the pX-III and pX-IV regions of HTLV-I; and a 2.3-kbp fragment containing the pX-I and pX-II regions. The 2.3-kbp DNA was further cut with the HhaI enzyme to generate a 400-bp, pX-I fragment and a 545-bp, pX-II fragment.

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 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. Genomic DNA of NC37 cells, after digestion with the enzyme Mbo-I, under conditions of partial digestion, as described by Maniatis et al. (Molecular Cloning: Laboratory Manual, 1982), was fractionated on a 0.5% agarose gel (preparative) by electrophoresis at 40 volts for approximately 24 hours. DNA fragments, ranging in size from 20 to 45 kbp, were cut out of the gel and electroeluted into a dialysis bag containing one-half strength electrophoresis buffer. Samples of DNA were then phenol-extracted 3X, chloroform-extracted 3X, and then ethanol-precipitated.

The recovered MboI fragments of NC37 DNA were treated with calf intestinal alkaline phosphatase enzyme according to the standard procedure of Maniatis

et al., to remove terminal phosphate groups. After phenol and chloroform extractions, the DNAs were precipitated as before, and the final pellet was resuspended in sterile Tris-EDTA (pH 7.4) buffer at a concentration of about 0.5 mg/ml.

5. Cosmid Vector pJB8 DNA: The purified DNA arms of the cosmid vector pJB8 were purchased from a commercial company (Amersham).

6. Ligation of Synthetic 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 the Molecular Cloning: Laboratory Manual. the micrograms of HindIII linkers (the 8-nucleotide, 10-nucleotide, or 12-nucleotide molecules) were used, and the incubation was carried out at 15°C for about 15 hrs.

DNA fragment ends were blunted by a filling-in reaction, using the "Klenow" fragment of E. coli DNA polymerase I (for 5'-protruding ends) or bacteriophage T4 DNA polymerase enzyme (for 3'-protruding ends), using standard blunting conditions.

Ligation of the phosphorylated synthetic linker to a DNA fragment was done using 2 µg of linker in the 10 µl volume described above in a ligation reaction with 0.5 µg of insert DNA in a final volume of 20 µl, and 2 µl of T4 DNA ligase enzyme. The reactions were allowed to proceed at 15°C overnight. The ligated DNAs were subjected to digestion with HindIII restriction enzyme in excess ($\geq$10 units per µg of DNA) for several hours. The digested DNAs were fractionated on 0.8% or 1% agarose gels, and the DNA fragments recovered as described before.

7. Preparation of a Cosmid Library of NC37 DNA: Three µg of the MboI partials of NC37 cell DNA (20-45-kbp range) were ligated to 0.15 µg of each of the cosmid pJB8, BamHI-generated, DNA arms, in a volume of 20 µl, and under standard ligation conditions. The ligated material was used to package bacteriophage λ particles as described by Amersham. The resulting packaged material was then used to transform the E. coli derivative, LE392. Bacterial cells were grown in LB broth at 37°C.

8. Screening of Cosmid Library and Plasmid Clones: To screen the cosmid library, bacterial colonies grown on LB plates containing 50 µg/ml of ampicillin (approx. 1 x 10^4 colonies per 150-mm plate) will be prepared and replicated as described in the method of Hanahan and Meselson (Gene 10: 63-67, 1980). Nitrocellulose filters containing recombinant cosmids will then be hybridized (colony hybridization) to a nick-translated, ^{32}P-labeled, exon-I probe of normal human c-myc, essentially as described by Grunstein and Hogness (Proc. Natl. Acad. Sci. USA 72: 3961-3965, 1975). Positive hybridizing clones will be picked and grown in liquid media for preparation of the recombinant DNA.

Plasmid clones of HTLV envelope and pX gene sequences were prepared as follows: For transformation of the E. coli derivatives DC646 and N483, ligation mixtures of the expression vectors, pJL6, pJLA15, or pCJX, and insert DNA fragments of

the envelope or pX gene sequences (approx. 1 µg of DNA) were brought to a 50 µl volume with 50 mM Tris-HCl, pH 7.5, 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_{590nm} 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 of cold, 50 mM $CaCl_2$. Upon mixing, 100-200 µl aliquots were removed to 85-mm NZYDT agar plates containing 50 µg/ml of ampicillin, spread evenly, and then incubated at 37°C overnight.

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 overnight at 37°C, mini plasmid preparations were made using the method of Birnboim and Doly (Nucl. 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 N4830 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.

9. Radiolabeling and Gel Electrophoretic Analysis of Bacterial Proteins: The plasmid clones, prepared in N4830, 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 0.2 A_{590nm} 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 10 min. Aliquots of 150 µl of the cells were removed and added to 15 µl of the above media containing 2.5 µCi [^{35}S] cysteine (>300 mCi/mmol), and incubated for a further 1.5 min. at 32°C or 41°C.

Following cell 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% βmercaptoethanol, 0.5% bromophenol blue dye) and resolved on a 10% SDS-polyacrylamide gel according to the procedure of Laemmli (Nature 227: 680-685, 1970). Protein bands were visualized by autoradiography after fixing and vacuum-drying of the gel.

Major Findings:

1. 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 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.

The envelope gene, which is about 1.4 kbp in size, codes for an envelope protein product of about 48,000 daltons, which is modified by glycosylation to a 62,000-dalton glycoprotein. The pX region, based on its four, small, open reading frames downstream of the envelope gene, could potentially code for polypeptides of 11,000, 10,000, 12,000, and 27,000 daltons, respectively, for pX-I to pX-IV, 5'- to 3', in this order.

Using the plasmid constructs listed below in expression assays, no protein products in the expected size range were detected in induced N4830 cells using a 10% SDS-polyacrylamide gel: (a) the PvuII to XbaI insert, harboring the entire envelope gene of HTLV-I, as well as the pX region, was not found to express a protein product in the 40,000- to 50,000-dalton range; and (b) neither did the 1.86-kbp PvuII to XbaI insert, express a protein product in the induced N4830 cell system.

From the above data one can assume that the envelope gene product may be made, but that it was toxic to the cells. Indeed, we have observed that these two plasmid constructs markedly inhibited the growth of these cells at 32°C. An alternative assumption could be that small deletions or point mutations in the U.S.-HTLV isolate may be present, and that the presumed open reading frame, based on the Japanese sequence data, is absent. In keeping with this, we have observed that certain restriction enzyme sites are missing in the U.S.-HTLV isolate.

By taking advantage of both unique and common restriction endonuclease sites within the envelope gene, several different plasmid clones have been constructed, utilizing HindIII linker sequences, in order to put the blunt-ended inserts in different reading frames. The results of the expression products using these constructs are forthcoming.

The pX region was digested with different restriction endonucleases to generate specific fragments for the construction of expression plasmids. Work on these plasmids is underway and results are in progress. Since HTLV has been reported to resemble both an acute and chronic leukemia virus (i.e., its role in in vitro cell transformation and the clonal origin of infected cells with respect to provirus integration), the pX region may prove to play an important role in the malignant transformation process.

2. The Cellular myc Oncogene of the Lymphoblastoid Cell Line, NC37: The c-myc locus of NC37 cells is rearranged and overexpresses c-myc mRNAs. Southern blotting techniques and hybridization of genomic NC37 DNA with specific c-myc ^{32}P-labeled, nick-translated DNA probes have revealed that the rearranged allele resides on an approximately 23.0-kb EcoRI restriction DNA fragment, whereas the normal allele resides on a 13.5-kbp fragment.

Possible mutations within the first intron, flanking the 3'-regions of exon-I of NC37 c-myc, are assumed based on restriction enzyme site polymorphism in this region. Comparison of an SstI enzyme blot of genomic NC37 DNA with several with a ^{32}P-labeled, nick-translated, intron-I probe, revealed differences and similarities between this cell line and other Raji lines, based on restriction fragment lengths. Each cell line showed substantial variability in its chromosomal break-point positions.

Previously, I had constructed a phage λ Charon 4A library of NC37 cell DNA (10-20-kbp range of EcoRI-digested DNA) and have located the normal c-myc allele by plaque hybridization screening. The clone λ NC4R, containing the 13.5-kbp EcoRI insert, was subcloned at the EcoRI and HindIII sites of pBR322 as a 8.3-kbp insert. This clone will be used for nucleotide sequence comparison analysis with the rearranged NC37 c-myc allele. The isolation of the rearranged allele is yet to be completed, and work is in progress to titer and screen the newly constructed, cosmid pJB8 library of partially digested BamHI DNA of NC37.

3. Transfection of NC37 genomic DNA into NIH/3T3 cells: In collaboration with Dr. Blair (Microbiology Section) we are conducting transfection experiments with NC37 cell DNA, and eventually will include its cloned alleles of the c-myc oncogene, to determine transforming activity.

4. Search for c-myc Oncogene in Slime Mold: The conservation of cellular oncogenes throughout evolution may have significant consequences for the understanding of the biological roles of these genes. Only a very small number of the 20 known cellular oncogenes have been isolated from lower eukaryotic organisms by molecular cloning. In collaboration with Dr. Blumberg (Molecular Control and Genetics Section), we are screening a genomic Dictyostelium discoideum (slime mold) phage library, using ^{32}P-labeled, nick-translated DNAs of both the chicken c-myc (exon-2) and the MC29 viral 3'-sequences as molecular probes. Some of this work is also done in collaboration with Dr. Watson of this laboratory.

Our results suggest that a 1.1-kbp, EcoRI DNA fragment of genomic D. discoideum DNA hybridizes weakly to the myc DNA probes; and we are currently using this fragment to clone into the phage λ Charon 21A at its EcoRI site.

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 protein of the human retrovirus, HTLV, to obtain specific antibodies raised against it. The development of diagnostic and clinical reagents in the treatment of HTLV-associated infections would become possible as a result of such an approach.

As an infectious, human, type-C retrovirus, HTLV and HTLV-variant associated viruses have been linked as the causative agents in such T-cell malignancies as cutaneous T-cell lymphomas (mycosis fungoides, sezary syndrome), adult T-cell leukemias, and the dreaded disease, AIDS. Moreover, HTLV 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 poses an epidemiological and clinical problem for many regions of the world.

In addition, several cellular oncogenes, presumably having normal physiological functions, when present unperturbed in their normal chromosomal orientation, have recently been shown to undergo rearrangement and chromosomal translocations (Burkitt's lymphoma tissues and cell lines), somatic point mutations (lung and bladder carcinoma tissues of patients), or amplification (human neuroblastoma cell lines and primary leukemia of cultured cells from a patient with promyelocytic leukemia). The role of the c-myc oncogene in the genesis of human tumors is still uncertain, but the association of this oncogene with human Burkitt's lymphomas, in which chromosomal aberrations involving chromosome numbers 8 and 14 have been reported, provides an interesting model for continued study of this system.

Finally, from an evolutionary standpoint, it is important to identify and isolate the common precursor (primordial) myc oncogene in lower eukaryotic species. By identifying its structural and biological roles in such organisms, we may further our understanding of its evolutionary functions in higher eukaryotes, especially their physiological function in humans.

Proposed Course:

Ongoing and proposed experiments will involve: (1) expression of portions of the envelope gene of HTLV-I in bacteria; (2) expression of the regions of the pX sequences that may code for a putative transforming protein(s) in HTLV-I; (3) utilization of these abundantly produced proteins in raising specific antibodies in rabbits to both env and pX genes; (4) use of these antibodies in the development of radioimmunoassays (RIAs) for the quick and efficient screening of antisera from HTLV-infected patients and for identifying those at risk in endemic regions; (5) isolation of the rearranged c-myc allele of NC37 cells and its characterization by restriction endonuclease mapping and nucleic acid sequencing; (6) comparison of sequence data of the first exon and intron of both the normal and rearranged c-myc molecular clones to identify point mutations, deletions, or insertions; (7) development of a model for the activation of NC37 c-myc and identification of the causative agent responsible for conferring tumorigenicity on this cell line; and (8) cloning of the putative D. discoideum c-myc locus and its characterization by restriction enzyme mapping and sequencing. This gene will then be used as a lower eukaryotic, c-myc probe to identify further genes in organisms of other lower phyla.

Publications:

Flordellis, C. S., Kan, N. C., Psallidopoulos, M. C., Samuel, K. P., Watson, D. K. and Papas, T. S.: A cellular gene homologous to v-mht is expressed in chicken and human cells. In Ahmad, F., Black, S., Schultz, J., Scott, W. A. and Whelan, W. J. (Eds.): Advances in Gene Technology: Human Genetic Disorders. Miami, ICSU Press, 1984, pp. 166-168.

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Watson, D. K., Psallidopoulos, M. C., Samuel, K. P., Dalla-Favera, R. and Papas, T. S.: The myc genes. In Prasad, U., Levine, P. and Pearson G. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Malaysia, University of Malaysia Press, 1983, pp. 253-262.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05221-04 LMO

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

DNA Recombination in Prokaryotic and Eukaryotic Systems

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

PI:	S. E. Bear	Staff Fellow	LMO	NCI
Others:	D. L. Court	Research Biologist	LMO	NCI

COOPERATING UNITS *(if any)*

Laboratory of Experimental Pathology, NIADDK, NIH, Bethesda, MD (B. Carter); Molecular Genetics, Inc., Minnetonka, MN (L. Enquist); Dept. Microbiology & Immunology, Univ. Michigan School of Medicine, Ann Arbor, MI (D. Friedman)

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 spece provided.)*

The interaction of a phage-encoded topoisomerase I with specific sites to allow DNA recombination is being studied by: 1) genetic analysis of the inhibition of this reaction by various topoisomerase I mutants, and 2) biochemical analyses using attachment sites cloned onto a plasmid vector. Functional domains of the protein and recombination intermediates are being identified.

A new role for integration host factor (IHF) has been defined. This accessory factor of λ site-specific cleavage is also involved in the packaging of λ DNA.

Control regions of the genome of HSV-1 have been identified in the dDNA that occurs as reiterated sequences in virion particles. An origin of replication and packaging sites are evident. When λ HSV hybrid clones are transfected into eukaryotic cells with HSV helper, substantial editing of the λ sequences is seen.

PROJECT DESCRIPTION

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

S. E. Bear	Staff Fellow	LMO	NCI
D. L. Court	Research Biologist	LMO	NCI

Objectives:

A. To examine the site-specific cleavage and rejoining reactions of λ DNA. This has been approached by studying the control of phage-encoded and host-encoded proteins. The phage-encoded protein, Int, is a type I topoisomerase. The Int protein is required for the site-specific, DNA recombination event that occurs when λ DNA integrates into, or is excised from, the E. coli chromosome. The integration event occurs between a specific site on the λ chromosome, attP, and a site on the E. coli chromosome, attB, and results in an integrated prophage flanked by virus/host hybrid sites, attL on the left and attR on the right. In addition to the phage-encoded Int protein, integration and excision require host factors (himA and hip). Excision, the recombination event between attL and attR that results in excision of the prophage also requires a second phage-encoded protein, Xis. These recombination events are multi-step processes involving: (1) recognition by the topoisomerase and host factors of the DNA substrates, (2) binding of the proteins, (3) synapsis of the two DNA substrates, (4) cleavage of the DNA, and (5) recombination of the DNA molecules and release from the synapse. A chi-form, or Holliday structure, has been proposed to be the intermediate form of the two DNA molecules during site-specific recombination. To study the different steps of the recombination event and to identify the proposed intermediates of recombination, we are utilizing bacteriophage with mutations in the int gene. The role of host-encoded factors has been examined in another λ DNA cleavage reaction, the packaging of λ DNA.

B. To examine the possibility that control regions in the genome of herpes simplex virus type 1 (HSV-1) present in the DNA of defective HSV-1 particles can be used in eukaryotic cloning ("shuttle") vectors.

Methods Employed:

A. Standard genetic, biochemical, and recombinant DNA techniques were employed to study the phenomenon of negative complementation: the inhibition of wild-type int by a mutant int. This inhibition is not observed with all point mutants or with some deletion mutants. In some cases, even if the mutant int protein cannot promote recombination alone, the mutant can act to inhibit the wild-type int from completing the recombination. A set of 36 int mutants, previously generated, are being tested for their negative complementation potential in both integration (PxB) and excision (LxR). Also, in place of the wild-type int, we are using three "super int" products (from int mutants that produce a more active int) in the same genetic tests. In this way, the mutants are grouped genetically

according to their inhibition phenotype. Prototype int mutants will be selected from each group of negative-complementing phage, and the DNA from the inhibited recombination event subjected to a biochemical analysis to detect recombination intermediates in the following way: The specific sites for recombination, attP and attB, have been cloned in inverted orientation in the plasmid vector, pBR322. In addition, the hybrid attachment sites attL and attR have also been cloned in inverted orientation in pBR322. These cloned sites act as substrates for the recombination event in vivo (integration, PxB; excision, LxR). Int (mutant and wild-type) and Xis proteins are provided by a phage infection of the cells carrying either plasmid. The host factors, himA and hip, are provided by the E. coli cells. Recombination results in DNA rearrangements in the plasmid and can be demonstrated by restriction endonuclease digestion of the plasmid DNA and examination of the DNA fragments after gel electrophoresis. An intermediate of the recombination event will also be detectable by a characteristic migration in gel electrophoresis.

B. The 9.5-kb, Eco RI dDNA fragment (major class I) and the phage vector (λgtWES.B) carrying the 9.5-kb, Eco RI fragment (λWES::12-7) have been described previously (Z01CP04882-06 LMV and Z01CP05102-03 LMV). An HSV DNA fragment lacking an origin of replication site (not derived from dDNA populations) has also been cloned into bacteriophage vectors. The hybrid plasmid (pBR325::12-7) has also been constructed in this laboratory.

To test for an origin of replication and a possible packaging site, this hybrid phage or plasmid DNA is transfected into VERO cells along with helper HSV. When HSV plaques are evident (48 hrs), both total DNA and DNA from herpes virus capsids are isolated. Supernatants are passaged, and after 1, 3 and 5 passages, the virion DNA is isolated again. These DNAs are restricted with Hpa I and electrophoresed in agarose. The gels are dehydrated and hybridized to either a ^{32}P-labelled λ or pBR325 vector DNA. Clones containing an origin of replication and a packaging sequence will be perpetuated. This results in an amplification of the adjoining vector sequences.

Major Findings:

A.1. 7/36 phage with int mutations negatively complement an LxR reaction by the biochemical analysis, 3/36 strongly, negatively complement an LxR reaction by the genetic analysis, and 11/36 are moderately inhibitory.

A.2. 3/36 phage tested with int mutations negatively complement a PxB reaction by the genetic analysis. 5/36 phage show moderate inhibition.

B. DNA isolated from HSV-1 virions shows λ sequences only in the group transfected with the λ hybrid carrying the 9.5-kb Eco RI insert. It was also observed that much of the λ DNA has been deleted and rearranged, and that about one-half of the 9.5-kb HSV insert is lost. This serves to elucidate more definitively the sequences required for the replication and packaging of these hybrid clones. Plasmid sequences are also detected at the fifth passage in the experimental group transfected with pBR325::12-7. However, no major rearrangements were seen

in the plasmid DNA amplified and detected at the fifth passage. The amplified pBR325::12-7 DNA could retransform E. coli. Amplified λ/12-7 DNA could not be packaged into phage heads using in vitro packaging extracts.

Significance to Biomedical Research and the Program of the Institute:

A. DNA recombination is similar throughout eukaryotic and prokaryotic life forms. Integration of viral DNA into the genome of its cellular host is seen, not only in prokaryotic bacteriophage, but in the eukaryotic DNA and RNA tumor viruses. In the RNA tumor viruses, these recombination events are key elements in the disruption of the cellular physiology that leads to the generation of tumors. In addition, chromosomal anomalies resulting from such recombination events as translocations and deletions, have been identified in a wide range of disease states. An understanding of the process of DNA recombination in prokaryotes provides a basis for work in eukaryotic systems.

B. These studies indicate that an origin of replication and packaging sequences are present in the DNA that is represented as a concatemer in defective HSV particles. In addition, "deletion mutants" of the original phage:HSV clone are rapidly generated, just as defective (deletion) particles are generated from full-length HSV. An understanding of the mechanism of the generation of these deletion mutants will contribute to the understanding of the replicative life cycle of HSV. This will also contribute to the effective use of HSV replication origin and packaging sequences as part of eukaryotic cloning vectors.

Proposed Course:

1) Examine by the biochemical analysis for recombination intermediates. 2) Clone the mutant int gene into an expression vector to make high levels of the protein. With purified protein, experiments can be designed to determine at which step in the recombination process the different int mutants are inhibitory. These experiments may lead to the determination of active sites on the Int protein, since the genetic lesion has been physically mapped. The DNA sequence changes of these mutants will also be determined. 3) Isolate mutations which revert the negative complementation phenotype. This will identify functions involved in the inhibition. This project will terminate when Dr. Bear leaves the NIH on June 21, 1984.

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. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05238-03 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Transforming Genes of Acute Leukemia Viruses and Their Cellular Homologues

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

PI:	D. K. Watson	Senior Staff Fellow	LMO	NCI
Others:	T. S. Papas	Acting Chief	LMO	NCI
	T. T. Chen	Expert	LMO	NCI
	S. J. O'Brien	Geneticist	LVC	NCI
	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
	K. P. Samuel	Visiting Associate	LMO	NCI
	C. Flordellis	Visiting Fellow	LMO	NCI

COOPERATING UNITS *(if any)*
Biochemistry Department, Georgetown University, Washington, DC (J. Chirikjian)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.10	1.10	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 the MC29 transforming gene (v-myc). 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. Using synthetic peptides as antigens, we have prepared myc-specific antisera. Immunoprecipitation of cellular extracts has allowed isolation of p110 (the MC29 transforming protein) from non-producer Q8 cells and p55 from chicken embryo fibroblasts. The overlapping tryptic peptide fingerprint pattern suggests that these two proteins have a common composition, offering the first conclusive evidence that defines p55 as the chicken cellular myc gene product.

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.

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
T. T. Chen	Expert	LMO	NCI
S. J. O'Brien	Geneticist	LVC	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
K. P. Samuel	Visiting Associate	LMO	NCI
C. Flordellis	Visiting Fellow	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.

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 (Method 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. Analysis of chicken c-myc sequences 5' to the myc-specific exons allows identification of additional exons in the normal cellular gene which have been replaced by Δgag in v-myc.

2. Thirteen additional non-gag nucleotides of MC29 have been localized upstream from the 3'-most coding exons of chicken c-myc. This defines the probable recombination points between the helper virus and the transduced cellular sequence. The existence of a perfect splice donor sequence at the 3' border of this chicken sequence suggests that it is an exon. These 13 nucleotides are not conserved in human c-myc.

3. Northern analysis of polyA+ RNA from chicken fibroblasts has allowed identification of three possible exons 5' to the two viral homologous chicken exons. In addition, by examining the nucleic acid sequence, it is possible to identify consensus splice donor signals with all three of these presumptive exons.

4. The recombination points of ALV LTR insertions have been identified by analysis of sequence data.

5. The human c-myc gene contains three exons as defined by Northern blot analyses. The 5'-most exon contains all of the expected transcription initiation signals and a single open reading frame. Although this exon is

conserved between mouse and human sequences, it is not present in the chicken or fish sequence upstream from the v-myc homologous regions.

6. 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. This library was also used to obtain fish c-ets and c-mht genes.

7. Using multiple positions from within the predicted myc protein, we have synthesized and employed as antigens a series of synthetic decapeptides to elicit antibody response in rabbits. The antibody derived from the 5'exon peptide successfully immunoprecipitated a p110 and a p55 protein from Q8- and CEF-labeled cellular extracts, respectively.

8. By comparing tryptic peptide analyses of immunoprecipitated p55 protein and p110 protein, we have established that p55 is indeed the normal chicken myc gene product. We have confirmed the precise site of initiation of translation for the immunoprecipitated, normal myc protein by microsequencing the amino acid termini.

9. 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.

10. DNA prepared from panels of hamster X human and mouse X human somatic cell hybrid clones, which have lost specified human chromosomes, were used for analyses to determine the chromosomal localization of ets.

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. The sequence of chicken c-myc 5' to v-myc-specific sequences will be further analyzed. The sequence of chicken myc cDNA will be determined and compared to the previously determined sequence of the chicken genomic clone. This comparison will allow clear identification of the cellular exons and the cellular initiation signal, thus allowing assignment of the amino acids of the normal cellular gene.

2. In addition to antibodies against synthetic peptides, the expressed human c-myc protein will be isolated from gels and used as antigen.

3. The extent of homology between the c-onc genes of fish, chickens, and humans will be assessed by heteroduplex analysis of cloned DNA.

4. Chicken and human genomic ets clones will be further characterized by restriction enzyme and sequence analyses. A cDNA library prepared from CEF polyA+ will be used to isolate several ets cDNA clones.

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 will also be used as antigen to elicit antibody production.

6. Cellular transformation assays will also be conducted using v-ets DNA in order to define sequences essential for tumorigenesis.

7. 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.

Publications:

Flordellis, C. S., Kan, N. C., Psallidopoulos, M. C., Samuel, K. P., Watson, D. K. and Papas, T. S.: A cellular gene homologous to v-mht is expressed in chicken and human cells. In Ahmad, F., Black, S., Schultz, J., Scott, W. A. and Whelan, W. J. (Eds.): Advances in Gene Technology: Human Genetic Disorders. Miami, ICSU Press, 1984, pp. 166-167.

Papas, T. S., Kan, N. C., Watson, D. K., Flordellis, C. S., Psallidopoulos, M. C., Lautenberger, J., Samuel, K. P. and Duesberg, P.: Myc-related genes in viruses and cells. In Levin, A., Topp, W., Vande Woude, G. F. and Watson J. D. (Eds.): Cancer Cell. New York, Cold Spring Harbor Press, 1984, pp. 153-163.

Papas, T. S., Watson, D. K., Reddy, E. P., Duesberg, P. H. and Lautenberger, J. A.: Nucleotide sequence analysis of the chicken c-myc focus reveals homologous regions with the transforming gene of MC29. In Rich, M. A. (Ed.): Leukemia Reviews International. New York/Basel, Marcel Dekker, Inc., 1983, pp. 300-302.

Watson, D. K., Psallidopoulos, M. C., Samuel, K. P., Dalla-Favera, R. and Papas, T. S.: The myc genes. In Prasad, U., Levine, P. and Pearson G. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Malaysia, University of Malaysia Press, 1983, pp. 253-262.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05239-03 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

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 Fellow	LMO	NCI
Others:	T. S. Papas	Acting Chief	LMO	NCI
	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
	C. Flordellis	Visiting Fellow	LMO	NCI
	J. P. Bader	Research Microbiologist	LMO	NCI
	U. Rovigatti	Visiting Scientist	LMO	NCI

COOPERATING UNITS *(if any)*
Department of Biology, University of California, Berkeley, CA (P. H. Duesberg)

LAB/BRANCH
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SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.20	1.20	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 provirus of MH2 has been molecularly cloned. Its 5.2-kb genome has the genetic structure 5'-Δgag(2.0 kb)-mht(1.3 kb)-myc(1.3 kb)-c(0.4 kb)-poly(A)-(0.2 kb)-3'. myc is the onc-specific sequence shared by the MC29 subgroup, including MC29, CMII, and OK10 viruses, while mht is a cell-derived, MH2-specific sequence. The nucleotide sequence of 3.5 kb from the 3' end of Δgag to the 3' end of cloned, proviral, MH2 DNA has been determined. The following results were obtained: (i) Δgag-mht forms a hybrid gene with a contiguous reading frame of 2682 nucleotides that terminates with a stop codon near the 3' end of mht. The 969 nucleotides of mht, including the stop codon, are 80% sequence-related to the onc-specific raf sequence of MSV 3611 (94% homologous at the deduced amino acid level). (ii) The myc sequence is preceded by an RNA splice acceptor site shared with the cellular proto-myc gene, beyond which it is colinear up to a 3' termination codon and 40 noncoding nucleotides with the myc sequences of MC29 and chicken proto-myc. It seems that myc forms, together with a 5' exon derived from the partial gag gene, a second MH2-specific gene. It is concluded MH2 contains two genes with oncogenic potential: the Δgag-mht gene, which is closely related to the Δgag-raf transforming gene of MSV 3611, and the myc gene, which is closely related to the transforming gene of MC29. Our studies indicate that the molecularly cloned MH2 DNA transforms chicken embryo fibroblasts on transfection with helper DNA, or on superinfection with helper virus. A transforming virus was recovered from the supernatant of these transformed cells. T1 oligonucleotide analysis of the RNA extracted from this virus identified the virus to be MH2. Experiments using deletion mutants in the Δgag, mht, and myc genes are in progress in order to elucidate the oncogenic functions of these genes.

PROJECT DESCRIPTION

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

N. C. Kan	Visiting Fellow	LMO	NCI
T. S. Papas	Acting Chief	LMO	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
C. Flordellis	Visiting Fellow	LMO	NCI
J. P. Bader	Research Microbiologist	LMO	NCI
U. Rovigatti	Visiting Scientist	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 homologues, 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 introduce functionally modified oncogenes to specific target cells in an effort to analyze and alter the function of their normal counterparts. The techniques of molecular cloning, DNA sequence analysis, and site mutagenesis will be used to implicate specific nucleotides in the transformation process.

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 was 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 integrated proviral genome of the avian carcinoma virus, MH2, has been cloned. Its 5.2-kb genome has the genetic structure 5'-Δgag(0.2 kb)-mht (1.3 kb)-myc(1.3 kb)-c(0.4 kb)-poly(A)(0.2 kb)-3'.

2. Sequences related to the viral mht gene (v-mht) have been shown to be present in normal human, mouse, and chicken DNAs.

3. The cellular mht (c-mht) genes are expressed in normal chicken and human cells. In both species the size of the cellular mht-related mRNA is 3.8 kb.

4. The 3.5 kb of nucleotide sequence from the 3' end of Δgag to the 3' end of cloned, proviral, MH2 DNA has been determined. The following results have been obtained: (a) Δgag-mht forms a hybrid gene with a contiguous reading frame of 2682 nucleotides that terminates with a stop codon near the 3' end of mht. The 969 nucleotides of mht, including the stop codon, are 80% sequence-related to the onc-specific raf sequence of MSV 3611 (94% homologous at the deduced amino acid level); (b) The myc sequence is preceded by an RNA splice acceptor site shared with the cellular proto-myc gene, beyond which it is colinear up to a 3' termination codon and 40 noncoding nucleotides with the myc sequences of MC29 and chicken proto-myc. It seems that myc forms, together with a 5' exon derived from the partial gag gene, a second MH2-specific gene; and (c) The entire polymerase and envelope genes of MH2 have been replaced by the cellular sequences, and myc is followed by the 3'-terminal, retroviral c region of about 400 nucleotides.

5. It has been concluded that MH2 contains two genes with oncogenic potential: the Δgag-mht gene, which is closely related to the Δgag-raf transforming gene of MSV 3611; and the myc gene, which is closely related to the transforming gene of MC29.

6. The molecularly cloned MH2 DNA has been shown to transform chicken embryo fibroblasts on transfection with helper DNA, or on superinfection with a helper virus. A transforming virus was recovered from the supernatant of these transformed cells. T1 oligonucleotide analysis of the RNA extracted from this virus identified the virus to be MH2.

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:

1. In order to elucidate the oncogenic functions of the Δgag, mht, and myc genes in MH2, I have constructed deletion mutants in vitro covering different portions of the MH2 genome. The mutated DNA molecules will be used in the DNA-mediated transfer experiments into primary cultures of chicken embryo fibroblasts or into mammalian tissue culture cells, such as NIH 3T3 cells. The DNA transfection experiments will be performed in collaboration with U. Rovigatti and J. Bader.

2. Biochemical methods will be employed to detect the integrated MH2 genome in transformed tissue culture cells, including cells transformed by MH2 deletion mutants. These methods include the DNA blot hybridization technique developed by E. Southern (J. Mol. Biol. 98: 503-517, 1975), and the DNA cloning technique. RNA blot hybridization and immunoprecipitation methods will be used to investigate the expression of deletion mutants in MH2-transformed cells.

3. An attempt is underway to insert the viral mht gene into the expression vectors, pJL6 and pJLA16, in order to express the v-mht gene in E. coli. Antibodies raised against the expressed v-mht gene will be used to immunoprecipitate the P100 protein in MH2-transformed cells. This experiment should provide direct proof that the P100 protein is a Δgag-mht hybrid protein, which does not contain any myc sequence. Anti-mht and anti-gag antibodies will also be used to investigate the expression of MH2 deletion mutants in tissue culture cells.

4. 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.

Publications:

Flordellis, C. S., Kan, N. C., Psallidopoulos, M. C., Samuel, K. P., Watson, D. K. and Papas, T. S.: A cellular gene homologous to v-mht is expressed in chicken and human cells. In Ahmad, F., Black, S., Schultz, J., Scott, W. A. and Whelan, W. J. (Eds.): Advances in Gene Technology: Human Genetic Disorders. Miami, ICSU Press, 1984, pp. 166-167.

Kan, N. C., Flordellis, C. S., Garon, C. F., Duesberg, P. H. and Papas, T. S.: Avian carcinoma virus MH2 contains a transformation-specific sequence, mht, and shares the myc sequence with MC29, CMII and OK10 viruses. Proc. Natl. Acad. Sci. USA 80: 6566-6570, 1983.

Kan, N. C., Flordellis, C. S., Mark, G. E., Duesberg, P. H. and Papas, T. S.: A common onc gene sequence transduced by avian carcinoma virus MH2 and by murine sarcoma virus 3611. Science 223: 813-816, 1984.

Kan, N. C., Flordellis, C. S., Mark, G. E., Duesberg, P. H. and Papas, T. S.: Nucleotide sequence of avian carcinoma virus MH2: Relation of MH2-specific sequences to other oncogenic avian and to murine sarcoma viruses. Proc. Natl. Acad. Sci. USA 81: 3000-3004, 1984.

Kan, N. C., Flordellis, C. S., Mark, G. E., Lautenberger, J. A. and Papas, T. S.: A unique cellular sequence was independently transduced by avian carcinoma virus MH2 (v-mht) and a murine sarcoma virus (v-raf). In Ahmad, F., Black, S., Schultz, J., Scott, W. A. and Whelan, W. J. (Eds): Advances in Gene Technology: Human Genetic Disorders. Miami, ICSU Press, 1984, pp. 188-189.

Lautenberger, J. A., Kan, N. C., Court, D., Pry, T., Showalter S. and Papas, T. S.: High-level expression of oncogenes in Escherichia coli. In Papas, T. S., Chirikjian J. and Rosenberg, M. (Eds): Expression of Cloned Genes in Prokaryotic and Eukaryotic Cells: Gene Amplification and Analysis. New York, Elsevier/North Holland, Inc., 1983, Vol. 3, pp. 147-174.

Papas, T. S., Kan, N. C., Watson, D. K., Flordellis, C. S., Psallidopoulos, M. C., Lautenberger, J. A., Samuel, K. P. and Duesberg, P.: Myc-related genes in viruses and cells. In Watson, J. D., Vande Woude, G. F., Levine, A. and Topp, W. (Eds): Cancer Cell. New York, Cold Spring Harbor Press, 1984, pp. 153-163.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05286-03 LMO

PERIOD COVERED

October 1, 1983 to September 30, 1984

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)* Wistar Institute, Philadelphia, PA (Carlo Croce); Laboratory of Molecular Carcinogenesis, Dana-Farber Cancer Institute, Boston, MA (C. Cooper); Molecular Mechanisms of Carcinogenesis Laboratory, Basic Research Program, Litton Bionetics, Inc., Frederick, MD (G. Vande Woude, M. Dean)

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.)*

The HOS cell line, which was originally derived from a human osteosarcoma, is non-tumorigenic and can be transformed to anchorage-independent growth and tumorigenicity by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or 7,12 dimethylbenz(a)-anthracene (DMBA). It was shown previously that DNA prepared from the MNNG-HOS, transformed cell line will transform NIH 3T3 cells in DNA transfection assays, whereas DNA prepared from either HOS cells or DMBA-HOS cells failed to induce foci. The MNNG-HOS transforming gene (met), which has been cloned in several overlapping lambda clones, totaling 40 kb of the human sequence, shows no homology with the known members of the ras oncogene family nor with the viral oncogenes mos, myc, myb, src, erb, sis, rel or with the B-lym gene. There has been no rearrangement of met gene sequences within MNNG-HOS cells when compared with HOS cell DNA, human placental DNA or DNA from several human tumors. Cloned probes specific for the met gene detect a major species of polyadenylated RNA isolated from MNNG-HOS NIH 3T3 transformants. Using somatic cell hybrids, the human met transforming gene has been mapped to chromosome 7.

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 identify, isolate, and characterize a transforming gene detected by transfection of NIH 3T3 cells with DNA from a chemically-transformed, human cell line, MNNG-HOS. To analyze the normal, cellular homolog of this gene and to determine the mechanism by which the oncogenic potential is activated.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells. Construction of representative, genomic DNA libraries in λ replacement vectors, and screening of 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.

Major Findings:

1. The Transforming Gene Present in the Chemically-Transformed Human Cell Line, MNNG-HOS, has been Cloned in Overlapping λ Phage. It was shown previously that DNA prepared from the MNNG-HOS, transformed, human cell line will transform NIH 3T3 cells in DNA transfection assays, whereas DNA prepared from the parental HOS cells failed to induce foci. The MNNG-HOS-transformed NIH 3T3 cells would induce tumors in nude mice and secondary transformants could be derived from either restriction tumor or primary transformant DNA. Analysis of the alu hybridizing restriction fragments in secondary transfectants revealed a common pattern. Initially, the Blur 8 probe, specific for human, highly repeated DNA, was used to isolate an alu hybridizing, EcoRI fragment which was, in turn, used as a probe to isolate adjacent regions of the transforming gene (spanning a total of 40 kb) from a partial Sau 3A library from a secondary NIH 3T3 transformant. Comparison of the human DNA present in cell lines derived from individual foci has identified 26 kb of human DNA which is invariably present. This is in agreement with transfection studies using size fractionated, partially digested DNA from a secondary, NIH 3T3 transformant, which demonstrated that transforming activity was associated with DNA from 26-30 kb in length.

2. The MNNG-HOS Transforming Gene (met) is not Related to the ras Oncogene Family. Hybridization of cloned met gene sequences to cloned viral and cellular oncogenes showed that the MNNG-HOS transforming gene is not homologous to the known members of the ras oncogene family, B-lym, or the viral oncogenes, mos, myc, myb, src, sis, or rel. Furthermore, the restriction map of the met gene is distinct from that of N-ras or B-lym, and from the human homologs of Ha-ras, Ki-ras, mos, myc, abl, sis and fes.

3. The MNNG-HOS Cell Line Shows an Aberrant Transcription Pattern Compared with the Parental HOS Cell Line when Probed with a met Gene-Specific Probe. Multiple species of polyadenylated RNAs of 8 kb, 9 kb, and 11 kb hybridizing to met gene-specific probes were detected in HOS, MNNG-HOS, and DMBA-HOS cells, as well as in several human transformed cell lines. In contrast, a unique polyadenylated RNA species of 6.5 kb is present in NIH 3T3 transformants. This corresponds to an additional message present within MNNG-HOS cells, which is absent from HOS cells. Met gene sequences are not detectably rearranged in MNNG-HOS cells or NIH 3T3 transformants, when compared to HOS cells. This suggests that a small deletion, insertion or point mutation in MNNG-HOS cells may be responsible for the altered expression pattern of the met gene.

4. The met Transforming Gene is Mapped on Chromosome 7. In collaboration with Dr. C. Croce, Southern hybridization analysis of a panel of mouse x human somatic cell hybrids showed that the met gene maps to chromosome 7. The met gene was further localized to chromosome 7 from 7p 11.4 to 7 qter by analysis of two additional somatic cell hybrids, one of which retained only human chromosome 7, and the other only a portion of chromosome 7 from 7p 11.4 to 7 qter.

Significance to Biomedical Research and the Program of the Institute:

Characterization of a dominant, cellular, transforming gene in human cells transformed in vitro by MNNG may eventually lead to the elucidation of one of the mechanisms of transformation of cells by chemical carcinogens. The action of proto-oncogenes has been associated with both normal cell development and the development of neoplasia. The identification of the met gene will, therefore, allow us to study the function of an apparently new, human, transforming gene in both the process of cell transformation and its role in normal cell growth and development. Since several human neoplastic disorders have characteristic aberrations of chromosome 7, analysis of such cell lines using met gene-specific probes will assess whether this gene is associated with any characteristic abnormalities in chromosome 7 or, potentially, with tumor development.

Proposed Course:

The structure and expression of met gene-specific transcripts within MNNG-HOS, HOS cells and NIH 3T3 transformants will be further analyzed using conventional R-loop and DNA duplex analysis. Met gene-specific transcripts will also be compared within several human cell lines to determine if there is any correlation between aberrant transcription of this gene and specific neoplasias. The precise chromosomal location of the met gene will be determined by standard in situ hybridization techniques, and cell lines with specific chromosome 7 aberrations will be analyzed to determine whether there has been any rearrangement of met gene sequences. cDNA clones containing all or part of the met gene will be isolated from existing human cell cDNA libraries and by construction of such libraries from an NIH 3T3 transformant. These clones will be used to obtain the DNA sequence of the met gene. Analysis of its sequence by comparison with existing sequences in data banks will determine its relatedness to known eukaryotic genes. Expression of met gene cDNA clones in bacteria and use of synthetic peptides derived from cDNA sequence data will be used to raise antisera against

met gene protein. In vitro translation and hybrid-arrested translation using immunoprecipitation and conventional translation systems will provide further information on the met gene protein.

Publications:

Cooper, C. S., Blair, D. G., Oskarsson, M. K., Tainsky, M. A., Eader, L. A. and Vande Woude, G. F.: Characterization of human transforming genes from chemically-transformed, teratocarcinoma, and pancreatic carcinoma cell lines. Cancer Res. 44: 1-10, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05288-03 LMO

PERIOD COVERED

October 1, 1983 to September 30, 1984

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 are being used to resolve oligonucleosomes specifically derived from actively transcribed genes in order to determine their protein composition.

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 histones, as well as the DNA, and RNA species.

Major Findings:

A. Differentiation specific genes are 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 functions in Dictyostelium are completely inactive in growing cells and require a very specific aspect of cellular interaction to be activated, we are interested to know whether these genes exist in a DNase I-sensitive or -insensitive configuration in chromatin. In particular, we are interested to know if these genes require a change in their 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 shutoff of transcription of the late genes, as well as to rapid degradation of the 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 differentiation are packaged with respect to the DNase I-sensitive configuration.

Nuclei from 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 either expressed actively in the growing cells or only expressed during late development. The rate at which these fragments disappeared as a function of DNase I digestion was measured. Under conditions of very low ionic strength, where the higher order chromatin structure is lost and the DNA exists as a 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 differences in the sensitivity of these two classes of genes to DNase I. Finally, using spot blots to analyze the rate of DNase I digestion of the genes to non-hybridizable fragments, we still detect no differences in sensitivity of expressed and nonexpressed genes, even when the highly transcribed rRNA genes are compared with the differentiation-specific genes. These results indicate that the differentiation-specific genes are in a preactive, DNase I-sensitive configuration in the chromatin of growing cells, even though they are not transcriptionally active in these cells. Thus, transcription of these genes late in development does not require the kind of alteration in chromatin structure which is defined by conversion of a gene from the DNase I-insensitive state to a -sensitive state.

B. Use of micrococcal nuclease to detect alterations in 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 of 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 the preactive, DNase I-sensitive configuration (described in part A). Unlike the very precise nucleosome repeat ladder observed in higher eukaryotes, the Dictyostelium pattern is somewhat more diffuse and irregular. Higher level resolution of the repeat ladder on acrylamide or formamide, high percentage agarose gels reveals discontinuities and irregularities in the repeat pattern that are not observed in the chromatin of higher cells. This irregular repeat pattern has been analyzed for individual genes which fall into 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 transcrition at the time when cell contact is formed. The differentiation-specific genes which are transcriptionally inactive in the growing cells from which the chromtin was prepared, give a very precise repeat ladder with unit length of 175 nucleotides and resemble the pattern 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 to arise as a result of cuts, not only between nucleosomes, but also at precise sites within the core particle. These cuts within the core particle generate bands which are 50, 70, 90 or 110 base pairs longer than an integral multiple of the 175 base pair repeat. The genes which are transcribed at a higher rate show the same nonintegral repeat as the genes expressed only at a very low level, however, the bands are now superimposed on a background smear. Thus, micrococcal nuclease digestion of chromatin detects structural features which are unique to genes that are actively transcribed, and between preactive, but nontranscribed genes and actively

transcribed genes. Whether this state is the result of interaction of the nucleosomes with RNA polymerase alone, or association of nucleosomes on genes which are being transcribed with additional proteins or histone variants, is a question which we are in a position to address as described below.

C. Two-dimensional analysis of nucleoprotein complexes may allow identification of proteins specifically associated with oligonucleosomes derived from actively transcribed genes. 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 major 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 non-integral repeat length and is likely to have arisen from a cut within the core particle. We are currently devising conditions under which we can electrotransfer the DNA's 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 non-integral 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 RNA polymerase alone is sufficient to distort the structure of the mononucleosome to allow the internal cuts observed in the core particles by micrococcal nuclease digestion.

D. Construction of a genomic library and cloning of different classes of developmentally controlled genes for correlating structural organization with sequence maps. So far, our analysis of the structural organization of the actively transcribed genes and the inactive, developmentally controlled genes has been carried out using cDNA clones which are specific probes for the protein-coding portions of the genes. These probes do not allow us to analyze structures associated with the adjacent control regions of these genes. We have, therefore, constructed an Eco RI partial library in λgt wes B4 and have used our various cDNA clones to screen it. To date, we have obtained genomic clones encompassing the coding region and three to four kilobases to the 5' and 3' ends of two constitutively expressed genes, two developmentally specific genes which are not transcribed in the growing cells, and one of the class of genes which is transcribed at a very low level in growing cells and induced to a high level of expression late in development. Since Dictyostelium genes are not known to have large introns, we believe these clones should encompass the coding and flanking regions of these genes. We are currently mapping these clones and hope to use them to define

boundaries of different chromatin structural domains relative to the coding and control sequences for the genes. Additionally, we would like to identify sequences which are not in nucleosomes, origins of DNA replication, and attachment points of the DNA to the nuclear matrix.

Significance to Biomedical Research and the Program of the Institute:

Abnormalities in differentiation and developmental gene expression are characteristic of the malignant cell. Because of the unique features of its developmental program, Dictyostelium provides a powerful and simple system for exploring mechanisms which control eukaryotic developmental gene expression. Fifty-five percent of the Dictyostelium genome is transcribed during vegetative growth and another 25% is expressed during differentiation; thus, Dictyostelium provides a unique opportunity to explore the structural organization of chromatin which is actively transcribed.

Proposed Course:

The structural organization of a gene in chromatin may be a vital component of the controls governing its activation during development and in response to other stimuli. Actively transcribed genes in higher eukaryotes are a minor percent of the total chromatin; and the changes associated with transcriptional activation appear to be quite subtle, possibly even varying for genes transcribed at different rates or by different polymerases. As a result, very little is actually known about the structure of nucleosomes associated with active genes. A key question is whether nucleosomes from active genes are even able to coil into the higher order solenoid structure. Our studies have identified a structural organization unique to genes which are actually being transcribed. As described in part C of Major Findings, features of this organization potentially allow us to specifically resolve on polyacrylamide gels oligonucleosomes derived from genes which are being actively transcribed. We propose to pursue this fractionation in order to analyze the protein composition of the oligonucleosomes from the active genes. We anticipate one of two results. Either the proteins comprising the oligonucleosomes from active genes will be identical to those found on inactive genes or we will find histone variants, histone modifications, or nonhistone, chromosomal proteins associated with the active nucleosomes or in the linker regions between these nucleosomes. In the latter case, we will utilize several biophysical techniques to determine if such modifications prevent coiling of the oligonucleosomes into the higher order solenoid structures or if they result in a more open, half nucleosome-like structure. Either of these modifications might facilitate the passage of RNA polymerase. If there are no differences in the composition of the oligonucleosomes from active and inactive genes, then this will establish that passage of RNA polymerase, itself, is sufficient to distort the structure of the nucleosome, giving rise to the altered structure described in part B of Major Findings. This would argue that the DNase I-sensitive configuration of nucleosomes associated with active genes is, together with the appropriate exposure jof the control sites, sufficient for gene transcription. We have also been isolating and mapping genomic clones of these genes so that we can extend our structural studies to analyze the protein interactions and structural organization at control sites on these genes.

Masakazu Oyama, a postdoctoral student in my group, (see Project Number Z01CE05361-01 LMO) is in the early stages of developing a transformation system which we hope to use together with in vitro mutagenesis to genetically define the sites at which the various steps in the regulation of these genes occur.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05294-03 LMO

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Isolation and Characterization of a Human Transforming Gene from a Teratocarcinoma

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 present in 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.

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 initial goals of these studies were the isolation by molecular cloning and analysis of the activated transforming gene in passaage 330 PA-1 cells. Further studies as to the biological effects of this gene on this or other human cells in culture was the second objective of this work.

Methods Employed:

DNA-mediated transfection was performed by the standard calcium phosphate precipitate method. NIH 3T3 cells were either carried in culture for 14-21 days and inspected for foci of morphologically altered cells, or injected into athymic nude mice after transfection, and the mice inspected weekly for tumor formation.

The genomic library was prepared using DNA from a secondary focus, partially digested with Sau 3A I which was fractionated on a sucrose density gradient in the 17-23-kbp range. This purified DNA was ligated into the BamHI arms of the λ phage vector, EMBL-3.

DNA sequence analysis was performed by the chemical degradation method of Maxam and Gilbert (Methods Enzymol. 65: 497-559, 1980).

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 form tumors in athymic nude mice only after long latent periods, 17-30 weeks. 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 oncogene is correlated with the increased level of tumorigenicity observed in late cell culture passages.

A study of the human DNA sequences present in PA-1 secondary foci by Southern blot analysis revealed no sequences related to oncogenes of the Harvey or Kirsten sarcoma viruses, other than those present in the normal mouse genome. However, a human ras^N probe revealed related sequences in DNA from PA-1

secondary foci. A representative genomic library was constructed from DNA from a PA-1 secondary focus partially digested with the restriction enzyme Sau 3A I, using the bacteriophage vector, EMBL-3. This genomic library was screened with a ras^N probe and phage clones were isolated which contained this activated ras^N on two RI fragments, each 7.0 kb. These were cloned onto the plasmid vector, pBR322, to reassemble the gene. This plasmid clone, pMT-J, was assayed for biological activity in the NIH 3T3, DNA-mediated, transformation assay. This construct was found to efficiently transform NIH 3T3 cells ($3\text{-}8 \times 10^3$ ffu/μg).

Since other human ras genes have been found to be activated by a point mutation at either amino acid position 12 or 61, the DNA sequence of the first two exons of the transforming gene was analyzed. The DNA sequence of the second exon was found to perfectly match that of the normal human ras^N. However, DNA sequences 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. This, therefore, demonstrates that ras^N, like ras^H, can be activated by a point mutation at the codon for position 12. Previously, only a change at amino acid 61 had been reported, but the human ras^H has been found to be activated at either position 12 or 61. 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. Seventy-five percent 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.

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 has 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, therefore 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. Since this transforming gene can be correlated with tumorigenicity, the role of this gene in the stages of progression of carcinogenesis can be investigated.

Proposed Course:

The teratocarcinoma cells, PA-1, were malignant in the patient from which they were isolated, but are non-tumorigenic in athymic nude mice. These cells become tumorigenic with passage in culture; and this can be correlated with the presence of an activated ras^N transforming gene. Future studies will involve the introduction of the ras^N into early, non-tumorigenic passages of PA-1 cells or other human, non-tumorigenic cell lines to determine if the presence of this gene is sufficient for tumorigenicity or are other alterations, genetic or epigenetic, required. In addition, the nature of the gene sequences from early passage PA-1 DNA giving rise to the non-ras^N, NIH 3T3 cell tumors will be investigated to seek to isolate additional transforming genes from PA-1.

Publications:

Cooper, C. S., Blair, D. G., Oskarsson, M. K., Tainsky, M. A., Eader, L. A. and Vande Woude, G. F.: Characterization of human transforming genes from chemically-transformed, teratocarcinoma, and pancreatic carcinoma cell lines. Cancer Res. 44: 1-10, 1984.

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 (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05295-03 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

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. K. Oskarsson	Chemist	LMO	NCI
	M. A. Tainsky	Senior Staff Fellow	LMO	NCI
	B. M. O'Hara	Visiting Fellow	LMO	NCI
	M. Park	Visiting Fellow	LMO	NCI
	K. J. Dunn	Bio. Lab. Tech. (Micro.)	LMO	NCI

COOPERATING UNITS *(if any)* Molecular Mechanisms of Carcinogenesis Laboratory, Basic Research Program, Litton Bionetics, Inc., Frederick, MD (G. F. Vande Woude, A. Seth, M. Dean) Nucleic Acids and Protein Synthesis Laboratory, Program Resources, Inc., Frederick, MD (M. Zweig, S. D. Showalter, L. A. Eader).

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 mouse cellular homolog (c-mos-Mo) of the Moloney murine sarcoma virus (MSV) transforming sequence (v-mos) can be activated by linking it to MSV, long-terminal-repeat (LTR) sequences. The human cellular homolog (c-mos-Hu) of mos does not transform even when linked to LTR sequences. Certain recombinants between c-mos-Hu and v-mos which contain sequences derived from both parents, are able to transform NIH 3T3 cells following DNA transfections, but at reduced efficiencies relative to v-mos. Active recombinants containing the 3' portion of c-mos-Hu express high levels of mos protein product, which can be detected by peptide antisera specific for the carboxy-terminus of c-mos-Hu. The pattern of activity observed in c-mos-Hu/v-mos recombinants is consistent with the presence of several functional domains within mos. In c-mos-Hu two of these appear to interact to prevent the expression of a transforming potential.

A dominant ras-K gene isolated from the human pancreatic carcinoma cell line, PANC-1, contains a G→A transitional mutation within the 12th codon of the coding sequence. The active ras-K gene encodes an aspartic acid rather than a glycine at position 12. An activated ras-N has also been identified in DNA obtained from a gastric adenocarcinoma. The gene has been cloned from the primary human tumor and the activating lesion localized within the first two coding exons.

PROJECT DESCRIPTION

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

D. G. Blair	Research Chemist	LMO	NCI
M. K. Oskarsson	Chemist	LMO	NCI
M. A. Tainsky	Senior Staff Fellow	LMO	NCI
B. M. O'Hara	Visiting Fellow	LMO	NCI
M. Park	Visiting 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. Functional domains of c-mos^{Hu} can be identified which affect the transforming activity of the gene product. Previous results have indicated that the human homolog of mos, c-mos^{Hu}, was inactive in DNA transfection assays. Certain recombinants containing both c-mos^{Hu} and v-mos were active in transforming NIH 3T3 cells in DNA transfection assays, but the levels of activity varied from approximately 20% to approximately 0.2% of the level observed for the v-mos parental constructs. Both active and inactive constructs will express mos gene products when inserted into a bacterial expression vector, suggesting that the block does not lie at the expression level. The pattern of activity suggests a model which proposes that interactions between different domains of the mos polypeptide are required for activity. Evolutionary sequence divergence has led

to a situation where certain regions of the protein will interact when both are encoded by either human or mouse mos sequences, but not when they are encoded by different sequences. The construction of hybrids between equivalent gene sequences separated by a wide evolutionary distance has allowed us to investigate the functional diversions of gene sequences whose separation was previously only determinable on a nucleotide sequence level.

2. Cells transformed by certain c-mos^{Hu}/v-mos recombinants express mos-specific proteins at high levels in comparison to v-mos-transformed cells. Peptide antisera which specifically recognize either the v-mos or c-mos^{Hu} carboxy-terminal peptide sequence have been prepared. These sera recognize bacterially expressed v-mos and v-mos/c-mos^{Hu} hybrid proteins using either Western blotting or immunoprecipitation techniques. NIH 3T3 cells transformed by recombinant mos constructs which contain human-derived 3' sequences express a mos gene product of approximately 35,000 daltons at a level equivalent to that observed in cells acutely infected with Moloney murine sarcoma virus. In contrast, reciprocal recombinants containing 3' v-mos sequences express barely detectable levels of mos product. Mos-specific RNA is detected at similar levels in both types of transformants. The level of mos product could reflect selection for high levels of expression of a poorly-transforming gene product encoded by hybrids containing 3' human mos sequences.

3. The active ras^{K} oncogene cloned from the human pancreatic carcinoma cell line, PANC-1, contains an aspartic acid in place of glycine at position 12. We had previously described an active ras^{K} sequence which was transferable from the human pancreatic carcinoma cell line, PANC-1. Sequence analysis of the first coding exon of the ras^{K} gene cloned from a secondary transfectant of PANC-1 revealed only one difference in comparison to previously published sequences of the normal human ras^{K} gene. A G→A transition has resulted in a glycine (GGT) to aspartic acid (GAT) change in the predicted sequence for the ras^{K} protein. This change represents a transition as opposed to a transversion mutation and is the first report of this type of mutation at the 12th position in the human Kirsten ras oncogene.

4. Several primary human tumor DNAs contain transfectable oncogenes when analyzed in the nude mouse tumor assay. We had previously reported that the nude mouse could be used to detect and select transformed NIH 3T3 cells from a transfected cell population. We have screened DNA samples isolated from fresh surgical human tumor specimens in order to eliminate the possible effects of cell culture or oncogene activation. We have also increased the sensitivity of the assay by cotransfecting with tumor DNA and pSV2 neo plasmid and then selecting cells which have taken up the plasmid and have become resistant to the neomycin analog, G418. This selected population, most or all of which will also contain transfected human DNA, is then injected into nude mice to select for transformed cells. Utilizing this system, we have identified and isolated an activated ras^{N} sequence from a gastric adenocarcinoma. This gene has been cloned into bacteriophage lambda and a clone containing the 5' segment of the gene is active when ligated to the 3' normal portion of the ras^{N} gene. In addition, an unidentified active oncogene has been transferred through two rounds of transfection from a nasopharyngeal carcinoma and a carcinoma of the

prostate. These genes do not appear to be known members of the human ras family and studies to identify and clone these sequences are in progress.

Significance to Biomedical Research and the Program of the Institute:

The process by which inactive sequences with oncogenic potential are "turned-on" to express this potential is one of the central questions of human carcinogenesis. Such activation events have been shown to be due to quantitative changes at the level of gene expression through gene amplification and rearrangement, and promoter insertion. In addition, they have been shown to be due to qualitative changes arising through point mutations or gene rearrangements. Our increasing understanding of how these events occur should enable us to effect and manipulate these events both in vivo and in vitro. It should allow us to reproduce and study the process of spontaneous or chemically-induced activation in vitro, to identify normal genetic sequences with oncogenic potential, and to define the range of events which could lead to the activation of these sequences.

DNA transfection with human tumor DNA has allowed the identification and isolation of specific human DNA sequences which may be involved in initiation and maintenance of the transformed phenotype in human cancer. Screening techniques based on tumor formation in nude mice represents a definitive, biologically significant, selection system. It offers the potential of recognition of transforming oncogenes with morphological manifestations which may be difficult to detect. It allows the utilization of other recipient cell lines in which morphological transformation is not apparent. Transforming DNA sequences, once detected by tumor induction, can be identified, cloned and analyzed by conventional techniques.

The development of human recipients for analysis of cloned oncogenes and for selection of transforming sequences present in tumor cell genomes represents an important validation for the oncogenic potential of genes isolated through selection and screening of rodent cells. It will allow for the direct analysis in vitro of the process of oncogenesis in normal human diploid cells.

Proposed Course:

Specific recombinants between c-mos^{Hu} and v-mos will be constructed and analyzed to extend and verify the localization of specific domains of mos. The localization of and the level of protein expression in cells transfected by inactive hybrid constructs, as well as various active constructs, will be analyzed to further characterize the function of mos and the role of its level of expression in transformation. Hybrid mos constructs will be introduced into human cells to determine if human mos has an oncogenic potential in human cells which differs from its ability to transform rodent cells. Specific regions of c-mos^{Hu} mutagenized in vitro by various techniques will be analyzed by transfection to determine if specific codon mutations will activate the transforming potential.

The nature of the activating mutation in the cloned ras^{N} oncogene will be identified. Cultures of peripheral blood lymphocytes from the same individual will be established, and the normal ras^{N} homolog will be cloned and compared to the

activated isolate. The nature of the non-ras oncogenes transfected from the nasopharyngeal carcinoma and the prostate carcinoma will be analyzed. Sensitivity of the transforming activity to restriction nucleases, the pattern of human repeat sequences conserved, and the hybridization to known oncogenes will be used to identify the specific genes involved. The genes will be cloned and their lesions analyzed. Screening for active onc genes utilizing the nude mouse assay with NIH 3T3 cells will continue. Analysis of neuroblastoma and breast carcinoma DNA samples as part of ongoing collaborations will be continued.

The ability of cloned oncogenes to transform diploid human fibroblasts will be measured by utilizing microinjection and bacterial protoplast fusion to introduce these genes into low passage human fibroblasts. The properties of cells carrying and expressing the ras, mos, abl and myc oncogenes will be analyzed.

Publications:

Blair, D. G., Wood, T. G., Woodworth, A. M., McGeady, M. L., Oskarsson, M. K., Propst, F., Tainsky, M., Cooper, C. S., Watson, R., Baroudy, B. M. and Vande Woude, G. F.: Properties of the mouse and human mos oncogene loci. In Levin, A., Topp, W., Vande Woude, G. F. and Watson, J. D. (Eds.): Cancer Cell. New York, Cold Spring Harbor Press, 1984, pp. 281-289.

Cooper, C. S., Blair, D. G., Oskarsson, M. K., Tainsky, M. A., Eader, L. A. and Vande Woude, G. F.: Characterization of human transforming genes from chemically-transformed, teratocarcinoma, and pancreatic carcinoma cell lines. Cancer Res. 44: 1-10, 1984.

Wood, T., Blair, D., McGeady, M. L. and Vande Woude, G.: Sequences involved in the activation of the transforming potential of a normal cellular gene, c-mos. In Gluzman, Y. and Shenk, T. (Eds.): Enhancers and Eukaryotic Gene Expression. New York, Cold Spring Harbor Press, 1984, pp. 110-117.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05342-02 LMO

PERIOD COVERED

October 1, 1983 to September 30, 1984

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 spece provided.)*

Characterization of the Moloney murine sarcoma virus v-mos protein has been hindered by expression of extremely low levels in transformed cells. In order to analyze the v-mos gene product, a eukaryotic expression system is being developed in which v-mos protein will be produced at elevated levels. Specifically, plasmid vectors have been constructed into which v-mos has been placed under the control of the murine, beta-globin, major promoter in various arrangements. This plasmid DNA containing v-mos and plasmid DNA containing the gene coding for the enzyme, adenine phosphoribosyl transferase (aprt), have been simultaneously introduced into murine erythroleukemia cells lacking aprt (MEL aprt-) using calcium phosphate-mediated DNA coprecipitation or spheroplast fusion. Upon induction of differentiation, it is predicted that the exogenously added betaglobin promoter will be activated to express v-mos protein at elevated levels. This system will provide a method by which to explore the nature of the v-mos protein both in vivo, in these MEL cells, and in vitro, after isolation and purification. In addition, such a powerful eukaryotic expression system would be generally applicable for use in studies of other gene products.

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 develop a system for the above purpose, which is suitable to use for high-level expression of other gene products.

Methods Employed:

The v-mos gene from the plasmid pHT10 and the mouse β major globin gene from λ gt WES.MBG2 (Tilghman, S.M. et al., PNAS 74: 4406-4410, 1977) were used to construct, in pBR322, plasmids containing the v-mos gene under the control of the β-globin major promoter in various arrangements using standard in vitro recombinant DNA techniques. Each of the plasmid DNAs has been introduced simultaneously with the selectable marker gene, adenine phosphoribosyl transferase, 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 is extracted from expanded single cell colonies and used in DNA blotting experiments to identify v-mos-containing clones. Those cells into which v-mos has been successfully introduced without rearrangements have been induced to differentiate. Upon activation of the exogenously added β-globin promoter, elevated expression of v-mos is examined at the RNA level by dot blot and Northern analyses and at the protein level by immunofluorescence and immunoprecipitation.

Major Findings:

Cell colonies with an aprt$^+$ phenotype have been obtained after cotransfection of MEL aprt$^-$ cells with a 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 indicates the presence of unrearranged v-mos sequences in the aprt$^+$ clones. Three to four days post-induction with sodium butyrate, hemoglobin production can be detected by benzidine staining, thus indicating that the cells have been successfully induced to differentiate. Analysis of mRNA populations indicates 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. Preliminary examination of aprt$^+$ cells containing v-mos sequences using trypan blue staining suggests that a reduction in viability occurs upon induction as compared with cells containing aprt alone.

Significance to Biomedical Research and the Program of the Institute:

There are several advantages of a eukaryotic expression system which utilizes the inherent biological mechanism of induction of differentiation in MEL cells. First, the protein product of a specific gene can be expressed as desired by treatment of cells with inducing agents. Second, upon induction of erythroleukemia cells, globin represents 10% of the total cytoplasmic proteins expressed. This observation is evidence of the strength of the activated globin promoters. Third, the background of cellular proteins is low due to a decrease in macromolecular synthesis upon induction. Therefore, this expression system has 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 will facilitate in vitro biochemical analyses of the v-mos gene product by providing significant quantities of protein with which to work. In addition, since mos produced in a bacterial expression system under the control of bacteriophage promoters is available, the nature of eukaryotically expressed mos and prokaryotically expressed mos can be compared. Of particular interest is how specific modifications which occur in eukaryotic protein processing, but not in prokaryotic cells, may affect the properties of mos. Moreover, this system will permit the determination of the in vivo localization of v-mos within MEL cells and the effect of v-mos upon the differentiation program of MEL cells, upon transformation, and upon cell viability. Finally, the ability of the various plasmid constructs to express v-mos will provide insight into those regions within the β-globin genome which are required for activation of globin expression during induction.

Proposed Course:

Each of the plasmid constructions will be introduced into MEL aprt$^-$ cells by spheroplast fusion. Multiple cell colonies will be picked, single cell cloned, expanded and examined for v-mos DNA and expression of v-mos RNA and protein after induction. Upon determining the optimum conditions for activation of v-mos expression, we will investigate the biochemical and biological effects of v-mos expression in MEL cells and the nature of the mos product.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05343-02 LMO

PERIOD COVERED: October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*: The Transforming Genes of Acute Leukemia Viruses and Their Cellular Homologs

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

PI:	C. Flordellis	Visiting Fellow	LMO	NCI
Others:	T. S. Papas	Acting Chief	LMO	NCI
	N. C. Kan	Visiting Fellow	LMO	NCI
	K. P. Samuel	Visiting Associate	LMO	NCI
	D. K. Watson	Senior Staff Fellow	LMO	NCI
	J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
	T. W. Pry	Microbiologist	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:
1.60	1.10	0.50

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The v-mht forms, with the Δgag sequences of MH2, a fused gene which is in all likelihood a transforming gene. Sequences related to the v-mht oncogene are present in the genomes of normal chicken and of many mammalian species (including man). This oncogene is expressed as a unique RNA species of approximately 4 kb. Studies of the chicken mht have been initiated to determine its real transcriptional and translational boundaries.

PROJECT DESCRIPTION

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

C. S. Flordellis	Visiting Fellow	LMO	NCI
T. S. Papas	Acting Chief	LMO	NCI
N. C. Kan	Visiting Fellow	LMO	NCI
K. P. Samuel	Visiting Associate	LMO	NCI
D. K. Watson	Senior Staff Fellow	LMO	NCI
J. A. Lautenberger	Senior Staff Fellow	LMO	NCI
T. W. Pry	Microbiologist	LMO	NCI

Objectives:

The purpose of this investigation is to determine the functional relationship between onc genes and their normal cellular homologs. Structural analysis along with study of RNA transcripts of these genes, from both normal and transformed cells, will allow us to better understand their biological functions.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cells by treatment with proteinase K followed by phenol-chloroform extraction. Preparation of vector DNA from phage λ derivatives by phenol extraction of CsCl-banded phage.

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose gels and/or polyacrylamide gels.

3. Preparation of DNA probes using purified MH2-specific DNA to be nick-translated using E. coli DNA polymerase.

4. Southern blot analysis using nitrocellulose filters.

5. Isolation of phage from the libraries containing virus-related sequences by hybridization of cDNA 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).

7. Subcloning of isolated c-MH2 DNA fragments into pBR322.

8. DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol. 65: 499-560, 1980).

9. RNA extraction from cultured cells using guanidine hydrochloride extraction. Isolation of RNA after cellular fractionation. Separation of polyA-RNA by several (two to three) cycles of purification through oligo(dT) cellulose. Analysis of RNA on formaldehyde-agarose gels by Northern analysis.

Major Findings:

1. The nucleotide sequence of MH2 has revealed the presence of two cell-derived sequences in its genome: a v-myc region with 90% homology (deduced at the amino acid level) to the v-myc of MC29; and a 1.3-kb (v-mht) unique sequence placed in phase with the Δ-gag region, having a potential coding capacity for a product of 90,000 daltons. The oncogene, v-mht, has been found to be highly homologous to the murine v-raf oncogene.

2. Southern blot analysis has enabled the detection of v-mht-related sequences in the genome of normal chickens and in the genome of mammalian species, including man.

3. Northern blot analysis of polyA-containing, total cellular RNA has allowed the identification, in normal chicken fibroblasts, of an mht-specific RNA species 4.0 kb in length. In the human cell line studied (HL-60, Daudi) an mht-related RNA species of 3.7 kb was detected.

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 homologs is, therefore, of immense importance to better understand the mechanism of the leukemic process.

Proposed Course:

We will analyze: (1) The chicken c-mht portion homologous to the extreme 5' end of the v-mht, using: (a) heteroduplex analysis between a chicken c-mht clone and proviral MH2 DNA, and (b) restriction enzyme characterization and sequence analysis to determine the probable recombination point between the helper virus and the cellular sequences; and (2) The chicken c-mht locus upstream from the extreme 5' end of v-mht-related sequences, by: (a) S1-mapping analysis to precisely determine the site of initiation of transcription in normal and cultured cells transformed with MH2. The same question will be addressed by characterizing the ds-cDNA constructed extension of the extreme 5' v-mht as primer; (b) sequencing the upstream portion of the genome to determine the real transcription and translation domains of the c-mht oncogene; and (c) subcloning portions of the upstream portion into expression vectors (pJL6 and its derivatives) and using the expressed polypeptides as antigens to elicit mht-related antibodies in order to bring down and characterize the product of c-mht in both normal and transformed cells.

Publications:

Flordellis, C. S., Kan, N. C., Psallidopoulos, M. C., Samuel, K. P., Watson, D. K. and Papas, T. S.: A cellular gene homologous to v-mht is expressed in chicken and human cells. In Ahmad, F., Black, S., Schultz, J., Scott, W. A. and Whelan, W. J. (Eds.): Advances in Gene Technology: Human Genetic Disorders. Maimi, ICSU Press, 1983, pp. 166-167.

Kan, N. C., Flordellis, C. S., Garon, C. F., Duesberg, P. H. and Papas, T. S.: Avian carcinoma virus MH2 contains a transformation-specific sequence, mht, and shares the myc sequence with MC29, CMII and OK10 viruses. Proc. Natl. Acad. Sci. USA 80: 6566-6570, 1983.

Kan, N. C., Flordellis, C. S., Mark, G. E., Duesberg, P. H. and Papas, T. S.: A common onc gene sequence transduced by avian carcinoma virus MH2 and by murine sarcoma virus 3611. Science 223: 813-816, 1984.

Kan, N. C., Flordellis, C. S., Mark, G. E., Duesberg, P. H. and Papas, T. S.: Nucleotide sequence of avian carcinoma virus MH2: Relation of MH2-specific sequences to other oncogenic avian and to murine sarcoma virus. Proc. Natl. Acad. Sci. USA 81: 3000-3004, 1984.

Kan, N. C., Flordellis, C. S., Mark, G. E., Lautenberger, J. A. and Papas, T. S.: A unique cellular sequence was independently transduced by avian carcinoma virus MH2 (v-mht) and a murine sarcoma virus (v-raf). In Ahmad, F., Black, S., Schultz, J., Scott, W. A. and Whelan, W. J. (Eds.): Advances in Gene Technology: Human Genetic Disorders. Miami, ICSU Press, 1984, pp. 188-189.

Papas, T. S., Kan, N. C., Watson, D. K., Flordellis, C. S., Psallidopoulos, M. C., Lautenberger, J., Samuel, K. P. and Duesberg, P.: Myc-related genes in viruses and cells. In Levin, A., Topp, W., Vande Woude, G. F. and Watson, J. D. (Eds.): Cancer Cell. New York, Cold Spring Harbor Press, 1984, pp. 153-163.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05344-02 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

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)*

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 spece provided.)*

In order to develop a transfection system with human cells in which the effect of cellular DNA from human tumors and previously cloned onc genes can be assayed, we have screened a number of non-tumorigenic human cell lines for several characteristics suiting them for use as recipients in such a transfection system. The SV80 cell line and a hybrid between HeLa and a normal diploid fibroblast can both be transfected for cloned selectable markers with a high efficiency. They have a cryptic tumorigenic potential, that is, under certain rare circumstances, they will form tumors in nude mice.

SV80 cells were transfected with mixtures of a selectable marker, guanine phosphoribosyl transferase (gpt) and cloned onc genes. Introduction of MSV or EJ sequences by cotransfection with the selectable marker did not render the cells tumorigenic nor result in the obvious formation of foci, although individual cells showing expression of the MSV p30 antigen showed morphological alterations and virus could be rescued from the MSV-transfected cells. In a preliminary experiment, transfection using a mixture of MSV DNA and adenoviral E1a and E1b DNA, did result in tumor formation.

The HeLa/fibroblast hybrid line could be cotransfected at low efficiency in comparison with rodent cell lines. Cells cotransfected with EJ DNA did not become tumorigenic nor did they show any morphological alteration. Messenger RNA studies to date indicate that the non-tumorigenic hybrids do not show suppression of H-ras or myc expression, when compared to tumorigenic derivatives isolated from them by others.

We have cloned a 5' EcoRI fragment of an N-ras gene from a human gastric adenocarcinoma. This N-ras fragment is active in transfection assays on NIH 3T3 cells when ligated to the 3' EcoRI fragment from the normal human gene.

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.

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:

Presence of Active MSV Sequences or EJ Bladder Gene Sequences is Not Sufficient to Render SV80 Cells Tumorigenic. It was found that the SV80 cell line could be efficiently transfected with the gpt marker as determined by selection in culture and by Southern analysis. Five to ten percent of the transfected cells also picked up MSV DNA when the gpt and MSV DNAs were mixed prior to transfection. Despite the presence of the proviral p30 antigen and the fact that the transfected provirus could be rescued using FeLV infection, the cells did not form tumors in nude mice nor did they form obvious foci in culture. This suggests that these cells are resistant to the action of the mos gene, an unexpected finding given their partially transformed nature. Subclones of this population have been obtained which are close to 100% positive for expression of the p30 antigen.

Transfection of SV80 cells with MSV DNA and the adenoviral 5 E1a gene coupled to an E1b/β galactosidase hybrid gene did result in tumor formation in a single preliminary experiment. The explanted tumor has thus far been examined for the presence of MSV DNA, which it contains in high levels. SV80 cells transfected with the EJ gene did not cause tumors nor did they show any obvious morphological alterations.

Transfection of Hybrid Cells with EJ DNA Does Not Render Them Tumorigenic. ESH20 cells, hybrids of HeLa cells and normal human diploid fibroblasts were found to be transfectable for the gpt marker. They did not, however, cotransfect the MSV DNA. They do become morphologically altered and do cause tumors following infection with MSV/FeLV, indicating a potential for tumorigenicity following successful transfection. We did succeed in cotransfecting the EJ gene. These cells did not cause tumors in nude mice. Because of the difficulty in cotransfecting these cells, we are preparing a series of in vitro, ligated DNAs which should ensure successful cotransfer of the desired onc genes. We have available to us two non-tumorigenic hybrids of HeLa and normal diploid fibroblasts and four tumorigenic lines derived from them. The level of expression of ras^H- and myc-related sequences in the non-tumorigenic hybrids was compared to the levels in the tumorigenic derivatives and in the HeLa parent. This was done in the hope that the non-tumorigenic nature of the hybrids would correlate with suppression of cellular onc gene expression when compared to the tumorigenic members of the family. No consistent suppression of these genes was observed.

An Active Fragment of N-ras Has Been Cloned From a Human Gastric Adenocarcinoma. We have cloned a portion of an N-ras gene directly from a human gastric carcinoma. This gene segment, when joined to the remaining, normal segment in vitro, causes NIH 3T3 cells to form foci in transfection assays.

Significance to Biomedical Research and the Program of the Institute:

A number of cellular genes have been isolated which cause NIH 3T3 cells to form foci in monolayer culture and to form tumors in nude mice. The NIH 3T3 cells are suitable for this purpose presumably because they have proceeded to a penultimate step in the process of transformation and may need only one additional change to render them transformed. Also, they are efficient recipients in DNA transfection, a process by which new genes can be introduced into large numbers of cells. However, there are two major drawbacks to the use of this recipient line. Being of murine origin, it is questionable what role the genes which transform them plays in human systems. Also, they appear to be relatively more sensitive to some classes of cellular transforming genes, i.e., members of the ras family. There are many primary human tumors and tumor cell lines which do not transform them and these may possess transforming genes inactive in NIH 3T3 cells. For these reasons, we are seeking to develop focus and tumor formation assays using human cell lines. These lines must be efficiently transfectable, which human lines generally are not, and they must be non-tumorigenic, but with some indication that they have progressed to a condition which is recognized as being close to transformed. Using such lines, it may be possible to detect new oncogenes which are not transformable on NIH 3T3 cells. These

cells could also be used to confirm the functionality and possible interactions of previously isolated genes in human cells. Any genes detected in this system can be cloned using established procedures.

A number of ras genes which are active in transfection assays on NIH 3T3 cells has been cloned from human sources. These include mainly ras^K genes and a small number of ras^H and ras^N genes. All of these possess an alteration in the 12th or 61st amino acid codon. Because of the relative paucity of information on ras^N genes, we are determining the nature of the alteration in a ras^N gene which we have found to be active in transfection assays on NIH 3T3 cells. This information will be particularly pertinent as the gene has been isolated directly from tumor material rather than from NIH 3T3 transfectants or human cell lines and because we have available the normal tissue from the same patient as a source of the normal gene.

Proposed Course:

The inability of transfected mos sequences to render SV80 cells tumorigenic will be confirmed using the cell subclones. This will involve Southern and Northern analyses, rescue of the proviral MSV using FeLV or GALV, and transfection of the MSV DNA in the SV80 cells into NIH 3T3 cells. Protein analysis has been undertaken. Once we have identified cell subclones containing expressed functional mos sequences, experiments will then be carried out to determine what further alterations are necessary to render these cells tumorigenic. These include treatment with 5-azacytidine, tumor promoters, and various carcinogens, or transfection with other onc genes.

A series of in vitro ligations is being prepared using combinations of the gpt gene with MSV DNA, a particularly active ras^H gene isolate (T24), a myc proviral isolate, the adenoviral E1a-E1b/β galactosidase construct and the E1a gene alone. These will be transfected into SV80 cells and ESH20 cells. Following Southern analysis of isolated cell clones, tumorigenicity and morphological alterations will be tested for, as will the continued functionality and expression of the transfected sequences. These experiments should further elucidate the events necessary to render SV80 cells tumorigenic and they will also help to define the steps needed to render ESH20 cells tumorigenic.

The analysis of mRNA populations in the hybrid family will continue, using as probes, ten onc genes now collected in our laboratory. This will allow us to determine if the non-tumorigenic nature of the hybrids correlates with suppression of any particular cellular onc gene, thus suggesting a simple mechanism for the suppression.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05347-02 LMO

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Gene Regulation Mediated by Transcription Initiation and Termination

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

PI:	M. Zuber	Visiting Fellow	LMO	NCI
Others:	D. L. Court	Research Biologist	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

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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.)*

Regulation of early gene expression in phage λ is mediated by termination and antitermination of transcription. Towards a better understanding of the transcription termination/antitermination mechanism, the Rho-dependent, tRI terminator, along with its regulatory sequences, was cloned onto a plasmid. Deletions of various lengths into the terminator segment were generated in vitro; the deletion end points were sequenced. Deletions that remove the terminator (or part of it) express a distal gene at higher levels than when transcription terminates. Interestingly, a region far upstream to the tRI terminator and its regulatory sequences is shown to be required for the process of transcription termination. This new, and as yet unreported, DNA region is believed to be a possible Rho protein interaction site. The regulatory factor, N, antiterminates transcription from nutR, a site located in the tRI region. In order to study the effect of the same deletions on the λ N protein-mediated, transcription antitermination activity, a Rho-independent, tI terminator was cloned in between the tRI terminator and the distal structural gene. N protein was provided from a single copy of λ on the E. coli chromosome. Interestingly, this amount of N allowed complete antitermination at tRI alone. The combination of tRI and tI, however, was only read through 10% of the time. In other contexts, tI alone is read through 100% when acted upon by N.

Gene regulation at the level of transcription initiation was studied with the λ pRE promoter. The λ pRE promoter requires positive activation by the λ cII protein. Mutations generated in vitro in this promoter relieved it from its dependence on cII protein for activation. In addition, cII now repressed the cII-independent character of the mutant.

PROJECT DESCRIPTION

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

M. Zuber	Visiting Fellow	LMO	NCI
D. L. Court	Research Biologist	LMO	NCI

Objectives:

We are chiefly interested in gene regulation in phage λ at the level of transcription initiation and termination. Studies involve examination of the regulatory functions and DNA sequences which affect initiation and termination.

Transcription antitermination positively regulates the early gene expression in phage λ. The antitermination machinery comprises both phage- and host-encoded proteins. The phage-encoded N gene product somehow modifies the transcription process at a specific sequence on the phage genome called "nut" site (for N utilization) so that the RNA polymerase can read through the transcription terminator signal at the Rho-dependent, tRI terminator site. A host protein nusA is also involved in the termination/antitermination process.

Two types of terminators exist: those that can terminate with RNA polymerase alone and those that require a host protein, Rho, in addition. Although the structure of Rho-independent terminators is well understood; very little is known about the Rho-dependent terminators. The project we have undertaken emphasizes experimentation towards a better understanding of the transcription termination/antitermination mechanisms at the Rho-dependent, tRI terminator.

Methods Employed:

Standard microbial, genetic, biochemical and recombinant DNA techniques.

1. In vitro generation of deletions in DNA using ExoIII+S1 or Bal 31.

2. DNA sequencing techniques of Maxam & Gilbert (PNAS 74: 560-564, 1977).

3. Standard cloning techniques: phosphatase, filling in, S1 treatment, ligation transformation.

4. Construction of the bacterial strain following standard genetic techniques.

5. Galactokinase enzyme assay.

Major Findings:

1. The Rho-dependent, tRI terminator of phage λ along with two regulatory sequences, box A and nutR, were cloned into a plasmid. The box A and nutR sequences are the proposed sites for host nusA action and for λ N action,

respectively. The tRI terminator reduces transcription between the gal promoter and the galactokinase gene on the plasmid to 20-30% of the level without termination.

2. In vitro deletions extending to different lengths into the transcriptional regulatory sequences were made with exonuclease III and S1 endonuclease. DNA sequencing determined the deletion end points.

3. The deletion mutants were cloned onto the vector (see 1.) to examine the effect of the terminator and the respective deletions on the expression of the galactokinase gene downstream. Deletions that remove the terminator (or part of it) express the distal gene at higher levels than when transcription terminates at the terminator. A new, and as yet unreported, DNA region far upstream to the tRI terminator and its regulatory sequences was shown to be required for transcription termination.

4. A pair of bacterial strains was constructed. One expressed λ N product from a prophage. The other lacked λ N function. The effect of N function on the normal terminator and the deletions is examined.

5. N protein completely relieves the tRI terminator activity. A second terminator, t_I, placed distal to tRI is not antiterminated completely by N.

6. Mutations were generated in vitro in the promoter for repressor establishment of lambda (pRE). One of these mutations relieved this promoter from its dependence on the positive activator protein, cII.

7. The presence of cII represses the cII-independent promoter.

Significance to Biomedical Research and the Program of the Institute:

The expression of certain genes is turned on in cancer cells. 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. Information learned here is applied to problems concerning cancer development through interaction with other groups in the Laboratory of Molecular Oncology.

Proposed Course:

1. Studies are in progress to determine in further detail the newly-recognized DNA region necessary for termination activity at tRI and to determine if this new DNA sequence is a possible Rho interaction site.

2. To study why the N protein completely relieves the tRI terminator activity, while only a partial antitermination is achieved under tRI + t_I conditions.

3. To transfer all of the deletions, with and without the t_I terminator, onto the chromosome, and to study the effect of the deletions on the expression of the distal structural gene in single copy.

4. Studies are now in progress to study the termination activity of deletions generated from the 3' end of the tRI terminator.

5. To clone the t_I terminator in between these 3' deletions and the structural gene downstream, and to study the antitermination activity of N on these deletions.

6. To transfer the cII-independent pRE mutation to λ from the plasmid to study its effect on λ biology.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05359-01 LMO

PERIOD COVERED

October 1, 1983 to September 30, 1984

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. Shih	Research Chemist	LMO	NCI
	L. Ulsh	Microbiologist	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.30	1.0	0.30

CHECK APPROPRIATE BOX(ES)

☐ (a) 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 ras oncogene, which is responsible for tumorigenesis, has been cloned into an E. coli expression vector, and its gene product can be obtained in a large amount which is sufficient for biochemical and enzymological analyses. Using this protein, we will be able to characterize the biochemical activity of ras oncogene products in growth control and receptor-mediated signal transfer. We will also make monoclonal antibodies against the synthetic peptides, which are expected to be able to detect the mutations in ras genes. These antibodies will be a powerful tool for diagnosis of cancer at the biochemical level.

PROJECT DESCRIPTION

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

S. Hattori	Visiting Fellow	LMO	NCI
T. Shih	Research Chemist	LMO	NCI
L. Ulsh	Microbiologist	LMO	NCI

Objectives:

Recent progress in DNA-mediated gene transfer techniques (transfection) revealed that mutations in ras oncogenes are one of the causes of cellular transformation into the cancerous state. Therefore, it is of primary importance to characterize the biochemical properties of the ras oncogene product, p21. For this purpose, the ras oncogene product expressed in E. coli will enable one to obtain unambiguous data, since this system is completely devoid of any eukaryotic cellular protein.

It is also important to develop biochemical systems for the diagnosis of cancer. Towards this end, we are attempting to obtain monoclonal antibody which can recognize a mutation in the ras oncogene, and then to develop potential serological procedures applicable to tumor diagnosis.

Methods Employed:

Immunoprecipitation is being employed for the detection of ras gene products and cellular protein(s); these may have specific interaction(s) with ras oncogene products. We are making site-specific monoclonal antibodies to precisely determine the active site of the protein. These antibodies should also be useful in the diagnosis of specific tumors where ras oncogenes are expressed.

Site-specific mutagenesis of the cloned ras gene in expression vectors is being employed. This will clarify the function and biochemical properties of the ras oncogene product.

Major Findings:

The ras oncogene product expressed in E. coli was purified to apparent homogeneity by gel filtration and affinity chromatography. The yield and the purity of the protein were both sufficient for biochemical characterization. The purified protein was also used as an antigen to obtain specific monoclonal antisera.

Significance to Biomedical Research and the Program of the Institute:

Although the mutations in ras oncogenes have been identified as one of the cause of cellular transformation, little is known about the mechanism of transformatio at the biochemical level. The p21 coded for by ras oncogenes has the unique properties of guanine nucleotide binding and autophosphorylation in the presence of GTP. Therefore, a more detailed understanding of the biochemical properties

of ras oncogene products will give us some insight into how a cell is malignantly transformed and how cell growth is controlled by its interaction with this oncogene protein.

Proposed Course:

The proposed course is to pursue the biochemical function of the ras oncogene product(s) and its role in cellular transformation and in the control of cell growth. The project will be approached using the techniques of recombinant genetics coupled with hybridoma-monoclonal immunology.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05360-01 LMO

PERIOD COVERED
November 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Identification of Genes and Gene Products Responsible for Cell Transformation

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
	N. C. Kan	Visiting Fellow	LMO	NCI
	D. G. Blair	Research Chemist	LMO	NCI
	T. S. Papas	Acting Chief	LMO	NCI

COOPERATING UNITS *(if any)*
Pediatric Oncology Department, Sloan Kettering Memorial Hospital, New York, NY (L. Helson); Univ. North Carolina, Chapel Hill, NC (G. Haughton and C. Pennel); Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (F. Tamanoi).

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Cellular Transformation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.05	1.05	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.)*

Oncogenes appear to be involved in several kinds of cancer in animals and man. An understanding of their function(s) and their alterations in neoplasia is still required. Toward this goal, we are studying the oncogenes present in MC29 and MH2 viruses following genetic, biochemical and physiological approaches. Deletion mutants of the mht and myc oncogenes have been constructed (see Project Number Z01CE05239-03 LMO) and their transformation efficiency is measured in transfection assays. The role of different portions of these oncogenes in transformation can, therefore, be assessed and correlated with the expression of truncated, oncogenerelated proteins. Such proteins have been identified in transformed/transfected cells by using specific antibodies. Similar antibodies enable us to immunoprecipitate the cellular counterparts of viral oncogenes in normal or in malignant cells. A more physiological characterization of the myc oncogene is also obtained, taking advantage of the known DNA binding properties of the myc protein. Different DNA's are utilized in order to measure specific and/or non-specific binding of the the myc protein. The possible role of oncogenes and protooncogene abnormalities in non-virally-induced cancers has also been studied in human neuroblastoma and mouse lymphoma.

PROJECT DESCRIPTION

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

U. Rovigatti	Visiting Scientist	LMO	NCI
J. P. Bader	Research Microbiologist	LMO	NCI
N. C. Kan	Visiting Fellow	LMO	NCI
D. G. Blair	Microbiologist	LMO	NCI
T. S. Papas	Acting Chief	LMO	NCI

Objectives:

To identify portions of viral and cellular oncogenes related to MC29 and MH2 virus oncogenes which are necessary for cell transformation. To identify transforming viral or cellular oncogenes in the NIH/3T3 cell transfection assay. To identify oncogene-related proteins in transformed/transfected cultured cells and in tumor cell lines. To express oncogene-related proteins in bacteria with plasmid expression vectors and to raise antibodies against such proteins. To study alterations in the cellular myc oncogene occurring in different human and mouse tumors.

Methods Employed:

A. Proviral DNA sequence cloning in bacterial plasmids and generation of deletions by restriction enzyme cutting and ligation or by exonuclease treatment.

B. Transformation of cells in culture by avian and murine sarcoma viruses. Transforming virus rescue by superinfection with helper viruses or by fusion with replication-competent cells. Selection of transformed cells by growth in suspension and by morphological criteria.

C. Transfection assays into chicken embryo fibroblasts by superinfection with helper viruses or by cotransfection with cloned helper virus DNA. Micromanipulation of single cells/foci.

D. Southern blot analysis of transfected clones and of animal or human tumors. Northern blot analysis of oncogene expression in transformed/normal cell lines.

E. Expression of oncogene-related proteins in bacteria by using plasmid expression vectors. Immunoprecipitation of proteins and polyacrylamide gel electrophoresis analysis.

F. Primary culture of normal cells. Tumor cell culture and tumor cell line formation.

G. DNA binding assay for oncogene products. Binding assay for specific DNA sequences.

Major Findings:

The efforts of many laboratories in the last ten years have associated genetic elements called oncogenes with neoplastic transformation. Even if the involvement of viral and cellular oncogenes in particular tumors appears to be well established, we still know very little about how oncogenes work. Many efforts in the Laboratory of Molecular Oncology are concentrated on establishing the role(s) and function(s) of the viral and cellular myc oncogene(s) in neoplastic transformation. Toward this goal, our particular project aims to identify properties and define functions of the myc oncogene in transformation by following three complementary strategies:

1) Transforming sequences related to the oncogenes of MC29 and MH2 have been analyzed in the transfected clones. We are particularly interested in the proteins encoded by such oncogenes. MC29 and MH2 viruses both contain myc oncogene related proteins, which are linked to a portion of the gag protein only in the case of MC29. The expression of such proteins can then be monitored by using specific antibodies against different portions of the oncogene protein. Such oncogene-specific antibodies have been and are being developed. We have recently expressed in bacteria a human cDNA clone containing cellular myc-related sequences; antibodies are being prepared against this expressed onc gene product. Antibodies against such polypeptides should enable us to study normal and abnormal c-myc-related proteins expressed in human cells.

2) Several animal and human tumors have been analyzed for the presence of altered c-myc sequences, using Southern blot and DNA-mediated transfection assays. We have focused our attention on human neuroblastoma and myeloid leukemia cell lines, where we find that the c-myc oncogene is amplified, i.e., it is present in several copies/genome. Amplification at the DNA level is studied in relationship to the increased expression of the c-myc protein(s). A complementary assay has been performed by transfecting the same human tumor cell DNA into NIH/3T3 cells and, also, into our particular strain of NIH/3T3 cells. Recently, we have obtained a similar finding, also in a mouse lymphoma, where c-myc sequences appear to be amplified and rearranged. In vivo and in vitro studies of this lymphoma and its c-myc-related proteins are now being performed, and cloning and sequencing of the amplified human and mouse c-myc genes are in progress.

Significance to Biomedical Research and the Program of the Institute:

The last ten years have seen revolutionary developments in cancer research, which has culminated in the association of several different oncogenes, present in retroviruses, with animal and human cancers. Even if this association between oncogenes and cancer is well-documented, we still lack much knowledge of the mechanism(s) by which viral oncogenes might induce malignant transformation or the mechanism by which proto-oncogenes might trigger neoplasia. The system we are using in order to approach these problems is quite unique and well-characterized. The LMO was the first laboratory to clone and sequence the oncogenes present in MC29 and MH2 viruses. The genetic approach we utilize (i.e., generation of deletion mutants) should answer important questions about the mechanism of carcinogenesis of individual oncogenes (mht, myc), or about the cooperative effects between two oncogenes, such as is found in the MH2 virus.

Furthermore, biochemical and physiological approaches will characterize these oncogene products, which should shed some light on the mechanism(s) of carcinogenesis. The search for a specific function, for example, for a non-random DNA binding site of the myc protein, is still unresolved and an important goal of the Laboratory and the field of molecular oncology. Finally, the study of oncogene involvement in naturally-occurring tumors could clarify the pathogenesis of nonvirally-induced animal and human cancers, and has important implications for the possible diagnosis, and ultimate control of neoplastic diseases.

Proposed Course:

Genetic approaches have been very fruitful in oncogene studies and we expect to obtain results using powerful recombinant genetic tools: transfection studies of deleted oncogenes should provide us with important answers about oncogenetic mechanisms and the usage of gene expression vectors is providing us with several valuable probes (i.e., the human c-myc protein and specific antibodies against it). Biochemical and physiological assays are being designed for the myc-related proteins present in transformed or uninfected cells, as well as for such proteins expressed in bacterial expression vectors. The analysis of human neuroblastomas and leukemias and of mouse lymphomas has been initiated and will be extended; with the characterization of genetic abnormalities, at the gene expression level, both biochemically and physiologically.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05361-01 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

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)*

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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 very simple model system, the cellular slime mold, Dictyostelium discoideum, is being used to study mechanisms which control developmental gene activation during normal differentiation. Post-aggregation Dictyostelium cells transcribe an additional 26% of their genome, which is not expressed in earlier pre-aggregation-stage cells. Our previous studies have indicated that cell-cell interaction is a necessary prerequisite for the synthesis and stability of the late messenger RNAs. Following activation, the actual rate of transcription and the subsequent stability of many of the messenger RNAs transcribed off of this portion of the genome are further regulated by a cyclic AMP-mediated process. In these studies we have better defined the nature of the requirement for cell-cell interaction and have further attempted to develop conditions under which we can use transformation and in vitro mutagenesis to identify control sites involved in cAMP mediated regulation of developmental gene expression.

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, these terms: 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 Z01CE05288-03 LMO), we wanted to develop a system which would allow us to better define the biochemical nature of these phenomena. As a graduate student in Japan, I worked with K. Okamoto and I. Takeuchi to develop such a system. 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 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 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) Analysis of conditions which promote differentiation, and demonstration that these conditions induce prespore- and prestalk-specific mRNA accumulation.

1) Cells must pass through a specific period during early development in order to differentiate in shaken culture: Cells starved and plated for development for two hours 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. These cells also fail to accumulate any of the late mRNA species, indicating that the failure to differentiate is a transcription-level event. By contrast, cells plated for development for 4-6 hours (late rippling stage--but prior to the formation of tight cell contacts) will differentiate and express late mRNAs provided that cAMP is present and the cells in the suspension culture can form clumps. Thus, during the interval between 2 and 6 hours of development, the cells acquire the competence to differentiate. A cAMP-dependent protein kinase has recently been identified and is thought to be synthesized during this period. A question of considerable interest is whether cAMP functions to regulate the transcription rate and subsequent mRNA stability via a protein kinase intermediate. Experiments utilizing specific inhibitors of the cAMP protein kinase are planned to determine if the synthesis of this enzyme is the basis of the competence to differentiate that is acquired between 2 and 6 hours.

2) The NH_4^+ ion can relieve the requirement for multicellularity (clump formation): Cells which are competent to differentiate require both cAMP and clump formation. Disruption of clump formation, even in the presence of cAMP, prevents differentiation. However, if differentiation-competent cells supplemented with cAMP and maintained as single cells are additionally supplemented with 30mM $(NH_4)_2SO_4$, both prespore and prestalk mRNAs can be induced to accumulate. 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 NH_4^+. Thus, it is not surprising that NH_4 should play a role in coordinating developmental gene expression with the metabolic state of the cell.

3) An additional class of stalk-specific genes is not inducible by either NH_4^+ or cAMP: The majority of differentiation-specific, late genes have fallen into two categories. One group contains prespore genes and genes common to both spore and stalk pathways, whose transcription is initiated by the cAMP-NH_4^+ controls discussed above. A second, somewhat less frequently found class of differentiation specific genes is expressed at a very low level in growing cells and induced by the cAMP-NH_4^+ controls to accumulate only in the prestalk cells. We have now identified a third class of late genes which are stalk-specific and do not respond to either cAMP or NH_4^+. In fact, accumulation of mRNA for this class of genes is specifically blocked when developing cells are dissociated into the suspension culture regardless of whether the cells are supplemented with cAMP, NH_4^+, or allowed to form clumps. Very preliminary experiments utilizing conditons for priming the cells for differentiation that cause the clumped cells to secrete a slime sheath, however, does result in accumulation of this class of stalk-specific mRNA. This is in agreement with earlier morphological evidence that indicates that, while prespore and prestalk differentiation can occur in the

suspension culture conditions, induction of genes required for the terminal stages of stalk cell differentiation require additional components or are inhibited by the culture conditions. Interestingly, in in vivo development, it is the stalk cells and not the spore cells which remain encased in the slime sheath during terminal differentiation; thus, the coordinate ability to induce slime sheath and express this late class of stalk genes may not be mere coincidence.

4) Possible identification of conditions which may induce pathway-specific gene expression: The three requirements that we have identified: passage of cells through the appropriate time interval in early development, cAMP, and NH_4^+, are all necessary for expression of most differentiation-specific genes. However, they do not appear to impart pathway specificity. Both prespore- and prestalk-specific genes are stimulated equally. Earlier work in Japan has indicated that prespore-specific antigens and enzymes can be induced by $MgSO_4$, while expression of prestalk proteins is inhibited. Additionally, Ca^+ was shown to inhibit prespore differentiation. Experiments are in progress to determine if pathway-specific mRNAs can be induced to accumulate under these ionic conditions. Preliminary results have indicated that, while Ca^+ may inhibit the expression of prespore specific proteins, prespore mRNAs accumulate normally in the presence of Ca^+; thus, this may represent a post-transcriptional block.

C) Development of a transformation system for identification of control regions which allow prespore and prestalk cells to respond to environmental stimuli.

Since we have identified culture conditions where appropriately primed, single cells can be induced by NH_4^+ or cAMP to synthesize prespore- and prestalk-specific mRNAs, and since we have now obtained genomic clones encoding these genes and their flanking regions (see Project Number Z01CE05288-03 LMO), we are in a position to start to use in vitro mutagenesis to identify the control sites responsible for this part of the regulation of the expression of these genes. In preliminary experiments, we have used a bacterial $CaCl_2$ transformation procedure to introduce into Dictyostelium cells the dominant, selectable marker encoding resistance to the drug G418, carried on the SV40-derived vector, pSV2-Neo. Maintenance of this plasmid by the Dictyostelium cells is very dependent upon growth conditions. Cells grown on synthetic media cannot maintain the plasmid efficiently. In nature, Dictyostelium grows very rapidly on bacteria. We have, therefore, isolated a mutant of E. coli, K12, resistant to 80 μg/ml of G418. When Dictyostelium cells transformed with pSV2-Neo are plated directly onto a lawn of G418-resistant E. coli on agar plates containing 80 μg/ml of G418, amoeba resistant to G418 grow and form single colonies, which leave a round clearing in the bacterial lawn resembling a very large bacteriophage plaque. Our preliminary results indicate that Dictyostelium cells are transformed with the pSV2-Neo vector at a frequency of 1 cell in 2,000. Mock-transformed cells, which have received pBR322 or λ DNA, form colonies at a ten-fold lower frequency of approximately 1 cell in 20,000. We have also adapted the bacteriophage plaque lifting procedure to the Dictyostelium colonies and can detect DNA in the Dictyostelium plaques, which is present at 200 copies per cell. We have not yet tried to detect lower copy number sequences. We feel, however, that we potentially have a rapid way to determine biochemically that the G418-resistant phenotype results from maintenance of the pSV2-Neo plasmid. Encouraged by these preliminary

results, a student who will be joining us for the summer will continue to develop this transformation system. It has been found for other lower eukaryotic cells that the viral RSV LTR can be utilized five to ten-fold more efficiently as a promoter than the SV40 sequences. We are planning to try this, as well as other vector systems, including the BPV episome, to try to improve the efficiency of the transformation system.

Significance to Biomedical Research and the Program of the Institute:

Abnormalities in differentiation and developmental gene expression are characteristic of the malignant cell. 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 and proportion regulation, can control the expression of different groups of coregulated genes during differentiation. It is important to note that teratocarcinoma cells are very prone to such regulation, being malignant in one environment and yet differentiating normally in another.

Proposed Course:

We have now determined that multicellularity, itself, is not necessary for differentiation-specific gene expression, but that the elevated concentration of NH_4^+, which is found in the tightly contacted cell clumps, together with cAMP, is sufficient to induce late gene expression, provided that the cells have passed through a short interval of time early in development. Our future experiments will be directed towards 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 transacetylase (CAT) or β-galactosidase. If we can get the cells to express CAT or β-galactosidase in response to cAMP and NH_4^+, we will then be able to utilize in vitro mutagenesis to identify the sites on the genes where these controls act. In conjunction with 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 NH_4^+, can coordinately regulate both the transcription rate and the stability of these mRNAs. If we can define conditions in our culture system which will induce spore-specific or stalk-specific differentiation, we should be able to utilize the same techniques to identify additional controls involved in determining pathway-specific differentiation.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE08718-06 LMO

PERIOD COVERED
October 1, 1983 to September 30, 1984

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:	S. E. Bear	Staff Fellow	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)

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:
0.70	0.70	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.

PROJECT DESCRIPTION

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

D. L. Court	Research Biologist	LMO	NCI
S. E. Bear	Staff Fellow	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) nonterminating. 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, 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.

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 clone the genes

for RNaseIII, Pnpase, and RNaseI of E. coli. (4) To isolate t_I termination mutants. (5) To analyze the mechanism of post-transcriptional control mechanisms, i.e., endo- and exo-ribonuclease control systems in E. coli. (6) 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. (In Press)

Court, D., Schmeissner, U., Bear, S., Rosenberg, M., Oppenheim, A. B., Montanez, C. and Guarneros, G.: Control of λ int gene expression by RNA processing. In Hamer, D. H. and Rosenberg, M. J. (Eds.): Gene Expression, ICN-UCLA Symposia on Molecular and Cellular Biology. New York, Alan R. Liss, Inc., 1983, Vol. 8, pp. 311-326.

Lautenberger, J. A., Court, D. L. and Papas, T. S.: High level expression of Escherichia coli of the carboxy-terminal sequences of the avian myelocytomatosis virus (MC29) v-myc protein. Gene 23: 75-84, 1983.

Lautenberger, J. A., Kan, N. C., Court, D. L., Pry, T., Showalter, S. and Papas, T. S.: High-level expression of oncogenes in Escherichia coli. In Papas, T. S., Rosenberg, M. and Chirikjian, J. G. (Eds.): Gene Amplification and Analysis: Expression of Cloned Genes in Prokaryotic and Eukaryotic Cells. New York, Elsevier/North Holland, Inc., 1983, Vol. 3, pp. 147-174.

Mizusawa, S., Court, D. and Gottesman, S.: Transcription of the SulA gene and repression by LexA. J. Mol. Biol. 171: 337-343, 1983.

Schmeissner, U., McKenney, K., Rosenberg, M. and Court, D.: Removal of a terminator structure by RNA processing regulates int gene expression. J. Mol. Biol. (In Press)

Schmeissner, U., McKenney, K., Rosenberg, M. and Court, D.: Transcription terminator involved in the expression of the int gene of phage lambda. Gene (In Press)

ANNUAL REPORT OF

LABORATORY OF MOLECULAR VIROLOGY

NATIONAL CANCER INSTITUTE

October 1, 1983 through September 30, 1984

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 cellular proteins 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 effects of viral infection on cellular control mechanisms in transformation and in lytic responses; 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.

Among the primary objectives in the Laboratory of Molecular Virology is the elucidation of signals associated with gene expression. 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 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. Experiments have been designed in an attempt to elucidate the mechanism by which the activator/enhancer sequences function.

A principal interest in our laboratory for the future will be the definition of 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.

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 against "aberrant" cells by the immune system.

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, may be a tolerogenic form of the class I antigens, acting as a blocking factor to regulate the function of cytotoxic T-cells in the process of immune surveillance. Studies are in progress to confirm this hypothesis by using this secreted class I antigen to modulate T-lymphocyte recognition of tumor cells.

A considerable effort has been directed towards obtaining nucleotide sequences of murine and human oncogenes. 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 isolate proto-oncogenes and to study their structure and expression in growing and differentiating cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05101-06 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one lina batwaen 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:	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:
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 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 this multifunctional SV40 T-antigen.

PROJECT DESCRIPTION

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

Gilbert Jay	Chief, Cell Physiology Section	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 primary human epidermal keratinocytes by the combined action of SV40 and the ras transforming genes. Nature (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05214-04 LMV

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 cherecters 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:	George Khoury	Chief	LMV	NCI
Others:	John Brady	Expert	LMV	NCI
	Laimonis Laimins	Staff Fellow	LMV	NCI
	Nadia Rosenthal	Staff 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:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

An unusual and unique eukaryotic transcriptional control element which markedly increases the transcriptional activity of eukaryotic genes is the activator or enhancer element. We first characterized the enhancer elements in the genomes of simian virus 40 (SV40) and the Moloney sarcoma virus (MSV) and demonstrated that these sequences act in a host-cell specific fashion. This implies a potential for tissue-specific gene expression. The core activity of the enhancer has been localized to a very short stretch of nucleotides within the SV40 72 bp repeat. In attempting to identify cellular enhancer elements, we have investigated sequences in the putative control regions (the long terminal repeats or LTRs) of endogenous retroviruses. Certain of these cloned sequences appear to manifest low levels of enhancer activity.

PROJECT DESCRIPTION

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

George Khoury	Chief	LMV	NCI
John Brady	Expert	LMV	NCI
Laimonis Laimins	Staff Fellow	LMV	NCI
Nadia Rosenthal	Staff 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.

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. Although we have confirmed the association of proteins with the GC-rich sequences, we have been unable to find proteins which specifically interact with the 72 bp repeats.

3. Long terminal repeats (LTR)-like sequences have been indentified in endogenous, retrovirus-like elements. The control regions of these LTR-like sequences have been shown to contain enhancer elements.

Significance to Biomedical Research and the Program of the Institute:

The understanding of the molecular mechanisms leading to the initiation of transcription of eukaryotic genes is important in the understanding of transforming genes and cellular differentiation.

Proposed Course:

These projects will continue with an emphasis on the identification of the mechanism by which enhancer elements control gene expression.

Publications:

Hamer, D. H. and Khoury, G.: Introduction. In Gluzman, Y. and Shenk, T. (Eds.): Enhancers and Eukaryotic Gene Expression. New York, Cold Spring Harbor Laboratory, 1983, pp. 1-15.

Laimins, L. A., Kessel, M., Rosenthal, N. and Khoury, G.: Viral and cellular enhancer elements. In Gluzman, Y. and Shenk, T. (Eds.): Enhancers and Eukaryotic Gene Expression. New York, Cold Spring Harbor Laboratory, 1983, pp. 28-37.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05216-04 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
DNA Sequences of Important Tumor Virus Genetic Elements

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

PI:	Ravi Dhar	Visiting Scientist	LMV	NCI
Others:	Amelia Nieto	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-related genes have been isolated from yeast Saccharomyces cerevisiae using mammalian Harvey ras DNA as a probe. These have been named c-ras-sc-1 and c-ras-sc-2. We have obtained a complete DNA sequence of both the yeast ras genes. The DNA sequence predicts encoded polypeptides of 40,000 daltons for c-ras-sc-1 and 41,100 for c-ras-sc-2. Of their N-terminal 90 amino acids, the yeast ras genes retain more than 80% of homology with other mammalian ras genes. Their c-termini, however, have diverged.

We have used c-Ha-ras and c-Ki-ras probes to study the expression of these genes at the transcriptional level in a developmental rat mammary gland system. We have observed an increase in the concentration of c-Ha-ras RNA in the mammary gland during gestation, whereas during lactation it drops to about one-fifth of that level. The levels of c-Ki-ras RNA remains constant during the development stage.

PROJECT DESCRIPTION

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

Ravi Dhar	Visiting Scientist	LMV	NCI
Amelia Nieto	Visiting Fellow	LMV	NCI
Richard Koller	Biologist	LMV	NCI

Objectives:

Our major objectives are:

1) To determine the DNA sequence of yeast ras genes and to correlate the amino acid sequence with potential biological functions.

2) To determine the evolutionary relationship among different ras genes.

3) To study transcriptional regulation of the ras genes in growing and differentiating tissues.

Methods Employed:

Recombinant DNA technology; Maxam and Gilbert DNA sequencing; Southern and Northern blot analysis.

Major Findings:

The two ras-related yeast genes c-ras^{sc-1} and c-ras^{sc-2} encode polypeptides of 40,000 and 41,000 daltons, respectively. The N-terminal 90 amino acids from both yeast genes share 80% homology with ras genes of other vertebrates. The majority amino acid differences in this region represent conservative changes that would not affect secondary structure. The c-ras^{sc-1} and c-ras^{sc-2} genes encode 7 additional amino acids at their N-termini and 117 and 129 additional c-terminal amino acids, respectively, relative to the homologous proteins of higher eukaryotes.

The ras-encoded amino acids 12 and 61 are known mutation sites for activation of ras oncogenes in different tumors. The yeast ras genes encode glycine at amino acid 12 and glutamine at amino acid 61 analogous to the amino acids found at these positions in normal mammalian p21.

We have analyzed the transcripts of both Ha- and Ki- ras genes in various tissues during differentiation. The concentration of Ha-ras transcripts increases during pregnancy in the mammary gland and falls during lactation, whereas the concentration of Ki-ras transcripts remains constant during mammary gland development. We have analyzed RNA in MTW9 tumors during active growth and regression and have found no changes in the concentration of Ha- and Ki-ras transcripts.

Significance to Biomedical Research and the Program of the Institute:

Activation of ras genes has been associated with certain human tumors. Analysis of these genes and their transcriptional regulation should provide a better understanding of their potential role in human cancer.

Proposed Course:

We plan to study the expression of yeast c-ras genes under the control of an inducible regulatory element which allows for the overproduction of the gene product; any phenotypic changes associated with overproduction will be studied.

Publications:

DeFeo-Jones, D., Scolnick, E., Koller, R. and Dhar, R.: ras-related gene sequences identified and isolated from Saccharomyces cerevisiae. Nature 306: 707-709, 1983.

Dhar, R., Nieto, A., Koller, R., DeFeo-Jones, D. and Scolnick, E.: Nucleotide sequences of two ras^H-related genes isolated from yeast Saccharomyces cerevisiae. Nucleic Acids Res. 12: 3611-3618, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05217-04 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

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

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:
1.0	1.0	0

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

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

The agnoprotein is a late SV40 gene encoding a 61 amino acid polypeptide of unknown function. Mutant C1-5, engineered by a site-directed mutagenesis, is a 2-base insertion at the unique HpaII site located within the agnogene. Analysis of plaques formed by transfection of DNA from mutant C1-5 gives evidence of a second mutation. These revertants which occur at high frequency result from base alterations at or near the site of the original mutation and appear to acquire a functional agnoprotein.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations 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

Objectives:

To study the function of the simian virus (SV40) agnoprotein.

Methods Employed:

Cesium-chloride-ethidium-bromide equilibrium centrifugation; restriction endonuclease analysis; immunoprecipitation; SDS-polyacrylamide gel electrophoresis; immunofluorescence.

Major Findings:

Rapid growth of plaques arising from transfection of C1-5 DNA after an apparent lag period suggested the possibility that reversion may have taken place. These plaques were found to contain second site mutations at or near the site of the original agnoprotein mutation in vivo. These revertants induced a functional agnoprotein as demonstrated by immunofluorescence, which was localized predominantly in the perinuclear and cytoplasmic regions. The agnoprotein observed in revertants seemed to migrate somewhat faster in SDS-polyacrylamide gel electrophoresis than that produced by wild-type SV40.

Significance to Biomedical Research and the Program of the Institute:

SV40 replication in mutant C1-5 is dependent upon "reversion." Our observations suggest that the agnoprotein plays an important role on the efficient SV40 life cycle. Further investigation of these mutants will hopefully provide an understanding of the role of the agnoprotein in SV40 gene expression.

Proposed Course:

We will investigate the alterations observed in each "revertant" by DNA sequencing. This will include analysis of the biological and biochemical properties of the revertants. Characterization of the acquired functional agnoprotein synthesized by these mutants will be performed. A search for an endogenous agnoprotein equivalent in cells will be initiated.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05219-04 LMV

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 charecters 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 professionel personnel below the Principel 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.

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, E., Jay, F. and Jay, G.: Comparison of different synthetic ribosome binding sites for the efficient expression of eukaryotic proteins in Escherichia coli. In Papas, T. S., Rosenberg, M. and Chirikjian, J. (Eds.): Expression of Cloned Genes in Prokaryotic and Eukaryotic Vectors. New York, Elsevier/North-Holland, Inc. 1983, pp. 89-101.

Jay, E., Rommens, J., and Jay, G.: Synthesis of mammalian proteins in bacteria. In Cheremisinoff, P. N. and Ouellette, R. P. (Eds.): Biotechnology Handbook. Ann Arbor, Butterworth (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05220-04 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 charactars or lass. Title must fit on one line between the borders.)*
Studies on the Structure and Function of Cell Surface Antigens

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

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Michel Kress	Visiting Fellow	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:
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 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 Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Michel Kress	Visiting Fellow	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 (Proc. Natl. Acad. Sci. USA 79: 4947, 1982). 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 between these genes (J. Biol. Chem. 258: 13929, 1983). The availability of such specific probes forms the basis for future studies directed towards defining the mechanism of regulation of 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 (Nature 306: 602, 1983). 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 significanly 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 (Nature, in press). 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 have 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:

Cosman, D., Khoury, G. and Jay, G.: Three classes of mouse H-2 messenger RNA distinguished by analysis of cDNA clones. Nature 295: 73-76, 1982.

Kress, M., Barra, Y., Seidman, J. G., Khoury, G. and Jay, G.: Functional insertion of an Alu type 2 repetitive sequence in class I genes from the H-2D subregion of the mouse major histocompatibility complex. Nature (In Press)

Kress, M., Glaros, D., Khoury, G. and Jay, G.: Alternative RNA splicing in the expression of the H-2K gene. Nature 306: 602-604, 1983.

Kress, M., Liu, W. -Y., Jay, E., Khoury, G. and Jay, G.: Comparison of class I (H-2) gene sequences: Derivation of unique probes for members of this multigene family. J. Biol. Chem. 258: 13929-13936, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05253-03 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Early Control Region of BKV

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

PI:	Nadia Rosenthal	Staff Fellow	LMV	NCI
Others:	George Khoury	Chief	LMV	NCI
	Michel Kress	Visiting Fellow	LMV	NCI

COOPERATING UNITS *(if any)*
Department of Microbiology, University of Heidelberg, West Germany (P. Gruss)

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	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 enhancer element of human papovavirus BKV has been characterized by testing BKV enhancer subclones in a transient expression assay. Like its monkey counterpart, simian virus 40 (SV40), the BKV enhancer activates transcription of heterologous genes in human, monkey or mouse cells. Although the BKV and SV40 enhancer sequences are different, certain shared oligonucleotide motifs may be responsible for the similar function of the two enhancer elements. Studies are in progress to characterize further the contribution of these homologous sequences to enhancer activity.

PROJECT DESCRIPTION

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

Nadia Rosenthal	Staff Fellow	LMV NCI
George Khoury	Chief	LMV NCI
Michel Kress	Visiting Fellow	LMV NCI

Objectives:

To analyze the role of BKV enhancer sequence components in enhancer function.

Methods Employed:

Isolation and cloning of specific DNA fragments; construction and characterization of SV40/pBR322 vectors; growth and isolation of cloned molecules from bacterial and animal cell cultures; mRNA extraction from virus-infected cell cultures; restriction enzyme cleavage of DNA; transfer of DNA or RNA by blotting techniques; hybridization of radioactive DNA probes to DNA or RNA blots; protein extraction from cell cultures; immunoprecipitation.

Major Findings

The enhancer element of BKV has been defined and its function characterized in human, monkey and mouse cells. The genomes of BKV and SV40 show extensive similarity except in the enhancer regions. However, comparison of the BKV and simian virus 40 (SV40) enhancer elements reveals that certain DNA oligonucleotide stretches (such as a "core" region, and GC-rich motifs) are conserved but are differently arranged in a background of unrelated sequences. The presence of similar oligonucleotide stretches in other viral and cellular enhancers suggests that they are necessary for enhancer function. We have designed experiments to test the role of these common sequences in the activation of heterologous genes by testing individual sequence components of both the BKV and SV40 enhancer regions for similar function. The advantages of this approach stem from the very close evolutionary relationship of these two viruses. By replacing a similar component of one viral regulatory element with its counterpart from the other virus, we have constructed vectors which should establish whether enhancer function relies on these common DNA sequences.

Significance to Biomedical Research and the Program of the Institute:

The presence of enhancer elements in both viral and cellular genes suggests a common mechanism of gene activation. Studies on enhancer function are therefore important not only for the elucidation of the mechanisms of viral infection and transformation, but also for our understanding of cellular gene regulation in normal and transformed cells.

Proposed Course:

The results of our initial studies on enhancer sequence components will direct the construction of further deletion and point mutations to pinpoint the crucial nucleotides responsible for enhancer activity.

Publications:

Rosenthal, N., Kress, M., Gruss, P. and Khoury, G.: BK viral enhancer element and a human cellular homolog. Science 222: 749-755, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05254-03 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

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)*
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:
1.0	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 in the laboratory. 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 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 other transcriptional regulatory elements, such as promoter sequences responsible for tissue-specific expression of insulin. In addition, upstream sequences may contain signals inhibitory for transcription. Ongoing studies will further map and characterize the potential inhibitory region and investigate their mode of action.

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 expression and regulation of insulin genes in fibroblasts and insulinoma cells.

Methods Employed:

RNA analyses by blot 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 inhibitory segments may 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 metabolism 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: Z01CE05255-03 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

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

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

PI:	George Khoury	Chief	LMV	NCI
Others:	Gordon Hager	Chief, Hormone Action and Oncogenesis Section	LEC	NCI
	Michael Ostrowski	Senior Staff Fellow	LEC	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:
0.7	0.5	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.)*

The mouse mammary tumor virus genome (MMTV) is of particular relevance to the study of gene regulation because its transcription is regulated by an interaction with a hormone-receptor complex. We have begun to analyze the nucleotide signals responsible for this regulation in two types of experiments. In short-term assays, the MMTV long terminal repeat sequences (LTRs) were coupled to the prokaryotic gene, chloramphenicol-acetyltransferase (CAT), which is a sensitive and accurate indicator of gene expression. Intact LTR sequences or deletion mutants thereof were introduced into tissue culture cells in the presence or absence of dexamethasone in an attempt to decipher those sequences responsible for hormone responsive regulation. In a second type of assay, based on transformation using similar constructs which had the transforming Harvey ras gene (p21) in the position analogous to that of CAT, we determined the transformation efficiency of the constructs harboring various mutants of these regulatory signals, in the presence or absence of steroid hormone. In general, the results of these two types of assays were in good agreement. Deletion of sequences from the 5' end of the LTR resulted in a general decrease in gene activity in the presence of the hormone, but an increase in activity in the absence of hormone. One possibility under investigation is that negative regulatory sequences suppress gene expression in the absence of the specific inducer (dexamethasone).

In a parallel set of studies, we have confirmed data from other laboratories, indicating that the upstream control signals of MMTV can regulate the activity of other heterologous promoters in a hormone-dependent fashion.

PROJECT DESCRIPTION

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

George Khoury	Chief	LMV	NCI
Gordon Hager	Chief, Hormone Action and Oncogenesis Section	LEC	NCI
Michael Ostrowski	Senior Staff Fellow	LEC	NCI

Objectives:

These studies are directed at an understanding of the manner by which hormone responsive target sequences in DNA are triggered to provide for gene activation at the transcriptional level.

Methods Employed:

Construction of chimeric genes; deletion mutagenesis; DNA transfection; protein assays.

Major Findings:

In the presence of an exogenous enhancer, deletion of MMTV LTR sequences leads to an increased constitutive expression from the MMTV transcriptional unit. Futhermore, our studies indicate that certain promoter elements of MMTV (the TATA box and cap site) are not necessary for induction.

Significance to Biomedical Research and the Program of the Institute:

Steroid hormone control of certain inducible genes is an important aspect of gene regulation which concerns a basic biological mechanism and has important implications for a number of disease states.

Proposed Course:

Site directed mutagenesis should allow a detailed analysis of the hormone inducible control region within the MMTV LTR and may provide for an understanding of positive or negative regulatory sequences within in.

Publications:

Kessel, M. and Khoury, G.: Induction of cloned genes after transfer into eukaryotic cells. In Papas, T. S., Rosenberg, M. and Chirikjian, J. G. (Eds.): Expression of Cloned Genes in Prokaryotic and Eukaryotic Cells. New York, Elsevier/North-Holland, Inc., 1983, pp. 234-260.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05354-02 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

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

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:
1.0	1.0	0

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

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

The activated form of the human proto-oncogene c-Ha-ras (EJ or T24) that was cloned from a cell line established from a bladder carcinoma is capable of transforming NIH 3T3 cells in culture. The DNA sequences involved in the control of expression of this gene are being defined. The techniques of S1 mapping and primer extension will determine the 5' end of the processed transcript. To analyze the DNA sequences that constitute the promoter element, segments 5' to the coding region of the gene will be cloned into a recombinant plasmid vector and assayed for their ability to initiate expression of a foreign gene by RNA and protein production in a transient assay system.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations 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

Objectives:

This study is directed at an examination of the DNA sequences that control expression of the human proto-oncogene, c-Ha-ras, and the corresponding oncogene isolated from a bladder carcinoma.

Methods Employed:

Isolation and cloning of specific fragments; construction of recombinant vector molecules; extraction of mRNA; S1 nuclease analysis; primer extension; Northern and Southern blotting analysis of RNA and DNA; immunoprecipitation.

Major Findings:

A human tumor cell line (T24) that was established from a bladder carcinoma has been shown to contain a gene that is capable of morphologically transforming NIH 3T3 mouse cells. Analysis of this gene has shown that it is the human cellular homologue (c-Ha-ras) of the transforming gene present in the Harvey murine sarcoma virus. Comparison of the c-Ha-ras oncogene from the tumor cell line with a normal human c-Ha-ras proto-oncogene indicates that the activation of the proto-oncogene is the result of a single base pair change in the amino acid coding region of the gene. However, the normal c-Ha-ras gene is capable of morphologically transforming NIH 3T3 cells if its level of expression is elevated through the use of a retroviral LTR promoter element. It is therefore important to gain an understanding of the nucleic acid signals that are involved in the control of the expression of the c-Ha-ras gene.

DNA sequences 5' to the coding region of the activated c-Ha-ras gene have been cloned into the recombinant plasmid $pA_{10}cat_{3M}$ at a site immediately 5' to the CAT coding sequences. This plasmid contains the coding sequences of the gene, chloramphenical-acetyltransferase, but does not contain any portion of a known eukaryotic promoter element. Transformation of this plasmid into monkey CV-1 cells followed by transient assay of CAT gene enzymatic activity has shown that a 1.25 kb fragment of c-Ha-ras DNA is capable of inducing levels of CAT activity 10-fold greater than the control plasmid, $pA_{10}CAT_{3M}$, without any insert.

Primer extension and S1 nuclease analysis will pinpoint the transcription initiation site of the c-Ha-ras gene in the T24 cell line. With these results to guide us, the c-Ha-ras promoter element will be characterized by deletion mutagenesis using CAT vectors and the transient assay system.

A second series of experiments has been initiated that is designed to examine the morphological transformation properties of the c-Ha-ras oncogene. Results of other investigators have demonstrated that two distinct oncogenes are required to transform a primary (non-established) rodent cell. Using primary rat embryo fibroblasts (REF), we have reproduced these findings and observe that maximal transformation of these cells is dependent upon the action of two oncogenes, c-Ha-ras and myc (the myc gene is the transforming gene that is present in the avian retrovirus, MC29). However, we have also observed that primary REF cells can be transformed, using the c-Ha-ras gene alone, at a much lower efficiency than when the two oncogenes are used together.

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. Information regarding the function of this gene or the control of its expression could be important to an understanding of neoplastic transformation.

Proposed Course:

The cellular transformation studies will be continued by examining the effects of expression levels of c-Ha-ras on the efficiency of transformation of primary REF cells. The c-Ha-ras gene will be fused to a variety of promoter elements which should result in various levels of c-Ha-ras expression. It is possible that the requirement of the second oncogene (myc) can be circumvented by increasing the levels of c-Ha-ras expression.

We also plan to examine the oncogene(s) required to transform primate cells to see if a different class (or classes) of genes can transform rodent cells but not primate cells. A human cell line, WI38 (primary embryonic lung fibroblast), and a monkey cell line, CV-1 (established kidney cell), will be used. Our preliminary results show that CV-1 cells cannot be transformed by the c-Ha-ras gene, the myc gene, or a combination of these two. This result suggests that the transformation of primate cells may occur by different mechanisms than rodent cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05355-02 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 oherecters 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 Investigetor.) (Name, title, laboratory, and institute affiliation)*

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Michel Kress	Visiting Fellow	LMV	NCI
	Yves Barra	Guest Researcher	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:
1.7	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.)*

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 it. 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 (Cell 36: 139, 1984). This class I gene is expressed only in the liver (Proc. Nat. Acad. Sci. USA 79: 4947, 1982) and encodes a secreted class I antigen (Cell 34: 189, 1983). 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 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 Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Michel Kress	Visiting Fellow	LMV	NCI
Yves Barra	Guest Researcher	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:

Kress, M., Cosman, D., Jay, E., Khoury, G. and Jay, G.: Molecular cloning and expression of a gene that encodes a novel transplantation-related antigen. In Pearson, M. L. and Sternberg, N. O. (Eds.): Gene Transfer and Cancer. New York, Raven Press, 1984, pp. 337-344.

Kress, M., Cosman, D., Khoury, G. and Jay, G.: Secretion of a transplantation-related antigen. Cell 34: 189-196, 1983.

Maloy, W. L., Coligan, J. E., Barra, Y. and Jay, G.: Detection of a secreted form of the murine H-2 class I antigen with an antibody against its predicted carboxy-terminus. Proc. Natl. Acad. Sci. USA 81: 1216-1220, 1984.

Mellor, A., Weiss, E. H., Kress, M., Jay, G. and Flavell, R. A.: A non-polymorphic class I gene in the murine major histocompatibility complex. Cell 36: 139-144, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05390-01 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

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
	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
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the spece provided.)*

We have studied the regulatory mechanisms responsible for the expression of MHC class I genes which encode the classical transplantation antigens. We have observed that hypomethylation of these class I genes leads to their repression and suggest that this may be the molecular basis for tumor cells to escape immune surveillance.

PROJECT DESCRIPTION

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

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Kenichi Tanaka	Visiting Associate	LMV	NCI
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:

Using cloned cells derived from F9 teratocarcinoma stem cells that have been differentiated by treatment for varying periods of time with retinoic acid, we have been able to reconstruct the various stages of induction of the H-2K gene during development. We have observed that the level of activation of this gene correlates well with the level of DNA methylation; low levels of induction during early stages of differentiation are accompanied by hypermethylation of the H-2K gene in one chromosome and high levels of induction at later stages of differentiation by hypermethylation of H-2K genes in both chromosomes. That hypermethylation of the H-2K genes responsible for their activation is demonstrated by our ability to reverse this induction process through treatment of cells with azacytidine, an inhibitor of DNA methylation.

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 identify agents that can either block hypomethylation or induce hypermethylation of DNA.

Publications:

Tanaka, K., Appella, E. and Jay, G.: Developmental activation of the H-2K gene is correlated with an increased level of DNA methylation. Cell 35: 457-465, 1983.

Tanaka, K., Ozato, K., Jay, G., Parnes, J. R., Ramanathan, L., Seidman, J. G., Chang, K. S. S. and Appella, E.: Control of H-2 antigen and β2-microglobulin gene expression in mouse trophoblast cell clones. Proc. Natl. Acad. Sci. USA 80: 5597-5601, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05391-01 LMV

PERIOD COVERED
October 1, 1983 through September 30, 1984

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

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:
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.)*

We previously identified an 11-base DNA sequence, 5'-G-G-T-A-C-C-T-A-A-C-C-3', which is important in the control of SV40 late RNA expression in vitro and in vivo (Brady et al., Cell 31: 625-633, 1982). Subsequently, a series of mutants with deletions extending from SV40 map position 0 to 300 was prepared by nuclease BAL 31 treatment. Our transcription studies demonstrated that, in addition to the promoter domain near map position 300, there are essential DNA sequences between nucleotide positions 74 and 95 that are required for efficient expression of late simian virus 40 (SV40) RNA. Included in this SV40 DNA sequence were two of the six GGGCGG hexamers from the SV40 21 bp repeat sequences and an 11-nucleotide segment which showed strong homology with the upstream sequences required for the efficient in vitro and in vivo expression of the histone H2A gene. This upstream promoter sequence supports transcription with the same efficiency when it is moved 72 nucleotides closer to the major late cap site.

Using in vitro competition studies, we demonstrated that promoter-containing DNA fragments, which harbor neither the SV40 early or late -25 transcriptional control signals (TATA box) or the major RNA initiation sites, effectively compete for essential transcriptional factors required for SV40 transcription. The ability to compete for transcriptional factors is dependent upon sequences located within the 21 bp repeats. In addition, using an SV40-adenovirus 2 recombinant DNA, we demonstrate that the SV40 21 bp repeat region can stimulate in vitro transcription from the heterologous adenovirus 2 major late promoter (Ad2 MLP). In the absence of contiguous SV40 transcription control sequences, the 21 bp repeats are capable of initiating transcription, in a bidirectional manner, from proximally located sequences.

PROJECT DESCRIPTION

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

John Brady	Expert	LMV	NCI
Janet Duvall	Biological Aid	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.

Major Findings:

1. DNA sequences between nucleotide position 74 and 95, within the simian virus 40 (SV40) 21 base pair repeats, are required for SV40 early and late transcription in vitro. These sequences show strong homology to sequences required for efficient expression of the histone H2A gene.

2. The upstream sequence remains active when it is moved 72 nucleotides closer to the major late RNA initiation site.

3. The SV40 21 base pair repeat, independent of other transcriptional control elements, effectively binds transcriptional factors required for early and late transcription.

4. The 21 base pair repeats can stimulate transcription from a heterologous adenovirus-2 major late promoter.

5. In the absence of contiguous transcriptional control sequences, the 21 base pair repeats are capable of initiating transcription, in a bidirectional manner, from proximally located sequences.

Significance to Biomedical Research and the Program of the Institute:

During cellular transformation, the expression level 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:

Our in vitro analysis of the SV40 late promoter provides the basis for a series of future experiments:

1. Using D loop site-specific mutagenesis, a series of base substitution mutants will be constructed in the SV40 21 base pair 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 base pair units are functionally identical or represent distinct transcriptional regulatory units.

2. Our present data suggest that the upstream regulatory sequence is a bi-directional polymerase II transcriptional unit. Using recombinant DNA technology, the SV40 21 base pair repeats will be positioned in both orientations at various distances upstream from the early and late SV40 promoter. The mutants will then be analyzed for transcriptional efficiency.

3. To determine if the SV40 21 base pair repeats will augment transcription from heterologous promoters, the SV40 transcription control unit will be positioned upstream of the adenovirus major late, ovalbumin, β-globin and histone H2A promoters.

4. We will determine whether the H2A sequence can functionally substitute for the homologous SV40 21 base pair repeat.

5. 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 functionality.

Publications:

Brady, J., Radonovich, M., Thoren, M., Das, G. and Salzman, N. P.: The SV40 major late promoter: An upstream DNA sequence required for efficient in vitro transcription. Mol. Cell. Biol. 4: 133-141, 1984.

Mishoe, H., Brady, J., Lancz, J. and Salzman, N. P.: In vitro transcription initiation by purified RNA polymerase II within the adenovirus 2 major late promoter region. J. Biol. Chem. 259: 2236-2242, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05392-01 LMV

PERIOD COVERED: October 1, 1983 through September 30, 1984

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	Guest Researcher	LMV	NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Molecular Virology

SECTION: Virus Tumor Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	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 early simian virus 40 (SV40) gene product, large tumor (T) antigen, is responsible for the initiation of viral DNA replication and the autoregulation of early gene expression through direct protein-DNA interactions. We investigated the role of T-antigen in late viral gene expression, independent of its function in amplifying templates through DNA replication. SV40 DNA was transfected into BSC-1 and COS-1 cells and in the presence of inhibitors of DNA replication. Electrophoretic immunoblot analysis indicated that both the onset and the extent of SV40 late gene expression is increased in COS-1 cells, which constitutively express SV40 T-antigen. Blot hybridization analysis of poly(A)-selected RNA demonstrated that the level of synthesis of the major late structural protein, VP-1, in COS-1 cells was due to increased transcription. Similar results were obtained when plasmids that contain the SV40 late gene but lack both the origin for viral DNA replication and the early gene coding region were transfected onto COS-1 cells. Using lines of SV40-transformed monkey kidney cells that express altered T-antigens, we found that enhanced expression of the late gene product is correlated with the ability of T-antigen to bind SV40 DNA. Cotransfection in CV-1 and HeLa cells of a recombinant DNA plasmid containing the SV40 large T-antigen and a plasmid containing the SV40 late gene region induces transcription from the SV40 late promoter. These results indicate that large T-antigen plays a role in the stimulation of late viral gene expression.

In vivo promoter competition studies suggest two important aspects of T-antigen-induced SV40 late transcription. First, the transcriptional activation requires direct binding of T-antigen to sites I and II. Second, T-antigen activation of SV40 late transcription may require removal of negative transcriptional control factors from the SV40 late promoter.

PROJECT DESCRIPTION

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

John Brady	Expert	LMV NCI
George Khoury	Chief	LMV NCI
Mary Loeken	Guest Researcher	LMV NCI

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.

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 are 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 gene at the transcriptional level. The transcriptional activation appears to be mediated, in a trans-acting mechanism, by 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. Our present data suggest that SV40 T-antigen binding at sites I and II is critical for transcriptional activation of late gene expression. Site-specific mutagenesis of the DNA control region will be used to determine the critical sequences. We are presently testing a number of T-antigen amino acid substitution mutants to determine the critical areas of T-antigen required for binding to the regulatory sequence and postulated amino acid

sequences required for interaction with host cellular transcriptional factors.

2. 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.

3. 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., Bolen, J. B., Radonovich, M., Salzman, N. and Khoury, G.: Stimulation of simian virus 40 late gene expression by simian virus 40 tumor antigen. Proc. Natl. Acad. Sci. USA 81: 2040-2044, 1984.

Brady, J., Laimins, L. A. and Khoury, G.: Stimulation of gene expression by viral transforming proteins. In Levine, A., Vande Woude, G., Topp, W. and Watson, J. D. (Eds.): Cancer Cells. Cold Spring Harbor, Spring Harbor Laboratory, 1984, pp. 105-110.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05393-01 LMV

PERIOD COVERED

October 1, 1983 through September 30, 1984

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:
1.0	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.)*

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.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations 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:

Ca^{++}-phosphate transfection of eukaryotic cells; enzymatic assay for chloramphenicol-acetyltransferase (CAT); S1 RNA analysis; 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 the pathogenic action associated with retroviruses.

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 mutation will be constructed to identify specific sequences involved in enhancer activity.

Publications:

Laimins, L. A., Gruss, P., Pozzatti, R. and Khoury, G.: Characterization of enhancer elements in the long terminal repeat of Moloney murine sarcoma virus. J. Virol. 49: 183-189, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05394-01 LMV

PERIOD COVERED

November 1, 1983 through September 30, 1984

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:	George Khoury	Chief	LMV	NCI
	Su-yun Chung	Senior Staff Fellow	LMV	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The tissue-specific expression of immunoglobulin κ-light chain genes and heavy chain genes is associated with the enhancer sequences in these genes. Our objective is to reconstitute the tissue-specific expression of these genes extrachromosomally using a bovine papilloma virus (BPV) vector and somatic cell fusion techniques. The possibility of obtaining episomes actively exressing immunoglobulin genes should allow the study of the mechanism of tissue-specific enhancer function.

PROJECT DESCRIPTION

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

Chou-zen Giam	Visiting Fellow	LMV	NCI
George Khoury	Chief	LMV	NCI
Su-yun Chung	Senior Staff Fellow	LMV	NCI

Objective:

This project is directed towards the study of specific enhancer elements active in B-lymphocyte and T-lymphocyte. It should provide information about the mechanism of tissue-specific enhancer function. The present approaches include:

1. Construction of BPV-immunoglobulin gene plasmids and introduction of these plasmids into mouse L-cells and myeloma cells. We will assay gene expression in these cells.

2. Construction of HTLV-LTR plasmids with chloramphenicol acetyltransferase (CAT) gene as an assayable marker and their introduction into T-lymphocytes.

Methods Employed:

Recombinant DNA techniques; somatic cell fusion techniques; gene expression using transient and permanent assays; RNA and protein analysis.

Major Findings:

We have generated the recombinant DNA molecules needed for these experiments. Further studies will be directed toward elucidation of the nucleotide sequences governing tissue-specific gene expression and the mechanism by which they are recognized.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the mechanism of tissue-specific enhancer function is important in understanding of cell differentiation.

Proposed Course:

The project will continue to emphasize the identification of biologically important macromolecules mediating tissue-specificity of enhancers in B-cells and T-cells.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF VIRAL CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1983 to September 30, 1984

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 with special emphasis on delineating the roles, mechanisms and regulation of action of oncogenic viruses, virus-related and virus-associated genetic sequences, and gene products. Research efforts are 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 host genetic and immune systems are studied to elucidate the mechanisms of natural cancer control, especially in virus-related cancers. 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 present in all vertebrates (even in certain invertebrates, like Drosophila, and lower eukaryotes, like yeast) which have been identified in two ways. The first was the discovery that certain acute transforming retroviruses contained genomic regions which were homologous to genes found in normal cellular DNA and which, themselves, were capable of causing tumors or transforming fibroblasts in vitro. The second route of detection came from batch transfection of mouse 3T3 cells with DNA from human tumors. The subsequent isolation of specific human DNA segments from derived transformants formed the basis for a new group of "transforming genes" or oncogenes. 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

ometime obscure (much to the chagrin of active workers in the area). Within the last year, 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. 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 800 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 (SAIDS) and in FeLV-infected cats (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. Developing the human gene map of oncogenes and other loci which participate in human cancers. Molecular cloning, high resolution cytogenetic procedures and gene mapping panels of somatic cell hybrids between rodent and human cells have been employed in an extensive series of mapping experiments to genetically locate a variety of human cellular genes which participate in neoplastic transformation. Within the last two years, the human gene map has experienced a large increase in the number of neoplasia loci which have been mapped to specific chromosomal positions. Of the 27 specific human loci which have been chromosomally mapped to date, 11 (40%) of these have been assigned by the LVC scientists and their collaborators. The construction of this extensive human map has played an important role in the resolution of early genetic events in neoplastic transformation in man.

The ras gene family in man consists of five distinct cellular genes: Ha-ras-1, Ha-ras-2, Ki-ras-1, Ki-ras-2, and N-ras. Three of these are functional loci with intervening sequences, while the other two are apparently intronless pseudogenes which lack transforming activity (in 3T3 cells) even after ligation to potent LTR promoters. The human ras genes represent a family of transforming genes most frequently isolated from human tumors by the NIH-3T3 transfection assay. We have

mapped four of the human ras loci to four different human chromosomes and the fifth, N-ras, has been located on a fifth chromosome by a different laboratory.

Two other human oncogenes, ets and rel, have also been studied using molecular clones of the retroviral locus and DNA extracts of hybrid DNA. Both of these proto-oncogenes are near firm assignments of chromosomal positions in man. Of all the oncogene loci studied in man to date, only three (ras, raf, and ets) have apparent pseudogenes. The generation and/or transposition of these pseudogenes may be very ancient since, in at least the case of one family (ras), the presence of a homologous chromosomal linkage group of the pseudogenes has been retained in the cat genome. Multiple families of DNA segments homologous to retroviral genomes have been cloned and characterized. Of these, over 30 different proviral segments have been followed in hybrid panels and the position of each in the human genome is being determined with special attention to neighboring oncogenes and other neoplastic loci.

2. The raf oncogene and its cellular homologs. The v-raf transducing retrovirus was originally isolated in this laboratory from the murine sarcoma virus, 3611-MSV. The raf oncogene is a murine homolog which was independently captured in the avian MH2 transforming virus. Humans have two copies of this proto-oncogene, c-raf-1, a functional locus containing at least eight exons and seven introns, and an intronless pseudogene with chain termination signals in each reading frame. The two raf loci mapped to human chromosomes 3 and 4, respectively, using our human hybrid panel. The 5' and 3' ends of the coding portion of this gene have been identified. Conditions have been determined for its expression as a transforming gene in vitro. The intracellular location of its normal gene products has been analyzed. The c-raf gene products have been purified to homogeneity from mouse cells. Mouse and human raf-specific antisera were obtained by use of raf sequence-derived synthetic peptides, as well as genetically engineered raf protein. Transformation of cells by the raf oncogene is enhanced in vitro and in vivo in the presence of a second oncogene, v-myc. A combination of both oncogenes was discovered in the genome of the avian carcinoma virus, MH2, suggesting a role for this specific pair of genes in the development of natural carcinomas.

3. Isolation, characterization and transmission of a primate type D retrovirus associated with acquired immune deficiency syndrome in macaques (SAIDS). Macaque monkeys at four Regional Primate Research Centers (RPRC) have an immune deficiency disease called simian AIDS (SAIDS) characterized by lymphocytopenia, opportunistic infections, and an unusual tumor termed retroperitoneal fibromatosis (RF). A novel, type D retrovirus has been isolated after cocultivation of RF tissues obtained from several species of macaques housed at the University of Washington RPRC. This isolate, designated SAIDS-D/Washington, morphologically transforms various rodent cell lines, and can be distinguished antigenically and by hybridization from all known primate retroviruses. There are multiple copies of partially related nucleic acid sequences in Old World monkey cellular DNA; these studies suggest an origin for SAIDS in langur monkey cell DNA. SAIDS-D virus is being biologically and molecularly cloned and the amino acid sequences of the viral polypeptides and the restriction maps of the viral genomes are being compared to those of other type D retroviruses. The finding that SAIDS-D virus seems to be present almost exclusively in animals with RF and SAIDS constitutes preliminary evidence for attributing the etiology of SAIDS to this isolate. In preliminary experiments, the disease was transmitted by inoculation of macaques with cell-free viral filtrates. Vaccination experiments will attempt to elicit a protective antibody response to subsequent challenge with cloned live virus. The

viral etiology of simian AIDS has important implications as a model for the etiology, prevention, treatment, and molecular biology of immunosuppressive diseases, and as a marker for the identification, quarantine, and eventual control of AIDS in man.

No SAIDS-D viruses have been isolated from various human AIDS lymphocytes, nor is there evidence of nucleic acid sequences related to SAIDS-D virus in the DNAs of various AIDS tissues or cells. Magnesium-dependent reverse transcriptase activity can be detected, however, after addition of fresh human T-lymphocytes to lymphocytes from AIDS patients. This viral activity is not related immunologically to the SAIDS-D viral proteins as determined by radioimmunoassays. The identification of a virus associated with simian AIDS will provide a useful primate model for the prevention and treatment of human immunosuppressive diseases.

4. Identification, isolation, and molecular cloning of novel classes of "oncogenes" which specify sensitivity to tumor promoters or which transform promotable (P+) JB6 murine cells but do not transform 3T3. The JB6 mouse epidermal cell model system was developed to study the mechanism(s) of tumor promotion. The JB6 cells have apparently been initiated and partially promoted, but require further promotion to become neoplastic. These cells are stably nontumorigenic and anchorage-dependent, and in response to a variety of tumor promoters, become irreversibly tumorigenic and anchorage-independent. Stable promotion-sensitive (P^+) and promotion-insensitive (P^-) variants isolated from the original uncloned JB6 cell population have turned out to be valuable for testing a series of hypotheses for biochemical and molecular events required in the process of promotion of neoplastic transformation.

Promotion-sensitivity behaves as a dominant genetic trait transferable by DNA transfection. DNA from P^+, but not from P^- cells, when transfected into P^- cells, yielded about a fivefold increase in promotion of anchorage-independence in response to TPA. Genes for promotability are sensitive to the restriction endonucleases, EcoRI and HindIII, but not to BglII and BglIII. BglII was utilized to prepare P^+ DNA fragments that were used to establish a genomic plasmid library in the pcD-x, Okayama-Berg expression vector. Using a sib-selection technique and the gain-of-promotion-sensitivity assay, genes that specify sensitivity to promotion of neoplastic transformation by tumor-promoting phorbol esters have now been cloned. Two independently active, nonhomologous clones, designated pro-1 and pro-2, have been isolated and are being characterized. The pro-1 gene shows no homology with 11 different oncogenes.

DNA from TPA-induced, JB6-derived tumor cells, when transferred to JB6 P^+ cells, produces anchorage-independent transformation (in the absence of TPA). This transforming activity is not detected after transfection into NIH 3T3 cells and differs from P+ activity on the basis of restriction enzyme sensitivity. Cloning of the gene is underway. JB6 P^+ cells may be useful recipients for the detection of some classes of transforming genes not detectable using the NIH 3T3 assay.

5. A role for the oxygen radical, superoxide anion in the promotion of neoplastic transformation of JB6 cells by phorbol esters and the associated decrease in cell surface trisialoganglioside. Two lines of evidence implicate the superoxide anion in promotion-relevant signal transduction following phorbol ester interaction with its receptor. The first is that exposure of P^+, but not P^-, JB6 cells to TPA results in a greater than 50% decrease in the activity of superoxide dismutase (SOD). This would be expected to lead to elevation of the superoxide anion. The

second line of evidence implicating the superoxide anion as a signal transducer is that addition of exogenous SOD inhibited promotion of transformation by the phorbol ester, TPA. The superoxide anion acts during the first one to two hours following phorbol ester receptor (c Kinase) binding, suggesting a tight coupling of the two events.

Tumor-promoting phorbol esters and other promoters produce a 90% decrease in surface trisialoganglioside (G_T) synthesis in promotable cells, but not in nonpromotable variants. This suggests that the G_T switch in response to TPA may be one of a few critical, required events in tumor promotion. Selection of JB6 cells for resistance to the G_T response to TPA coselected for promotion resistance arguing further for a causal relationship of G_T response to promotion of neoplastic transformation. Decreased G_T synthesis was also characteristic of mouse epidermal tumor cells transformed by chemicals, but not by the viral oncogene, mos, suggesting a possible neoplasia-maintenance function for decreased G_T after chemical transformation.

Evidence suggesting an oxidative mechanism for the loss of G_T has been obtained. When JB6 P^+ cells are oxidized by sodium metaperiodate under conditions that oxidize surface G_T sialic acids, this produced both decreased G_T synthesis and promotion of transformation, suggesting that an oxidative pathway for the loss of G_T can be both possible and promotion-relevant.

6. Urinary growth factors in human neoplasia. The urinary protein of a patient bearing a highly malignant brain tumor (astrocytoma, grade IV) was adsorbed selectively on trimethylsilyl-controlled pore glass (TMS-CpG) beads to yield a high molecular weight (HMW) human transforming growth factor (hTGF). In its apparent molecular size (28,000 M_r), receptor binding, immunologic behavior, and clonogenic activity, the HMW hTGF was indistinguishable from HMW human epidermal growth factor (hEGF). A form of HMW hEGF was previously reported to be present in low concentrations in normal human urine. Thus, rather than being uniquely of glioblastoma tumor cell origin, the HMW TGF/EGF growth factor may reflect a host response to tumor burden.

In separate in vitro experiments, an α-type transforming growth factor (TGF_α) is produced at high levels by rat embryo cells transformed by the Snyder-Theilen strain of feline sarcoma virus. In addition, a β-type TGF that is independent of cell transformation is present in conditioned cell culture medium. This extracellular type of TGF_β activity was also present in conditioned medium of transformation-defective mutant rat cells, and in batch extractions made directly from fetal calf serum alone. Consequently, it may derive from the fetal calf serum component of the medium used for cell culture.

7. Genetic analysis of integration sites of HTLV. T-cell leukemia/lymphoma virus (HTLV-I) is a unique, exogenous retrovirus which has been isolated from patients with certain T-cell leukemias and lymphomas in southern Japan, the Caribbean basin, southeastern United States, and elsewhere. The virus is capable of rapid transformation of T-cells in vitro, but the mechanism(s) of transformation both in vitro and in vivo is unclear. Two panels of somatic cell hybrids have been constructed by fusion of rodent cell lines with the HTLV-infected cell lines, Hut 102 and MJ. These panels allow for the chromosomal localization of HTLV proviral integration sites, as well as for any human gene for which an assay or cloned molecular probe exists. Southern analyses of different passages of Hut 102 and MJ cells with cloned HTLV probes have demonstrated that HTLV integration is

progressive during in vitro cell culture. Analyses of somatic cell hybrids has revealed that the 20 or more proviral integrations present in late passage, HTLV-infected cell lines are dispersed among multiple chromosome homologs. Preliminary data indicate that two proviral integrations visualized in early passage Hut 102 cells are located on chromosomes 4 and 20. In addition, the structural gene for T-cell growth factor (TCGF) has been localized to chromosome 4 in normal human lymphocytes. The data suggest that the gene encoding TCGF is not operative in HTLV-induced malignant transformation.

In related studies, it has been demonstrated that virtually all HTLV-I-infected T-cell lines express novel, human HLA antigenic determinants which are in addition to and distinct from the HLA phenotype of autologous HTLV-negative B-cell or T-cell lines. A genetic analysis of the novel HLA expression indicated that the antigen was not encoded by the host cell HLA locus, but rather was encoded by the HTLV genome. When the participation of the HLA locus in T-cell function is considered, the homology between a viral protein and an HLA class I protein may be of great biologic significance in the pathogenesis of HTLV-induced neoplasias.

8. Generation of new transforming retroviruses from murine lung carcinoma. New, transforming, murine, type C viruses obtained in this laboratory were characterized with respect to their genome structure and their ability to transform cells in vitro and in vivo. A lung carcinoma-associated virus (LCAV, CI-1-4) was molecularly cloned and shown to have a mink cell focus-forming (MCF)-type structure. Transformed mink lung cells harboring this virus were sensitized to epidermal growth factor (EGF), suggesting that the mechanism of transformation by LCAV may involve sensitization of alveologenic lung cells to EGF. Two of the molecularly cloned viruses, CI-3 and CI-4, were also tested in vivo and found to be oncogenic, inducing disease with a latency period of 4 to 14 months. The disease spectrum includes stem cell leukemia, erythroleukemia and lung adenoma. This is the first example of pathogenic, in vitro-generated MCF-MuLV. Due to the long latency period involved, this system should be useful for the identification of intermediate steps in carcinogenesis.

9. Alignment of the feline and human linkage maps predicted and later proved a striking cytogenetic homology between the primate and Felidae families. Comparison of linkage relationships of homologous loci provides an opportunity to examine the status of genome organization in animal models of cancer and human inborn errors. We have prepared a biochemical genetic map of the domestic cat, including more than 45 loci over the past few years. Included in this map are several classes of loci which participate in human diseases and cancers: (1) endogenous cellular DNA sequences homologous to retroviral RNA genomes; (2) the homologs of cellular proto-oncogenes; (3) growth factor receptors; (4) restriction genes which delimit viral replication; (5) cell surface antigens, including the major histocompatibility complex; (6) integration sites of retroviruses; and (7) structural genes for lysosomal enzymes involved in human inborn errors for which there are feline models. Comparative analysis of the feline and human gene maps revealed a striking conservation of linkage association to the extent that approximately 25% of the human genome can be aligned band for band to the corresponding chromomere in the feline genome. The striking concordance of feline and primate genetic maps has two major aspects of biological significance: first, the evolutionary implications are noteworthy since the chromosome organization has maintained some semblance of order despite 80 million years of divergence (between primates and felids); and second, the comparative genetics has a predictive value, since once a gene has been located in the cat, a strong suggestion as to the position of a

homologous locus in man can be made. This aspect may be especially important in identifying mammalian genes (like retroviruses or controlling elements) capable of transposition during mammalian evolution. Eight lysosomal enzymes have been chromosomally assigned in the cat and molecular clones of their human counterparts are being developed in anticipation of gene delivery to feline disease models.

10. Embryo collection, freezing, and transfer in mice. Extensive progress was made in improving postfreezing viability of mouse embryos. This project is concerned with the long-term banking of a resource of more than 140 inbred, congenic, outbred and mutant stocks and strains of mice. Technology is now in use to permit viable recovery of 50 to 80% of all mouse embryos frozen (a two- to fourfold improvement over the rates of 1982). Because each of the mouse strains represents a unique genetic entity, this project provides an invaluable opportunity for comparative evaluations of embryo survival rates after freeze-thawing. During the past year, a comprehensive study was completed involving the frozen storage of over 18,000 embryos. The results indicated that the capability of embryos to survive cryopreservation varied markedly among strains, and was at least partially dependent on genotype. Frozen-thawed embryos were biologically viable as embryo transfer to recipient, surrogate females produced pregnancies and live-born litters. Survival rates of 80% with certain standard inbred strains are routinely achieved. Additional preliminary studies have been initiated with the embryos of cat and miniature swine, which hold much promise as animal models. Efforts have primarily concentrated on developing expertise in domestic animal models with eventual application to nondomestic species. Such techniques can improve reproductive potential and eventually allow microinjection of molecularly cloned genes which participate in transformation and inborn errors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE04825-11 LVC

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 chrerecters or less. Title must lit on one line between the borders.)*
Studies of the Nature and Control of Endogenous and Exogenous Retroviruses

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

PI:	Daniel K. Haapala	Microbiologist	LVC	NCI
Others:	Raoul E. Benveniste	Medical Officer	LVC	NCI

COOPERATING UNITS *(if any)*
Department of Microbiology, Georgetown University School of Medicine and Tropical Diseases, Washington, DC (K. J. Denniston); Department of Virology, Institut Pasteur, Paris, France (J. C. Chermann, L. Montagnier)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	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 nature of endogenous virus control by host cell function has been pursued in various systems, but with a continuing emphasis on the domestic cat model. These studies have served to distinguish two levels of control, i.e.: (1) control of replication, and (2) control of expression of viral transformation in cells replicating viruses. Information acquired and indicator cell systems developed during these studies have been applied to other systems, including AIDS and SAIDS.

PROJECT DESCRIPTION

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

Daniel K. Haapala	Microbiologist	LVC	NCI
Raoul E. Benveniste	Medical Officer	LVC	NCI

Objectives:

To characterize and utilize viral nucleic acids and proteins as diagnostic and analytical tools for probing the nature and mechanism(s) of viral oncogenesis. To study different virus-cell interactions to determine the nature of the control mechanism(s) operant in eukaryotic cells.

Methods Employed:

Standard biochemical, biophysical, cell and virus culture methods were used. These included ultracentrifugal, chromatographic, restriction enzyme, Southern transfer, nucleic acid hybridization, molecular cloning, gel filtration, electrophoretic, immunological, and in vitro protein synthesis techniques.

Major Findings:

1. Studies of the intracellular control of an endogenous virus: The use of feline embryonic cells as a model system. Feline embryo fibroblast (FEF) cells have been characterized as being "permissive" or "restrictive" for the endogenous cat virus, RD-114. Although neither cell type totally restricts virus replication, there is a quantitative difference in the total proportion of a population producing virus after de novo infection. The restrictive cells characteristically control virus replication in some manner, so that a small proportion of the population becomes producers and this proportion is maintained over long-term subculture. Permissive embryonic cells, on the other hand, are readily infected and the total population produces virus. A major effort to define the restrictive cell phenotype is further warranted because of another property; they restrict transformation acquired by sarcoma virus pseudotyped by RD-114 virus. No focus formation occurs in restrictive cells producing murine sarcoma virus (MSV) (RD-114), although the transforming virus produces normal amounts of RNA and protein. We have also tested a natural recombinant virus of cats in the system. This virus, which has gag genes identical to RD-114 virus, has a novel envelope. It is subject to the same intracellular restriction as RD-114, indicating that the target of restriction is the virus gag gene, as reported for Fv-1 restriction of mouse viruses.

2. The development of a biological assay for primate viruses. As reported last year, the French lymphadenopathy associated virus (LAV) isolated from pre-AIDS human subjects, does not form foci in the cat S^+L^- cell, PG-4. This observation has been extended to other S^+L^- cells of other species. We have included the simian isolate, $SAIDS_1$ in these studies and found that it also fails to form foci or rescue MSV from S^+L^- cells of several species. We now know that the LAV and SAIDS viruses are both type D, not type C viruses. Additional studies have shown

that other primate type D retroviruses do not register in S^+L^- cells and, therefore, the results with AIDS and SAIDS are not surprising.

3. The biological and biochemical nature of the SAIDS virus. Following initial reports of morphological changes induced by the SAIDS virus, an effort was made to quantify and characterize this activity. Detailed studies indicate that no associated transforming virus, analogous to replication-defective recombinant viruses of mice and cats, were found. The morphological changes induced by SAIDS follow one-hit kinetics and are not dependent on an associated, exogenously added type C virus, as shown in independent studies using flat, indicator lines, including species in which the SAIDS virus replicates. "Foci" of SAIDS-induced morphological variants were subcultured and failed to form agar colonies. The results obtained to date are all consistent with a cytopathic effect being responsible for morphologic changes induced by the SAIDS virus. This may have an important bearing on the nature of the disease induced by this virus.

Significance to Biomedical Research and the Program of the Institute:

The use of viral proteins and nucleic acids as markers to study oncogenesis is essential. Increasing the number and availability of such markers will increase our ability to understand the oncogenic process. The question of how a cell organizes vast amounts of genetic material continues to be of fundamental importance. Cancer-causing viruses provide a tool which can be used to examine the control of a few gene products. Our studies on the intracellular control of endogenous, vertically transmitted, cat viruses in FEF cells could be a unique model for future studies of endogenous human viruses. The confirmed association of LAV with AIDS makes continued study of this virus mandatory.

Proposed Course:

The potential of the recombinant viruses of mice to promote disease calls for continued study of the recombinant cat virus, including details of its env and LTR sequences. The type D retroviruses will also be studied at the molecular level. The SAIDS virus isolated in the LVC is currently being cloned. The clones will be compared to other isolates and molecular recombinants will be studied in appropriate biological indicator systems in order to characterize the SAIDS disease. In collaboration with the French, molecular and biochemical studies of LAV will be conducted using the tools and techniques derived from the studies reported above.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE04868-09 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Generation of New Transforming Mouse Type C Viruses

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

PI:	U. R. Rapp	Chemist	LVC	NCI
Others:	C. Majumdar	Expert	LVC	NCI
	P. Sutrave	Visiting Fellow	LVC	NCI
	C. C. Harris	Medical Officer	LHC	NCI
	J. D. Minna	Senior Scientist	MOB	NCI
	M. Goldsborough	Geneticist	LVC	NCI
	S. J. O'Brien	Geneticist	LVC	NCI

COOPERATING UNITS *(if any)* Lab. Viral Immunobiology, Litton Bionetics, Inc., Frederick, MD (S. Oroszlan and A. Schultz); Genentech, Inc., San Francisco, CA (H. Oppermann and P. Seeburg); Lab. Cell Biology, NIMH, NIH, Bethesda, MD (T. Bonner); Harvard Medical School, Boston MA (P. Leder); PRI, Frederick, MD (M. Gunnell)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.4	1.6

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

New, transforming, murine, type C viruses were characterized with respect to their genome structure and their ability to transform cells in vitro and in vivo: (1) A lung carcinoma-associated virus (LCAV, CI-1-4) was molecularly cloned and shown to have a mink cell focus-forming (MCF)-type structure. Transformed mink lung cells harboring this virus were sensitized to epidermal growth factor (EGF), suggesting that the mechanism of transformation by LCAV may involve sensitization of alveologenic lung cells to EGF. Two of the molecularly cloned viruses, CI-3 and CI-4 were also tested in vivo and found to be oncogenic. The nucleic acid sequence of CI-3 virus showed that the entire envelope gp70 was derived from an endogenous dual-tropic env gene. Comparison of the CI-3 viral gp70 and LTR sequences with those of class I and class II MCF MuLV established its unique structure. (2) A new oncogene, raf, from the murine sarcoma virus, 3611-MSV, was molecularly cloned, sequenced, and used for the isolation of its human cellular homologs, which were mapped on human chromosomes 3 and 4. The translational products of this virus were P90 and P75 polyproteins which contained viral p15 and p12 structural proteins fused to a tumor gene product lacking tyrosine-specific phosphokinase activity. The two polyproteins differ only by the fact that P90 is in a glycosylated form, whereas P75 is myristilated. Synthetic peptide sera, as well as a polyvalent, anti-v-raf, protein serum, identify a 60K, cytosolic, c-raf-1 protein in human cells. This size agrees well with the size predicted from the nucleotide sequence of the human c-raf-1 proto-oncogene. The raf protein was purified for amino acid sequence determination from mouse fibroblast cells in which the c-raf proto-oncogene was activated by an LTR insertion and subsequently amplified.

Transformation of cells by the raf oncogene is enhanced in vivo and in vitro in the presence of a second oncogene, v-myc. A combination of both oncogenes was discovered in the genome of the avian carcinoma virus, MH2, suggesting a role for this specific pair of genes in the development of natural carcinomas.

PROJECT DESCRIPTION

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

Ulf R. Rapp	Chemist	LVC	NCI
Chirabrata Majumdar	Expert	LVC	NCI
Pramod Sutrave	Visiting Fellow	LVC	NCI
Curtis C. Harris	Medical Officer	LHC	NCI
John D. Minna	Senior Scientist	MOB	NCI
Mindy Goldsborough	Geneticist	LVC	NCI
Stephen J. O'Brien	Geneticist	LVC	NCI

Objectives:

To isolate and characterize "transforming genes" that have been incorporated into the genome of nontransforming type C viruses and to employ these new viruses for the development of immunological reagents directed against the products of their human homologs.

Methods Employed:

Directly transforming retroviruses generally are characterized by the presence of cell-derived, transformation-specific sequences that have been incorporated into the retroviral genome. We have developed an in vitro system that has allowed, for the first time, the systematic isolation of new and tissue-specific, transforming type C viruses. The starting materials were cells chronically infected with murine leukemia virus (MuLV) after induction of endogenous type C virus with iododeoxyuridine (IUDR). Such virus stocks contained minority component viruses with specific toxicity or transforming activity for selected target cells. They could be obtained by cloning virus from the progeny of acute infections of highly permissive cells, such as chemically transformed C3H/10T1/2 cells or SC-1 cells. Tumors that developed upon inoculation of such selected virus stocks into newborn mice were established in culture and used as sources of specific tumor-inducing viruses. Cloned virus stocks which induced specific tumors were further purified and characterized by molecular cloning in phage vectors, the nucleic acids of their acquired oncogenes were sequenced, and the human and mouse cellular homologs were isolated. The deduced amino acid sequences from one new oncogene, v-raf, and its active, human cellular homolog, c-raf-1, were used for the generation of synthetic peptide antisera. In addition, v-raf protein was made by use of expression vectors, and polyvalent antisera were raised that specifically react with v-raf and c-raf proteins. These immunological reagents were then employed for the purification of c-raf-1 proteins from mouse and human cells.

Major Findings:

1a. Derivation of directly transforming type C virus from cell cultures. Alveologenic lung carcinoma-inducing virus, which had a latency period of 6 to 12 months, was isolated in vitro by selection of virus with the ability to transform mink lung cells from IUDR-induced C3H/MuLV. Transformed mink lung cells were cloned in soft agar and a particular clone of productively transformed cells was

the source of lung carcinoma virus. These cells produce a replicating, recombinant mink cell focus-forming (MCF) class of MuLV and contain, in addition, persistent, unintegrated viral genomes in circular, as well as linear forms. Subcloning of these cells in soft agar showed continued segregation of producer and nonproducer transformed, as well as revertant, cells. We have cloned the unintegrated, circular provirus from productively transformed mink lung cells. The recombinant genome of the CI-3 virus, described below, was preexistent as a minority component in the IUDR-induced population of C3H/MuLV, since an identical genome was isolated by molecular cloning from this stock.

1b. Analysis of the env gene of molecularly cloned MCF/MuLV recombinants isolated in vitro which are capable of transforming cells in culture. 2408 nucleotides of CI-3 DNA, including the MCF envelope gene, have been sequenced and compared to ecotropic AKV and dualtropic Moloney MCF sequences. The recombination junctions are within the polymerase gene, less than 50 nucleotides upstream from the MCF-specific BamHI site, and 15 nucleotides to the 5' terminal of the gp70/p15E cleavage site. The nature and location of the recombinant junctions favor a mechanism involving DNA exchange during reverse transcription. The 597-nucleotide, Prp15E sequence contains five base changes relative to AKV; the U3 sequences are identical to those of AKV (T1 #101 is absent). The MCF-specific, glycoprotein region lies within the amino-terminal 216 amino acids. In contrast, the carboxy-terminal 158 amino acids exhibit an overall homology of 87% to ecotropic AKV sequences, conserving the locations of apolar stretches, cysteine and proline residues, and glycosylation sites. This recombinant appears to be a mirror image of those viruses described by Thomas and Coffin (J. Virol. 43: 416-426, 1982) which arise early during the development of leukemogenic MCF in vivo.

The SFFV-like deletion of CI-4 has removed 696 nucleotides which code for three of the six glycosylation sites of gp70 and the extensive hydrophobic regions surrounding the gp70/p15E junction. The preexistent reading frame is uncompromised; the carboxy-terminal p15E sequences remain. Interestingly, this deletion is flanked by a direct repeat (TGGTANCGGGA). Mice infected with CI-4 virus develop, within three to nine months, malignant lymphomas of granulocytic, as well as lymphocytic lineage. Frank erytholeukemia was observed at low incidence in mice more than 12 months postinjection.

2a. A new histiocytoma/fibrosarcoma-inducing virus. An acute transforming virus was isolated from mice inoculated with a virus obtained by IUDR induction of methylcholanthrene-transformed C3H/10T1/2 cells. This virus, designated 3611-MSV, transforms embryo fibroblasts and epithelial cells in culture. Mice inoculated with 3611-MSV at birth develop tumors within four weeks which contain several distinct, mesenchymal cell types with fibroblasts as the predominant component. This new virus isolate resembles previously described mammalian acute transforming viruses in that it is replication-defective, requiring a type C helper virus for successful propagation both in vitro and in vivo. Several nonproductively transformed clones have been isolated by endpoint transmission of 3611-MSV to mouse or rat cells. Pseudotype virus stocks obtained from such clones transform cells in vitro, are highly oncogenic in vivo, and exhibit host range and serologic properties characteristic of the helper virus. The major 3611-MSV translational product has been identified as a 90,000 (P90) molecular weight (M_r) polyprotein with amino-terminal MuLV gag gene proteins, p15 and p12, linked to an acquired, sequence-encoded, nonstructural component. In contrast to gene products of many

previously described mammalian transforming viruses, the 3611-MSV-encoded polyproteins lack detectable protein kinase activity. Additionally, 3611-MSV-transformed cells resemble those of the chemically transformed cell line, C3H/MCA-5, from which 3611-MSV was originally derived, in that they do not exhibit overall elevated levels of phosphotyrosine.

2b. DNA sequence of v-raf. The nucleotide sequence of 1.5 kilobases (kb) encompassing the transforming gene (v-raf) of 3611-MSV has been determined. v-raf sequences were found to have been inserted into the p30 region of an ecotropic MuLV, with the concomitant deletion of the 2.4 kb extending to the middle of the polymerase gene. A five-nucleotide direct repeat exists at each end of the v-raf sequences. A single nucleotide deletion, ten bases upstream from the acquired sequences, places the oncogene in an open reading frame terminated by an amber triplet approximately 180 nucleotides from the 3' onc/MuLV junction. Sequences typical of eukaryotic promoters are not found as part of the inserted oncogene. From the deduced amino acid sequence, a hybrid gag-raf polyprotein would have a molecular weight of approximately 75 kilodaltons. Consistent with the gag-x structure, we find that only the P75 polyprotein is modified by the fatty acid myristate, whereas only the P90 polyprotein is glycosylated. Comparison of the deduced v-raf amino acid sequence with other oncogenes revealed domains homologous to v-src and v-mos.

2c. Deletion mapping of v-raf. Subclones of 3611-MSV containing deletions from either the 5' or 3' end of v-raf were tested for transforming activity in transfection assays. A subclone of the viral genome containing the 5' long terminal repeat (LTR), gag region, and the entire oncogene was treated with Bal-31 exonuclease to generate the deleted clones. In clones containing deletions from the 3' end it was determined, by sequence analysis, that 220 nucleotides could be removed from v-raf while still maintaining transforming ability; a clone missing 260 nucleotides did not transform. The normal termination codon for v-raf is located 180 nucleotides in from the 3' end. The studies on the 5' end showed that no more than 35 nucleotides could be removed before a loss in transforming ability was observed. Protein data on the 3'-deleted clones show that both P75 and P90 are being synthesized, indicating that the carboxy end is not responsible for the size difference in the proteins.

2d. The human and mouse cellular homologs of the raf/mil oncogene. We have identified two loci in the human genome which are related to the raf/mil oncogene. One of these, c-raf-2, is a processed pseudogene. The active gene, c-raf-1, contains 11 exons which are homologous to v-mil, nine of which are also homologous to v-raf. These exons span more than 21 kb and account for 1.26 kb of coding sequence and 0.96 kb of 3' untranslated sequence, the 3' end of the message having been defined by cDNA cloning. Since the message in humans is 3.5 kb, there is another 1.3 kb from the 5' end of the message yet to be accounted for. Comparison with the mouse cellular homolog reveals extensive homology in the 2-kb region immediately 3' of the poly (A) site. The function of this conserved region is unclear, but it might represent a transcription termination site located substantially 3' of the poly (A) site.

The biological activity of c-raf-1 has been demonstrated by transfection using DNA constructs in which portions of v-raf have been replaced by the corresponding c-raf-1 sequence. Thus, point mutations are not required to activate the gene. However, it is possible that the gene is activated by removal of its 5' end.

A mechanism of transduction is suggested by the sequences of c-raf-1 at the points corresponding to the helper-virus-oncogene junctions of v-raf. At the 5' junction there is a 20-nucleotide sequence which is identical in c-raf-1 and v-raf and which matches 16 of 19 corresponding bases of the Moloney p30 sequence. At the 3' junction there is a stretch of six nucleotides which are identical in c-raf-1, v-raf and MoLV polymerase. Allowing for some divergence in the untranslated sequences of the mouse and human homologs, this 3' homology region might be eight to ten nucleotides in the mouse homolog. This region of the mouse homolog is currently being sequenced. Taken together, these homologies indicate that the raf gene was transduced by homologous recombination between helper proviral DNA and the raf cellular homolog.

2e. The human homologs of the raf/mil oncogene are located on human chromosomes 3 and 4. Two human genes that are homologous to both the murine transforming gene (oncogene) v-raf and the chicken transforming gene v-mil have been mapped by means of human-rodent somatic cell hybrids to human chromosomes previously devoid of known oncogenes. One gene, c-raf-2, which appears to be a processed pseudogene, is located on chromosome 4. The other gene, c-raf-1, which appears to be the active gene, is located on chromosome 3 and has been regionally mapped by chromosomal in situ hybridization to 3p25. This assignment correlates with specific chromosomal abnormalities associated with certain human malignancies.

Significance to Biomedical Research and the Program of the Institute:

The goal of this research is the isolation of new, cell-derived transforming functions from mouse and human cells. Research from several laboratories over the past several years has demonstrated that spontaneous and chemically induced tumors from mouse and man may have activated oncogenes identical to those of directly transforming retroviruses. These important findings emphasize the relevance of retroviral oncogenes for an understanding of human malignancy. Another recent advance made in the study of mouse and primate retroviruses was the finding of endogenous human type C virus genes. The previous demonstration of exogenous human T-cell leukemia virus (HTLV), therefore, also emphasizes the importance of another class of transforming mouse retroviruses, the MCF class, which are recombinants between exogenous and endogenous MuLVs and includes SFFV, as well as our lung carcinoma virus. The new human oncogenes, c-raf-1 and c-raf-2, are now being studied for a potential role in human malignancy.

Proposed Course:

1. Work with lung carcinoma-associated MCF MuLV. The present emphasis will continue on the detailed characterization of viral genomes of nonproducer, transformed mink lung epithelial cells, as well as of the virus-induced lung adenocarcinoma cells. Although the viral genome, which persists as an unintegrated provirus in productively transformed mink cells, has been purified by molecular cloning, it is still possible that nonproducer, transformed mink cell subclones, which have been isolated, may yet be found to contain an integrated viral genome which may be either a virus-cell recombinant or could act by a "promoter-insertion" mechanism. Since the yield of virus rescue by superinfection with helper virus is extremely low, conventional approaches (e.g., the preparation of transforming virus-specific DNA probes or isolation of subgenomic size, proviral DNA from Hirt extracts of acutely infected cells) are not readily applicable. Attempts are in progress to

select transformants which are more susceptible to rescue. In addition, the recently obtained subclones of lung carcinoma-associated virus will be used to isolate integrated provirus of mouse origin by molecular cloning.

2. Confirmation of the etiological relationship of virus to murine lung tumors. In order to definitively establish whether the viral genome (molecularly cloned from mink lung cells transformed in vitro) is indeed the causative agent in the induction of lung tumors, we will pursue the following lines of research: (1) tumor induction in NFS/N mice with transfected, molecularly cloned virus in the presence and absence of various helper MuLVs, and (2) transfection and selection of transformed foci with DNA from (a) the transformed mink lung cells that were the source of the MCF class of viruses, CI-2,3 and CI-4, described above, and (b) cellular DNA from the induced lung carcinoma, as well as lung carcinoma cell lines.

3. Completion of the characterization of c-raf. Both mouse and human c-raf genes will be analyzed. The 5' end of the cellular gene will be determined with the help of c-raf cDNA, which we have already characterized. The transcriptional regulation of the c-raf-1 gene will be studied in vitro and conditions will be defined that affect its expression in vivo.

The potential involvement of c-raf in certain human tumors will continue to be tested in two ways: (1) Its position relative to chromosomal rearrangements involving human chromosome 3 is being determined in human small cell lung carcinoma cell lines that have specific deletions on chromosome 3 and in a familial renal carcinoma (in collaboration with Dr. Phillip Leder) which is specifically associated with a translocation involving chromosome 3. (2) The search for human tumors with a high level of c-raf expression will be continued.

A potential physiological role for c-raf, as well as the genetics of its regulation will be studied in mice. We will first screen inbred mouse strains for expression-polymorphism by determining the levels of c-raf transcription in various tissues of fetal and adult animals. Strains with different expression levels will then be used in genetic crosses to examine number, dominance or recessiveness and chromosomal map position(s) of the regulatory gene(s) involved.

4. Completion of v-raf characterization. v-raf will be further characterized in respect to its origin, mode of acquisition, potential involvement in MCA-5 transformation, location of the gene product of its cellular homolog, and in regard to functional domains by site-specific mutagenesis.

The origin of v-raf will be investigated by a search for a polymorphic marker that may distinguish the gene in different mouse strains. In the absence of such a marker the virus stock that went into the NSF/N mice in which the original tumor developed will have to be examined. We may also decide to determine the frequency of v-raf repair in various differentiated cell types in order to pinpoint a probable cell type in which the original transduction may have occurred.

The question of raf involvement in MCA-5 transformation will be examined, first by analysis of its expression in these cells. Preliminary experiments (dot blot

hybridization) show it to be expressed, perhaps at elevated levels, relative to the untransformed C3H/10T1/2 parent cell line. Another line of experiments, employing mainly Southern blot analysis, will determine whether or not c-raf is amplified, rearranged or structurally altered in MCA-5 cells. Preliminary experiments indicated some differences in the size of fragments hybridizing to v-raf DNA between MCA-5 and normal mouse cell DNAs. We will also determine, by transfection of MCA-5 DNA onto untransformed C3H/10T1/2 cells, whether transforming DNA has a restriction pattern compatible with c-raf.

A functional analysis of v-raf will include site-specific mutagenesis of v-raf DNA. We currently plan to use, as a strategy for mutagenesis, a technique developed in Dr. Wallace's laboratory (Wallace, R. B. et al., Nucleic Acids Res. 9: 3647-3656, 1981) which uses synthetic oligonucletide-directed mutagenesis for producing specific point mutations in cloned DNA.

5. The cooperative interaction between v-myc and v-raf oncogenes will be studied by use of appropriate DNA constructs. 3611-MSV DNA will be altered by addition of v-myc DNA and the transforming potential of these constructs tested in vitro and in vivo.

6. Characterization of additional viral isolates. Finally, we would like to begin work on some of the other acutely transforming, perhaps tumor gene transducing, virus isolates that we have previously isolated and which are currently held in reserve. It is particularly important to be able to analyze these additional isolates for several reasons: (1) they might contain additional, unique oncogenes; and (2) their structure might give clues to their origin and also may help determine the reproducibility of our isolation procedure for new oncogenic retroviruses. It is hoped that some of this information will also help in our understanding of the mechanisms of oncogene transduction which, in turn, may allow us to improve the frequency of future in vivo and in vitro isolations.

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 (In Press)

Bonner, T. I., Kirby, S., Gunnell, M., Sutrave, P., Mark, G. and Rapp, U. R.: Structure and biological activity of the human cellular homologues of the raf oncogene. Nature (In Press)

Bonner, T. I., O'Brien, S. J ., Nash, W. G. and Rapp, U. R.: The human homologs of the raf/mil oncogene are located on human chromosomes 3 and 4. Science 223: 71-74, 1984.

Goldsborough, M. D., Mark, G. E., Borchert, P. A. and Rapp, U. R.: Localization of the transforming sequences of the oncogene v-raf by transfection with BAL-31 generated subgenomic fragments. J. Virol. (In Press)

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Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., Jr. and Stephenson, J. R.: Structure and biological activity of v-raf, a new oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA 80: 4218-4222, 1983.

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Rapp, U. R., Reynolds, F. H., Jr. and Stephenson, J. R.: Isolation of new mammalian type-C transforming viruses. In Pearson, M. L. and Sternberg, N. L. (Eds.): Progress in Cancer Research and Therapy: Gene Transfer and Cancer. New York, Raven Press, 1984, Vol. 30, pp. 169-178.

Sutrave, P., Bonner, T. I., Rapp, U. R., Jansen, H. W., Patschinsky, T. and Bister, K.: Nucleotide sequence of avian retroviral oncogene v-mil: Homologue of murine retroviral oncogene v-raf. Nature 309: 85-88, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05124-07 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Retrovirus Expression in Primate Placenta

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

PI:	K. J. Stromberg	Medical Director	LVC	NCI
Others:	R. E. Benveniste	Medical Officer	LVC	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	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.)*

An examination of various rhesus monkey (Macaca mulatta) organs has shown type C viral antigen expression preferentially in the placenta (Stromberg, K. and Huot, R., Virology 122: 365-369, 1981). Separate cocultivations of isolated primary trophoblasts from ten rhesus monkey placentas with cell lines from heterologous mammalian species led to rapid isolation of type C rhesus retroviruses in four of ten cases. These four retrovirus isolates have been designated MMC-2 through MMC-5. Distinction of these viral isolates from the initial rhesus isolate (MMC-1) and the previous isolate from the stumptail monkey, Macaca arctoides, (MAC-1) could be made by host range studies and liquid DNA hybridization, but not by limited restriction endonuclease digestion. Specifically, the cellular DNA from rhesus isolates MMC-2 through MMC-5 melted 0.7oC to 1oC lower than either MAC-1 or MMC-1. Using our hybridization conditions, it was not possible to distinguish between MAC-1 and the originally reported isolate from rhesus, MMC-1. Both MAC-1 and MMC-1 were obtained in single, long-term cocultivation experiments (over seven months). The present isolates, MMC-2 through MMC-5, were detected in two to five weeks. The prompt detection of type C particle expression following cocultivation of rhesus trophoblast cells with heterologous cell lines demonstrates that trophoblast, as a differentiated cell type, is relaxed for complete retroviral expression. It remains to be seen if other primate species, including higher apes and man, will yield infectious retrovirus using this approach.

PROJECT DESCRIPTION

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

Kurt J. Stromberg	Medical Director	LVC	NCI
Raoul E. Benveniste	Medical Officer	LVC	NCI

Objectives:

First, to study type C viral expression in rhesus placenta to establish conditions whereby endogenous retroviruses might be more readily isolated from primate species. The aim has been to establish that there is a tissue-specific preference for expression of endogenous type C virus in primate placenta and to apply principles established in the rhesus system to primate species from which retroviruses have not been isolated, including man.

Methods Employed:

Rhesus retroviral expression was scored by radioimmunoassay (RIA) of the p26 antigen from Macaca arctoides type C virus (MAC-1). Clarified pellets (100,000 x g) of cell and organ culture supernatants were screened for DNA polymerase activity using rAdT synthetic templates. Cellular DNA was isolated from cell lines infected with the various isolates and hybridized to a ^{3}H-DNA transcript prepared from the MAC-1 isolate, and melting curves were performed.

Major Findings:

1. Retroviral isolation from primate trophoblasts. The preferential expression of Macaca arctoides type C retrovirus (MAC-1) p26 antigen in rhesus trophoblast has been documented (Stromberg, K. J. and Huot, R., Virology 122: 365-369, 1981). Antigen expression was detected in 16 out of 16 placental specimens, but not in ten other different fetal organs from each of eight selected animals. The levels of antigen detected in placenta ranged between 2 and 218 ng/mg of protein with a correlation between lower antigen expression and term gestation or parity greater than ten. A ten-fold higher level of antigen expression was detected at the external surface of the placenta near the decidua in comparison to the remainder of the placenta towards the amniotic surface. Thus, even within the placenta, there was a preferred site for endogenous retroviral antigen expression. Separate cocultivations of isolated trophoblasts from ten rhesus placentas using three indicator cell lines (A549, FEC and CF2Th) led to rapid isolation in feline embryo cells (FEC) of type C rhesus retroviruses in four of ten cases. These four retrovirus isolates have been designated MMC-2 through MMC-5. Five of the remaining six sets of cocultivations grew simian foamy virus and were discontinued. With all four retroviral isolates, p26 expression was detected in the cell monolayers between two and five weeks, and Mn^{++}-dependent DNA polymerase activity was evident in the culture supernatants between five and nine weeks after initiation of cocultivation. Distinction of these viral isolates from the initial rhesus isolate (MMC-1) and the previous isolate from the stumptail monkey, Macaca arctoides (MAC-1), could be made by liquid DNA hybridization, although not by limited restriction endonuclease digestion. Both MAC-1 and MMC-1 were obtained in single,

long-term cocultivation experiments (over seven months). The present isolates, MMC-2 through MMC-5, were detected in two to five weeks. Consequently, primary trophoblast cells represent a useful differentiated cell type for isolation of infectious retrovirus from this primate species.

The buoyant density of all the purified new Macaca mulatta isolates is 1.14 cm^3 and all have a type C morphology by electron microscopy. The host ranges of MAC-1 virus isolated from Macaca arctoides and two of the new retroviral isolates from Macaca mulatta are different. In agreement with previous data, MAC-1 replicates well in cells of dog, cat, or human origin. MMC-2 grew only in the feline cell line, while MMC-3 grew in both the feline and canine cell lines. Neither isolate replicated in the human cell line, A549, under the conditions employed. Thus, these new rhesus isolates can be distinguished from the previously isolated stumptail virus on the basis of host range differences. Radioimmunoprecipitation assay over a 100-fold concentration range of competing p26 antigen revealed no distinction in p26 among MAC-1 and MMC-1 or MMC-2 through MMC-5. Furthermore, homologous radioimmunoassays, kindly performed by Dr. Charles Benton, formerly at the Frederick Cancer Research Facility, with monospecific antisera to MMC-1 p26 and purified MMC-1 p26 antigen, when compared with purified virus extracts of MMC-1 through MMC-5, again showed parallel competition curves. These results are not unexpected, inasmuch as previous radioimmunoassays with the major internal structural protein of endogenous primate viruses have shown that closely related species cannot be readily distinguished.

In contrast, nucleic acid hybridization studies have been shown to detect differences among the virogenes of closely related species of Old World monkeys. Type C viruses isolated from three species of baboons (Papio cynocephalus, P. hamadryas, P. papio) have been shown to differ in the thermal stabilities of their nucleic acid sequences by 2-4 ^{o}C, whereas several independent isolates from one species of baboon have nearly identical thermal stabilities ($<0.5^{o}C$ difference). It is a property of genetically transmitted retroviruses that related nucleic acid sequences can be found in evolutionarily related species. The differences in thermal stability among the baboon isolates correlate well with the results expected based on phylogenetic differences between these primate species as determined by the DNA homology of their non-repetitive cellular genes.

Consequently, cellular DNA from cell lines infected with MAC-1, MMC-1, and MMC-2 through MMC-5 was hybridized to a complementary DNA probe prepared from MAC-1 virus RNA. The cellular DNA from rhesus isolates MMC-2 through MMC-5 melted $0.7^{o}C$ to $1.0^{o}C$ lower than the homologous hybrid. The lower melting temperatures obtained are consistent with the base-pair mismatching of the viral genomes. The data, therefore, suggest that these M. mulatta viruses can be distinguished from the previously described M. mulatta isolates. Using our hybridization conditions, it is not possible to distinguish between the viral isolate from stumptail macaque (MAC-1) and the originally reported isolate from rhesus (MMC-1). DNA reassociation studies of macaque cellular DNA show that this genera of primates is more closely related phylogenetically than are the various species of the baboon genus Papio. The closer degree of nucleic acid sequence homology among the endogenous, genetically transmitted stumptail and rhesus viral isolates as compared to the baboon viral isolates is thus expected based on the overall relationships among these primate species.

A restriction endonuclease cleavage map of the MMC-1 proviral genome integrated in canine cell line DNA has been reported. We compared the restriction maps of MAC-1, MMC-1, and MMC-2 through MMC-5 genomes integrated in human, canine, and feline cells, respectively. The nick-translated probe used was prepared from cloned DNA representing the internal EcoRI 4.7-kilobase (kb) fragment from colobus virus, a closely related type C virus. Restriction enzyme digests with EcoRI, BamHI, HindIII and SacI did not reveal any differences between the stumptail or macaque viral genomes. Thus, even though the MMC-2 through MMC-5 viral genomes possess a 0.7°C to 1.0°C nucleic acid base-pair mismatch with respect to MAC-1, those sites are not represented by the loci defined by these four restriction enzymes.

Each of the cocultivations of rhesus trophoblasts with feline cells led immediately to cell fusion with formation of large aggregates of multinucleated cells. Since human trophoblast in organ culture, as opposed to monolayer culture, produces a 10^4 increase in chorionic gonadotropin production, considerable effort was made to isolate rhesus retrovirus by seeding a monolayer of FEC cells with rhesus placental tissue in organ culture. This procedure did not induce fusion of cells within the FEC monolayer, or result in expression of viral p26 antigen or reverse transcriptase activity. Consequently, the intimate juxtaposition and resultant fusion of permissive indicator cell lines with rhesus trophoblast seem to be essential for isolation of infectious retrovirus. Trophoblasts cocultivated with either the human line, A549, or the canine line, CF2Th (from which no virus was recovered), did not result in multinucleations or fusion of the two cell types. Paradoxically, the syncytial formation between trophoblast and indicator cell lines may hasten the appearance of contaminating simian syncytium forming (foamy) viruses, as was the case in five of ten placentas in this study, and is reported also to be 50% in baboon placentas. In any event, the prompt detection of type C particle expression following cocultivation of rhesus trophoblast cells with heterologous cell lines demonstrates that trophoblast, as a differentiated cell type, is relaxed for retroviral expression. Using these techniques, we have isolated several endogenous rhesus retroviruses that can be distinguished by host range and nucleic acid hybridization criteria from the previously isolated viruses from stumptail and rhesus macaques. It remains to be seen if other primate species, including higher apes and man, will yield infectious retrovirus using these approaches.

Significance to Biomedical Research and the Program of the Institute:

The retroviral part of this project has been directed toward use of the rhesus model system to determine factors which influence expression and which might enhance isolation of retroviruses from higher primates. Clearly, rhesus trophoblast is a preferential tissue source for isolation of endogenous retroviruses, and these techniques can be extended to other primates, including man, from which endogenous retroviruses have not yet been isolated. The relevance of retroviruses to carcinogenesis in higher primates might be clearer if endogenous primate retroviruses could be more readily isolated from them.

Proposed Course:

Because of other research interests, and limited technical help and resources, the approach to primate retroviral isolation using cocultivation with trophoblast cells will be discontinued. Relevant stocks remain frozen down for future use.

Publications:

Stromberg, K. J. and Benveniste, R. E.: Efficient isolation of endogenous rhesus retrovirus from trophoblast. Virology 128: 518-523, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05150-05 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Chemical Carcinogenesis and Cocarcinogenesis In Vitro

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

PI: U. R. Rapp Chemist LVC NCI

Others: None

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:
2.0	0.4	1.6

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

An in vitro system was established for the quantitative chemical transformation of rat epithelial cells (RC-E). These cells can also be transformed by C3H MuLV in conjunction with 12-O-tetradecanoylphorbol-13-acetate (TPA). Transformed cell clones obtained from soft agar generally were virus nonproducers. The purpose of these experiments was to sequence-label TPA-promotable cellular tumor genes and, thus, make possible their isolation by molecular cloning. RC-E cells have also appeared to provide the first system for two-stage carcinogenesis in culture with ethylnitrosourea (ENU) as carcinogen and TPA as tumor-promoter. The observation of cocarcinogenesis of RC-E cells with MuLV and TPA promoted a search for factors homologous to TPA in normal sera. Such a transforming factor was found and purified from normal mouse serum.

PROJECT DESCRIPTION

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

U. R. Rapp Chemist LVC NCI

Objectives:

To develop improved in vitro assays for 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 carcinogenesis 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 cocarcinogenesis experiments to widen the repertory of tumor genes for screening; (3) attempts to identify genes promotable by 12-O-tetradecanoylphorbol-13-acetate (TPA) by sequence-labeling with murine leukemia virus (MuLV) genomes; and (4) isolation of transforming gene(s) from extensively characterized, chemically transformed cells (i.e., MCA5) by transfection and molecular cloning. It was demonstrated recently that MCA5 cell DNA contains, or yields upon transfection, an active version of the oncogene Ki-ras. The evidence for its involvement in chemical transformation of C3H/10T1/2 cells, however, is weak, resting solely on its activity in the transfection assay which may be selectively sensitive to a subset of cellular oncogenes. We, therefore, plan to include in our studies flat revertants derived from MCA5 cells as part of a genetic analysis of oncogene activation in MCA-induced C3H/10T1/2 cell transformation. Moreover, we will be using untransformed C3H/10T1/2 Cl 8 cells, the parent of MCA5, as the recipient for DNA transfection.

Major Findings:

1. Use of epithelial rat cells for transformation in vitro with ethylnitrosourea (ENU) and TPA. Rat embryo epithelial (RC-E) cells were transformed in vitro using ethylnitrosourea (ENU) as a carcinogen either by itself or in conjunction with the tumor promoter, TPA. The frequency of transformation in the absence of TPA was 5×10^{-4} at 10 g/ml of ENU. Growth of ENU-treated cells in TPA-substituted medium increased the transformation frequency eightfold. Morphologically transformed cells were isolated from individual colonies growing in soft agar and analyzed for the production and deposition of pericellular glycoproteins. The ENU-transformed cells were able to retain pericellular matrix structures, as were the nontransformed RC-E control cells. Cell surface labeling revealed differences in the glycoprotein patterns between the cells. The ENU-transformed cells produced fibronectin and procollagen type I as their major glycoproteins. Procollagen type I was produced and deposited in the cell layers in altered form, i.e., procollagen 1-trimer, as shown by polypeptide analysis. The results indicate that ENU can induce malignant transformation of RC-E cells in culture and modify their production and deposition of pericellular glycoproteins. Clonal lines of RC-E cells transformed by ENU and TPA will be compared with those transformed by MuLV and TPA (see below). This comparison will include the restriction enzyme sensitivity pattern of transforming DNA from both sets of transformed cells.

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 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 chemically transformed mouse cells.

Evidence from transfection experiments with chromosomal DNA has shown (Shih et al., Proc. Natl. Acad. Sci. USA 76: 5714, 1979) that chemical transformation of cells may be achieved by the activation of single tumor genes. Similarly, an oncogene-transducing retrovirus has been isolated from IUDR-induced chemically transformed cells. These "activated" cellular oncogenes may be altered as a consequence of carcinogen treatment or may represent derepressed normal genes. In order to study this question, recombinant DNA technology is being used to isolate such a gene(s) from transformed cells. Once this transforming gene(s) is available, it will be possible to isolate its normal counterpart by screening a gene library made from normal cellular DNAs. 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 chemically transformed cells. The total

cellular DNAs from MCA5 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 vector, 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 5×10^5 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). A mixture of recombinant DNAs isolated from the entire gene library is used to transfect untransformed C3H/10T1/2 Cl 8 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 other cellular genes. In order to isolate the recombinant containing this gene(s), we will follow a subselection procedure. The entire gene library is divided into ten sub-libraries, each containing one-tenth the number of the recombinants in the original library. The DNA mixture isolated from each of these sub-libraries is assayed for its biological activities. The sub-libraries, containing the transforming gene(s), are identified and should represent a tenfold enrichment. Similarly, the positive sub-libraries will again be divided into ten subdivisions and the mixture of DNAs from each subdivision will be assayed for the presence of the transforming gene(s). After six to seven cycles of enrichment, single clones, each containing a specific transforming gene, may be isolated.

2. TPA enhancement of transformation of mouse MMCE and rat FRE cells by IUDR-induced C3H MuLV.

The basis for the observed transformation in conjunction with TPA may either be the presence of directly transforming virus in the IUDR-induced virus population, or result from a two-stage carcinogenesis with MuLV as the mutagen/carcinogen. Epithelial rat cells transformed by IUDR-induced C3H MuLV, in conjunction with TPA, are presently being analyzed for MuLV sequences in the cellular genome and also for RNA transcripts initiated from within the viral genome. Transformed cells which contain only one copy of complete or defective MuLV will be chosen for further experiments.

3. Use of cocarcinogenesis for the isolation of new acutely transforming mouse type C viruses.

The observation has been made that NFS/N mice, inoculated with MuLV as newborns and receiving butylnitrosourea (BNU) at four weeks of age, developed virus-positive lung tumors at high incidence. Some of these lung tumors yielded viruses which, in turn, induced lung tumors and peritoneal tumors in the absence of BNU. BNU, by itself, induced virus-negative lung tumors, as well as lymphomas. MuLV, in the absence of BNU, induced lymphomas with a long latency period. We plan to investigate whether lung tumor-derived, in addition to peritoneal tumor-derived, MuLV contains a directly transforming component which has acquired a tumor gene and also can induce tumors in other target tissues in MuLV-infected mice. The isolation of additional transforming mouse type C viruses is expected.

4. Screening of chemically transformed C3H/10T1/2 fibroblast and MMCE and RC-E epithelial cells for the expression of previously characterized viral oncogenes.

Publications:

Hautanen, A., Rapp, U. R. and Keski-Oja, J.: Transformation of cultured rat epithelial cells by ethylnitrosourea: Consistent chromosome rearrangements associated with transformation. Cancer Genet. Cytogenet. (In Press)

Keski-Oja, J., Alitalo, K., Hautanen, A. and Rapp, U. R.: Transformation of cultured epithelial cells by ethylnitrosourea: Altered expression of type I procollagen chains. Biochim. Biophys. Acta. 803: 153-162, 1984.

Rapp, U. R., Gunnell, M. and Marquardt, H.: Normal mouse serum contains peptides which induce fibroblasts to grow in soft agar. J. Cell. Biochem. 21: 29-38, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05180-04 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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
	Kurt J. Stromberg	Medical Director	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:
0.7	0.4	0.3

CHECK APPROFRIATE BOX(ES)

☐ (a) 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 within the mammalian genome. Among the Felidae, only six small species of cats have been found to contain nucleic acid sequences related to RD-114 and FeLV viruses. Studies with subgenomic probes from the endogenous RD-114 virus have shown conservation among fragments in the gag and pol viral regions of domestic cat DNA. These same fragments are also found in multiple copies in cell DNA from the other five RD-114-containing cat species. The leopard cat from Southeast Asia, Felis bengalensis, does not contain either RD-114 or FeLV viral sequences in its DNA. The offspring of matings between the leopard and domestic cats thus afford a unique opportunity to study the segregation of both sets of virogenes in the progeny of backcrosses of F1 hybrids to the virogene-negative parent. Liquid hybridization studies with radioactively labeled DNA probes prepared from RD-114 and FeLV viruses reveal that F1 hybrids contain approximately one-half of the number of virogene copies present in the domestic cat parent. When restriction digests of cell DNAs from backcross offspring were analyzed with probes specific for RD-114, different individuals exhibited different combinations of viral fragments. Analogous results have been obtained with subgenomic FeLV probes. These results demonstrate that viral sequences from both endogenous families are dispersed on multiple chromosomes throughout the cat genome. Segregation of different RD-114 sequences in cat x rodent somatic cell hybrids has confirmed this conclusion. 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 derived by molecular hybridization techniques - it differs in many respects from the classical relationships derived by anatomical considerations and morphometric measurements.

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
Kurt J. Stromberg	Medical Director	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. Characterization of retroviruses isolated from various macaque species. An examination of various rhesus monkey (Macaca mulatta) organs has shown a preference for type C viral antigen expression in the placenta. Separate cocultivations of isolated trophoblasts from ten rhesus monkey placentas with cell lines from heterologous mammalian species led to rapid isolation of type C rhesus retroviruses in four of ten cases. These four retrovirus isolates have been designated MMC-2 through MMC-5. Distinction of these viral isolates from the initial rhesus isolate (MMC-1) and the previous isolate from the stumptail monkey, Macaca arctoides (MAC-1), could be made by liquid DNA hybridization, although not by limited restriction endonuclease digestion.

Both MAC-1 and MMC-1 were obtained in single, long-term, cocultivation experiments (over seven months). The present isolates, MMC-2 through MMC-5, were detected in two to five weeks. Consequently, primary trophoblast cells represent a useful differentiated cell type for isolation of infectious retrovirus from this primate species.

2. Characterization of endogenous, retroviral gene sequences in crosses between domestic cat and leopard cat. The domestic cat, Felis catus, contains 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.

3. The transfer of retroviruses from primates to felines as a marker for studies of evolutionary rates. Earlier work had 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 four to six 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.

4. 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 being 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 since they are equidistant from a phylogenetic perspective from all other carnivores. In collaboration with Dr. S. O'Brien, the giant panda was shown to belong to the Ursidae family. The pandas, raccoons, and bears were also studied from a variety of evolutionary perspectives, utilizing DNA hybridization, isozyme mapping, and immunological distance, and compared to previously obtained data on karyology and comparative anatomy.

Significance to Biomedical Research and the Program of the Institute:

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. The viral sequences are, therefore, subject to the same evolutionary processes as the cellular DNA sequences.

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.

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.

Publications:

Benveniste, R. E.: Retroviruses as tools in molecular evolution. In MacIntyre, R. J. (Ed.): Evolutionary Biology. New York, Plenum Press (In Press)

Stromberg, K. J. and Benveniste, R. E.: Efficient isolation of endogenous rhesus retrovirus from trophoblast. Virology 128: 518-532, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05330-02 LVC

PERIOD COVERED
October 1, 1983 to September 30, 1984

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:	K. J. Stromberg	Medical Director	LVC	NCI
Others:	W. R. Hudgins	Chemist	LVC	NCI

COOPERATING UNITS *(if any)*
Laboratory of Molecular Virology and Carcinogenesis, Litton Bionetics, Inc., Frederick, MD (L. E. Henderson); Division of Endocrinology, Vanderbilt University, Nashville, TN (D. N. Orth); Oncogen, Seattle, WA (D. R. Twardzik)

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:
1.0	0.2	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.)*

The urinary protein of a patient bearing a highly malignant brain tumor (astrocytoma, grade IV) was adsorbed selectively on trimethylsilyl-controlled pore glass (TMS-CpG) beads to yield a high molecular weight (HMW) human transforming growth factor (hTGF). The growth factor activity had an approximate Mr of 28,000, similar to that of a high molecular weight form of human EGF (HMW hEGF) previously reported to be present in low concentrations in normal human urine. Following surgical resection of the tumor, no appreciable HMW hTGF activity was detectable in a comparable 48-hour urine collection. HMW hTGF generated a competitive binding curve similar to that of standard small molecular weight hEGF, and parallel to HMW hEGF. In its apparent molecular size, receptor binding, immunologic behavior, and clonogenic activity, the brain tumor-associated HMW hTGF was indistinguishable from HMW hEGF. Thus, rather than being uniquely of glioblastoma tumor cell origin, the HMW TGF/EGF growth factor may reflect a host response to tumor burden.

An alpha-type transforming growth factor (TGF_α) is produced at high levels by rat embryo cells transformed by the Snyder-Theilen strain of feline sarcoma virus. In addition, a beta-type transforming growth factor is present in conditioned cell culture medium that is independent of cell transformation. This TGFβ had an approximate Mr of 12,000 and eluted at 37% acetonitrile during high performance liquid chromatography. This extracellular type of TGF activity was also present in conditioned medium of transformation-defective mutant rat cells, and in batch extractions made directly from fetal calf serum alone. Consequently, it may derive from the fetal calf serum component of the medium used for their growth in cell culture.

PROJECT DESCRIPTION

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

Kurt J. Stromberg	Medical Director	LVC	NCI
William R. Hudgins	Chemist	LVC	NCI

Objectives:

To study the expression of a family of urinary growth factors in human cancer patients with respect to their histologic type and their extent of tumor burden, to develop novel growth factor isolation procedures employing hydrophobic adsorption, and to use rats, and nu/nu rats bearing human tumors as model systems.

Methods Employed:

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) it addresses 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; and (4) if the HMW GF was a tumor cell product, and not simply a host factor which responded to tumor burden, a differential concentration might be found between urine and cerebral spinal fluid.

The presence of growth factor(s) was assessed by stimulation of growth of normal rat kidney cells (NRK) in soft agar, and competition with ^{125}I-labeled epidermal growth factor (EGF) for binding to membrane receptors. 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, use of TMS-CpG beads resulted in a rapid one-step, 225fold reduction of protein with significant 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. Similarly, control and FeSV-induced, tumor-bearing Fischer rats, strain 344, were used for comparison of urinary growth factor profiles.

Major Findings:

1. Human brain tumor-associated urinary high molecular weight (HMW) growth factor may be identical to HMW epidermal growth factor. High molecular weight growth factor (HMW GF) was adsorbed from the urine of a patient bearing a glioblastoma multiforme by TMS-CpG beads. Following a second cycle of TMS-CpG adsorption, HMW GF had a molecular weight of approximately 28,000 using Bio-Gel P-100 chromatography. Following an estimated 95% surgical resection of the tumor, no

growth factor activity of this sort was detectable in a comparable 48-hour urine collection. This brain tumor-associated HMW GF activity contained only about 20% of the radioreceptor activity of the standard small molecular weight human epidermal growth factor (SMW hEGF), while maintaining full immunoreactivity to the SMW hEGF. Consequently, the HMW GF associated with brain tumor burden in this patient appears similar to the high molecular weight form of human EGF (HMW hEGF) previously reported in low concentration in normal human urine. Thus, the HMW GF may be of host cell, rather than uniquely of tumor cell, origin.

For further comparison, the HMW GF from the pooled preoperative urine of ten patients with malignant astrocytoma was isolated by TMS-CpG beads, sequential Bio-Gel P-10 and P-100 chromatography, and reverse phase high pressure liquid chromatography (HPLC). No distinction could be made between the HMW EGF from normal urine purified by Dr. David Orth at Vanderbilt University and tumor-associated HMW GF in respect to biochemical behavior during purification, molecular size, or clonogenic activity in soft agar. However, HMW GF was undetectable in 5 liters of control urine, whereas 210 mg was obtained in pure form from 26 liters of tumored urine.

2. Partial characterization of a TGFβ-like polypeptide that is present in conditioned medium independent of cell transformation. Fischer rat embryo cells transformed by the Snyder-Theilen strain of feline sarcoma virus (ST-FeSV FRE Cl 10) release a TGFα into the cell culture medium. Addition of exogenous mouse EGF to the various fractions during purification reveals a TGFβ type of growth factor of 12,000 M_r that elutes on HPLC at approximately 37% acetonitrile. The demonstration of this TGFβ-like activity in conditioned medium of rat cells nonproductively infected with transformation-defective, Abelson MuLV mutants, which do not secrete TGFαs, indicate its presence to be independent of TGFα production. Two hundred and fifty ml of fetal calf serum, equivalent to what would be present in 2.5 liters of Dulbecco's MEM used for cell culture growth of ST-FeSV FRE Cl 10 cells or transformation-defective, Abelson MuLV mutant cells, was acid ethanol-extracted and evaluated for growth factor activity after Bio-Gel P-30 chromatography. A peak of soft agar activity was detected at approximately 12,000 M_r only after an addition of 2 ng of exogenous EGF. Consequently, this extracellular TGFβ-like activity may derive from the fetal calf serum component of the culture medium used for cell growth.

3. Fischer rats bearing FeSV-induced tumors have urinary growth factors of similar molecular weight to those in FeSV-transformed cells in culture. Growth factors promoting growth in soft agar have been partially purified from the urine of Fischer rats, strain 344, bearing Snyder-Theilen FeSV-induced tumors. TMS-CpG beads effectively adsorbed soft agar growth-promoting activity from the rat urine which eluted quantitatively between 15 and 25% acetonitrile to provide a one hundredfold purification. After Bio-Gel chromatography clonogenic activity eluted in a molecular weight range that was similar to ST-FeSV-transformed cells in cell culture. Currently, a comparison is being made between urine samples from control Fischer rats and those bearing FeSV-induced tumors.

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 GF and other relevant growth factors, such as TGFα, is an essential step in this process.

Proposed Course:

Quantitative procedures for large-scale HMW GF isolation and characterization using pooled preoperative urines from patients with malignant astrocytomas are currently being developed. This will assist in identifying the HMW GF in tumored urine as identical to HMW hEGF and establishing it to be of host cell, rather than uniquely of tumor cell, origin. The long-term objective is to follow individual bulk urine collection and concentration on TMS-CpG beads with high pressure liquid chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis using silver staining of gels to quantify the relative presence of each member of the family of urinary growth factors (HMW hEGF/HMW GF, SMW hEGF, TGF and TGF). 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 on the quantity and type of urinary growth factors produced.

Publications:

Tralka, T. S., Yee, C. L., Rabson, A. B., Wivel, N. A., Stromberg, K. J., Rabson, A. S. and Costa, J. C.: Murine type C retroviruses and intracisternal A-particles in human tumors serially passaged in nude mice. J. Natl. Cancer Inst. 71: 591-599, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05333-02 LVC

PERIOD COVERED
October 1, 1983 to September 30, 1983

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

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:
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.)*

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 and a leopard. The mtDNAs have been digested with 17 different restriction enzymes and cleavage maps have been constructed for four of them (lion, cheetah, domestic cat and clouded leopard). The intra- and interspecific variations of these four 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.

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

Objectives:

1. The construction of mitochondrial DNA (mtDNA) cleavage maps from certain index species (domestic cat, cheetah, lion and clouded leopard) 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 four feline species. mtDNA has been isolated from four index cat species: the domestic cat, cheetah, lion, and clouded leopard. An average of 43 restriction enzymes has 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 four 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 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 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.

Significance to Biomedical Research and the Program of the Institute:

This work complements the in-depth biochemical and genetic studies of the domestic cat initiated by Dr. S. J. O'Brien (see Project Number Z01CE05385-01 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 is an important animal model of infectious retroviruses in natural populations (FeLV) and is a fertile system for oncogene isolation. No species, except chickens, 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 must be added to our "index species". 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., Goldman, D., Knight, J., Moore, H. D., Wildt, D. E., Bush, M., Montali, R. J. and Kleiman, D.: Giant panda paternity. Science 223: 1127-1128, 1984.

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 Vitro (In Press)

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)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05358-01 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

The Role of Retroviruses in Simian Acquired Immune Deficiency Syndrome (SAIDS)

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

PI:	K. J. Stromberg	Medical Director	LVC	NCI
Others:	R. E. Benveniste	Medical Officer	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); Litton Bionetics, Inc., Frederick, MD (H. Rabin, L. E. Henderson, S. Oroszlan); 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:
1.0	0.4	0.6

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

A novel type D retrovirus was isolated by cocultivation of explants of fibromatous tissue from a rhesus monkey (Macaca mulatta) with immunodeficiency and retroperitoneal fibromatosis (RF). This type D virus, isolated from a macaque with simian acquired immunodeficiency syndrome (SAIDS-D/Washington), is exogenous and is partially related to the Mason-Pfizer and the langur monkey type D viruses. The SAIDS-D/Washington virus can be distinguished from all other primate retroviruses by antigenicity and molecular hybridization. Nucleic acid hybridization studies reveal that the origin of the SAIDS-D isolate may reside in Old World monkey (subfamily Colobinae) cellular DNA. Foci which suggest a transformed phenotype have been noted after SAIDS-D/Washington infection of some continuous cell lines.

A biochemical and immunological characterization of SAIDS-D/Washington viral proteins is in progress. The SDS-PAGE profiles of polypeptides among type D retroviruses (MPMV, langur endogenous virus, SAIDS-D/Washington) from Old World monkeys are similar, but differences become evident upon HPLC separation of viral polypeptides and subsequent amino acid sequence analysis. By radioimmunoassay of p10 and glycoprotein antigens, the SAIDS-D/Washington retroviral isolate from RF tissue can be distinguished from two type D viruses isolated at the California and New England Regional Primate Research Centers from blood of monkeys with SAIDS.

Although some inoculated macaques are viremic, and control macaques are not, no solid tumors of RF, nor immunologic abnormalities diagnostic of SAIDS, have been observed in a cell-free transmission study of eight M. nemestrina after three months post-inoculation of SAIDS-D/Washington virus.

PROJECT DESCRIPTION

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

Kurt J. Stromberg	Medical Director	LVC	NCI
Raoul E. Benveniste	Medical Officer	LVC	NCI

Objectives:

To study aspects of the retroviral association to acquired immune deficiency syndrome in a naturally-occurring simian model system (SAIDS), including (1) presumed viral etiology through isolation of retroviruses from macaques with SAIDS and viral transmission of SAIDS with cell-free filtrates from virus-producing cultures, (2) immunological and biochemical characterization of retroviral proteins from SAIDS viral isolates, and (3) prevention of SAIDS through vaccination of susceptible primate colonies.

Methods Employed:

Tissues and fluids from the several species of macaques with clinical signs of SAIDS at the Washington Regional Primate Research Center were cocultivated with heterologous mammalian cells that had previously been shown to support the replication of a wide variety of primate viruses. Detection of virus release into the conditioned medium of these cocultivations was made by a sensitive, reverse transcriptase assay. Characterization of resulting viral isolates was made by standard virological, biochemical, and immunological techniques.

Major Findings:

1. Isolation of SAIDS-D/Washington virus. To examine the etiology of SAIDS at the Washington Primate Center, retroperitoneal fibromatosis (RF) tissue from a juvenile male rhesus monkey with immunodeficiency was cocultivated with various heterologous mammalian cells. After only two weeks, a Mg^{2+}-dependent, reverse transcriptase activity was detected in the conditioned medium from dog thymus cells. Electron microscopic examination of the virus revealed typical type D retroviral particles, indistinguishable from Mason-Pfizer monkey virus (MPMV), in that intracytoplasmic A particles were common, and budding occurred by envelopment of preformed A particles. Budding of virions with incomplete nucleoids was also observed, and characteristic of MPMV, nucleoids of the mature virions were cone-shaped. This isolate, designated SAIDS-D/Washington, can be distinguished from all other type D primate retroviruses (MPMV, the endogenous virus of langurs [PO-1-Lu], and the endogenous virus of squirrel monkeys [SMRV]) by antigenicity and molecular hybridization.

2. Characterization of SAIDS-D/Washington viral isolate. The cells which supported SAIDS-D virus growth remained fibroblastic and no cytopathic effect or morphological evidence of transformation was 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 SAIDS-D 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 MPMV. Lysed virus pellets of SAIDS-D, 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. The envelope protein of SAIDS-D appears immunologically distinct from MPMV since neither it, PO-1-Lu, nor SMRV competed in the MPMV gp70 assay. SAIDS-D virus was also tested and found 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 preliminary radioimmunoassays of p10 and glycoprotein antigens the SAIDS-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. The SAIDS-D/Washington isolate is distinct from other primate retroviruses by comparison of nucleic acid sequence homology and thermal stability, but is partially related (36 to 38%) to MPMV and the endogenous langur virus from Southeast Asian macaques. No homology was detected to the DNA of the New World, type D isolate from squirrel monkeys. As to thermal stability, the hybrids formed between SAIDS-D viral DNA transcripts and the cellular DNA of MPMV-or langur virus-infected cells melt approximately $12^{o}C$ lower than the homologous hybrid. The cellular DNA of several primate species was also tested for nucleic acid sequence homology to the SAIDS-D virus, and the highest degree of homology was obtained with DNA from the Colobinae subfamily of Old World monkeys, in particular, langur DNA (51%). Nucleic acid hybridization studies also show that the SAIDS-D virus from M. mulatta is present in a fibroma of a second macaque species, M. nemestrina, from the same primate colony.

3. Viral transmission of SAIDS. Sixteen M. nemestrina were divided into matched groups by age, sex, and origin (colony- or feral-born) for a study of SAIDS disease transmission by inoculation of SAIDS-D/Washington virus, prepared as a cell-free filtrate of 500x-concentrated, conditioned, cell culture medium. One ml of virus, at an end-point dilution of 10^{6} to 10^{7} infectious particles per ml, was inoculated IM and IV, or IP and IV, into eight macaques. No solid tumors of RF, nor definite immunologic abnormalities diagnostic of SAIDS have been observed after three months. However, whereas eight of eight control M. nemestrina were non-viremic, one of eight experimental animals was viremic at three and five weeks postinoculation as determined by cultivation of blood with an appropriate indicator cell line and assay for supernatant, reverse transcriptase activity. One of the other eight experimental animals died at five and one-half weeks post-inoculation. Pathologic examination revealed a lymphoid depletion pattern similar to that observed in animals with RF.

Significance to Biomedical Research and the Program of the Institute:

Simian AIDS represents a paradigm of human acquired immunodeficiency syndrome. 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, in respect to the origins and natural history of retroviral-induced disease, the SAIDS-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 the same geographic area.

Proposed Course:

The discovery of infectious retroviruses associated with SAIDS appears to be a general finding, and may have implications for human AIDS. Whether SAIDS-D/Washington is associated with AIDS in man will be determined by examination of appropriate human material. In any case, the ability to grow SAIDS-D virus to high titers in heterologous mammalian cells, and the availability of specific immunologic and molecular probes will allow a determination of the prevalence, and pattern of transmission of this agent among primates. An immediate objective of our effort is to determine the nature of the viral pathogenicity of SAIDS more precisely. For example, does MPMV itself cause a SAIDS-like disease after inoculation in macaques? Is biologically cloned SAIDS-D/Washington virus pathogenic? Do these viruses infect macaque thymus-derived lymphocytes in vitro? Can a purely lymphotropic retrovirus be isolated from the peripheral blood of SAIDS-affected macaques?

Publications:

Stromberg, K. J., Benveniste, R. E., Arthur, L. O., Rabin, H., Giddens, W. E., Jr., Ochs, H. D., Morton, W. R. and Tsai, C.-C.: Characterization of exogenous type D retrovirus from a fibroma of a macaque with simian AIDS and fibromatosis. Science 224: 289-292, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05382-01 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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:	N. H. Colburn	Expert	LVC	NCI
Others:	M. I. Lerman	Visiting Scientist	LEP	NCI
	K.-T. Yao	Guest Researcher	LVC	NCI
	T. Shimada	IPA Fellow	LVC	NCI
	G. A. Hegamyer	Health Science Officer	LVC	NCI

COOPERATING UNITS *(if any)*

Litton Bionetics, Inc., Frederick, MD (M. Cohen); University of Texas System Cancer Center, Smithville, TX (T. Slaga); Cancer Research Laboratory, The University of Western Ontario, London, Ontario, Canada (D. Denhardt)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.3	2.9	2.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The aim of this research is to identify and characterize genes whose activity is involved in the progression of cells from the normal to the neoplastic phenotype in mice and humans. Genes that specify sensitivity to promotion of neoplastic transformation, by tumor-promoting phorbol esters in mouse epidermal JB6 cells, have now been cloned. By carrying out a sib selection on a genomic plasmid library of DNA from promotion-sensitive cells, two independently active clones containing active segments of 1.1 and 3.8 Kb, and designated pro-1 and pro-2, have been isolated. The gene, pro-1, is structurally unrelated to any of 11 of the known oncogenes. This should not be surprising if it is the case that oncogenes are involved in maintenance of the tumor cell phenotype, while pro genes specify events in induction of neoplasia. In addition, a novel transforming activity not detectable after DNA transfection into NIH 3T3 cells has been identified in the DNA of tumor cells derived from JB6 cells by TPA exposure. The future course of this investigation of mouse genes will focus on using unique pro gene probes to determine whether the basis for resistance of promotion-resistant JB6 cells or tumor promotion-resistant rodents (in vivo) involves altered structure or altered expression of pro genes. The functions of pro genes will be sought. The nature of the interaction between pro genes, which may function only when their expression is induced by tumor promoters, and transforming genes, which may be expressed constitutively once induced or activated, will be investigated. Currently a biological assay is being developed for measuring the activity of the dominant gene that specifies the human cancer-prone trait Basal Cell Nevus Syndrome (BCNS). This assay will be used to clone the BCNS gene.

PROJECT DESCRIPTION

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

Nancy H. Colburn	Expert	LVC	NCI
Michael I. Lerman	Visiting Scientist	LEP	NCI
Kai-Tai Yao	Guest Researcher	LVC	NCI
Tomiko Shimada	IPA Fellow	LVC	NCI
Glenn Hegamyer	Health Science Officer	LVC	NCI

Objectives:

To elucidate the nature of genetically determined events that are causally related to preneoplastic progression in mice and humans. To clone the genes involved in tumor promoter-induced progression to the tumor cell phenotype in JB6 mouse epidermal cells. To characterize the structure, function and mode of regulation of these genes. To develop a biological assay for the gene that specifies the cancer-prone trait Basal Cell Nevus Syndrome. To clone the Basal Cell Nevus gene.

Methods Employed:

Gene cloning techniques using "search" and "rescue" strategies. DNA-calcium-phosphate DNA transfection followed by assay for gain of sensitivity to promotion of anchorage-independence by the tumor promoter, TPA. Sequencing the cloned genes by the Maxam and Gilbert technique. Computer-aided analysis of their structure and possible function. Southern and Northern transfer techniques to analyze genome organization and expression of the pro genes. Restriction mapping techniques are also utilized.

Major Findings:

1. Genes that specify sensitivity to promotion of neoplastic transformation by tumor-promoting phorbol esters in mouse epidermal JB6 cells have now been cloned. The cloning included establishment of a genomic plasmid library of DNA from promotion-sensitive (P^+) cells in the BamHI site of the Okayama-Berg expression vector pcD-X and isolation of active clones by Lederberg's sib-selection technique, using as the assay for biological activity the gain of promotion sensitivity after DNA transfection.

Two independently active clones, p26 and p40 (inserts of 3.4 Kb and 12 Kb, respectively) were isolated and characterized. They showed equal specific activity (per mole gene) compared to the specific activity of the total P^+ DNA. Stated differently, when DNA concentration during transfection was varied, the activity of the total P^+ DNA was no longer detectable at 1 μg per dish, while the activities of p26 and p40 were still detectable at 10-100 pg per dish.

2. These promotion-sensitive genes, contained in p26 and p40, are different from each other. EM heteroduplex analysis and hybridization between the functional segments, p26 and p40, failed to show any structural homologies between them, thus indicating that they represent different genes. This suggests that sensitivity to

promotion of neoplastic transformation can be specified by at least two genes, either of which can act alone to determine the same phenotype.

3. The p26 pro gene, designated pro-1, is different from 11 of the known viral oncogenes. Southern blot hybridization under stringent conditions using the active segment of p26 as a ^{32}P-labeled probe showed that pro-1 is not related to abl, fes, fms, mos, myc, myb, 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. Determination of the degree of structural relationship of these oncogenes to the p40 gene (pro-2) is underway.

4. Restriction mapping and transfection of segments of the inserts located the active genes to 1.1-Kb and 3.8-Kb segments of p26 and p40, respectively. This demonstrates that the cloned genes are functionally active and do not need the SV-40 promoter of the pcD-x vector. They also do not need anymore of the mouse sequence than the 1.1- and 3.8-Kb sequences, respectively. Sequencing of the p26 gene (the 1.1-Kb segment) is now nearly complete.

5. Pro genes appear to exist in transcriptionally active configurations. The active genes (pro-1 and pro-2), show DNase I hypersensitivity, (i.e., sensitivity to low concentrations of DNase I under conditions that produce little effect on DNA size) suggesting that they exist in transcriptionally active configurations in the chromatin of both P^+ and transformed cells. This implies that these genes are being or have been transcribed into mRNA.

6. 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 p26 P transfectants are sensitive to promotion of transformation by TPA at similar concentrations, but unlike the parental cells, they are insensitive to promotion by epidermal growth factor. These P transfectants are, like the parental P cells, sensitive to the antipromoter, retinoic acid, but 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.

7. A new 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). This 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 detectable using the NIH 3T3 assay.

8. This transforming sequence appears to be different from pro genes. 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^- cells, suggests that this transforming activity is specified by a gene different from pro genes.

9. A human sperm library contains a sequence homologous to a subclone of pro-1 as a single copy. Hamster and baboon DNAs also showed low copy numbers of pro-1 homologs, while rat DNA showed a high copy number. This suggests that while the pro-1 gene is apparently repeated in certain species, it may exist in a cellular or inactive form in normal cells of other species, including humans.

10. Pro-1 homology was found for DNAs from about half of some 14 different human tumors. The human tumor DNAs that were homologous to pro-1 (under stringent conditions) included some but not all carcinomas, fibrosarcomas and leukemias tested. The possibility that pro-1 activity could be significant in the etiology of certain classes of human tumors is being investigated.

11. Fibroblasts of Basal Cell Nevus Syndrome (BCNS) patients but not age-matched normal fibroblasts can be induced to escape senescence by transfection of pro-1 DNA. After transfection of p26 plasmid DNA, BCNS fibroblasts, but not age-matched normal fibroblasts, have undergone at least four more population doublings than untransfected controls which have now undergone senescence. This suggests that pro-1 may be able to function as an "immortalizing" gene for certain human cells. It further suggests that pro-1 can complement the BCNS gene to specify cellular immortalization, a change that has been postulated to be one event in the process of neoplastic transformation.

12. BCNS cells, but not matched normal fibroblasts, can be induced to form morphologically transformed foci by transfection of the v-myc oncogene. This may constitute the much-needed biological assay which will permit the cloning of the BCNS gene.

13. Promotion-sensitive and promotion-resistant JB6 cells do not differ on the basis of DNA methylation, either in response to TPA or in basal methylation. This has been reported by Bondy and Denhardt (Carcinogenesis 4: 1599-1603, 1983). University of Western Ontario (see Cooperating Units).

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.

Proposed Course:

Answers to the following questions will be sought after subcloning unique probes from one or both pro genes. (1) Are JB6 P^- cells promotion-resistant because: (a) They lack pro (P^+) genes (or homologs of pro genes)? (b) They have pro genes but they are rearranged? (c) They have pro genes but copy number is insufficient? (d) They have pro genes but they are not transcribed? (e) They

have pro genes that are both transcribed and translated, but the products are inactive due to lack of an activating point mutation? (2) Are skin tumor-promotion-resistant (by TPA) strains of mice resistant for one of the above reasons a-e? Likewise for promotion-resistant species like hamsters? (3) Can pro genes and/or gene expression be detected in skin during the process of mouse skin two-stage carcinogenesis? At what stage? (4) What is the nucleotide sequence of pro-1 (p26)? Of pro-2 (p40)? Is it related to the sequences of any known genes? (5) What are the function(s) of pro genes? (6) What is the structure and function of the pro homologs in human cells? (7) What is the range of human cells that can be immortalized by pro-1?

In addition, other proposed objectives include the following: (1) to clone the novel transforming gene found in TPA-induced tumor cells and to determine how it interacts with the pro genes to bring about expression of tumor cell phenotype, and (2) to continue to work out the assay for biological activity of the BCNS gene and to clone the gene that specifies cancer predisposition in human Basal Cell Nevus Syndrome.

Publications:

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, J. M., Greaves, M. and Rowley, J. D. (Eds.): Genes and Cancer. New York, Alan R. Liss, Vol. 17 (In Press)

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 (In Press)

Colburn, N. H., Talmadge, C. B. and Gindhart, T. D.: Transfer of sensitivity to tumor promoters by transfection of DNA from sensitive into insensitive mouse JB6 epidermal cells. Mol. Cell. Biol. 3: 1182-1186, 1983.

Sobel, M. E., Dion, L. D., Vuust, J. and Colburn, N. H.: Tumor-promoting phorbol esters inhibit procollagen synthesis at a pretranslational level in JB-6 mouse epidermal cells. Mol. Cell. Biol. 3: 1527-1532, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05383-01 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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:	N. H. Colburn	Expert	LVC	NCI
Others:	Y. Nakamura	Guest Researcher	LVC	NCI
	B. Smith	Guest Researcher	LVC	NCI
	T. D. Gindhart	Expert	LEP	NCI

COOPERATING UNITS *(if any)*

University of Texas Medical School, Galveston, TX (R. Fleischman and M. Brysk)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.8	2.4	1.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The goal of these studies is to determine the required biochemical events occurring between the interaction of tumor promoters with the plasma membrane and the activation of effectors of neoplastic transformation. Candidate second messengers include protein phosphorylation, reactive oxygen generation, and calcium mobilization. Recent evidence indicates that the free radical superoxide anion, but not hydrogen peroxide or singlet oxygen, 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. We have previously reported that the major epidermal cell surface ganglioside, trisialoganglioside (GT), shows substantially decreased net synthesis after tumor promoter exposure in promotion-sensitive (P+), but not in promotion-resistant (P-), JB6 cells. This observation led to the hypothesis that GT is an oxidative target of oxygen radicals generated after TPA exposure, and that this oxidation leads to decreased GT synthesis and promotion of transformation. Predictions of this hypothesis have been confirmed in a recent article demonstrating that sodium metaperiodate ($NaIO_4$), a known oxidizer of cellular GT sialic acid, produced both decreased GT synthesis and promotion of transformation. Recent evidence indicates that promotion of transformation in JB6 cells requires both extracellular calcium and calcium mobilization from bound pools. It is postulated that certain calcium-dependent enzymes, including the phorbol ester receptor, protein kinase C, play a regulatory role in tumor promotion. The proposed course will be concerned with: (1) determining the promotion-relevant, membrane lipid targets of reactive oxygen generated by phorbol esters, (2) characterizing the decrease in SOD by tumor promoters, (3) elucidating the calcium-dependent processes that regulate tumor promotion, and (4) understanding how these signal transduction events regulate the expression of pro genes.

PROJECT DESCRIPTION

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

Nancy H. Colburn	Expert	LVC	NCI
Yoshiyuki Nakamura	Guest Researcher	LVC	NCI
Bonita Smith	Guest Researcher	LVC	NCI
Thomas Gindhart	Expert	LEP	NCI

Objectives:

To determine the required biochemical events occurring 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 include protein kinase C-catalyzed protein phosphorylation, reactive oxygen generation, and calcium mobilization.

Methods Employed:

Assay of the effects of various modulators of reactive oxygen on promotion of neoplastic transformation (anchorage-independence) by TPA. Assay of the effects of these modulators on the expression of anchorage-independence by tumor cells. Determination of ganglioside oxidation by dinitrophenyl hydrazine derivatization and thin layer chromatography. Assay of effects of various modulators of calcium uptake and mobilization on the promotion (by TPA) and expression (without TPA) of the tumor cell phenotype as measured by anchorage-independent growth in 0.33% agar. Assay of calcium-dependent phospholipid-dependent protein kinase (C kinase) activity. (See Project Number Z01CE15273-03 LEP)

Major Findings:

1. The free radical superoxide anion, but not hydrogen peroxide or singlet oxygen, appears to be an essential mediator of promotion of neoplastic transformation in JB6 mouse epidermal cells. Bovine superoxide dismutase (SOD), which removes the superoxide anion, inhibits the induction of transformation (anchorage-independence) by TPA in JB6 cells, but not expression of transformation by tumor cells derived from JB6. Promotion is also specifically inhibited by the SOD mimetic, copper diisopropylsalicylate (Cu[II]DIPS), but not by the hydrogen peroxide eliminators, catalase or glutathione peroxidase, or by a singlet oxygen quencher. Copper DIPS and its congeners may, therefore, be useful in cancer prevention.

2. The activity of the superoxide anion is critical during the first two hours after the phorbol ester interacts with its cell surface receptor (C-kinase). Delay of the addition of SOD by one hour results in no loss of promotion-inhibitory activity, but delay by two 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 leading to transformation.

3. Hydrogen peroxide, on the other hand, acts to inhibit expression of the tumor cell phenotype by MNNG- and TPA-induced mouse epidermal tumor cells. Addition of highly purified catalase (which removes hydrogen peroxide) to any of several tumorigenic, mouse epidermal cell lines derived by exposure to methyl nitro nitrosoguanidine (MNNG) or TPA, produced enhanced yields of anchorage-independent colonies.

Catalase addition increases the yield of TPA-induced colonies from JB6 promotion-sensitive (P^+) cells, but this can be accounted for at the level of enhanced expression of the tumor cell phenotype. These observations suggest cause for optimism that the elevation of hydrogen peroxide will selectively inhibit the growth of tumor cells.

4. The cell surface, promotion-relevant trisialoganglioside (GT) may be a target of oxidation by the transformation promoter, sodium metaperiodate, but, possibly, not by radicals generated by TPA. We have previously shown that TPA and other tumor promoters produced a substantial decrease in the synthesis of cell surface trisialogangliosides in P^+, but not promotion-insensitive (P^-) JB6 cells. Low G_T synthesis also characterizes tumorigenic transformants of JB6 and other chemical transformants. The following hypothesis was tested: that TPA and certain other tumor promoters produce elevated reactive oxygen, which produces oxidation of cell surface gangliosides, which, in turn, causes decreased ganglioside synthesis, and, consequently, promotion of neoplastic transformation. Initial evidence supports this hypothesis for promotion of transformation by the cell surface sialic acid oxidant, sodium metaperiodate ($NaIO_4$), but leaves the question unanswered for superoxide generators. Measurements of sialic acid oxidation by derivatization of the aldehyde formed with dinitrophenyl hydrazine demonstrated that G_T oxidation in the sialic acid does occur after cell-free exposure of G_T to sodium metaperiodate, but not to potassium superoxide. The critical, unanswered question is whether oxidized G_T can be found after exposure of cells to TPA.

5. Extracellular calcium is apparently required for TPA-induced promotion of transformation in JB6 cells. Both the divalent ion chelator, ethylene glycol-bis(β-aminoethylether)-N,N-tetraacetic acid (EGTA), and the calcium channel blocker, lanthanum, (low concentrations) inhibited TPA-promoted transformation, but not the expression of anchorage-independence by tumor cells. Hence, the process of induction of transformation in JB6 cells requires movement of calcium into the cells by TPA. This suggests that promotion involves a calcium-dependent event such as the action of a calcium-dependent enzyme.

6. Calcium binding and/or mobilization at the cell membrane may be required for promotion of transformation. At high, but nontoxic, concentrations at which it stimulates calcium mobilization from bound to free forms, lanthanum acts as a promoter of transformation in JB6 P^+ cells. This suggests that calcium-mobilization from bound to free forms may even be sufficient for triggering second stage promotion of neoplastic transformation (to which JB6 P^+ cells appear to be analogous).

7. Some 18 endogenous substrates have been identified for the calcium-dependent, phospholipid-dependent protein kinase C in JB6 cells. (See Project Number Z01CE15273-03 LEP) One or more of these substrates is expected to be promotion-relevant and could turn out to be related to products of genes that specify promotion-sensitivity. (See Project Number Z01CE05382-01 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 on the market. These signals that may regulate the expression of "preneoplastic progression" genes can very likely be controlled in ways that will result in increased latent periods for cancer.

Proposed Course:

(1) Attempts will be made to determine, via several, independent approaches, whether the promotion-relevant targets of reactive oxygen are DNA or membrane lipids and, if the latter, which lipids? In particular: (a) Follow up the cell-free determinations of oxidative products produced from gangliosides by exposure to $NaIO_4$ or certain radical-generating agents, with a demonstration of ganglioside oxidation products produced in cells after exposure to $NaIO_4$, TPA, or other superoxide-generating agents. (b) Follow up the initial observation that TPA produces a substantial decrease in SOD in promotion-sensitive, but not in promotion-resistant, cells with detailed time course studies. Determine whether G_T blocks this event. (c) Regarding DNA as the target, Gensler and Bowden recently published (Carcinogenesis 4: 1507-1511, 1983) the finding that the promotion of transformation in JB6 cells could be dissociated from DNA strand-break production by radical-generating tumor promoters. The Bowden laboratory is pursuing this further by examining other kinds of DNA damage. (2) Elucidate the calcium-dependent processes (reflected in sensitivity to a decrease in the extracellular calcium pool) that are required for the process of promotion of neoplastic transformation by TPA. (3) Determine the nature of the calcium-dependent process that inhibits TPA-promoted transformation. Is it the elevation of G_T, via its synthesis and/or its degradation? (4) Determine whether certain combinations of interferons discriminate in their growth-inhibitory action between preneoplastic JB6 P^+ cells and paired neoplastic cells.

Publications:

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 (In Press)

Colburn, N. H., Srinivas, L., Hegamyer, G. A., Dion, L. D., Wendel, E. J., Cohen, M. and Gindhart, T. D.: The role of specific membrane and gene level changes in the mechanism of tumor promotion: Studies with promoter resistant variants. In Borzsonyi, M., Yamasaki, H. and Hecker, E. (Eds.): The Role of Cocarcinogens and Promoters in Human Experimental Carcinogenesis. Lyon, International Agency for Research on Cancer Scientific Publications (In Press)

Fleischmann, W. R., Newton, R. C., Fleischmann, C. M., Colburn, N. H. and Brysk, M. M.: Discrimination between nonmalignant and malignant cells by combinations of IFN-γ plus IFN-$\alpha\beta$. J. Biol. Resp. Modif. (In Press)

Srinivas, L. and Colburn, N. H.: Preferential oxidation of cell surface sialic acid by periodate leads to promotion of transformation in JB6 cells. Carcinogenesis 5: 515-519, 1984.

Srinivas, L. and Colburn, N. H.: Reduced trisialoganglioside synthesis in chemically but not mos-transformed mouse epidermal cells. Cancer Res. 44: 1510-1514, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05384-01 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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:	Ulf R. Rapp	Chemist	LVC	NCI
	Douglas R. Lowy	Deputy Chief	DB	NCI
	Robert C. Gallo	Chief	LTCB	NCI
	Takis S. Papas	Acting Chief	LMO	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	Mitchell H. Gail	Med. Stat. Inves.	CNDTS	NCI

COOPERATING UNITS *(if any)* LBI, Frederick, MD (M. Cohen); 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); Johns Hopkins Hospital, Baltimore, MD (K. Smith); PRI, Frederick, MD (W. G. Nash, R. F. Bauer, Jr.)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	0.8	1.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Mapping panels of somatic cell hybrids between rodent and human cells have been employed in an extensive series of mapping experiments to genetically locate a variety of human cellular genes which participate in neoplastic transformation. The hybrids, which were made with either normal or neoplastic human parent cells, segregate human chromosomes in different combinations. Seven distinct classes of neoplasia-related genes (with examples) have been studied and these include: (1) cellular proto-oncogenes which are sequentially homologous to retroviral or transfectionally active oncogenes (raf-1,2, Ha-ras-1, Ha-ras-2, Ki-ras-1, Ki-ras-2, ets, and rel); (2) growth factors (IL-2 or TCGF); (3) growth factor receptors (EGFR); (4) endogenous cellular DNA sequences homologous to retroviral RNA genomes (ERV1,2,3...); (5) integration sites for retroviruses (BEVI, HTLV-I); and (6) restriction genes which delimit retroviral replication in mammals. Within the last two years, the human gene map has experienced a large increase in the number of neoplasia loci which have been mapped to specific chromosomal positions. Of the 27 specific human loci which have been chromosomally mapped to date, 11 (40%) of these have been assigned by the Genetics Section scientists and their collaborators. The construction of this extensive human map has played an important role in the resolution of early genetic events in neoplastic transformation in man. The genetic analysis of each of these loci has been considered in the perspective of the entire genome, of the rapidly advancing human gene map, and of the nonspecific chromosomal rearrangements characteristic of human malignancies. The emerging human gene map has provided, and 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
Ulf R. Rapp	Chemist	LVC	NCI
Douglas R. Lowy	Deputy Chief	DB	NCI
Robert C. Gallo	Chief	LTCB	NCI
Takis S. Papas	Acting Chief	LMO	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Mitchell H. Gail	Med. Stat. Inves.	CNDTS	NCI

Objectives:

1. The augmentation of the human gene map with loci which have direct or indirect connections to the processes of neoplastic transformation in man or in other vertebrate model systems. The specific genes under study fall into six general groups: (a) cellular proto-oncogene loci, (b) growth factors, (c) receptors for growth factors and retroviruses, (d) endogenous cellular DNA sequences homologous to retroviral RNA genomes, (e) chromosomal integration sites for chronic transforming retroviruses, and (f) restriction genes which delimit retroviral replication.

2. The combined application of somatic cell genetics and electrophoretic resolution of cellular DNA sequences following digestion with specific restriction endonucleases to the biology of retroviral integration, excision and transposition in human cells.

3. 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.

4. The development of new approaches to the understanding of genetic control of carcinogenesis. These goals involve the identification and characterization of genetic targets (cellular genes) of carcinogenesis.

5. 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 oncogenes. The ras gene family in man consists of five distinct cellular genes: Ha-ras-1, Ha-ras-2, Ki-ras-1, Ki-ras-2, and N-ras. Three of these are functional loci with intervening sequences, while the other two are apparently intronless pseudogenes which lack transforming activity (in 3T3 cells) even after ligation to potent LTR promoters. The human ras genes represent a family of transforming genes most frequently isolated from human tumors by the NIH-3T3 transfection assay. We have mapped four of the human ras loci to four different human chromosomes and the fifth, N-ras, has been located on a fifth chromosome by a different laboratory. The structure and chromosomal positions of the two "processed" pseudogenes suggest that their generation probably involved a cDNA copy of a processed mRNA.

The raf oncogene is a murine homologue which was independently captured in the avian MH2 transforming virus. Humans have two copies of this proto-oncogene, c-raf-1, a functional locus containing at least eight exons and seven introns (see Project Number Z01CE4868-09 LVC), and an intronless pseudogene with chain termination signals in each reading frame. The two raf loci mapped to human chromosomes 3 and 4, respectively, using our human hybrid panel. Two other human oncogenes, ets and rel, have also been studied using molecular clones of the retroviral locus and DNA extracts of hybrid DNA. Both of these proto-oncogenes are near firm assignments of chromosomal positions in man. Of all the oncogene loci studied in man to date, only three (ras, raf, and ets) have apparent pseudogenes. The generation and/or transposition of these pseudogenes may be very ancient since, in at least the case of one family (ras), the presence of and homologous chromosomal linkage group of the pseudogenes has been retained in the cat genome (see Project Number Z01CE05385-01 LVC).

2. Endogenous retroviruses. Within the last two years, three independent laboratories, using molecularly cloned probes, have identified three, apparently different, families of human DNA sequences which are related to replication-competent retroviruses isolated from other mammals. The groups include: (a) a small group distantly related to Mo-MuLV and BaEV, described by M. Cohen et al., (b) a larger family of 30-50 segments related to Mo-MuLV, described by M. Martin et al., and (c) 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 since specific transcripts can be detected using Northern analysis. The hybrid panels have also been used to examine the chromosomal location of the Martin family of 30+ copies. These 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.

3. The chromosomal organization of the 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) was assigned to chromosome 5 and its pseudogene, hDHFR-ψ4 to chromosome 3.

Since DHFR is known to be capable of DNA amplification, we designed an experiment to test which of the five genes participated in the amplification (methotrexate-mediated) process. In the one human cell line examined, the results were definitive insofar as only the functional gene was amplified, while none of the four "pseudogenes" were amplified. Finally, since DHFR-ψ1 is sequentially identical to the composite exon sequence of the functional gene, we hypothesized that the pseudogene formation, probably via a cDNA copy of a processed mRNA, may have been a recent event in human populations. By this logic, we searched for a restriction fragment length polymorphism (RFLP) of the DHFR-ψ1 locus in natural human populations. One was detected and upon extensive restriction enzyme analysis, was shown to represent (as expected) a polymorphism for an empty site of pseudogene insertion in a manner reminiscent of endogenous retroviral polymorphisms in chickens and cats (see Project Number Z01CE05385-01 LVC). The presence/absence of the intronless pseudogene represents a novel form of human DNA polymorphism.

4. Genetic analysis of the human Y chromosome-specific family. 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 which has been under study is the 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: (a) a Y-specific family which is repeated 7500 times per haploid genome and which comprises over 40% of all Y chromosome sequences; and (b) NYS for non-Y-specific family which 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.

5. Miscellaneous accomplishments. In collaboration with Dr. Mitchell Gail, a novel mathematical statistic was developed which is designed to estimate the number of members of a particular gene family which exists in the human genome, based upon the frequency of single, double, triple...hit isolations of the same

genes in a cloning experiment from a recombinant phage library. This statistic has been used to estimate the total number of human proto-oncogenes, the number of endogenous human retroviral segments and of other gene families. The neoplastic/oncogene genetic map of man has been assimilated from participating laboratories and is included in the 1984 edition of Genetic Maps (S. J. O'Brien, Editor).

Significance to Biomedical Research and the Program of the Institute:

The characterization of identified loci which participate in cell transformation has two important applications: (1) as the raw material for the dissection of developmental genetic analysis of the cellular events which lead to neoplastic transformation, and (2) as possible targets for carcinogens in screening protocols. The specific understanding of the developmental genetic sequence which characterizes the neoplastic event is necessary for any meaningful attempt to correct and to 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 which 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 are under development 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 Nienhius, A. W.: The chromosomal organization and assignment of the human dihydrofolate reductase genes: Dispersion, selective amplification and polymorphism. Proc. Natl. Acad. Sci. USA (In Press)

Barile, M. F., Grabowski, M. W., Stephens, E. B., O'Brien, S. J., Simonson, J. M., Izumikawa, K., Chandler, D. K. F., Taylor-Robinson, D. and Tully, J. G.: Mycoplasma hominis - tissue cell interactions: A review with new observations on phenotypic and genotypic properties. Sex. Trans. Dis. 10: 345-354, 1983.

Bonner, T. I., O'Brien, S. J., Nash, W. G. and Rapp, U. R.: The human homologs of the raf oncogene are located on human chromosomes 3 and 4. Science 223: 71-74, 1984.

O'Brien, S. J.: An abridged human gene map including: (1) oncogenes, (2) other neoplasia related loci, and (3) biochemical loci whose homologous counterparts have been assigned in other species. In O'Brien, S. J. (Ed.): Genetic Maps. New York, Cold Spring Harbor Press, 1984, Vol. 3, pp. 451-456.

O'Brien, S. J. (Ed.): Genetic Maps. New York, Cold Spring Harbor Press, 1984, Vol. 3, 583 pp.

O'Brien, S. J., Bonner, T. I., Cohen, M., O'Connell, C. and Nash, W. G.: Mapping of an endogenous retroviral sequence to human chromosome 18. Nature 303: 74-77, 1983.

O'Brien, S. J., Nash, W. G., Goodman, J. L., Lowy, D. R. and Chang, E. H.: Dispersion of the ras family of transforming genes to four different chromosomes in man. Nature 302: 839-842, 1983.

Roderick, T. H., Lalley, P. A., Davisson, M. T., O'Brien, S. J., Womack, J. E., Creau-Goldberg, N., Echard, G. and Moore, K. L.: Comparative gene mapping in mammals: Report of the international committee. Cytogenet. Cell Genet. 37: 312-339, 1984.

Seibert, K., Shafie, S. M., Tricke, T. J., Whang-Peng, J. J., O'Brien, S. J., Toney, J. H., Huff, K. K. and Lippman, M. E.: Clonic variation of MCF-7 breast cancer cells in vivo and in vitro. Cancer Res. 43: 2223-2239, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05385-01 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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:	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, D. A. Gilbert); Program Resources, Inc., Frederick, MD (W. G. Nash, R. F. Bauer, Jr.); University of California, San Diego, CA (J. S. O'Brien); Mt. Sinai School of Medicine, New York, NY (R. Desnick)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.6	0.4	3.2

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.)*

A combination of technical advances (cell fusion, high resolution G-banding, and molecular cloning) has contributed to an accelerated advance in genetic analysis in mammals. Comparison of linkage relationships of homologous loci provides an opportunity to examine the status of genome organization in animal models of cancer and human inborn errors. We have prepared a biochemical genetic map of the domestic cat, including over 45 loci over the past few years. Included in this map are several classes of loci which participate in human diseases and cancers: (1) endogenous cellular DNA sequences homologous to retroviral RNA genomes, (2) cellular proto-oncogene homologues, (3) growth factor receptors, (4) restriction genes which delimit viral replication, (5) cell surface antigens including the major histocompatibility complex, (6) integration sites of retroviruses, and (7) structural genes for lysosomal enzymes involved in human inborn errors for which there are feline models. Comparative analysis of the feline and human gene maps revealed a striking conservation of linkage association to the extent that approximately 25% of the human genome can be aligned band for band to the corresponding chromomere in the feline genome. The natural history of the endogenous retroviruses and the oncogene homologues during the mammalian radiations has been interpreted in the context of genomic evolution which, in several ways, has recapitulated the chronic processes of neoplastic transformation in man. Eight lysosomal enzymes have been chromosomally assigned in the cat and molecular clones of their human counterparts are being developed in anticipation of gene delivery to feline disease models. The genetic status of related species of the Felidae has revealed a rather recent, morphological divergence and the composite results on one nondomestic species have dramatically demonstrated the importance of abundant polymorphism at the major histocompatibility complex in mammals as a defense against adventitious, virological epidemics in natural populations.

PROJECT DESCRIPTION

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

Stephen J. O'Brien	Geneticist	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 extension of the biochemical genetic map of the domestic cat (Felis catus) with emphasis on genes which relate to the viral etiology of leukemia and lymphoma in the species. The specific classes of genes under study fall into seven general groups. The genes include: (1) endogenous cellular DNA sequences homologous to cDNA radioactive probes transcribed from retroviral genomic RNA, (2) chromosomal integration sites for exogenous retroviral insertion and persistence, (3) receptors on cell membranes which interact with viral glycoproteins to determine cell-species compatibility and viral host range, (4) restriction genes which 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 analysis of linkage and genomic organization between mammals which have received emphasis in genetics studies including man, mouse, cat, and nonhuman primates.

3. Development of immunological reagents for analysis of feline lymphoid- and myeloid-cell derivatives with specific emphasis on correlates to immune function, in vivo and in vitro.

4. Describe the molecular and chromosomal organization of endogenous retroviral families (specifically RD-114 and FeLV) in the domestic cat and its nondomestic relatives.

5. Development of baseline genetic data, molecular cloning, and embryological procedures for gene delivery of promotionally functional, lysosomal enzyme structural genes to feline models of human inborn errors.

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. Alignment of the feline and human linkage maps predicted and later proved a striking cytogenetic homology between the primate and Felidae families. Development of the feline genetic map in our laboratory revealed that 45 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 striking concordance of feline and primate genetic maps has two major aspects of biological significance: first, the evolutionary implications are rather significant since the chromosome organization has maintained some semblance of order despite 80 million years of divergence (between primates and felids); and second, the comparative genetics has a predictive value, since once a gene has been located in the cat, a strong suggestion as to the position of a homologous locus in man can be made. This aspect may be especially important in identifying mammalian genes (like retroviruses or controlling elements) capable of transposition during mammalian evolution.

2. Molecular genetic characterization of the RD-114 family of endogenous retroviral genes in the cat. RD-114 is a replication-competent, xenotropic retrovirus which 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, as well as to that 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 which 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 found to be dispersed on multiple feline chromosomes. The chromosomal locations of four RD-114 virogenes (REV1-4) were determined using a panel of rodent x cat somatic cell hybrids. One of these, REV1, is apparently a single locus on feline chromosome B3 which is inducible for replication-competent RD-114 virus.

3. Comparative genetic analysis of oncogenes in mammals. Molecular clones of each of 17 unique oncogenes have been obtained from initiating laboratories. Batch DNA extracted from the genetically characterized hybrids of the feline

hybrid panel has been used to assign feline oncogene homologues to cat chromosomes. Preliminary assignments have been obtained for myc, c-Ha-ras-1, c-Ha-ras-2, c-Ki-ras-1, c-Ki-ras-2, fes, and sis. 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 these five genes in the cat conform precisely to their predicted positions from aforementioned chromosomal homologies. Thus, the amplification and dispersion of both the functional and pseudogene members of this family have preceded the divergence of the carnivores and primates. Further, the genetic position of these oncogenes has been chromosomally inert (as opposed to transposition ally mobile, like retroviral families) for over 100 million years of mammalian evolution. A similar analysis of mouse/human oncogene linkage homologies from a similar perspective has reaffirmed this conclusion.

4. Genetic description of the major histocompatibility complex of the cat. A major histocompatibility complex (MHC) responsible for surgical allograft rejection and subsequent induction of alloantibodies has been defined in a number of laboratory mammals 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 Z01CE05389-01 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 using a two-stage cytotoxic antibody assay. The panel of sera was tested against 50 unrelated cats in a "cluster" analysis to subdivide the serological reagents. Each serum had a unique distribution among the 50 cats and no serum reacted with all of the cats. These results indicate an appreciable degree of allogeneic polymorphism at the MHC locus. Transmission tests of selected sera in cat families confirmed the notion that antigens were inherited in conformance with Mendelian expectations. The chromosomal location of the feline MHC has been determined by an FACS analysis of a feline rodent x feline somatic cell hybrid panel using one of the alloantisera derived from the grafts. The cluster analysis of the allogeneic sera is in progress and is expected to yield a more precise understanding of the genetic and immunological components of this locus.

5. A dramatic demonstration of the adaptive value of abundant genetic polymorphism, especially at the MHC for species' responses to viral epidemics: The case of the South African cheetah. A population genetic survey of over 200 structural loci had revealed that the South African cheetah (Acinonyx jubatus jubatus) has an extreme paucity of genic variability, probably as a consequence of a severe population bottleneck in its recent past. The genetic monomorphism of the species is extended to the MHC, since fourteen 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 of the species, (b) a high degree of juvenile mortality in captivity and in the wild, and (c) a markedly

high extent of spermatozoal abnormalities in cheetah ejaculates. The species-vulnerability of the cheetah was dramatically demonstrated by an epizootic of coronavirus-associated, feline infectious peritonitis (FIP) in an Oregon breeding colony in 1983. Exposure and spread of the FIP coronavirus, which has a very low morbidity (approximately 1%) in domestic cats, 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 reflect the pathogen's abrogation of the "associative recognition" (also called MHC restriction) of the cats' T-cell-mediated immune mechanism. As such, these results provide a biological example for understanding the adaptive significance of the extreme polymorphism at the MHC in outbred mammalian species.

6. Genetic analyses of structural genes for lysosomal enzymes in the cat. The domestic cat has provided a number of models of storage diseases which 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 which results in deficiency (in the parentheses) were: (a) β-glucuronidase (Mucopolysaccharidosis-VII), (b) α-galactosidase (Fabry's disease), (c) α-galactosidase (GM1-gangliosidosis), (d) β-mannosidase (mannosidosis), (e) β-glucosidase (Gaucher's disease), (f) fucosidase-A (α-fucosidosis), (g) β-hexosaminidase-A (Tay-Sach's disease), and (h) 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 Z01CE05389-01 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 for acquired immune deficiency syndrome. The availability of feline mutants which have biochemical deficiencies homologous to human inborn errors makes the feline genetic system even more useful for gene delivery protocols. Despite the rapid advances which have occurred in human molecular biology, the testing of gene delivery systems in animal models still remains a clinical and ethical imperative.

Proposed Course:

The continued pursuit of genetic mapping of oncogenes in both cat and man (see Project Number Z01CE05384-01 LVC) is anticipated. FeLV tumors and spontaneous cat tumors will be cytologically monitored for specific chromosomal rearrangements. The immunogenetic analysis of hematopoietic differentiation is anticipated using

monoclonal antibody reagents and functional in vitro assays. Gene delivery to feline and murine animal models of human genetic diseases is under development.

Publications:

O'Brien, S. J., Berman, E. J., Estes, J. D. and Gardner, M. B.: Murine retroviral restriction genes Fv4 and Akvr-1 are alleles of a single locus. J. Virol. 47: 649-651, 1983.

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: 1127-1128, 1984.

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 Vitro (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. Genetics (In Press)

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'Brien, S. J., Simonson, J. M., Razine, S. and Barile, M. F.: On the distribution and characteristics of isozyme expression in mycoplasma, ureaplasma and acholeplasma. Yale J. Biol. Med. (In Press)

Roderick, T. H., Lalley, P. A., Davisson, M. T., O'Brien, S. J., Womack, J. E., Creau-Goldberg, N., Echard, G. and Moore, K. L.: Comparative gene mapping in mammals: Report of the international committee. Cytogenet. Cell Genet. 37: 312-339, 1984.

Wildt, D. E., Bush, M., Howard, J. G., O'Brien, S. J., Meltzer, D., van Dyk, A., Ebedes, H. and Brand, D. J.: Diminished ejaculate quality in the South African cheetah. Biol. Reprod. 21: 1019-1025, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05386-01 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Genetic Analysis of HTLV Integration in Human Adult T-Cell Lymphoma

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
	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:
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.)*

Human T-cell leukemia/lymphoma virus (HTLV-I) is a unique, exogenous, horizontally transmitted retrovirus which has been isolated from patients with certain T-cell leukemias and lymphomas in southern Japan, the Caribbean basin, southeastern United States and elsewhere. The virus is capable of rapid transformation of T-cells in vitro, but the mechanism(s) of transformation both in vitro and in vivo is unclear. Previous avian and mammalian retrovirus isolates have been demonstrated to induce transformation by two different mechanisms; directly through the protein product encoded by an oncogene included in the retroviral genome or by alteration of the regulation of operative cellular loci by integration adjacent to normal cellular genes that are capable of causing transformation, when expressed (or repressed) in an unscheduled manner. Since HTLV-I does not carry an oncogene related to human DNA, the latter mechanism is a distinct possibility. We have utilized somatic cell hybrids constructed between rodent cells and various HTLV-I-infected cell lines to study the processes and consequences of HTLV-I chromosomal integration, as well as certain biologic phenomena observed in HTLV-I-infected cells. Integration in primary tumors was found to be monoclonal, but dynamic with as many as 20 secondary HTLV integrations occurring subsequent to tumorigenesis. The primary integration site of two HTLV-I-associated tumors have been chromosomally mapped and their sites are being examined from two perspectives: (1) to determine the specificity (or not) of targeted HTLV-I integration, and (2) the role of the chromosomal "neighborhood" of integration in the neoplastic transformation process.

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
Robert C. Gallo	Chief	LTCB	NCI

Objectives:

1. Determination of the chromosomal integration site(s) of 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 specific translocation breakpoints characteristic of specific neoplasias.

2. Application of somatic cell genetics to dissect certain biologic and immunologic phenomena associated with HTLV infection to determine their role in the pathogenesis of HTLV-induced neoplasia and immune suppression.

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 blotting, (5) radioimmune assays, and (6) fluorescent antibody staining and use of the fluorescent activated cell sorter (FACS).

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-infected cell lines, Hut 102 and MJ. The hybrids were characterized genetically by karyotypic analysis and by examination of 36 isoenzymes previously assigned to specific human chromosomes. Simultaneously, high molecular weight DNA was 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 dissecting 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 progressive 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. Analysis of somatic cell hybrids has revealed that the 20 or more proviral integrations present in late passage, HTLV-infected cell lines are dispersed among multiple chromosome homologs. Preliminary data

indicate that two proviral integrations visualized in early passage Hut 102 cells are located on chromosome 4 and/or 20.

2. The structural gene for T-cell growth factor (TCGF, also known as interleukin-2) has been localized to chromosome 4 in normal human lymphocytes. The gene is not rearranged on a chromosomal or molecular level in the HTLV-infected cell line, Hut 102. This data suggests that the gene encoding TCGF is not operative in HTLV-induced malignant transformation. The homologous TCGF locus in the domestic cat was assigned to chromosome B1, thus supporting linkage and high resolution G-trypsin banding data that indicate that this feline chromosome is partially homologous to human chromosome 4.

3. HTLV-I-infected T-cell lines express novel, human HLA antigenic determinants which are in addition to and distinct from the HLA phenotype of autologous HTLV-negative B-cell or T-cell lines. By examination of the panel of human/rodent somatic cell hybrids constructed with the HTLV-infected cell line, Hut 102, with a monoclonal antibody which specifically detects the novel HLA antigens, we have demonstrated that: (a) Expression of novel HLA antigens and normal class I antigens segregate independently in the hamster x Hut 102 hybrids. (b) The gene encoding the novel HLA antigens is not located on chromosome 6, where the MHC is located. (c) Expression of novel HLA antigens does not appear to correspond to the presence of any human chromosome except the X chromosome which is common to all hybrids tested. (d) All four hybrids expressing novel HLA antigens contained integrated provirus, while none of four hybrids lacking provirus expressed novel HLA antigens. This data suggests that a viral protein shares an epitope with certain HLA-A and -B antigens. Such homology between a viral protein and an HLA class I protein may be of great biologic significance in the pathogenesis of HTLV-induced neoplasias.

Significance to Biomedical Research and the Program of the Institute:

HTLV-I is the first virus demonstrated to be strongly associated with, and likely causative of, neoplasia in man. Characterization of HTLV and its biologic effects may help elucidate the molecular events that induce malignant transformation, as well as those events which permit the transformed cell to elude the immune response. Such advances could lead to the development of reagents useful for the diagnosis, treatment or prevention of certain human cancers.

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. RNA extracted from hybrids containing single chromosome homologs will be examined with cloned oncogene probes to screen for differential expression.

Publications:

Seigel, L. J., Harper, M. E., Wong-Staal, F., Gallo, R. C., Nash, W. G. 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.

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 (In press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05387-01 LVC

PERIOD COVERED
October 1, 1983 to September 30, 1984

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:	P. Sutrave	Visiting Fellow	LVC	NCI
Others:	U. R. Rapp	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. 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:
1.0	1.0	0.0

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

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

(1) The 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. (2) We have also determined the nucleotide sequence from an HgiAI site within the coding region of its oncogene v-myc to the KpnI site within the long terminal repeat (LTR) of MH2. 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 SPC region of the avian sarcoma virus, Y73, and the complete F3 and F1 segments of Rous sarcoma virus (RSV), strain SR-A. (3) The sequence of c-raf-2 reveals that it is a processed pseudogene. It lacks intervening sequences (introns), and has numerous insertions, deletions and termination codons in all reading frames. At the 3' end of the sequence is found a putative polyadenylation signal, the AATAA box. Nine bases following the AATAA box is a stretch of nine A residues which might represent the poly A tail. (4) 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.

PROJECT DESCRIPTION

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

Pramod Sutrave	Visiting Fellow	LVC	NCI
Ulf R. Rapp	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 (Methods Enzymol. 65: 499-560, 1980) 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 (Proc. Natl. Acad. Sci. USA 74: 5463-5467, 1977).

Major Findings:

1. c-raf-1 and c-raf-2, two different loci in the human genome related to the v-raf oncogene, have been characterized. Sequencing the c-raf-2 revealed that it is a processed pseudogene. The sequence contained termination codons in all three reading frames. Furthermore, it lacked the intervening sequences (introns) characteristic of the active gene. No direct repeats were found flanking the c-raf-2 pseudogene. A stretch of deoxyadenosine residue at the 3' end of the gene, following the polyadenylation signal site, was observed. The most important finding was a stretch of sequence homology between the pseudogene and MuLV. This homology, which is more apparent in c-raf-1, suggests a mechanism of transduction that involves homologous recombination.

2. 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 allowed us to conclude that the v-mil oncogene is 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.

3. 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 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.

4. Transforming ability of retroviruses containing the v-raf/mil and v-myc oncogenes. The genome of the avian carcinoma virus, MH2, contains two unrelated and independently expressed oncogenes, v-mil and v-myc. The v-myc oncogene is generally associated with the induction of B-cell neoplasms in both mammalian and avian species, whereas the oncogenic spectrum of v-mil has not yet been determined. Because of its close homology with v-raf (Jansen et al., Nature 307: 281-284, 1984 and Sutrave et al., Nature [In Press]), the previously isolated oncogene of 3611-MSV, we inferred that v-mil might share with v-raf the ability to transform fibroblasts and epithelial cells in culture and induce fibrosarcomas in newborns. In contrast, the combination of both genes in MH2 is associated with the induction of carcinomas in birds. Since it is possible that c-mil and c-myc were linked by chromosome rearrangement (similar to the 3p25-8q23 translocation in human salivary gland tumors [Bonner et al., Science 223: 71-74, 1984]) prior to their transduction in the ovarian tumor from which MH2 arose, this specific combination of two oncogenes may be a critical step in the development of natural tumors. In order to test the effect of v-myc on the pathogenicity of 3611-MSV, we have made constructs between 3611-MSV and MH2 viral DNAs. Whereas genomic DNA from MH2 did not transform NIH/3T3, C3H/10T1/2 or epithelial Mv-1-Lu cells in vitro, 3611-MSV viral constructs that were v-raf/mil +, v-myc - or v-raf/mil + and v-myc + did transform these cell lines. The specific activity of v-raf/mil +, v-myc + DNA was $\geq$ fivefold that of v-mil +, v-myc - DNA. Foci induced by constructs containing both oncogenes were larger and more homogeneous than those induced by v-raf/mil +, v-myc + DNA which contained morphologically transformed, as well as flat cells. In vivo pathogenicity tests are in progress to determine the oncogenic spectrum of these constructs in mice.

5. 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:

The majority of the human small cell lung carcinomas is associated with elevated levels of expression of the c-raf oncogene. The mechanism of activation of the c-raf oncogene is unknown. There may be mutational inactivation or deletion of one of the alleles, as observed in retinoblastomas and, recently, in Wilms tumor. Determining the mechanism of the elevated gene expression of c-raf would help in possibly understanding the development of these tumors.

Proposed Course:

A cDNA library will be made from human small cell lung carcinomas, which express elevated levels of the c-raf oncogene. Constructs will be made between cDNA and c-raf-1 to locate the altered region by studying their transforming ability in vitro. This region will then be sequenced to study the nature of the alterations involved in elevated expression or, possibly, the altered product of the c-raf oncogene which may result in tumorigenesis.

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 (In Press)

Sutrave, P., Bonner, T. I., Rapp, U. R., Jansen, H. W., Patschinsky, T. and Bister, K.: Nucleotide sequence of avian retroviral oncogene v-mil: Homologue of murine retroviral oncogene v-raf. Nature 309: 85-88, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CE05388-01 LVC

PERIOD COVERED
April 29, 1984 to September 30, 1984

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*
Molecular Basis of Retroviral Induced Neoplasias

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

PI:	James W. Casey	Senior Staff Fellow	LVC	NCI
Others:	Stephen J. O'Brien	Geneticist	LVC	NCI

COOPERATING UNITS *(if any)*
University of California, Davis, CA (M. B. Gardner); Louisiana State University Medical Center, New Orleans, LA (P. Deininger, D. Derse)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	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 utilization of many different animal models, all of which have been selected for a high incidence of neoplasia due to an infectious retrovirus, has provided an enormous body of information concerning the induction of neoplasia in both viral- and nonviral-induced cancer. We are focusing on three retroviral systems, all of which represent natural field infections (feline leukemia virus, bovine leukemia virus and equine infectious anemia virus) to better understand the molecular events that are responsible for tumorigenesis. We have isolated a new, defective feline virus which has transduced the oncogene myc. DNA sequence analysis of this isolate shows that the transduced myc oncogene is a P-30 fusion product. Proviral formation and transcriptional regulation studies on the bovine leukemia virus indicate that this virus readily forms provirus in a wide range of hosts, but requires host cell factors for a productive infection. These host cell factors are present only in a few cell lines and may be the critical rate-limiting step in the genesis of tumors. The equine infectious anemia virus is under study principally because of an antigenic relationship with the human retrovirus, HTLV-III, which is the principle candidate for the etiologic agent in the acquired immunodeficiency syndrome (AIDS). We have molecularly cloned this isolate and will proceed with nucleic acid hybridization studies to establish if a genetic relationship exists with human retroviruses.

PROJECT DESCRIPTION

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

James W. Casey	Senior Staff Fellow	LVC	NCI
Stephen J. O'Brien	Geneticist	LVC	NCI

Objectives:

The focus of our work is to understand the molecular basis of leukemogenesis utilizing animal retroviral systems. It is clear that no single, universal mechanism will evolve from these studies, and the logic of our multidisciplinary approach with three different retroviruses is to obtain a comprehensive picture of the primary molecular events that perturb the immune system and which ultimately progress to tumors. Specifically, the objectives of this project are: (1) to analyze a collection of feline viral-positive and viral-negative tumors for molecular alterations that occur in defined oncogenes, (2) to determine the nucleotide sequence of FeLV-myc and feline c-myc, (3) to analyze the infectivity (host range) of bovine leukemia virus (BLV) as assessed by provirus formation, (4) to determine the nucleotide sequence of the BLV long terminal repeat (LTR) and identify the putative promoters, (5) to determine the mode of gene regulation of the BLV using a transient gene expression assay, and (6) to molecularly clone the equine infectious anemia virus (EIAV) and determine its relationship with other retroviruses, including recent human HTLV-III isolates.

Methods Employed:

The following techniques were employed: (a) genomic molecular cloning in vectors, (b) DNA-mediated transfection, (c) cDNA library construction, (d) SV40 chloramphenicol acetyl transferase (CAT) assays to measure promoter/enhancer activity, (e) DNA sequence analysis using the Sanger shotgun technique, and (f) Southern blotting and mapping of eukaryotic genes.

Major Findings:

1. Isolation of a new, defective feline leukemia virus containing the oncogene myc from a T-cell lymphoma. Using the oncogene probes avian myc, avian myb, avian erb-B and murine abl, we screened 35 feline tumors of lymphoid origin. From these analyses, we detected a single aberrant band in myc, suggesting an alteration in the feline myc oncogene. We defined this additional myc-hybridizing band by preparing a bacteriophage lambda library and isolating all myc recombinant clones. We further found that the aberrant myc-hybridizing band was linked to feline leukemia viral LTRs. The end result of detailed restriction enzyme analysis showed that this tumor contained two identical, defective retroviruses which display all of the classical molecular features of avian and mammalian defective retroviruses.

These results are striking in that Neil et al. (Nature 308: 814-820, 1984) and Mullins et al. (Nature 308: 856-858, 1984) have independently isolated another feline, myc-transduced retrovirus which indicates that defective retroviruses can horizontally spread within a species. Furthermore, the fact that myc was

transduced on multiple occasions lends support to the importance of this gene in the genesis of lymphoid malignancy.

2. Determination of the nucleotide sequence of FeLV-myc. We have determined the nucleotide sequence of the myc portion of FeLV-myc 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 P-30 and the 5' portion of env. The entire pol gene was deleted in the process of transduction. We cannot yet ascribe a significance to the very slightly different amino acid substitutions that occur between feline v-myc and human c-myc. We will determine the nucleotide sequence of feline c-myc in order to measure amino acid changes that may confer additional neoplastic determinants on the feline v-myc protein.

One very striking feature is evident from our sequencing studies. That is, at the 5' feline v-myc, 3' P-30 junction, feline v-myc seems to have been transduced at the same position, or within a few nucleotides, of the transduction point for the oncogene fes, a related but distinctly different, defective feline retrovirus, FeSV. Furthermore, both of these retroviruses form P-30 fusion proteins within their respective oncogenes. These results beg the question of the importance of P-30 fusion in the mechanism of action of transforming genes.

3. Analysis of the host range and infectivity of BLV. A peculiarity of BLV is that, to date, there are only two established cell lines that produce appreciable amounts of this virus. We have isolated virus from the sheep cell line producer (FLK-BLV) and attempted infection of human RD-4, dog D-17, beagle, bovine, and bat cells, as well as the parental cell line of the producer FLK (sheep). After 16 weeks in culture, all infects were assayed for the presence of Mg^{++}- or Mn^{++}-dependent reverse transcriptase. In no case did we find any activity. We isolated DNA from each of these infects and analyzed the samples for the presence of BLV proviruses using a nick-translated, BLV representative probe (we previously cloned all four BLV proviruses from the FLK-BLV-producing cell line). We detected one to three proviruses in each cell line and, in all cases, they were random with regard to proviral integration sites. RNA was isolated from both the human RD-4 cell line and the dog D-17 cell line and analyzed for BLV-specific transcripts using Northern blot techniques. No bovine leukemia viral transcripts were observed. We estimate the sensitivity of our analysis to be one copy per ten cells. Our conclusion is that BLV can infect a wide range of cells in culture, in many of these cells proviral formation is blocked at the transcriptional level.

4. The nucleotide sequence of the BLV LTR. The nucleotide sequences of integrated BLV LTRs with their flanking virus and host DNA sequences have been determined. The BLV LTR is 531 bp in length and is bound by the dinucleotides 5'-TG....CA-3', which are part of a 3-bp inverted repeat. The integrated provirus is flanked by 6-bp direct repeats of cellular origin. A $tRNA^{pro}$ primer binding site is present immediately downstream of the 5' LTR. In addition to sequencing integrated proviral DNA clones, the nucleotide sequence of a cDNA clone representing the 3' end of genomic viral RNA was determined, thus revealing the RNA polyadenylation site and R:U5 boundary within the LTR. Unlike most other retroviruses, the consensus polyadenylation signal, "AATAAA", was not present preceding the BLV polyadenylation site. The RNA initiation and cap sites, defining the U3:R boundary, were located in the BLV LTR by S1 nuclease mapping. This site is

approximately 25 bp downstream of an A-T rich region, which probably encompasses a Goldberg-Hogness ("TATAA") box, and about 90 bp downstream of a potential "CCAAT" box. The BLV LTR possesses a U3 region of 204 bp, an unusually long R region of 240 bp, and a U5 region of 86 bp.

5. The mode of gene regulation of the BLV depends upon unique cellular factors. As stated in sections 3 and 4, BLV is capable of infecting cells and forming proviruses, but only rarely expresses its genetic information. Since LTRs are responsible for governing transcription of all retroviruses and the BLV LTR is uniquely different from most retroviruses, we have focused our attention on this region. We have linked the BLV LTR to the SV40 CAT system and measured transient expression of this construct after transfection onto different cell types. We used the SV40 CAT and SV40 Rous sarcoma virus LTR (RSV-CAT) as controls in all assays. The results show that the BLV LTR is inactive in all systems except for FLK-BLV cells and bat-BLV cells. As a control, we transfected SV40-BLV-CAT onto the parental FLK and bat cell lines and observed no activity. Thus, both BLV-producing cell lines, FLK-BLV and bat-BLV, contain transcriptional activating factors which support three- to fivefold greater activity than the RSV promoter.

6. Molecular cloning of the EIAV. Although it is too early to associate EIAV with a solid neoplasia, there is overwhelming evidence that EIAV is the direct causative agent of anemia. An ablation disorder involving macrophages is probably operative in this system. The similarity of EIAV to human HTLV-3 is based upon an antigenic relationship between the P-30 and envelope gene products. To precisely determine the genetic relationship between these two viruses, it is necessary to obtain well-defined EIAV hybridization probes. We have cloned four, full-length EIAV proviruses which appear different (polymorphic) from one another upon preliminary restriction enzyme analysis. These results may indicate that an antigenic variation similar to that which occurs in the feline FeLV A, B and C subtypes exists in this system. A high priority experiment will be to hybridize EIAV proviral DNA to DNAs from various human neoplasias, AIDS patients and HTLV clones.

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 to catalogue transforming events is at hand. Once these processes are fully understood, one can systematically begin to design new approaches to intervene in neoplastic progression.

Proposed Course:

The focus of our program is to use our molecular probes and recombinant DNA reagents to study: (1) the transforming mechanism of FeLV-myc, (2) the transcriptional regulation of the BLV LTR, and (3) the genetic relatedness of EIAV with human retroviruses. Our future emphasis will be to construct new cell lines which will support the growth and allow the identification of new retroviruses and transforming genes.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05389-01 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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. Schreive, L. L. Hall)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.0	0.6

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The objectives of this program are to increase the reproductive-endocrine-genetic data base of nondomesticated, wildlife species. Program strategies emphasize investigation of basic reproductive-endocrine-genetic factors which appear as the most critical prerequisites to the application of either artificial insemination or embryo transfer. The program employs a multidisciplinary approach targeted toward female and male reproduction genetics. The use of suitable, 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 cultivation, viable freezing, and micromanipulation of embryos of mouse, cat and miniature swine, which hold much promise as animal models. 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 not only allow rapid expansion of physiological-genetic norms for any given rare species, but 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	Physiologist	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, (3) hormonal induction of ovulation and sexual behavior using gonadotropic therapy, (4) surgical procedures for embryo recovery and transfer, (5) programmable freezing for cryopreserving embryos, (6) electroejaculation for semen collection, (7) light and electron microscopy for evaluation of spermatozoal viability and structural integrity, (8) dry ice-pelleting procedures for spermatozoal freezing, (9) in vitro establishment of primary cultures from embryos and skin biopsies, and (10) biochemical genetic procedures for determining extent and character of genetic variation in populations and for monitoring of paternity and maternity exclusion in embryo transfers.

Major Findings:

1. Hormonal evaluation of females. Radioimmunologic analysis of blood protein or steroid hormones allows the development and correlation of endocrine profiles, indices of pituitary-ovarian-adrenal activity and the comparative study of the influence of environmental factors, including stress, on gonadal-adrenal function.

(a) Further studies were conducted in the NIH strain of miniature pig currently used by the NCI Immunology Branch as an animal model for tissue transplantation. Our previous studies (Anat. Rec. 203: 55-65, 1982) indicated that the poor reproductive performance in this model was, in part, the result of a reduced ovulation rate caused by a high degree of selective inbreeding. Studies during 1983 established serum luteinizing hormone (LH), estradiol 17β and progesterone profile norms for this species, integrating these findings with ovarian events. The overall temporal relationships with respect to the cyclic rise and fall of these hormones were consistent with values reported for standard-sized pigs. However, parity appeared to markedly influence pituitary function, with multiparous females producing an earlier preovulatory LH release of lower magnitude, ovulating earlier than multiparous counterparts. This result currently is finding practical

application in determining optimally timed matings, thus ensuring adequate production of numbers of this model for future research use.

(b) Pituitary and gonadal responses to gonadotropin releasing hormone (GnRH) were studied in the adult, female cheetah (Acinonyx jubatus) and the results were compared to those of similarly treated males. The cheetah is considered ecologically endangered due to population deceleration in natural habitats and relatively poor reproductive performance under most zoological park management programs. An examination of the effects of GnRH was considered important, not only for expanding the data base, but also for future studies of natural, ovulatory LH release and the ancillary value of GnRH for timing ovulation and evaluating fertility potential or dysfunction. GnRH administered to anesthetized cheetahs caused elevations in serum LH. The magnitude of the GnRH-induced LH response was sex-dependent with males producing a twofold greater response than females. GnRH had no acute effect on ovarian estradiol-17β secretion. This study indicated that exogenous GnRH was effective in eliciting quantitatively weak LH response in an anesthetized, nondomestic felid.

(c) Little is understood of the effects of anesthesia and manipulation on adrenal function of female nondomestic species. The influence of several anesthetics (ketamine-HCl, halothane, methoxyflurane) and surgical manipulators (laparoscopy, skin grafting) on acute serum cortisol response in females of three species of wild Felidae were studied. Results demonstrated that anesthesia did not necessarily protect felids from eliciting adrenal responses to a manipulatory procedure. In general, serum cortisol in wild felids anesthetized with ketamine-HCl did not rise. Comparative observations suggested that laparoscopy induced a minor, short-term adrenal response. Corticol levels also rose in tigers and leopards subjected to laparoscopy, although this response could not be dissociated from the supplemental use of halothane. Results from a skin grafting study in the cheetah suggested that neither halothane nor methoxyflurane stimulated a cortisol response. It also was evident that a species-specificity existed in adrenal activity among wild felids under anesthesia, the order of activity leopards>tigers> cheetahs.

2. Ovulation induction in females. A prerequisite to successful artificial breeding or increased offspring numbers is the accurate control and timing of ovulation. Three studies have been concerned with hormonal induction of the ovulatory event.

(a) Considerable emphasis is placed on the cryobanking of various strains and stocks of NIH-maintained mice used as research models. A preemptive requirement of embryo collection is the administration of exogenous gonadotropins for timing ovulation, and thus optimizing the number of appropriately staged embryos capable of surviving freezing. Approximately 3000 female mice of five different strains received a standard gonadotropin treatment regimen, were mated, sacrificed and the oviducts were flushed. Resulting embryo numbers indicated that the ovarian response to a given gonadotropin treatment varied markedly within the species. At present, these differences can be attributed, in part, to specific variations in genotype. The data suggested that breeding strategies to produce various homozygous strains of mice did not necessarily compromise reproductive function. Inbred strains tested produced significantly greater numbers of four- to eight-cell embryos than similarly treated, outbred counterparts.

(b) Studies were initiated to determine the effectiveness of exogenous gonadotropins for increasing ovulation rate in the NIH-inbred miniature pig, a strain with a reduced litter size. Results indicated that the NIH-minipigs respond readily to hormonal therapy. Viable embryos collected one week after induced estrus indicated that small litter size was not likely the result of impaired fertilization, but rather due to an inherently low ovulation rate. The latter problem appeared accentuated by an unfavorable uterine environment, as detailed examination of the uteri of 22 females revealed that 17 animals had a form of cystic endometrial hyperplasia. If this condition results from nonpathogenic causes, the NIH-minipig additionally may serve as a suitable model for studying mechanisms of early embryonic loss as a consequence of inbreeding depression.

(c) The domestic sheep was employed as a model for developing estrus synchronization and embryo collection/transfer techniques for eventual application to endangered hoofed stock. Novel estrus synchronization treatments using vaginal progesterone, impregnated pessary and injections of various gonadotropic drugs were used to accurately control time of mating and ovulation. Comparative evaluation of pregnant mares' serum gonadotropin, human menopausal gonadotropin and follicle stimulating hormone demonstrated similar efficacy in producing ovulation as diagnosed by posttreatment laparoscopy and recovery of embryos six days after mating.

3. Embryo collection, culture, freezing and transfer. With improved understanding of basic reproductive relationships in nondomestic species, embryo storage and transfer eventually could play a valuable role in propagation. Program efforts have primarily concentrated on developing expertise in domestic animal models with eventual application to nondomestic species.

(a) Extensive progress was made in improving postfreezing viability of mouse embryos. This project, performed in collaboration with the Veterinary Resources Branch of the NIH, is concerned with the long-term banking of a resource of more than 140 inbred, congenic, outbred and mutant stocks and strains of mice. Technology is now in use to permit viable recovery of 50 to 80% of all mouse embryos frozen (a two- to fourfold improvement over the rates of 1982). Because each of the mouse strains represents a unique genetic entity, this project provides an invaluable opportunity for comparative evaluations of embryo survival rates after freeze-thawing. During the past year, a comprehensive study was completed involving the frozen storage of over 18,000 embryos. The results indicated that the capability of embryos to survive cryopreservation varied markedly among strains, and was at least partially dependent on genotype. Frozen-thawed embryos were biologically viable as embryo transfer to recipient, surrogate females produced 25 pregnancies and live-born litters.

(b) Embryo transfer in the domestic cat and sheep has profited from derived experiences in the mouse system. In the cat, ovarian activity and sexual behavior, including copulatory activity, continued to be successfully induced in approximately 75% of hormonally treated anestrus cats. Of 28 surgical flushes of the reproductive tract, embryos were recovered on 12 occasions (43%). Numbers of embryos collected per female ranged from 1 to 11 and a total of 54 embryos was identified. Immature embryos developed in culture and appeared morphologically normal after freeze-thawing. Although these efforts have demonstrated the feasibility of retrieving viable embryonic material from the domestic cat, the recovery

rates have been inadequate. Exogenous hormonal therapy in the cat caused ovarian hyperstimulation, including the production of cystic follicles. As a result, studies are in progress to assess blood steroid levels and uterotubal junction blockage in hormonally treated females, while simultaneously evaluating alternative methods for stimulating ovarian activity, including implantable, osmotic mini-pumps releasing GnRH. Embryo recovery and transfer in domestic sheep have been productive. A total of 116 embryos was collected from 32 ewes, and four lambs have been born after laparoscopic transfer to surrogate females. Additional pregnancies have been confirmed.

4. Semen collection and evaluation in males. The program places considerable emphasis on evaluating ejaculate quality of domestic and nondomestic species subjected to electroejaculation. Analyses were made of conventional traits, including volume, spermatozoal motility and progressive status, as well as morphological integrity. Interest in the latter characteristic was stimulated by our initial finding of an extraordinarily high (>70%) incidence of abnormal sperm forms in the cheetah. This finding appeared related to a striking lack of genetic polymorphism within the species. To further explore this phenomenon in other Felidae, spermatozoal morphology continues to be assessed in 28 species of wild felids. To date, the data indicate that ejaculates of most captive felids contain a relatively high proportion of morphologically deformed spermatozoa. Twenty of the 28 species evaluated averaged at least 36% abnormal spermatozoa per ejaculate. Thirteen species of felids produce aberrant sperm in the proportional range ($\pm$10%) of that detected in the cheetah. Therefore, it is possible that the high incidence of spermatozoal abnormalities in certain Felidae are either physiologically normal or may be attributable to factors associated with the captive environment. Related studies have emphasized documenting and publishing ejaculate characteristics for both captive and free-ranging nondomestic species, including the Dorcas gazelle, rhinoceros, giraffe and African elephant.

5. Semen handling and spermatozoa cryopreservation.

(a) The practical application of artificial insemination requires maintaining collected spermatozoa in a viable condition for indeterminate intervals prior to deposition into the female. In nondomestic species, little is known of seminal fluid constituents or their influence or fertilizing capacity or viability of spermatozoa. Our ongoing studies demonstrated that spermatozoal motility in wild felids and hoofed stock can be markedly improved by dilution of raw semen with certain tissue culture media. Further increases in longevity can be achieved by removing extraneous seminal fluid following low speed centrifugation.

(b) The zoological community at large has expressed considerable interest in the cryobanking of spermatozoa. The advantages of preserving genetic material from rare species has been well-documented. However, extensive preemptive studies designed to optimize sperm freezing efficiency are prerequisites to the large-scale banking of wildlife semen. Consequently, program strategies have emphasized examining various methods and diluents for freezing spermatozoa. Over 60 ejaculates from 18 species representing such diverse species as the clouded leopard, giraffe and African elephant have been frozen in two to seven cryoprotective diluents each. Thawed aliquots have been evaluated for percent motility, longevity and freeze-induced damage to acrosomal integrity. A definitive relationship has been established between type of diluent used and sperm ability to survive freezing.

Comprehensive efforts have resulted in recommendations for banking spermatozoa from rare species including the blesbok, Dorcas gazelle, onager, Pere David's deer, Eld's deer and African elephant.

6. Hormonal evaluation of males. Little information is available on male non-domesticated species, particularly baseline serum concentrations of protein or steroid hormones and their relationship to age, seasonality or fertility. Efforts to develop a data base could eventually provide indices for improved reproductive management and to evaluate the stressful influence of manipulatory procedures, including anesthesia and electroejaculation. Results demonstrated that the severity of the adrenal stress response was clearly related to the anesthetic drug used. Markedly different, specific responses occurred even among taxonomically related species. Efforts have concentrated primarily on the cheetah, although individuals or animal groups from 19 other species also were examined. The cheetah data demonstrated that electroejaculation of an anesthetized wild felid stimulated an acute adrenal response as indicated by elevated and peak serum cortisol levels immediately after electrical stimulation. This response was short-term as evidenced by rapidly falling serum cortisol levels following electroejaculation, and submaximal as indicated by greater cortisol concentrations in cheetahs treated with exogenous adrenocorticotropic hormone. Cortisol release had no effect on the release of testosterone or LH. Consequently, there was no evidence that electroejaculation-induced cortisol secretion exerted a modulating influence on testosterone or LH secretion or adversely affected reproductive function.

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 embry transfer/embryo freezing capabilities in wildlife species, initial research forts emphasize use of the laboratory mouse because of its elaborate backg gamete physiology. Technology now has been formulated which permits the of viable mouse embryos and offspring following freezing and liquid nitr age. These methods now are being used to cryobank the multitude of in murine genetic stocks at the NIH, thus offering biological insurance genetic drift or natural catastrophe. Interest in developing embryo capabilities in wild species of Felidae and ungulates has concentra

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 humans.

Proposed Course:

Efforts are scheduled to continue and expand several program areas. Particular emphases will be placed on: a comprehensive, comparative evaluation of freezing rates and alternative cryoprotectants for preserving mouse embryos; improving embryo recovery rates from the domestic cat, as well as the feasibility of accomplishing in vitro fertilization; adapting embryo transfer technology as developed for sheep to a species of nondomestic hoofed stock; expanding ejaculate characterizations of various wildlife species, particularly increasing sample sizes in the Felidae groups; completing the first major comparative study on stress-induced adrenal response among four taxonomically related wild felids.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05414-01 LVC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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:	R. E. Benveniste	Medical Officer	LVC	NCI
Others:	K. J. Stromberg	Medical Director	LVC	NCI
	F. Ruscetti	Senior Investigator	LMI	NCI

COOPERATING UNITS *(if any)* Univ. Washington Regional Primate Res. Center, WA(W. Giddens, W. Morton, H. Ochs, C. Tsai); LCI, NIAID, NIH, Bethesda, MD(A. Fauci); Pasteur Inst., Paris, France(J. C. Chermann, L. Montagnier); LBI, Frederick, MD(H. Rabin, L. Henderson, S. Oroszlan); PRI, Frederick, MD(L. Arthur); Univ. Alabama, AL(E. Hunter)

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.)*

At the University of Washington Primate Center, various macaque species show an immune deficiency syndrome (SAIDS) characterized by lymphocytopenia, opportunistic infections, and a fibromatosis tumor (RF). A type D retrovirus was isolated by co-cultivation of explants of RF tissue from a rhesus monkey (Macaca mulatta). This isolate, designated SAIDS-D/Washington, grows to high titers in a variety of primate and mammalian cells and morphologically transforms various rodent cell lines. The SAIDS-D virus can be distinguished from all other retroviruses by antigenicity and molecular hybridization, but is partially related to Mason-Pfizer and the langur monkey type D viruses. Nucleic acid hybridization studies reveal the presence of multiple copies of sequences partially related to the SAIDS-D virus in all Old World monkey cellular DNAs. The highest extent of homology is detected in langur monkey DNA. Since macaques and langurs cohabit Southeast Asia, that region may be a reservoir for SAIDS.

Eight polypeptides corresponding to gag and env viral regions have been purified by HPLC. Radioimmunoassays developed with these proteins reveal that SAIDS-D/Washington virus can be distinguished from all other primate isolates, including type D viruses isolated at the California and New England Primate Centers.

Additional SAIDS-D isolates have now been obtained from M. nemestrina, M. fuscata, and M. fascicularis with symptoms of SAIDS or with biopsy-confirmed RF. Eight macaques have been inoculated with SAIDS-D virus; to date one animal has died with symptoms of SAIDS, and one other macaque, known to be viremic, has developed the palpable abdominal nodules characteristic of RF. Vaccinations are being performed with disrupted virus and purified proteins in attempts to prevent SAIDS. The identification of a retrovirus associated with simian AIDS, the ability to cause the disease with this virus, and the prevention of disease by vaccination will have strong and immediate implications for the human disease and its control.

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 Ruscetti	Senior Investigator	LMI	NCI

Objectives:

To identify and characterize the causative agent of simian AIDS and to propagate it 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 immunological assays.

To examine the prevalence of the simian AIDS viral isolate 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 and purified viral glycoproteins. 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 isolate and propagate any retroviruses associated with human AIDS samples, including lymphocytes from patients with frank AIDS and pre-AIDS lymphadenopathy, as well as biopsy specimens from Kaposi's sarcoma.

Methods Employed:

Virus isolation was attempted using cell lines that have previously been employed for the isolation of other primate retroviruses. Fresh tumors, whole blood, or sera obtained from macaques was cocultivated with various cells and the supernatant assayed weekly for the presence of reverse transcriptase activity. Molecular cloning was performed using an 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 simian AIDS and fibromatosis. An acquired immunodeficiency syndrome, similar in certain respects to human 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.

To examine the etiology of SAIDS, RF tissue from an immunodeficient rhesus monkey was cocultivated with heterologous mammalian cells known to support the replication of a wide variety of retroviruses isolated from the following primate species: baboon, macaque, colobus, langur, owl monkey, and squirrel monkey. After two weeks in culture, a magnesium-dependent, reverse transcriptase activity was detected in the supernatant fluid of a dog thymus cell line (FCf2Th). Electron microscopic examination of the virus revealed typical, type D retroviral particles. "A" particles were also detected and cone-shaped nucleoids were seen in the mature virions. This isolate, designated SAIDS-D/Washington, grows to high titers in human, canine, rhesus, bat, and mink cell lines, releasing about 10^6 virus particles/ml of culture fluid.

Additional RF tissues and blood obtained from other macaque species such as M. nemestrina, M. fuscata and M. fascicularis have also yielded type D retroviruses after cocultivation. We have not found evidence of SAIDS-D viruses in healthy macaques from the colony.

2. Characterization of retroviruses isolated from macaques with simian AIDS. The various SAIDS-D isolates grow readily in a variety of heterologous mammalian cells, as well as in fibroblasts obtained from African green monkeys and various macaque species. The cells that support SAIDS-D virus growth remain fibroblastic and no evidence of transformation is observed. However, infection of the Raji cell line with the SAIDS-D virus leads to the formation of multinucleated, giant cells. Experiments are currently underway to determine whether the Raji or the dog thymus line is the most sensitive indicator for replicating virus. However, after infection of several rodent (mouse and rat) cell lines chosen for their flat morphology and ability to become transformed, foci of proliferating cells were observed in mouse NIH 3T3 cells and in rat NRK cells. These foci grow slowly on soft agar. Cell clones are being obtained and the cells will be inoculated into nude mice.

Since primates with simian AIDS are immunosuppressed, we are also examining if the SAIDS isolate will replicate in normal macaque lymphocytes. After infection, lymphocytes will be separated and any subpopulations of infected cells will be identified in a radioimmunoassay (RIA) for the virus core protein.

The SAIDS-D virus has also been biologically cloned by placing limiting dilutions of filtered supernatant fluid on dog thymus cells. Seven viral clones have been obtained; they will be examined for any variability in cell transformation potential.

3. Nucleic acid characterization of the SAIDS-D isolate. To further characterize the SAIDS-D virus, we compared the nucleic acid sequence homology and thermal stability between this isolate and the other known primate retroviruses. A

radioactively labeled, DNA transcript of SAIDS-D virus was prepared in an endogenous reaction; DNA transcripts >13S in size were generated. Hybridization to the cellular DNAs of cells infected with all known primate viruses and human T-cell leukemia virus (HTLV-I) showed that the SAIDS-D isolate is distinct from all other retroviruses, but is partially related (35%) to MPMV and the endogenous virus isolated from langur monkeys. No homology was detected to the cellular DNA of the New World type D virus isolated from squirrel monkeys. Reciprocal experiments showed that ^{3}H-labeled DNA transcripts prepared from MPMV and langur virus each hybridize 30% to the cellular DNAs of SAIDS-D virus-infected cells.

The extent of homology between SAIDS-D virus, MPMV, and langur virus was also examined by measuring the thermal stability of nucleic acid hybrids. The hybrids formed between SAIDS-D viral DNA transcripts and the cellular DNA of MPMV- or langur virus-infected cells dissociate 12°C lower than the homologous hybrid. Thus, the low degree of sequence homology between SAIDS-D viruses and the other Old World monkey type D viruses is probably due to an accumulation of base-pair mutations, rather than to a recombinational event involving a major portion of the MPMV virus genome.

The cellular DNAs of various primate species were also tested for homology to the SAIDS-D virus. The highest degree of homology is obtained with DNA from the Colobinae subfamily of Old World monkeys, especially langur DNA. The cellular DNAs from all members of the other subfamily of Old World monkeys (Cercopithecinae) hybridize 33-38% to the viral probe. Thus, all Old World monkeys contain nucleic acid sequences partially homologous to the SAIDS-D virus. When DNAs extracted from the tumor tissues of monkeys with SAIDS are examined, the presence of integrated SAIDS-D virus can be detected by the higher final extent of hybridization (100%). SAIDS-D viral sequences have now been detected in various fibromatous tissues from several species of macaques in the primate colony. The background of related nucleic acid sequences in all macaques precludes performing these kinds of studies by restriction enzyme analysis with molecularly cloned probes, since all macaque tissues yield multiple bands related to this class of viruses after digestion with various restriction enzymes. However, SAIDS-D virus is being cloned in order to obtain a restriction map and compare this isolate with the related langur virus and MPMV, as well as the other SAIDS isolates from the New England and California primate centers. An LTR-gag clone from MPMV (pMP6), obtained from Dr. Eric Hunter, is being used to clone the SAIDS-D/Washington virus into plasmid pBR322.

4. Immunological characterization and purification of SAIDS-D/Washington viral proteins. The viral proteins from the SAIDS-D virus have been purified by HPLC on Bondapak C_{18} columns after elution with acetonitrile and propanol gradients. In collaboration with Drs. L. Henderson and S. Oroszlan, the proteins of the SAIDS-D virus are being compared to the purified proteins obtained from MPMV, langur, and squirrel monkey type D viruses.

Eight polypeptides have been purified to homogeneity and their amino acid compositions and N-terminal sequences determined. The gag region of the virus is composed of p10, p12, p27, and p14 polypeptides (order from amino to carboxyl end of gag) and the envelope protein has been tentatively assigned the gp70, gp20, p22E, and p5 polypeptides. There is approximately a 10% amino acid difference between SAIDS-D virus and MPMV. This value is in good agreement with the 12°C thermal

stability reduction measured between these isolates, which corresponds to a 12 to 18% base-pair mismatch. Given the degeneracy of the genetic code, this would in turn correspond to approximately a 10% amino acid difference.

The purified SAIDS-D viral proteins have been inoculated into rabbits in order to raise specific antisera. Radioimmunoassays, performed in collaboration with Dr. L. Arthur, reveal that the SAIDS-D 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 gp70 and p10 radioimmunoassays. Sera from the primate colony at the University of Washington are being screened for antibodies related to this virus.

5. Epidemiology of simian AIDS at the University of Washington Regional Primate Research Center. In order to establish a correlation between the clinical status of the animals at the primate center and the presence of the SAIDS-D isolate, fresh blood or sera from live animals exhibiting clinical symptoms of SAIDS (opportunistic infections, progressive weight loss, diarrhea, anemia, retroperitoneal fibromatosis [RF]) are being cultivated with dog thymus cells in an attempt to isolate virus. The virus, if present, can be isolated after two to three weeks, so it is possible to screen large numbers of animals. We have received 60 coded sera samples; to date, virus has been isolated from seven animals (M. nemestrina, M. fuscata, M. fascicularis) with SAIDS and from all 15 tissues obtained at necropsy from animals that died of RF. Molecular hybridization studies reveal that these new, multiple isolates are very closely related to the original SAIDS-D virus. Further characterization will be done by comparison of restriction maps.

SAIDS-D viruses have not been isolated from macaques without symptoms of SAIDS, although the possibility of healthy carriers exists. Baboons are also housed at the same primate facility; no SAIDS-D viruses have been isolated from these animals.

6. Inoculation of macaques with SAIDS-D/Washington virus. Previous transmission studies at the University of Washington Primate Center, which used 1 ml of a 10% suspension of an RF tissue homogenate as the source of inoculum, resulted in RF in 3 of 14 primates at 109-175 days post-inoculation. In order to establish whether the SAIDS-D viral isolate is the etiologic agent of simian AIDS, eight macaques (M. nemestrina) were inoculated. All eight animals were virus antibody-negative before the study and were inoculated IV and IM with 10^6 virus particles. An identical number of age- and sex-matched controls was inoculated with culture fluid from the uninfected dog thymus cell line. All eight inoculated animals became viremic soon after inoculation. Five weeks after inoculation, one adult, feral monkey died after having had diarrhea and weight loss. At necropsy, histologic examination revealed the marked thymic and lymphoid atrophies characteristic of SAIDS. Two additional monkeys have developed the palpable abdominal nodules characteristic of RF. Primary and secondary antibody responses and lymphocyte blastogenesis assays of all 16 animals are being monitored.

Since the SAIDS-D viral isolate is related immunologically and by protein and nucleic acid sequence homologies to MPMV and langur type D viruses, these latter viruses will also be inoculated into macaques. If they do not cause RF or SAIDS, it should be possible to identify those portions of the SAIDS-D virus responsible

for disease. The colony in Washington is unusual in its high incidence of solid tumors (RF) associated with SAIDS, and the possibility of an oncogene associated with this virus will be examined.

7. Prevention of simian AIDS. At the University of Washington Primate Center, 15% of all deaths during the past three years were caused by simian AIDS; 70% of these cases also have an associated RF tumor. Morbidity and mortality figures at the other U.S. regional primate centers are similar to those for Washington State, although RF tumors rarely have been reported. 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 simian AIDS with other retroviruses. In view of the suspected association between human AIDS and T-cell lymphotropic viruses, we are attempting to isolate similar viruses from the primates in the Seattle colony. Specifically, normal lymphocytes from healthy macaques, as well as human T-cell lines that support the replication of HTLV, are being used as potential hosts for the presence of this class of viruses in the primate colony. Primate sera are also being screened for the presence of antibodies to HTLV; results to date have been negative.

9. Search for simian AIDS virus-related, nucleic acid sequences in human AIDS tissues. In collaboration with Dr. G. Quinnan, we have grown lymphocytes from seven patients with frank AIDS, as well as endothelial cells (factor VIII-positive) from six biopsies of Kaposi's sarcoma. No sequences related to the simian AIDS virus were detected in these cells, and no virus related to the simian AIDS type D isolate was obtained.

10. Identification of retroviruses in human AIDS. Lymphocytes from 30 patients with frank AIDS and 12 with pre-AIDS lymphadenopathy, as well as six endothelial lines established from biopsies of Kaposi's sarcoma were cultured with a variety of mammalian cells that previously have been used for the isolation of all known primate retroviruses. No reverse transcriptase activity, either magnesium- or manganese-specific, has been detected in culture fluid supernatants. However, peaks of reverse transcriptase activity are evident after an addition of normal human lymphocytes to lymphocytes from AIDS patients.

In collaboration with Dr. J. C. Chermann and Dr. L. Montagnier at the Pasteur Institute, a retrovirus isolated from a patient with lymphadenopathy was cultivated with various mammalian cell lines and normal, human T-lymphocytes in attempts to produce a continuous source of their virus. Peaks of reverse transcriptase activity are noted five to ten days after fresh lymphocytes are added to their cultures and attempts are being made to establish a continuous cell line releasing this virus. The virus from the Pasteur Institute is not related to the simian AIDS isolate as determined by radioimmunoassays to the major SAIDS-D virus core protein.

11. Simian AIDS as a model for human AIDS. Human AIDS is characterized pathologically by lymphoid depletion, depressed cellular and humoral immune functions, opportunistic infections and unusual neoplasias, particularly Kaposi's sarcoma,

and, less frequently, lymphomas. Simian AIDS at the University of Washington Regional Primate Center is characterized by persistent diarrhea, progressive weight loss, anemia, lymphocytopenia, chronic infections, and an unusual tumor, termed RF. Histologically, at necropsy, there is marked thymic and lymph node atrophies. Mitogen stimulation indices are reduced in animals with SAIDS. However, in SAIDS, unlike human AIDS, the helper/suppressor ratio of T-lymphocytes is unchanged. Since a type D retrovirus has been isolated from primates with SAIDS, and the disease reproduced by inoculation of monkeys, simian AIDS will provide a useful model for the prevention and treatment of human immunosuppressive diseases.

Significance to Biomedical Research and the Program of the Institute:

The isolation of a novel, type D retrovirus associated with simian AIDS and fibromatosis, the ability to grow the virus to high titers, and the finding that this virus seems to be present almost exclusively in animals with RF and SAIDS constitute important preliminary evidence for attributing the etiology of SAIDS to this isolate. Macaques have been inoculated with SAIDS-D/Washington virus, and preliminary evidence suggests that the animals are viremic. One of eight inoculated animals has died at five weeks post-inoculation with the clinical symptoms and histopathology at necropsy that are characteristic of SAIDS. Since it is possible that a passenger virus present in the original isolate is responsible for the disease, the biological and molecular clones of the virus will be inoculated into macaques.

The nucleic acid hybridization data suggest that the natural reservoir for simian AIDS 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 may have 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 simian AIDS, both as a model for immunosuppressive diseases and as a primate model for human AIDS. The ability to prevent simian AIDS 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 animals for future experiments with additional human and primate viruses.

The similarities between the immunosuppressive disease of primates (SAIDS) and human acquired immunodeficiency syndrome (AIDS) include both the clinical symptoms and various immunological parameters and tissue histology. The identification of a virus associated with simian AIDS, 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:

We will continue to identify those primates at the Washington colony 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.

The rodent cells transformed by the SAIDS-D virus will be examined for the presence of 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. Macaques will also be inoculated with biological and molecular clones of the SAIDS-D isolate to rule out the possibility that an unidentified, passenger virus is causing the disease. The related type D viruses, MPMV and langur monkey virus, will also be inoculated into macaques. Since 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.

Publications:

Stromberg, K. J., Benveniste, R. E., Arthur, L. O., Rabin, H., Giddens, W. E., Jr., Ochs, H. D., Morton, W. R. and Tsai, C.-C.: Characterization of exogenous type D retrovirus from a fibroma of a macaque with simian AIDS and fibromatosis. Science 224: 289-292, 1984.

ANNUAL REPORT OF

BIOLOGICAL CARCINOGENESIS BRANCH

NATIONAL CANCER INSTITUTE

October 1, 1983 to September 30, 1984

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 identifiy and evaluate new research trends relating to its program responsibilities.

The extramural activities of the Branch are accomplished through contractual agreements with universities, research institutes, and commerical organizations, and through traditional individual research grants, program project grants, new investigators awards, and conference grants with universities and research institutes. Currently, the Branch administers over 375 research activities with an annual budget of approximately 55 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 the 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, picorna-viruses, hepatitis B virus, and plant viruses. The Office of the Branch Chief serves as the focal point 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 biological research resources necessary for the extramural research effort. This 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 5,600 sera and tissue specimens and over 2,200 viral reagents were shipped to research laboratories from the inventory of frozen and stored biological reagents.

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 on the contract. There are currently six resource contracts functioning in the payback mode. These include two for production of viruses and viral reagents, three for animal resources, and one for specialized testing services. 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 budget in FY84 is estimated to be about 54.79 million dollars. It should be noted in the table that the Branch now administers 16 cooperative agreements at a level of 2.41 million dollars. These are all 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 now administers 12 contracts and 378 grants.

Two new RFAs, originally published by the BCB in FY83, were initiated and funded during this year. One RFA for traditional research projects (R01) sought to encourage research concerning hepatitis B virus and primary hepatocellular carcinoma. Eight new traditional R01 grants submitted under that RFA were funded in FY84 for a total of 1.082 million dollars. A second RFA for cooperative agreement applications (U01) issued in conjunction with the NIAID was designed to stimulate research on the infectious etiology of AIDS and Kaposi's sarcoma. Eleven new cooperative agreements were funded in FY84 for a total of 1.491 million dollars. Additionally, the Branch continued to administrate five other cooperative agreements initiated in FY83 that also dealt with etiologic studies of AIDS. Four of these continuing U01s were funded with DCE funds at the level of 0.871 million dollars.

Additionally, during FY84 the Branch formulated and presented to the DCE Board of Scientific Counselors (BSC) two concepts for new research initiatives which are to take effect in FY85. One initiative concerns bovine leukemia virus (BLV) research and is based on input from a BCB workshop held in May 1983 at NIH. The consensus of the workshop participants was that research on BLV needed stimulation and that an RFA should be developed encompassing virus-host interactions, molecular biology, and immunology. The concept was presented to the BSC at their March 1984 meeting and was approved for implementation as a cooperative agreement RFA. The second new research initiative deals with the human T-cell leukemia/lymphoma virus (HTLV), a type C RNA tumor virus, which has been shown to be etiologically associated with certain forms of human malignancies. This concept was based on a BCB workshop held in April 1984 at NIH, which is described below. The concept was presented to the BSC at their June 1984 meeting and was also approved for implementation as a cooperative agreement RFA.

During this reporting period the BCB sponsored two major workshops. One, referred to above, concerned HTLV, the first authentic human cancer virus. The workshop was held at NIH in Bethesda on April 12-13, 1984 and was chaired by Dr. Myron Essex. Members of the BSC attending, in addition to Dr. Essex, were Dr. Marcel Baluda and Dr. Charlotte Friend. The consensus of the participants was that NCI should stimulate additional studies in the following areas of HTLV research: 1) investigation of the viral genome of various substrains of HTLV including studies of the LTR, env and pX regions; 2) identification and characterization of viral genome protein products as a clue to determining if they are transforming proteins and to understand the functional activity of the resultant products; 3) investigations of virus integration sites in various systems and/or hosts to determine if the transforming function acts in the cis or trans mode; 4) investigations directed to characterizing the clinically relevant biological activities of the virus, especially its immunosuppressive and/or immunoregulatory effects on the host; 5) determination of the exact mode of horizontal transmission of the virus, including investigations of possible insect transmission; 6) studies in virus-host interactions including geographical localization, determination of host-range, endemic areas other than the Caribbean and Japan (i.e., Africa and the Far East) and localization and overlap of different types of HTLV virus; 7) characterization of HTLV-like viruses of non-human primates and determination if there is an evolutionary link to HTLV; and 8) investigation of the possible use of vaccines to prevent or suppress the horizontally transmitted HTLV associated diseases. A second BCB workshop was held in Rockville, Maryland on June 13, 1984. It concerned DNA viruses and human cervical carcinoma and was chaired by Dr. Marcel Baluda. It had as its objectives the determination of the current status of research on the relationship of herpes simplex viruses, human papilloma viruses and human cytomegalovirus to this malignancy and the elicitation of suggestions on whether additional fiscal resources should be devoted to the development of this area of research. The recommendations and discussions from that workshop are being evaluated for possible new initiatives.

Much fundamental information on biological carcinogenesis has been obtained 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 virus 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. The research highlights of the past year are presented here and in greater detail in the various section reports which follow.

The biological carcinogenesis research program has resulted in an increased understanding of the nature of the interactions between viruses and cells. An awareness of the value of animal tumor viruses as tools for the study of biological processes now exists which had not been perceived when the research program was begun. We now know that different viruses can cause cancer in different mammalian species ranging from mice to large domestic animals and monkeys, and that such viruses may be transmitted through germ cells as well as from animal to animal. Highly specific probes have detected virus-related components in human cells and elements of the human genome in viruses.

Recent studies have shown that oncogenic 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 to 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 homologues (c oncs) of viral transforming genes.

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 the cellular oncogene cannot transform truly normal cells without the assistance of some type of helper or initiator. Normal, pre-senescent cells appear refractory to transformation but such resistance completely disappears after immortalization by chemical and physical carcinogens. Other studies using transfection of the NIH/3T3 cell line identified an activated transforming gene in the DNA of six Burkitt's lymphoma cell lines as a human homologue of a transforming chicken gene found in chicken B-cell lymphoma DNA.

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. Recent findings have provided the first direct link between an oncogene and platelet derived growth factor (PDGF), a potent mitogen for cells of connective tissue origin. One study, using the avian system, explored the response of cells to PDGF and found that a novel modification of the product (protein kinase) of a cellular oncogene occurred within minutes and was accompanied by a 3- to 5-fold increase in its protein kinase activity. In another study, the simian sarcoma virus oncogene (v-sis) has been sequenced and its 28,000 molecular weight transforming protein product determined. A computer comparison of the N-terminal amino acid sequences of PDGF and of the transforming protein (p28) of v-sis has revealed an extraordinary degree of homology between PDGF and p28, implying that the two proteins have arisen from the same or closely related cellular genes. If so, activation of proto-oncogenes might be implicated as a step in the processes leading normal cells of certain tissues toward malignancy.

The oncogenes of small DNA tumor viruses, such as SV40 and 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 mapped. Specific biochemical activities and transformation functions have been assigned to discrete sections of the gene. The immortalization of cells in culture is a function of the amino terminal half of the tumor antigen protein. Induction and maintenance of the transformed phenotype in immortalized cells is a property of the carboxy-terminal half of the protein. It is now clear that these two functions are separable. Although the polyoma virus T antigens are not yet as well characterized, a recent observation involving the middle T antigen of 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 homologue 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 activity of the pp60c-src phosphokinase in vivo in a way similar to the transformation process of RSV.

Human T-cell leukemia/lymphoma virus (HTLV) is a novel exogenous retrovirus associated with mature T-cell malignancy in man. Extensive seroepidemiological

studies indicate that HTLV is present worldwide but is more prevalent in certain areas, including southwest Japan, the Caribbean, parts of South America, and the southeastern United States. There are now about a dozen isolates obtained from patients from different parts of the world. HTLV is distinguishable from all the animal retroviruses by immunological analysis, by analysis of nucleic acid homology, and by amino acid sequencing of structural proteins. The viral structural proteins that have been best characterized are the 19,000 dalton protein, for which a monoclonal antibody is available, and the major core protein, designated p24. Based upon immunological tests of their p24s, there are several subtypes of HTLV. Additionally, hybridization studies show that the DNA sequences of HTLV-II are distinct from those of HTLV-I. In contrast to HTLV-I, HTLV-II is associated only with a relatively benign disease (hairy-cell leukemia). Although HTLV is not closely related to any known animal retrovirus, it appears to be distantly related to bovine leukemia virus (BLV). This has been demonstrated by a weak serologic cross reaction between one of the minor viral proteins of HTLV and BLV. Recent studies have also shown some similarities in amino acid sequences of the HTLV major core protein, p24, with the p24 of BLV, reflecting a common evolutionary background. Evidence suggests that HTLV is not endogenous in humans but rather is acquired by infection. However, HTLV is not readily infectious and may require prolonged intimate contact for transmission. There are as yet no data regarding the presence or absence of a transforming component of HTLV akin to the acute transforming animal leukemia viruses. The HTLV-positive neoplastic T-cells do not carry an oncogene and it has been suggested that the virus may induce disease by activating cellular genes that are normally repressed.

Intensive studies to determine the microbiological etiology of acquired immune deficiency syndrome (AIDS) have centered on viruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus, hepatitis B virus (HBV), and the human T-cell leukemia/lymphoma virus (HTLV). The ubiquitous nature of CMV and EBV and the finding that these viruses are present in most, if not all, male homosexuals with or without AIDS has weakened the presumption of a significant etiological role for these viruses. Additionally, no definitive association of adenoviruses or HBV with AIDS has been established. In contrast, studies in several laboratories have focused attention on HTLV or HTLV-like viruses as a likely etiologic agent of this disease. Seroepidemiologic studies have provided convincing data on the specific association of an HTLV-type virus to AIDS. Antibodies directed against an HTLV-specific cell membrane antigen have been found in the majority of sera (70 to 80%) of AIDS patients examined, compared to the low incidence of 1% in matched homosexual controls. Similar seroepidemiologic surveys of asymptomatic adult hemophiliacs in Atlanta, Birmingham, Los Angeles and New York have shown that 11 to 19% carry serum antibodies against the HTLV-specific cell membrane antigen. This finding has suggested that hemophiliacs, a proportion of whom are known to develop AIDS, most likely get infected with the putative etiologic agent HTLV through the blood clotting factor they routinely receive as therapy.

Recently, a newly discovered subgroup or strain of the HTLV family of viruses, designated as HTLV-III, has been suggested as the primary causative agent of the AIDS immunodeficiency disease. The HTLV-III virus has been isolated from a total of 48 subjects including 18 of 21 patients with early AIDS symptoms,

26 of 72 adult and juvenile patients with overt disease, 3 of 4 clinically normal mothers of juveniles with AIDS, and from only 1 of 22 apparently normal homosexuals. The one individual found to be virus-positive subsequently developed AIDS. No HTLV-III was detected or isolated from 115 normal heterosexual volunteer subjects. The presence of serum antibodies directed against the p41 envelope antigen of the HTLV-III virus in a very high percentage of the AIDS patients (88%) and pre-AIDS subjects (79%) suggest that a reliable serological test for the detection and/or diagnosis of AIDS may soon be available.

In studies of the relationship of HBV to primary hepatocellular carcinoma (PHC), a rapid multisite monoclonal radioimmunoassay for measurement of human alpha-fetoprotein (AFP) using two high affinity monoclonal antibodies directed against distinct determinants of the protein has been developed. The test may be useful in the early identification, diagnosis, and monitoring of patients with primary hepatocellular carcinoma and other AFP-producing tumors in high risk populations. Additionally, it had been thought that viral replication is suppressed or inactive in many PHC patients and that HBV potential infectivity is presumably very low or absent in these individuals. However, recent studies indicate that when viral replication is present in hepatitis B surface antigen carriers $(HBsAg)^{+}$/anti-hepatitis B e antigen $(HBe)^{+}$, active liver disease is often found. In these individuals, active chronic liver disease appears to be related to continued replication and secretion of HBV and may occur in a much higher proportion of $HBsAg^{+}$/anti-HBe^{+} carriers than was previously suspected.

Investigations conducted during the past year on the human cellular analogue of the v-myc oncogene have shown that the human c-myc oncogene is located on the distal end of chromosome 8. In Burkitt's lymphoma (BL), chromosome rearrangement involving chromosome 8 is a common finding, with a reciprocal translocation frequently occurring between chromosome 8 and chromosome 14 which carries the immunoglobulin genes. Interestingly, the human c-myc oncogene is located in the same region that is translocated in BL. Since activation of the c-myc oncogene in chickens causes B cell malignancy, one may speculate on the possible involvement of the human c-myc oncogene in BL. More detailed studies on c-myc oncogene expression are ongoing to determine its role in transformation.

The ability to detect and diagnose cancer at an early stage of disease increases the probability of successful treatment. Results from a current multi-institution study indicate that certain anti-Epstein-Barr virus (EBV) antibodies are valuable markers for clinicians for the diagnosis of undifferentiated types of human North American nasopharyngeal carcinoma, including occult primary tumors. The IgA anti-virus capsid antigen antibody response is the most specific for this disease and of the greatest diagnostic value when used alone or in combination with an anti-early antigen test. In regard to prognosis, antibody titers to EBV immunofluorescence antigens do not vary greatly from those determined at diagnosis in patients that remain in clinical remission following initial therapy. In contrast, increases in antibody titers measured by immunofluorescence to the D component of the EA complex signal the presence of recurrent or active disease. However, antibodies measured by the antibody-dependent cellular cytoxicity (ADCC) assay continue to be the most predictive of disease course following therapy. Of

patients on study for at least two years, 75% of those with high ADCC titers at diagnosis have remained disease-free as opposed to approximately 35% in the low-titered group. In regard to survival, approximately 80% of the patients in the high-titered group have survived for longer than 3 years as opposed to approximately 55% in the low-titered group. These results strongly indicate that ADCC titers determined at diagnosis are predictive of disease course and can be employed to identify those patients who are candidates for more aggressive therapy.

Recent studies of the human wart viruses (HPV), members of the papillomavirus group, have led to speculation that these viruses have a pathogenic role in the development of certain human carcinomas particularly those of the anogenital tract. HPV DNA has been detected in an unusual wart disease, epidermodysplasia verruciformis, and studies reported this year have demonstrated the presence of HPV in several types of anogenital tumors, such as Bowenoid papulosis, carcinoma in situ, and verrucous carcinoma. Since many patients with anogenital wart disease do not develop genital malignancies, other factors, such as host immunity, co-carcinogens, and infection with other virus groups, may be involved in the process of malignant conversion.

With regard to papilloma viruses and human cervical cancer, the recent finding of the presence of HPV antigens by immunological and morphological criteria in a high percentage of patients with mild to moderate cervical dysplasia is interesting. About 400 patients were examined. Since such dysplasias are considered to be precursors of cervical carcinoma, a role for HPV in this oncogenic transformation is strengthened. A probable reservoir for the HPV's found in cervical lesions was the viruses derived from squamous papillomas of the male urethra. The DNA of the urethral HPVs have similar endonuclease restriction digestion patterns to cervically derived HPV DNA and both urethral and cervical lesions appear to derive from metaplastic squamous epithelium.

The hypothesis that human uterine or cervical carcinoma is associated with previous infection by the genital herpesvirus, herpes simplex virus type-2 (HSV-2), has recently been tested at the molecular level by the use of in situ cytological hybridization with cloned subgenomic fragments of HSV-2 DNA. HSV-2 specific RNA transcripts were detected in neoplastic cells of cervical tumors. Limited regions of the genome were transcribed, with one of these known to code for a DNA-binding protein. This protein, demonstrated in both transformed cells and cervical biopsy specimens, was shown to be identical with a previously reported DNA binding protein found in cervical carcinoma tissue. Thus, three viruses, human cytomegalovirus, the human wart virus, and HSV-2 have been implicated as playing some role in the induction of human cervical carcinoma.

TABLE I
BIOLOGICAL CARCINOGENESIS BRANCH
(Extramural Activities - FY 1984 - Estimated)

	No. of Contracts/Grants	$ (Millions)
Research Contracts	3	0.17
Research Grants	378	53.42
Traditional Project Grants (323 grants; $35.57 million)		
Conference Grants (10 grants; $0.07 million)		
New Investigator Research Grants (7 grants; $0.43 million)		
Small Business Innovative Research (1 grant; $0.003 million)		
Program Project Grants (21 grants; $14.94 million)		
Cooperative Agreements (16 grants; $2.41 million)		
Research Resources Contracts	9	1.20
TOTAL	390	54.79

TABLE II
BIOLOGICAL CARCINOGENESIS BRANCH
(Contracts and Grants Active During FY 1984)

	FY 84 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	$ (Millions)	No. of Grants	$ (Millions)
DNA Virus Studies I	2	0.17	85	14.03
DNA Virus Studies II	0	0	81	10.66
RNA Virus Studies I	1	0	98	12.78
RNA Virus Studies II	0	0	93	12.96
Office of the Chief	0	0	21	2.99
Research Resources	9	1.20	0	0
TOTAL	12	1.37	378	53.42

OFFICE OF THE BRANCH CHIEF

GRANTS ACTIVE DURING FY84

Investigator/Institution/Grant Number	Title
1. BASILICO, Claudio New York University 1 U01 CA 37295-01	Molecular Biology of AIDS Related Tumors
2. DESROSIERS, Ronald C. Harvard University 1 R01 CA 38205-01	Type D Retroviruses and Macaque Immunodeficiency
3. ESSEX, Myron E. Harvard University 1 U01 CA 37466-01	Association Between HTLV and AIDS
4. FINBERG, Robert W. Dana-Farber Cancer Institute 5 U01 CA 34979-02	Animal Models of AIDS
5. GARDNER, Murray B. University of California (Davis) 1 U01 CA 37467-01	Simian Acquired Immunodeficiency Syndrome - A Model for Human AIDS
6. GERMAN, James L., III New York Blood Center 1 U01 CA 37327-01	Chromosome Mutation in the Pathogenesis of AIDS
7. HASELTINE, William A. Dana-Farber Cancer Institute 5 R01 CA 19341-08	The Molecular Biology of Replication of RNA Tumor Viruses
8. HAYWARD, Gary S. Johns Hopkins University 1 U01 CA 37314-01	Interaction of EBV and CMV in AIDS and Kaposi's Sarcoma
9. HIRSCH, Martin S. Massachusetts General Hospital 5 U01 CA 35020-02	Viruses, Acquired Immunodeficiency and Kaposi's Sarcoma
10. HUANG, Eng-Shang Univ. of N.C. (Chapel Hill) 5 R01 CA 21773-06	Cytomegaloviruses and Human Malignancy
11. MC DOUGALL, James K. Fred Hutchinson Cancer Research Center 1 U01 CA 37265-01	Cytomegalovirus in AIDS and Kaposi's Sarcoma
12. MULLINS, James I. Harvard University 5 U01 CA 34975-02	Malignancy Associated Genetic Changes--Kaposi's Sarcoma

13.	POIESZ, Bernard J. SUNY Upstate Medical Center 1 U01 CA 37478-01	Acquired Immunodeficiency Syndrome: Association with HTLV
14.	RICH, Marvin A. AMC Cancer Research Center 1 R13 CA 38586-01	Conference on RNA Tumor Viruses in Human Cancer
15.	ROSENTHAL, Leonard J. Georgetown University 1 U01 CA 37259-01	Role of HCMV in KS Associated with AIDS
16.	SCHOOLEY, Robert T. Massachusetts General Hospital 1 U01 CA 37461-01	Human T-Cell Leukemia Virus; Virus-Host Interactions
17.	SUMAYA, Ciro V. University of Texas Hlth Sci Ctr 1 U01 CA 37477-01	Epstein-Barr Virus and Chromosomal Aberrations in AIDS
18.	VOLBERDING, Paul A. Univ. of Calif. (San Francisco) 5 U01 CA 34980-02	Studies of Acquired Immune Deficiency Syndrome
19.	VOLSKY, David J. Creighton University 1 U01 CA 37465-01	Studies of the Viral Etiology of AIDS
20.	YOHN, David Stewart Int Assoc. Comp Research on Leukemia 2 R13 CA 30226-02	International Symposium on Comparative Leukemia Research
21.	ZAIA, John A. City of Hope National Medical Center 5 U01 CA 34991-02	Role of CMV in the Acquired Immunodeficiency Syndrome

SUMMARY REPORT

DNA VIRUS STUDIES I

The DNA Virus Studies I program involves research on two groups of large DNA viruses, the herpes- and adenoviruses. In the DNA Virus Studies I component, extramural research is supported by two funding instruments. There are 85 research grants which utilize $14,030,000 in total cost. These include the traditional research grants, program project grants, conference grants, and new investigator grants. The major research emphasis lies in mechanisms of transformation which includes genome structure, function, and expression (80%); and virus-cell interaction (20%). In terms of the viruses being studied, 36% concern herpes simplex virus (HSV), 25% involve Epstein-Barr virus (EBV), 6% concern cytomegalovirus (CMV), 3% involve Marek's disease virus, and 30% involve adenoviruses. Two applied research contracts are also supported, for a total cost of $170,000. One of these contracts concerns the development of methods for the diagnosis and prognosis of nasopharyngeal carcinoma. The second contract involves efforts to intervene in CMV infections and neoplastic diseases in renal transplant patients.

The emergence of recombinant DNA and hybridoma technology has greatly aided investigators in searching for viral genomes and viral gene products in tissue samples and in transformed cells and in studying the mechanisms of transformation using a variety of model systems. The summary below indicates the progress of some of these studies supported by the DNA Virus Studies Program. These include the identification of transforming fragments in herpes simplex genomes, the development of immunological assays for the diagnosis and prognosis of nasopharyngeal carcinoma infections and the development of an in vitro DNA replication system for adenovirus replication.

Many herpesviruses have the ability to induce tumors in experimental animals and some occasionally exhibit this property in their natural hosts. For many years, herpes simplex type 2 virus (HSV-2) has been suspected of being a contributing cause of squamous cell cervical carcinoma. However, epidemiological and serological evaluation of a possible role for HSV-2 in this disease has been greatly complicated by (1) prior infection with the serologically cross-reactive HSV-1 virus, (2) the propensity of HSV-2 to estabilish long-term latent infections in ganglia innervating the genital region followed by periodic reactivation, and (3) the lack of a suitable animal model. Even the demonstration of viral specific DNA, RNA or antigens in preinvasive tumor cells by in situ hybridization and immunoelectron-microscopy could be explained by the preferential replication of reactivated virus in this tissue.

One method being used to resolve these issues has been the examination of the mechanism of morphological transformation by HSV-1 and HSV-2 in cultured cells. This strategy shows promise of providing information about (1) the presence of nucleic acid sequences and antigens from a possible viral "oncogene" in the tumor cells; (2) a possible indirect role for the virus, such as activation of cellular "transforming" genes or RNA tumor viruses; and (3) the identity of any viral genes, promoters and polypeptides that may be involved. Unlike SV40 and adenovirus, infection of almost any mammalian cell culture with HSV leads to productive, lytic infection and cell death.

To uncover the transforming potential of HSV in permissive cells, the lytic functions of the virus have to be inactivated, e.g., by UV irradiation. Transformed cell lines have also been demonstrated to arise from abortively infected cells; such cell lines have been established mostly from rodent cells and some of them have been shown to produce tumors in experimental animals. However, early attempts to demonstrate specific viral sequences in these transformed cells did not give consistent or reproducible results. Despite the common reports of cytoplasmic or cell surface viral antigens, no consensus of opinion has been achieved as to which, if any, viral polypeptides could be accurately described as tumor or transformation antigens.

One new approach to this problem has been to induce transformation by transfection with DNA fragments. Despite their relatively large size and complexity, the genomes of HSV-1 and HSV-2 have been dissected into well-defined, mapped segments with restriction enzymes. There has been some progress in describing the genome locations of individual immediate-early, delayed-early and late mRNAs and protein products. Therefore, several groups of investigators have attempted to identify specific fragments of HSV-1 and HSV-2 DNA which may be functionally active in initiating morphological transformation as assayed by transfection procedures. Although no definitive answers are as yet available, one transforming fragment, mtr-I, has been identified among HSV-1 strains and two transforming fragments, mtr-II and mtr-III, have been observed in HSV-2 DNA. In the case of HSV-2 strains, a situation analogous to that with adenoviruses may exist in that partial transformation may be achievable with one fragment, but both fragments may be needed for complete transformation (20, 29, 66, 74).

Another approach to this problem has been to study cells transformed by whole, inactivated HSV genomes for persistent viral products. Nucleic acid hybridization is used to detect HSV RNA and DNA. The quantitity and complexity of viral sequences changes with passage number of the cell lines, but in general very few copies or just a portion of the genome is present. When RNA transcripts expressed in the tumor cells were mapped by in situ hybridization using fragments of HSV-2 DNA, the results indicated that transcription was limited and came from three blocks of sequences. As mentioned above two of these have been implicated in transformation in vitro. However, no one sequence was invariably expressed in all of the HSV-positive specimens (57, 58).

Thus, current biochemical data does not permit an unambiguous determination of the mechanism by which HSV transformation occurs, i.e., whether HSV genomes carry true viral oncogenes, act by a promoter insertion mechanism or invoke some other process to activate cellular transformation proteins. Nevertheless, the evidence that this subgroup of herpesviruses is oncogenic and most likely could play some active role in tumor formation in humans is increasing. In particular, the finding of elevated antibodies to the viral specific 38kD polypeptide in sera of patients with cervical carcinoma, and the detection of the viral coded 128kD major DNA binding protein in tumor samples, which at least superficially correlated with the ability of the DNA fragments encoding these two proteins to initiate focus formation, provides

an intriguing basis for further studies. Significant additional progress may require the construction or selection of transformation-defective, host range mutant viruses that give aberrant focus formation phenotypes; it may also require the further development and utilization of well-characterized cell lines from human tumors.

A number of antigen complexes have been identified in Epstein-Barr virus (EBV)-infected cells, mainly by immunofluorescent procedures. These include two early antigens, EBNA (EBV-induced nuclear antigen) and EA (EBV-induced early antigen) and two late antigens, VCA (viral capsid antigens) and MA (cell membrane associated viral antigens). The EA complex has been further divided into diffuse (D) and restricted (R) components based on staining patterns with different human sera. Although most, if not all, of these antigens are now recognized to be complexes, little is known about the polypeptides that compose each of these antigens. The EA complex is of particular interest since antibodies to this complex are frequently present at high titers in patients with EBV-associated diseases as opposed to latently infected, but nondiseased control populations. The anti-EA reactivity in the sera of patients with infectious mononucleosis and nasopharyngeal carcinoma is directed primarily against the D component, whereas reactivity in sera of patients with African Burkitt's lymphomas is directed mainly against the R component. In addition, antibodies to the EA complex are of prognostic importance in patients with EBV-associated malignancies.

Epstein-Barr virus (EBV) markers are currently being applied to the diagnosis and prognosis of nasopharyngeal carcinomas (NPC) and occult tumors of the nasopharyngeal area. A total of 300 histopathologically confirmed NPC patients have been studied. When the serological profiles from NPC patients collected at diagnosis are related to tumor histopathology, a striking difference is noted between well-differentiated World Health Organization (WHO) 1 tumor (squamous cell carcinoma) versus WHO 2 and WHO 3 histopathological types. This has been particularly apparent with the IgG anti-EBV early antigen (EA) and IgA anti-EBV viral capsid antigen (VCA) antibody responses. Sera from patients with WHO 2 and WHO 3 tumors are positive for antibodies in both assays and generally at high titers. Approximately 85% of the sera from patients with WHO 2 and WHO 3 tumors are positive for IgA antibodies to VCA. In contrast, only 5-15% of sera from various control groups and approximately 15% of the sera from patients with WHO 1 tumors are antibody positive. Similarly, approximately 90% of the sera from NPC patients are positive for IgG anti-early antigen (EA) antibodies as opposed to 20-30% of the sera from control populations. The results indicate that the IgA anti-VCA test is a useful assay to the clinician for the diagnosis of certain histopathological types of NPC, including the occult form of the disease. Preliminary analysis indicates that the determination of both IgA anti-VCA and IgG anti-EA titers results in an even greater specificity, since many of the IgA antibody-positive control sera are negative for IgG anti-EA antibodies. Further studies are needed to resolve the questions of the 10-15% IgA antibody "false negative" NPC sera and the 10-15% "false positive" sera identified in the various control groups.

In contrast to previous retrospective studies, current prospective studies suggest that patients whose titers to EA and VCA antigens do not vary greatly from those determined at diagnosis, tend to remain in clinical remission following initial therapy. In contrast, increases in antibody titers measured by immunofluorescence to the D component of the EA antigen complex in both the retrospective and prospective studies have signaled the presence of recurrent or active disease. However, antibodies measured by the antibody-dependent cellular cytotoxicity (ADCC) assay (measuring anti-MA antibodies) continue to be the most predictive of disease course following therapy. Of patients studied for at least two years, 75% of those with high ADCC titers at diagnosis remain disease-free as opposed to approximately 35% in the low-titered group (33, 52, 87).

Recently three monoclonal antibodies to the EA complex have been prepared. One of them, R3, recognizes two major polypeptides of approximatley 50kD and 52kD; the other two, K8 and K9, precipitated an 85kD polypeptide. It has been possible to map the R3 polypeptide to the Bam HI M fragment. It was also determined that the R3-reactive polypeptide appears 4 hours earlier than the K8-recognized polypeptide. Use of these antibodies may be helpful in further defining the diagnosis and prognosis of NPC by measuring antibodies to specific viral antigens (39, 52, 79).

Other studies using co-transformation of mouse LTK^- cells with herpes simplex tk gene and cloned EBV DNA have mapped two different EBNAs. One is localized to the Bam HI M fragment and one to the Bam HI K fragment. Most patient antisera are reactive with both antigens, but it is possible to find patient sera that react with only one of the two antigens. The two antigens were studied during metaphase in their respective co-transformed LTK^+ clones. The K antigen was invariably found on the chromosomes, whereas the M antigen was present in granular form, but not attached to chromosomes. The products of both the Bam HI K and Bam HI M EBV DNA fragments localize in the nucleus, but to two different sites. Thus, these two EBV genes may prove useful in exploration and definition of signals which influence the transport and binding of proteins to chromosomal or non-chromosomal nuclear sites (44).

Cytomegalovirus is a member of the beta group of herpesviruses. CMV has been shown to transform cells, including human cells. It has been shown to be associated with Kaposi's sarcoma, although a causal relationship of the virus and the disease has not been established. A similarity between EBV and CMV is that both viruses usually cause self-limiting lymphoproliferative disorders in young adults and can presumably remain in a latent state in patients thereafter. While c-myc sequences have been established to be present in Burkitt's lymphoma cell lines, there is to date no evidence for their presence in herpes simplex-infected or-transformed cells. There is recent evidence suggesting the presence of v-myc oncogene-related sequences in the CMV genome. The verification of these observations could be of great importance in elucidating the mechanisms of latency and transformation of members of the herpesvirus group (71).

Interferon is being evaluated for use in renal transplant patients in two ways: first to prevent infection with cytomegalovirus (CMV) in seronegative

patients receiving a seropositive transplant and secondly to prevent virus reactivation in seropositive patients who are being immunosuppressed. Due to the wide distribution of this virus, a large number of patients are available for the reactivation studies. The preliminary findings indicate that 7 of 22 placebo patients developed CMV syndromes in contrast to one of 20 patients in the interferon treatment group. Opportunistic infections occurred only in the placebo group. There were two deaths in each group; the deaths in the placebo group were due to opportunistic infection, while those in the interferon-treated group were due to cardiomyopathy and a perforated intestinal diverticulum.

The second aspect of the study, that of patients susceptible to primary CMV infection, is more difficult to perform due to the scarcity of seronegative patients. To date 27 patients have been enrolled in these studies; 40 patients are necessary for statistically meaningful results. Since the initiation of these interferon studies, 12 of 189 non-interferon treated renal transplant patients have developed a variety of tumors, whereas no tumors have developed in the 41 interferon treated patients, with an average time of transplantation of 2½ years. Although the number of interferon-treated patients is small and the length of observation short, the results are encouraging (86).

The recent development of a DNA replication system that can initiate on adenovirus templates in vitro has made it potentially possible to study the transforming and lytic infections of this virus as well as to investigate the biochemistry of eukaryotic DNA replication. Although this goal has not yet been attained, it has been determined that both host-coded proteins and viral gene products are required for the initiation and elongation of adenovirus genomes. Adenovirus DNA replication depends on five components; three are virus coded: the DNA polymerase, a precursor to the covalently-linked terminal protein (pTP) and the DNA binding protein (DBP). Two additional factors (I and II) are present in uninfected nuclear extracts. Factor I may be a DNA binding protein; factor II is a DNA topoisomerase. Studies are underway to map the functional regions of the viral proteins so that the role of each of them in DNA replication can be better understood (35, 64).

Another approach to studying viral genes involved in both transformation and replication has been to study the E1 region of adenoviruses. Integration of both the E1A and E1B genes of adenovirus are necessary for complete transformation. The same E1A and E1B viral products produced during lytic infection are also produced in the transformed cell. Whether the E1A protein product activates the expression of the E1B gene to induce transformation or whether it has a completely independent transforming function remains to be determined. Researchers have approached the study of the E1 region by several methods. The study of the polypeptides has shown that E1 encodes two or more families of partially related polypeptides. One family, encoded by E1A, includes four to six polypeptides that are partially related. A second family, encoded by E1B, includes two major polypeptides, 20kD and 53kD that are highly related. E1B also encodes a major 19kD polypeptide that is unrelated to the 53kD/20kD family as it is translated in a different reading frame. Minor polypeptides, related to the 53kD/20kD

family, are also synthesized in infected cells. Purification of these polypeptides and preparation of monoclonal antibodies to them will allow for further analysis of their function including studies of specific enzymatic activity, studies with an in vitro transcription system, cell microinjection experiments to evaluate gene regulatory functions, and binding studies to identify specific interactions with nucleic acids and proteins (27, 61).

Other strategies for studying the function of this region include genetic methods such as inducing or constructing mutations in the E1 region followed by studies of the effects of these alterations on lytic infection and transforming activity (6, 28, 61).

A workshop on "DNA Viruses and Human Cervical Carcinoma" was held on June 13, 1984 with the objectives of determining the current status of research on the relationship of herpes simplex viruses, human papilloma viruses and human cytomegalovirus to this malignancy and the elicitation of suggestions on whether additional fiscal resources should be devoted to the development of this area of research. The recommendations and discussions from the workshop are being evaluated for possible new initiatives.

In conclusion, recombinant DNA technology has helped to define the genome segments of the herpesviruses and adenoviruses that are involved in transformation. Further studies should help to elucidate the mechanism(s) of transformation with the final aim being the control or reversal of this process.

DNA VIRUS STUDIES I

GRANTS ACTIVE DURING FY84

Investigator/Institution/Grant Number	Title
1. BERK, Arnold J. Univ. of Calif. (Los Angeles) 5 RO1 CA 25235-06	Biosynthesis of Adenovirus Early RNAs
2. BROWN, Nathaniel A. Univ. of Calif. (Los Angeles) 1 RO1 CA 35536-01	Human Lymphocytes Clonally Transformed by EBV
3. BUTEL, Janet S. Baylor College of Medicine 5 RO1 CA 25215-05	Tumor Virus Effects on Mammary Epithelial Cells
4. BUTEL, Janet S. Baylor College of Medicine 5 RO1 CA 22555-07	Biological Properties of SV40 Early Proteins
5. CALNEK, Bruce W. Cornell University 5 RO1 CA 06709-21	Studies on the Avian Leukosis Complex
6. CHINNADURAI, Govindaswamy St. Louis University 5 RO1 CA 31719-02	Genetic Analysis of Adenovirus 2 Early Genes
7. CHINNADURAI, Govindsawamy St. Louis University 5 RO1 CA 33616-05	Adenovirus 1p Locus: Role in Oncogenic Transformation
8. CLOUGH, Wendy G. Univ. of Southern Calif. 5 RO1 CA 23070-07	EBV DNA Synthesis in Transformed Lymphocytes
9. CLOUGH, Wendy G. Univ. of Southern Calif. 1 RO1 CA 35343-01A1	DNA Methylation in Lymphocytes
10. CONLEY, Anthony J. St. Louis University 5 RO1 CA 33101-02	Regulatory Functions of Herpes Simplex Gene Expression
11. COOPER, Neil R. Scripps Clinic and Res. Fdn. 5 RO1 CA 14692-11	Humoral Immunity to Viruses and Virus-infected Cells

12. COURTNEY, Richard J.
University of Tennessee
5 RO1 CA 24564-07
Studies of Purified Herpes Simplex Virus Glycoproteins

13. COURTNEY, Richard J.
University of Tennessee
5 RO1 CA 27870-03
Proteins of HSV-infected and Transformed Cells

14. COURTNEY, Richard J.
University of Tennessee
1 R13 CA 35993-01
Eighth International Herpesvirus Workshop

15. DARNELL, James E., Jr.
Rockefeller University
5 RO1 CA 16006-10
RNA and Growth Control in Animal Cells

16. DE MARCHI, Jeanette M.
Vanderbilt University
2 RO1 CA 20806-07
Induction by Cytomegalovirus of Cell DNA Synthesis

17. DESROSIERS, Ronald C.
Harvard University
5 RO1 CA 31363-03
Molecular Basis for Herpesvirus Saimiri Oncogenicity

18. EGGERDING, Faye A.
Univ. of Calif. (Los Angeles)
5 RO1 CA 25545-03
Regulation of Adenovirus 2 Transcription

19. FALK, Lawrence A., Jr.
Harvard University
5 RO1 CA 27225-03
Study of Human and Simian Lymphotropic Herpes Viruses

20. GALLOWAY, Denise A.
Fred Hutchinson Cancer Center
5 RO1 CA 26001-05
Herpesvirus Expression in Transformation and Latency

21. GALLOWAY, Denise A.
Fred Hutchinson Cancer Center
1 RO1 CA 35568-01
Molecular Studies on Herpesvirus Proteins

22. GALLOWAY, Denise A.
Fred Hutchinson Cancer Center
1 R13 CA 38135-01
Ninth International Herpesvirus Workshop

23. GAYNOR, Richard B.
Univ. of Calif. (Los Angeles)
2 RO1 CA 30981-04
Adenovirus 5 Mutants in Transforming Functions

24. GLASER, Ronald
Ohio State University
2 RO1 CA 29066-04
Epstein-Barr Virus DNA in Transfected Cells

25.	GLASER, Ronald Ohio State University 1 RO1 CA 36357-01A	Molecular Genetics of Epstein-Barr Virus
26.	GREEN, Maurice St. Louis University 5 RO1 CA 29561-26	Biochemistry of Animal Virus Multiplication
27.	GREEN, Maurice St. Louis University 5 RO1 CA 21824-07	Transforming Proteins of Three Human Adenovirus Groups
28.	HARTER, Marian L. Univ. of Med. & Dent. (NJ) 2 RO1 CA 28414-04	Function of Early Proteins Encoded by Adenovirus Type-2
29.	HAYWARD, Gary S. Johns Hopkins University 2 RO1 CA 28473-04	Cellular Transformation by DNA of Human Herpesvirus
30.	HAYWARD, Gary S. Johns Hopkins University 2 RO1 CA 22130-07	Structure and Regulation of Human Herpesvirus Genomes
31.	HAYWARD, S. Diane Johns Hopkins University 2 RO1 CA 30356-04	EBV Genome Expression: Localization of Specific Functions
32.	HELD, William A. Roswell Park Memorial Inst. 5 RO1 CA 27647-03	TK-mutants of Herpes virus and their Suppression
33.	HENLE, Werner Children's Hospital of Phila. 5 RO1 CA 33324-02	EBV Serology in Human Cancers and Immune Deficiencies
34.	HIRSCH, Martin S. Massachusetts General Hospital 5 RO1 CA 12464-13	Immune Reactivity and Oncogenic Virus Infections
35.	HORWITZ, Marshall S. Yeshiva University 2 RO1 CA 11512-15	Adenovirus DNA Synthesis and Polypeptide Assembly
36.	HOWETT, Mary K. Penn State Univ. (Hershey) 5 RO1 CA 25305-06	Modulation of the Tumorigenicity of Transformed Cells
37.	HYMAN, Richard W. Penn State Univ. (Hershey) 5 RO1 CA 16498-10	Malignancy and DNA Homology among the Herpesviruses

38. ISOM, Harriet C.
Penn State Univ. (Hershey)
5 RO1 CA 23931-06
Regulation of Differentiation in Hepatocytes in Vitro

39. KIEFF, Elliott D.
University of Chicago
2 RO1 CA 17281-09
EBV Interaction With Lymphoblasts in Vitro and in Vivo

40. KLEIN, George
Karolinska Institutet
5 RO1 CA 30264-03
Immune Effector Mechanisms in EBV-Carrying Patients

41. KLEIN, George
Karolinska Institutet
5 RO1 CA 28380-03
Epstein-Barr Virus Determined Nuclear Antigen (EBNA)

42. KNIPE, David M.
Harvard University
5 RO1 CA 26345-05
Genetics of Herpesvirus Transformation

43. LEWIS, James B.
Fred Hutchinson Cancer Res. Ctr.
5 RO1 CA 29600-02
Functions of Adenovirus Proteins in Transformation

44. MC DOUGALL, James K.
Fred Hutchinson Cancer Res. Ctr.
2 RO1 CA 29350-04
The Biology of Transformation Herpesvirus

45. MILLER, I. George, Jr.
Yale University
2 RO1 CA 12055-13
Studies of Epstein-Barr Virus

46. MILLETTE, Robert L.
Wayne State University
5 RO1 CA 21065-08
Herpes Simplex Virus Gene Regulation

47. MILLETTE, Robert L.
Portland State University
7 RO1 CA 39067-01
Herpes Simplex Virus Gene Regulation

48. MUNNS, Theodore W.
Washington University
5 RO1 CA 27801-05
Characterization of RNA/DNA in Oncogenic Systems

49. NONOYAMA, Meihan
Showa University Res. Inst.
5 RO1 CA 31949-03
Marek's Disease Virus: Transformation and Oncogenesis

50. PADMANABHAN, Radha K.
University of Kansas
1 RO1 CA 33099-01A
Structure and Functional Analysis of Adenovirus Genomes

51.	PAGANO, Joseph S. Univ. of NC (Chapel Hill) 2 PO1 CA 19014-07A	DNA Virus Genomes, Oncogenesis and Latency
52.	PEARSON, Gary R. Mayo Foundation 5 RO1 CA 20679-08	Epstein-Barr Virus-induced Membrane Antigens
53.	PEARSON, Gary R. Mayo Foundation 1 R13 CA 38272-01	First International Symposium on Epstein-Barr Virus and Associated Malignant Diseases
54.	PEARSON, George D. Oregon State University 2 RO1 CA 17699-08	Replication Map of an Oncogenic Virus
55.	PRUSOFF, William H. Yale University 5 RO1 CA 05262-23	Iododeoxyuridine, Iodo-DNA, and Biological Activity
56.	RAAB-TRAUB, Nancy J. Univ. of NC (Chapel Hill) 1 RO1 CA 32979-01A	EBV Transcription in Nasopharyngeal Carcinoma
57.	RAPP, Fred Penn State Univ. (Hershey) 1 RO1 CA 34479-01	Latency and Transformation by Herpesviruses
58.	RAPP, Fred Penn State Univ. (Hershey) 2 PO1 CA 27503-04	Herpesviruses and Neoplasia
59.	RASKA, Karel, Jr. Rutgers Medical School 5 RO1 CA 21196-07	Adenovirus T, Surface Antigens and Tumorigenicity
60.	REKOSH, David M. SUNY (Buffalo) 5 RO1 CA 25674-05	Adenovirus Early Gene Function and DNA Replication
61.	RICCIARDI, Robert P. Wistar Institute 5 RO1 CA 29797-03	Organization and Expression of Adenovirus Genes
62.	ROBERTS, Richard J. Cold Spring Harbor Laboratory 5 PO1 CA 13106-13	Cold Spring Harbor Laboratory Cancer Research Center
63.	ROBINSON, Robin A. University of Texas 1 R23 CA 36143-01A	Regulation of Cellular Gene Expression by HSV Icp-4

64. ROEDER, Robert G.
Rockefeller University
1 RO1 CA 34223-01
Regulation of Adenovirus Transcription

65. ROIZMAN, Bernard
University of Chicago
5 RO1 CA 08494-19
Mechanisms of Viral Infection in Relation to Cancer

66. SCHAFFER, Priscilla A.
Sidney Farber Cancer Institute
5 RO1 CA 20260-08
Transforming Genes of Herpes Simplex Virus

67. SCHIERMAN, Louis W.
University of Georgia
5 RO1 CA 30109-03
Immunogenetic Study of a Herpesvirus Induced Lymphoma

68. SILVERSTEIN, Saul J.
Columbia University
5 RO1 CA 17477-09
Molecular Biology of Herpes Virus

69. SPEAR, Patricia G.
University of Chicago
5 RO1 CA 21776-07
Herpesvirus Gene Expression in Transformed Cells

70. SPECTOR, David J.
Penn State Univ. (Hershey)
1 RO1 CA 34381-01
Regulation of Adenovirus Elb Gene Expression

71. SPECTOR, Deborah H.
Univ. of Calif. (San Diego)
1 RO1 CA 34729-01
Human CMV, Cell-Related DNA, Oncogenes and Kaposi Sarcoma

72. SPELSBERG, Thomas C.
Mayo Foundation
5 RO1 CA 25340-05
A New Class of Epstein-Barr Virus Nuclear Antigen

73. STROMINGER, Jack L.
Sidney Farber Cancer Institute
5 RO1 CA 24926-05
Study of Epstein-Barr Nuclear Antigen

74. STROMINGER, Jack L.
Sidney Farber Cancer Institute
5 PO1 CA 21082-08
Molecular Basis of Viral Oncogenesis

75. SUMMERS, William C.
Yale University
5 RO1 CA 13515-11
Genetic Study of Animal Viruses

76. THORLEY-LAWSON, David A.
Tufts University
2 RO1 CA 31893-03
Epstein Barr Virus Membrane Antigen

77. TIBBETTS, Clark J.
Vanderbilt University
2 RO1 CA 34126-02
Adenovirus Genome Expression/ Assembly/Transfection

78. TROY, Frederic A.
Univ. of Calif. (Davis)
5 RO1 CA 17327-08
Membrane-Bound Enzymes, Tumor Antigens and Malignancy

79. ULTMANN, John E.
University of Chicago
5 PO1 CA 19264-08
Viral Oncology Research Center Program

80. WAGNER, Edward K.
Univ. of Calif. (Irvine)
2 RO1 CA 11861-14
Control of Viral RNA Synthesis in Herpesvirus Infection

81. WENTZ, William B.
Case Western Reserve
5 RO1 CA 31973-02
Sexually Transmitted Disease in Uterine Carcinogenesis

82. WILLIAMS, James F.
Carnegie-Mellon University
5 RO1 CA 32940-02
Study of Adenovirus Transformation-defective Mutants

83. WILLIAMS, James F.
Carnegie-Mellon University
5 RO1 CA 21375-07
Genetic Analysis of Adenoviruses

84. WOLD, William S.
St. Louis University
5 RO1 CA 24710-05
Adenovirus 2 Coded Early Glycoprotein

85. ZIMMER, Stephen G.
University of Kentucky
5 RO1 CA 33434-02
Analysis of Defined Ad2 Transformed Cell Revertants

CONTRACTS ACTIVE DURING FY84

Investigator/Institution/Contract Number	Title
86. HIRSCH, Martin Massachusetts General Hospital NO1-CP4-3222	Clinical Trials of the Role of Interferon in Preventing Activation of Potentially Oncogenic Viruses in Organ Transplant Patients
87. PEARSON, Gary Mayo Foundation NO1-CP9-1006	Application of Epstein-Barr Virus Markers to Diagnosis and Prognosis of NPC and Occult Tumors of Nasopharynx Area in USA

SUMMARY REPORT

DNA VIRUS STUDIES II

The DNA Virus Studies II program of the Branch concerns the investigation of the major classes of small DNA tumor viruses: simian virus 40 (SV40), polyoma and papillomaviruses. In the component, there are 81 research grants with an estimated total funding of $10,665,571. Those 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. In terms of scientific areas, 45% of the grants deal with the structure and expression viral genes in animal cells, 23% deal with the biochemical properties and mechanism of action of viral gene products, 16% deal with the expression and function of cellular genes that are involved in the transformation process, 8% deal with the relationship of small DNA viruses to human cancers, and 8% deal with basic studies on the structure and expression of the eukaryotic cell genome that use the small DNA tumor viruses as model systems. In terms of the viruses being studied, 53% of the grants involve SV40, 21% mouse polyoma virus, 11% human or animal papillomaviruses, and 15% other viruses such as the human papovaviruses, BK and JC, adenoviruses, herpesviruses or parvoviruses.

Among the most notable advances in the DNA Virus Studies II program this year has been the continued progress in understanding the functions of both the tumor or "T" antigens of the papovaviruses, SV40 and polyoma, and the cellular proteins with which the T antigens interact to transform cells. In addition, the link between the papillomaviruses and certain dermatological and urogenital cancers, particularly cervical cancer, has been strengthened. These advances have come about due to the application of traditional biochemical, genetic and immunological analysis as well as the newer recombinant DNA and monoclonal antibody techniques. Detailed characterization of the papilloma viruses has recently been made possible by the new recombinant DNA methods. Highlights of this year's scientific progress are grouped below according to virus type.

A great deal of elegant work is beginning to reveal the functional properties of the tumor antigens from the SV40 virus. SV40 is a small DNA virus which was first discovered in the kidney cells of monkeys and was subsequently found to induce tumors in experimental animals and to transform primary cell cultures. Due to its small size (its DNA is only 5243 base pairs long and codes for five viral proteins) and ease of study in numerous permissive and non-permissive cells, SV40 has become one of the best described tumor virus systems at the molecular level. The entire nucleotide sequence is known as are the positions of the sequences which code for the origin of replication and the five viral proteins. Of these five proteins, the two tumor antigens, large T-antigen (T-Ag) and small t-antigen (t-ag), are transcribed and translated early during viral infection and are responsible for the transforming potential of this virus. Large T-Ag by itself can provide all the necessary functions to initiate and maintain transformation of cells, although small t-ag has an enhancing effect in certain circumstances and may be responsible for maintenance of some of the

phenotypic characteristics seen in transformed cells. These antigens are the subject of intense investigation as to their biochemical properties and mechanism of action. It has been shown that large T-Ag has numerous activities (thirteen have been described to date including initiation of DNA replication, binding to viral and cellular DNA, binding to the cellular protein p53, ATPase activity, self-aggregation, cell surface membrane association, etc.). Small t-ag, however, appears to function by binding cellular proteins. Recent work has focused on the further characterization of the various activities of T-antigens and their associated cellular proteins in order to sort out the specific protein functions and overall mechanism responsible for transformation.

One issue which has recently been explored using techniques of molecular biology is the cause of the inefficiency of transformation by SV40 tumor viruses in non-permissive cells. In a high multiplicity infection of rodent cells with SV40 virus, close to 100% of the cells transiently express a transformed phenotype, but then lose all signs of viral infection. This phenomenon of "abortive" transformation is poorly understood. Recent work, involving the construction of a highly efficient transforming "SV40 retrovirus," suggests that this phenomenon of abortive transformation is due to two factors: 1) inefficient integration of the viral genome into host chromosomes and 2) inefficient expression of the viral DNA in 90% of random insertion sites. This unusual retrovirus was constructed from a murine leukemia virus vector in which the "gag," "pol," and "env" (envelope) genes of the retrovirus were deleted and the coding sequences for the SV40 early proteins (both T antigens) were inserted. This engineered DNA was rescued from transfected cells and propagated using a helper virus to supply replicative functions. The resultant retrovirus, which is called MV40, morphologically transforms 100% of the rat or mouse cells within six days of culture. All colonies picked to date produce SV40 tumor antigens. In these virus stocks the SV40 early genes are under complete regulatory control of the murine retrovirus enhancer/promoter and polyadenylation signals. Thus, the rate limiting steps in SV40 transformation appear to be successful viral integration and efficient early gene expression. If enough tumor antigens are produced in infected cells, the cells will transform (9).

A variety of deletion and point mutations in the SV40 large T-Ag have been used to map the biochemical properties and transformation functions to specific regions of the T-Ag protein. The immortalization ability of T-Ag (the ability to make primary cell cultures grow and divide continuously, but not to express other phenotypic "transformed" traits such as the production of tumors in animals) has recently been localized to a specific group of nucleotides coding for the amino terminal half of the molecule. A subregion of this section of the amino terminal half of the protein has also been identified as the region responsible for the specific binding of T-Ag to the SV40 origin of replication; this observation suggests that immortalization may be strictly a change in some nuclear function such as transcription or replication. However, the frequency of successful immortalization by DNA coding only the amino terminal section of the T-Ag gene is only 2-6% of the frequency of immortalization by wild type DNA. Thus, the nucleotides encoding the carboxy terminal half of T-Ag must have functions which enhance

the frequency of immortalization. This enhancement function has also been recently localized to a specific region of 260 nucleotides and is most probably associated with nucleotides around the SV40 gene map position 0.243 m.u. (as suggested by various short deletion mutants at this site). Notably, the amino acids immediately proximal to the carboxy terminal end were found to be completely non-essential to the immortalization function, since SV40 DNA in which this DNA was deleted immortalized cells at the wild type rate. This same proximal carboxy terminal section has also been shown to contain the ATPase activity of T-Ag. Thus, these studies suggest that the ATPase activity of T-Ag is not essential to cell immortalization. However, previous work by others showed a strong correlation between the presence of ATPase activity and the ability of SV40 to fully transform cells. Together these observations suggest that the ATPase activity is necessary only for the expression of the full transformed phenotype in immortalized cells (72, 74).

In order to determine which transformed phenotypic characteristics were dependent on ATPase activity, the characteristics of cell lines immortalized by DNA sequences encoding only the amino terminal section of T-Ag were carefully examined. The results indicate that the amino terminal half of T-Ag is sufficient for the expression of the cell surface antigen, TSTA (tumor-specific transplantation antigen), and provides the ability to grow in low serum (2%) medium, although with a markedly slower generation time. The truncated T-Ag did not permit growth of transformed cells to high density, growth in anchorage-independent colonies, or formation of tumors in mice. By using other immortalized and minimally transformed cell lines derived from other deletions of T-Ag, it may be possible to localize regions responsible for conferring the fully transformed phenotype (72, 74).

In the biochemical area, significant progress has recently been made in defining the various biochemical activities of the T-Ag due to the availability of large amounts of T-Ag proteins (both wild type and mutant varieties) through the use of recombinant DNA techniques. In particular, a very specific molecular model for the binding of T-Ag to the SV40 origin of replication has been developed. By the use of DNase I sensitivity and methylation protection studies, the pentanucleotide sequence, (G/T)(Pu)GGC, has been identified as the recognition site of T-Ag binding. The SV40 origin of replication contains 13 of these recognition peptides arranged in three regions. Region I contains three pentanucleotide contact sites arranged as direct repetitions encompassing a span of 23 base pairs. In region II, four pentanucleotides were oriented as inverted repetitions. They also spanned a total of 23 base pairs. Region III had six recognition pentanucleotides arranged as direct repetitions in a space of 59 base pairs. DNase footprint and fragment assays of origin DNA-T-Ag binding led to the following conclusions: (1) a single recognition pentanucleotide is sufficient to direct the binding and accurate alignment of T-Ag protein on DNA; (2) the T-Ag protein binds within isolated region I or II in a sequential process leading to multiple overlapping areas of DNase protection within each region; and (3) the 23-base pair span of recognition sequences in region II allows binding and protection of a longer length of DNA than the 23-base pair span in region I (70).

The resulting molecular model developed to explain this data requires the orientation of T-Ag protein in a unique direction upon binding to the recognition pentapeptides. The model of T-Ag protein binding is supported by recent work on T-Ag protein binding to deletion mutants of the replication origin. This work also demonstrated that tight binding of three T-Ag molecules to the origin is not sufficient to initiate DNA replication. Binding studies have also shown a hierarchy of binding affinities to this section of origin DNA; region I has greater affinity for T-Ag than region II which has greater affinity than region III. Also, as predicted by the model, monomers of T-Ag protein bind 2-3 times more tightly than dimers and 5-10 times more tightly than trimers. Thus, for T-Ag binding to the SV40 origin, the monomer appears to be the functional unit. The function of T-Ag protein binding to regions I and II has also recently been examined in mRNA transcription assays. These results lend strong support to the hypothesis that the T-Ag protein binding to these sites contributes to the repression of SV40 early transcription in vivo (47, 70, 75).

The binding of T-Ag protein to non-origin DNA was also examined in both SV40, bacterial and human DNA samples. After footprint analysis, weak recognition pentanucleotides (in terms of binding affinity) were identified which were simple variants of the strong recognition pentanucleotide sequences. Thus, the tolerance for minor changes in the recognition sequence and the frequent occurrence of the recognition "consensus" pentanucleotide sequence offer an explanation for some of the features of the "non-specific" binding of T-Ag protein to DNA. In many cases, binding is evident only under low salt conditions. It is unlikely that such weak binding to single recognition sites could lead to negative regulation of gene expression. In contrast, even transient binding to DNA might possibly suffice to signal a positive regulatory event (70).

The role of T-Ag in the initiation of viral DNA replication is beginning to be clarified. First, using pulse-chase experiments it was found that T-Ag is preferentially found bound to newly initiated replicative intermediates (RI) of the SV40 genome. Surprisingly, p53, a cellular protein which is often found bound to T-Ag in transformed cells was not found in RIs by monoclonal antibody analysis. Second, although it has long been known that T-Ag can be phosphorylated at numerous sites on the protein, it has just recently been shown that purified T-Ag can catalyze a reaction with ATP leading to its O-adenylalation via a phospholdiester linkage with a serine residue. The reaction is reversible, but requires poly dT or SV40 DNA for reversibility. Since AMP exists at the end of all newly initiated SV40 DNA daughter L strands in vivo, the existence of the T-Ag adenylation reaction strongly suggests that it may have a role in priming the initiation of new viral DNA strands (47, 68).

The role of T-Ag in the maintenance of the transformed state has been partially clarified by the study of a mutant T-Ag derived from the SV80 cell line. This cell line was derived from a SV40 transformed line of human fibroblasts. The mutant T-Ag can maintain the transformed state of the cells and has normal ATPase activity, but has lost its abilities to bind tightly to the SV40 origin of replication and to initiate viral DNA synthesis. However, the mutant T-Ag has retained its ability to bind to

random double or single stranded DNA, but with a strong preference for supercoiled DNA. This result, along with results described above, suggest that once cell immortalization occurs, only the ATPase activity of T-Ag is needed for maintenance of the transformed phenotype (36).

SV40 T antigens are often found in association with cellular proteins. Since these cellular proteins may mediate the transformation process, the biochemical functions of these proteins are of great interest. Large T-antigen associates with a phosphoprotein of molecular weight 53K (p53 protein), while small t-antigen associates with at least two and possibly three cellular proteins. Characterization of the p53 protein is of great general interest because it appears to be a common element in many cellular transformation situations. It is expressed at high levels in cells transformed by at least two tumor viruses, SV40 and adenovirus, and by cells transformed by some carcinogenic chemicals.

The p53 protein has recently been shown to be a nuclear protein present at low levels in all cells examined. The protein appears to be evolutionarily conserved. After infection with SV40 with an active T-Ag gene, high levels of the protein appear and subsequently form a complex with T-Ag protein. The appearance or induction is highly dependent on the activity of T-Ag gene which suggests the T-Ag-p53 complex may mediate productive infection and transformation. However, since p53 appears in high levels in abortively infected nonpermissive cells, its presence in large amounts cannot be a sufficient cause for transformation. The mechanism of induction may vary with the type of cell. Both viral transformed and nontransformed cells have similar amounts of p53 mRNA suggesting that p53 protein turnover is inhibited in transformed cells. Murine embryonal carcinoma cells have levels of mRNA which decrease 10-20 fold upon differentiation into benign endoderm cells. Metabolic poisons which reduce ATP levels markedly increase the half life of p53 in nontransformed cells (4, 13, 45).

Pulse-chase experiments have shown that while newly synthesized T-Ag appears to be a monomer which slowly enters in complexes with p53, new p53 protein is synthesized exclusively on rough endoplasmic reticulum and is immediately taken up into T-Ag complexes. Detailed analysis of the formulation of T-Ag-p53 oligomers has revealed that it is not a random process, but an ordered sequential progression which apparently requires T-Ag to become more highly phosphorylated in order to become part of a T-Ag-p53 complex (4, 13, 34).

The small t-ag of SV40 appears to enhance transformation in certain cell lines but is not necessary for transformation. The t-ag protein has been recently purified in large amounts via cloning and expression in E. coli. At present, its only known biochemical activity is the dissolution of actin cables in nontransformed rat cells. The t-ag also binds two cellular proteins of 56kD and 32kD in molecular weight. These proteins are present in all cells investigated to date and their levels are not apparently changed by virus infection. Recently, a third protein of 20kD molecular weight has been found to associate with t-ag in the nucleus of cells. The 56KD protein has been found to be tubulin, which partially explains the actin cable dissolution effect as well as the recently found immunochemical staining of the cytoplasm by anti-t-ag antibodies. This staining appears as

an irregular network of cotton-wool-like deposits suggesting that the protein is associated with an organized structural network (e.g., Golgi or a component of the cytoskeleton). It has also recently been found that the 32kD protein binds S-adenosylmethionine (SAM) suggesting that it is an enzyme which uses this substrate. Labeling of 32kD protein with SAM does not require t-ag, but t-ag appears to preferentially bind to SAM labeled 32kD protein (48, 60).

The polyoma virus is similar to SV40 and its study has helped clarify the common features and possible variations in the transformation process for DNA viruses. The SV40 and polyoma virions are morphologically identical and they contain nearly identical amounts of DNA. However, their genomes are organized differently, particularly the early tumor antigen region responsible for transformation. Polyoma DNA codes for six proteins including three tumor antigens: large (T-Ag, 88kD), middle (mT-Ag, 55kD), and small (t-ag, 23kD). The relationship among the tumor antigens with regard to cellular transformation and induction in tumors is complex. The amino terminal end of the large T-Ag (which appears predominently in the nucleus) appears to be able to immortalize primary cells, whereas middle T-Ag is able to induce the transformed phenotype in previously immortalized cell lines. No specific function is known for small t-ag other than an occasional requirement in the transformation of certain cell lines. Biochemically, large T-antigen is known to bind to viral DNA and initiate viral DNA replication. Middle T-Ag appears to be associated with membraneous structures throughout the cell and to possess a specific tyrosine phosphokinase activity.

One nuclear function of the polyoma large T-antigen has been clarified recently. Transformation of cells requires the integration and amplification of the polyoma genome into the cell DNA in a head to tail fashion such that uninterrupted copies of the early gene region are produced. Recent studies with temperature-sensitive mutants of the large T-Ag strongly suggest that polyoma genome amplification and tandem integration is triggered by in situ replication initiated by large T-Ag binding at the origin of DNA replication. Unscheduled DNA replication leads to a high rate of recombination between homologous sequences which generates excision or amplification of viral genomic units depending on whether recombination occurs between parental or newly replicated DNA strands (4,5).

The polyoma middle T-Ag function has recently been characterized in studies using deletion mutants. As expected, protein gene products of middle T-Ag, which had carboxy terminal deletions as a result of premature termination, failed to become associated with cell membranes, failed to show the tyrosine-specific protein kinase activity associated with the wild-type middle T-Ag, and failed to transform mouse or rat cell lines. These results suggested that carboxy terminal amino acid sequences, particularly a hydrophobic domain implicated in membrane association, are important for protein kinase activity and cell transformation. Deletion mutations in the amino terminal region of the middle T-Ag gene produced truncated proteins which became associated with cell membranes, but failed to show the phosphokinase activity. Transfection with cloned DNAs coding for the truncated antigens failed to transform cells (23).

Middle T-Ag is also a phosphoprotein. The major sites of phosphorylation have been identified recently as tyrosine residues at amino acid sequence positions 250 and 315. Point mutations of these tyrosine residues to phenylalanine residues produce viruses with reduced phosphokinase activity (20% of wild type for the residue 315 mutant) and significantly decreased transforming ability (less than 1% of wild type) (7, 12, 38).

The biochemical characterization of the T-antigens and their associations with cellular proteins has been hampered by the lack of adequate amounts of the proteins. A number of laboratories have attemped to purify the T-antigens by cloning and expression in bacteria, but so far with limited success. Large quantities of a protein antigentically resembling middle T-antigen has been produced by immunoaffinity chromotography in several laboratories. The bacterial protein, however, lacks tyrosine phosphokinase activity. This surprising result is being actively investigated (23, 58).

Some recent work on middle T-Ag purification from infected mouse cells using a double immunoprecipitation precedure appears to be feasible but needs to be scaled up. Interestingly, the kinase active fraction (which is known to phosphorylate on serine/threonine residues) that has been purified by this procedure is 200,000 daltons in size. This result suggests that the kinase active middle T-Ag is associated either with itself or some cellular protein. Such associations have recently been identified (38).

A complex between middle T-antigen and a cellular protein kinase, pp60c-src, has been recently demonstrated. This observation is of great interest because pp60c-src is the cellular homologue of pp60v-src, the tyrosine protein kinase product of the Rous sarcoma virus (RSV) src gene. Thus, the mechanisms of transformation by polyoma and RSV may have common features. It seems possible that middle T-Ag may perturb the normal functioning of pp60c-src, and indeed the activity of the middle T antigen-associated pp60c-src has been found to increase significantly. It is not clear, however, whether pp60c-src is the only tyrosine kinase which can associate with middle T-Ag. In view of the homology in the catalytic domains of all known tyrosine kinases, it certainly seems possible that other cellular tyrosine kinases might be able to associate with middle T-Ag. Disruption of the function of these other kinases might also play a part in transformation (38).

The binding of polyoma large T-Ag to unique viral DNA sequence was characterized recently using a DNA fragment monoclonal immunoassay. It was found that the monoclonal antibody reacts with only a minor proportion of the large T-Ag molecules suggesting, as is the case with the SV40 large T antigen, that only a small, immunologically distinct subpopulation of polyoma large T-Ag is active in specific DNA binding. Using different restriction digests and polyoma mutants containing deletions in the viral regulatory region, it was determined that the polyoma large T-Ag binds specifically to three separable DNA regions. These include one adjacent to the minimum viral origin of replication, one spanning the restriction endonuclase Bgl I site, and one spanning the early mRNA cap site. The three fragments each contain tandem repeats of the sequence GPuGGC which has been

identified as the recognition site. Thus, as in the case of the SV40 T-antigen, polyoma large T-antigen interacts with those sequences involved in viral DNA replication and regulation of early gene transcription. However, unlike SV40 these binding sites are not adjacent on the viral genome, and furthermore the polyoma large T-Ag binds to these regions with similar affinities. The similarity of the polyoma large T-Ag recognition sequence to the recognition sequence found for SV40 T-Ag prompted a cross test of binding ability between heterologous binding sites and proteins. These studies showed that each large T-antigen specifically bound to its own DNA with at least a ten-fold greater affinity than to the other viral DNA. Furthermore, the relative affinities of the T-antigens for individual binding regions in a viral DNA differed. On the SV40 genome the ratio of region I, region II or region III binding was greater with SV40 T-antigen than with polyoma T-antigen. The affinities of polyoma T antigen for the polyoma origin and cap sites were about equal but greater than that for the Bgl I site. SV40 T-antigen bound specifically to the polyoma origin and Bgl I region but failed to bind detectably to the polyoma cap site. Since the polyoma origin and cap sites each contain three tandem repeats of the pentanucleotide, GAGGC, it is likely that some spacing of GPuGGC units or adjacent sequences are required for specific DNA binding of the SV40 T antigen (56).

The study of papillomaviruses is beginning to leave the descriptive/phenomenological phase and is entering into a more analytical phase due to the application of recombinant DNA techniques. These viruses have been known for many years to produce benign tumors (e.g. skin warts, condylomas) in both man and animals and are known to cause (or help cause) certain animal cancers which progress from an initial benign tumor. Thus, the possible role of these viruses in some human cancers has recently come under more active investigation. Sixteen different human papilloma viruses (HPVs) have been isolated in recent years, along with subvariants of each type. Most have been isolated from specific benign lesions. The best known ones, HPV-1 and -2, cause plantar and common warts, respectively. From a molecular biological perspective, the papilloma viruses are also unique among DNA tumor viruses in that they do not integrate into cellular DNA but transform cells as circular episomes. Their mechanism of transformation may be very different from the mechanism of papovavirus transformation. Thus, these viruses are of great clinical and scientific interest.

Recent work from a number of laboratories now show the presence of HPV DNA and HPV protein antigens in over 700 different tumors including cervical carcinoma in situ, cervical dysplasia, laryngeal papillomas, vulvar condylomas, verrucous carcinoma, Bowenoid tumors, and condyloma acuminato's (which have progressed to squamous cell carcinoma in the same lesion). Highly sensitive antigen detection has recently been made possible by the development of a highly sensitive immunocytochemical procedure. Various types of HPVs have been associated with certain tumors: HPV-6, is commonly seen in cervical carcinoma and dysplasia (HPV-3, -10, -11, and -16 are occasionally seen); HPV-5 is most commonly seen with the malignant transformation of epidermodysplasia verruciformus (HPV-8, and -10 are occasionally seen);

and HPV-6 is also seen in squamous papillomas of the respiratory tract. In most cases the viral DNAs that were detected were complete 5 megadalton genomic copies and were apparently not integrated into the host genome. Biopsies from patients with the same pathological type of lesions can contain different types of HPV. Multiple infections by different HPVs in the same patient was also observed. This multiplicity of HPVs in benign and malignant tumors has lead to the speculation that a differential prognosis may be associated with specific HPVs. In the case of laryngeal papillomas, HPV-6 variant 6c has been associated with a particularly severe and clinically less tractable disease. The presence of HPV DNA and antigens in these tumors strongly suggests, but does not prove, an oncogenic role for human papilloma viruses. Since many patients with wart diseases do not develop cancer, other factors such as host immunity, cocarcinogens and possibly other viral infections are probably involved (4, 24, 33, 40, 51).

Since a majority of squamous cell carcinomas of the cervix are preceded by clinical neoplastic precursors (cervical dysplasia), the recent finding that HPV DNA and antigens are found in a high precentage of mild and moderate cervical dysplasias strongly implicate HPVs in the genesis of cervical carcinoma. A similar situation holds for epidermodysplasia verruciformis patients who have a 30% chance of their diseases becoming malignant. A possible reservoir for cervical infection by HPV has been found. Squamous papillomas of the male urethra possesses HPV DNA which has a similar restriction endonuclease digestion pattern to cervically derived HPV. Also, urethral papillomas derive from the same type of tissue, squamous epithelium, as does cervical dysplasia. A multiplicity of HPV type genomes have been found to be associated with cervical dysplasias. Since not all dysplasias progess to carcinoma, it has been speculated that specific HPV types may be associated with the progression to cervical carcinoma. If future work validates this speculation, a clinical test may be developed to screen patients at risk (40).

Until recently, work on the mechanism of papilloma virus infection and possible oncogenicity have been very difficult due to the lack of in vitro cell systems which would either allow lytic growth or transformation. Numerous attempts have been and are continuing to be made to develop such systems for HPVs. Recent careful analysis of the reasons for the lack of success has shown that intact viral particles either do not adhere to or penetrate the membranes of the cell types so far tried. Only one papillomavirus (BPV-1 from cattle) has been successfully used in a murine cell transformation assay. Thus, a rough genetic map for this virus has become available and the nucleotide sequence has been worked out. (53)

BPV-1 transformed cells are being used to define the transforming functions of BPV-1 genes. Phenotypically normal revertants of BPV-1 transformed cells have been isolated and examined. BPV-1 DNA is still present, but in 10-fold reduced copy number. Such cells cannot be retransformed by BPV-1 virions. The structure of the BPV-1 DNA in these cells is being examined for mutations. Work with BPV-1 transformations will hopefully be a good model for the HPVs (41).

The use of recombinant DNA methodology has recently improved the chances for a detailed genetic and biochemical analysis of HPVs. Cloning of HPV DNA

has permitted the sequencing of the HPV-1a genome. The sequence of the HPV-3 genome is 30% complete. Knowledge of the nucleotide sequences has given us information about the probable amino acid sequences of HPV-coded proteins. Selected peptides have been synthesized for use in the production of antibodies. Most importantly, the problem of transforming cells in vitro may have been partially solved by the construction of plasmid vectors containing the HPV genome. This recombinant DNA has been transfected into selected human-derived cell lines. To date, eight cell lines have been derived from such transfections into HeLa cells using HPV-5 DNA plasmid recombinants. Preliminary data shows that the HPV-5 DNA is present in all the cell lines as whole copies and that mRNA, apparently specific for HPV-5 DNA, is being abundantly produced (31).

DNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY84

Investigator/Institution/Grant Number	Title
1. ALONI, Yosef Weizmann Institute of Science 2 RO1 CA 14995-10	Control of Gene Expression in Tumor Viruses and Cells
2. ALWINE, James C. University of Pennsylvania 5 RO1 CA 33656-02	Control of Late Gene Expression in DNA Tumor Viruses
3. ALWINE, James C. University of Pennsylvania 2 RO1 CA 28379-04	Regulation of DNA Tumor Virus Gene Expression
4. BASILICO, Claudio New York University 5 PO1 CA 16239-10	Biosynthesis in Normal and Virus-Transformed Cells
5. BASILICO, Claudio New York University 2 RO1 CA 11893-14	Cellular and Viral Control of Oncogenic Transformation
6. BENJAMIN, Thomas L. Harvard University 5 RO1 CA 19567-08	Mechanism of Cell Transformation by Polyoma Virus
7. BENJAMIN, Thomas L. Harvard University 5 RO1 CA 25390-06	Effects of HR-T Mutations on Polyoma Gene Expression
8. BERG, Paul Stanford University 5 RO1 CA 31928-03	Transduction of Genetic Information Related to Cancer
9. BOTCHAN, Michael R. Univ. of Calif. (Berkeley) 5 RO1 CA 30490-04	Transformation of Cells by SV40 Virus
10. BRADLEY, Margaret K. Dana-Farber Cancer Institute 1 R23 CA 38069-01	Nucleotide Binding Properties of SV40 Large T Protein
11. BROCKMAN, William W. Univ.of Michigan (Ann Arbor) 5 RO1 CA 19816-08	Role of SV40 Gene A in Cellular Transformation

12. CARMICHAEL, Gordon G.
University of Connecticut
5 RO1 CA 32325-02
Regulation of Polyoma Early Gene Expression

13. CARROLL, Robert B.
New York University
2 RO1 CA 20802-08
Biochemical Properties of the SV40 T Antigens

14. CHEN, Suzie
Columbia University
1 R23 CA 36319-01
Host Responses to In Vitro Mutated SV40

15. CHOW, LOUISE T.
University of Rochester
1 RO1 CA 36200-01
Human Papilloma Virus Gene Expression

16. CONRAD, Susan E.
Michigan State University
1 RO1 CA 37144-01
SV40-Induced Changes of Growth Regulation in Host Cells

17. CONSIGLI, Richard A.
Kansas State University
2 RO1 CA 07139-21
Studies in Polyoma Transformed Cells Virion Proteins

18. CROCE, Carlo M.
Wistar Institute
2 RO1 CA 16685-09
Mapping of Tumor Virus Genomes in Transformed Cells

19. DE PAMPHILIS, Melvin L.
Harvard University
2 RO1 CA 15579-10
Tumor Virus DNA Replication: A Probe Into Oncogenesis

20. DI MAIO, Daniel C.
Yale University
1 RO1 CA 37157-01
Analysis of Cell Transformation by Bovine Papilloma Virus

21. DI MAYORCA, Giampiero
Univ. of Med. & Dent. (NJ)
5 RO1 CA 25168-05
Transformation Genes of Simian Virus 40

22. DI MAYORCA, Giampiero
Univ. of Med. & Dent. (NJ)
5 RO1 CA 25169-05
BK Virus, A Human Papovavirus

23. ECKHART, Walter
Salk Institute for Bio. Studies
5 RO1 CA 13884-12
Viral Gene Functions and Regulation of Cell Growth

24. FARAS, Anthony J.
University of Minnesota
5 RO1 CA 25462-06
Human Papilloma Viruses and Malignant Disease

25.	FLUCK, Michele M. Michigan State University 5 RO1 CA 29270-03	Control of Gene Expression on Viral Transformants
26.	FOLK, William R. University of Michigan 5 RO1 CA 13978-12	Mammalian Cell Transformation by Oncogenic Viruses
27.	FOLK. William R. Univ. of Texas (Austin) 7 RO1 CA 38538-01	Mammalian Cell Transformation by Oncogenic Viruses
28.	FRIEDMANN, Theodore Agouron Institute 7 RO1 CA 37484-01	Cellular and Papovaviral Gene Expression
29.	FUJIMURA, Frank K. La Jolla Cancer Res. Found. 1 RO1 CA 37689-01	Regulatory Functions in Embryonal Carcinoma Cells
30.	GARCEA, Robert L. Dana-Farber Cancer Institute 1 RO1 CA 37667-01	Mechanisms in Polyoma Virus Assembly
31.	GHOSH, Prabhat K. Yale University 5 RO1 CA 32799-02	Regulation of Simian Virus 40 Transcription
32.	GRALLA, Jay D. Univ. of Calif. (Los Angeles) 5 RO1 CA 19941-08	Regulation of Transcription by DNA-Protein Complexes
33.	GREEN, Maurice St. Louis University 2 RO1 CA 28689-04	Human Papilloma Viruses
34.	GURNEY, Elizabeth T. University of Utah 2 RO1 CA 21797-06	Growth Control and Viral Gene Expression
35.	GUTAI, Mary W. Roswell Park Memorial Institute 2 RO1 CA 28250-04	SV40 DNA Replication and Recombination in Animal Cells
36.	HAGER, Lowell P. Univ. of Illinois (Urbana) 5 RO1 CA 17619-09	Biochemical Studies on T Antigen and Transformed Cells
37.	HALLICK, Lesley M. University of Oregon 2 RO1 CA 24799-04A	Psoralens as Probes for Viral Nucleoprotein Structure

38. HUNTER, Anthony R.
Salk Institute for Bio. Studies
2 RO1 CA 28458-04
Viral Transforming Proteins

39. KETNER, Gary W.
Johns Hopkins University
5 RO1 CA 21309-06
Transformation and Gene Regulation by Adenoviruses

40. LANCASTER, Wayne D.
Georgetown University
2 RO1 CA 32638-03
Papilloma Virus DNA and Antigens in Cervical Neoplasia

41. LANCASTER, Wayne D.
Georgetown University
5 RO1 CA 32603-03
Role of Papilloma Virus DNA in Cell Transformation

42. LANFORD, Robert E.
Baylor College of Medicine
1 RO1 CA 37105-01
SV40 T Antigen: Model for Nuclear Transport of Proteins

43. LEBOWITZ, Jacob
Univ. of Alabama (Birmingham)
5 RO1 CA 17077-09
Circular DNA Implications for Cancer and Drug Resistance

44. LEHMAN, John M.
University of Colorado
5 RO1 CA 16030-09
Pathology of Neoplastic Transformation

45. LEVINE, Arnold J.
SUNY (Stony Brook)
5 RO1 CA 28127-04
SV40 Cellular Antigens and Host Range

46. LEVINE, Arnold J.
Princeton University
7 RO1 CA 38964-01
SV40 Cellular Antigens and Host Range

47. LIVINGSTON, David M.
Dana-Farber Cancer Institute
5 RO1 CA 15751-10
Structure and Function of SV40 Nonvirion Proteins

48. LIVINGSTON, David M.
Dana-Farber Cancer Institute
5 RO1 CA 24715-06
Isolation and Function of Small SV40 T Antigen

49. MARTIN, Jonathan D.
Tulane University
5 RO1 CA 29631-03
Molecular Analysis of JC Virus Interaction With Cells

50. MERTZ, Janet E.
University of Wisconsin
1 RO1 CA 37208-01
Involvement of T Antigen in SV40 Late Gene Expression

51.	MOUNTS, Phoebe Johns Hopkins University 1 RO1 CA 35535-01	Analysis of Papilloma Virus in Laryngeal Papillomatosis
52.	NATHANS, Daniel Johns Hopkins University 5 PO1 CA 16519-09	Program on Molecular Biology of Viral Tumorigenesis
53.	NOONAN, Christine A. Baylor College of Medicine 5 RO1 CA 30925-03	Interaction of Human Wart Virus With Cultured Skin Cells
54.	OZER, Harvey L. Hunter College 5 RO1 CA 23002-08	Host Functions Related to Tumor Virus Infection
55.	POLLACK, Robert E. Columbia University 1 PO1 CA 33620-01	Directed SV40 Mutation: Cell and Molecular Consequence
56.	PRIVES, Carol L. Columbia University 5 RO1 CA 26905-05	Function and Expression of SV40 Viral Tumor Antigens
57.	ROBERTS, Bryan E. Harvard University 5 RO1 CA 27447-06	Organization and Expression of Genes in Viral DNAs
58.	ROBERTS, Thomas M. Dana-Farber Cancer Institute 5 RO1 CA 30002-03	Isolation of Polyoma T Antigens Synthesized in E. Coli
59.	ROMAN, Ann Indiana University 5 RO1 CA 29318-02	Regulation of Papovavirus Replication
60.	RUNDELL, Mary K. Northwestern University 5 RO1 CA 21327-06	Cellular Proteins Associated With SV40 T-antigen
61.	SCHAFFHAUSEN, Brian S. Tufts University 1 RO1 CA 34722-01	Products of the Transforming Genes of Polyoma Virus
62.	SHAH, Keerti V. Johns Hopkins University 2 RO1 CA 13478-13	Investigation of SV40-Related Infections of Man
63.	SHENK, Thomas E. SUNY (Stony Brook) 5 RO1 CA 28919-04	Structure and Function of DNA Tumor Virus Genomes

64. SHENK, Thomas E.
University of Rhode Island
1 R13 AI 21192-01
Gordon Conference on Animal Cells and Viruses

65. SHENK, Thomas E.
Princeton University
7 RO1 CA 38965-01
Structure and Function of DNA Tumor Virus Genomes

66. SOMPAYRAC, Lauren M.
University of Colorado
5 RO1 CA 34072-02
SV40 Deletion Mutants: Oncogenic Proteins

67. STEINBERG, Mark L.
New York University
2 RO1 CA 27869-04
Phenotypic Modulation of Human Infected Keratinocytes

68. TACK, Lois C.
Salk Institute
5 RO1 CA 37081-02
SV40 Chromosome: T-Antigen Complexes and Viral Function

69. TAMM, Igor
Rockefeller University
5 RO1 CA 18608-24
Virus-Induced Alterations in Animal Cells

70. TEGTMEYER, Peter J.
SUNY (Stony Brook)
2 RO1 CA 18808-10
Mechanisms of Viral Oncogenesis: SV40 Protein Function

71. TEGTMEYER, Peter J.
SUNY (Stony Brook)
5 PO1 CA 28146-04
Tumor Virus-Host Interactions

72. TEVETHIA, Mary J.
Penn State Univ. (Hershey)
5 RO1 CA 24694-06
Mutagenesis of Specific Regions of the SV40 Genome

73. TEVETHIA, Satvir S.
Penn State Univ. (Hershey)
2 RO1 CA 25000-06
Biology of SV40-Specific Transplantation Antigen

74. TJIAN, Robert T.
Univ. of Calif. (Berkeley)
5 RO1 CA 25417-05
The SV40 Tumor Antigen

75. TJIAN, Robert T.
Univ. of Calif. (Berkeley)
1 RO1 CA 34724-01
Autoregulation of SV40 Early Gene Expression

76. VARSHAVSKY, Alexander J.
Mass. Institute of Technology
5 RO1 CA 30367-03
SV40 Virus Structure and Replication

77. VILLARREAL, Luis P.
Univ. of Colorado Medical Center
5 R01 CA 25819-05

Gene Expression of A Small DNA Tumor Virus: SV40

78. WALTER, Gernot F.
Univ. of Calif. (San Diego)
1 R01 CA 36111-01

SV40 and Polyoma Virus Transforming Proteins

79. WEISSMAN, Sherman M.
Yale University
5 P01 CA 16038-10

Viral Transformation Molecular Basis-DNA Virus

80. WETTSTEIN, Felix O.
Univ. of Calif. (Los Angeles)
5 R01 CA 18151-08

Analysis of the Shope Papilloma-Carcinoma System

81. WILSON, John H.
Baylor College of Medicine
5 R01 CA 15743-11

Pathways of Information Exchange in Somatic Cells

SUMMARY REPORT

RNA VIRUS STUDIES I

The RNA Virus Studies I program primarily involves studies of murine and feline tumor viruses, and also lesser numbers of projects on primate, bovine and hamster tumor viruses. In the RNA Virus Studies I program extramural research is supported by two funding instruments, grants and contracts. The funding level of 98 grants was 12.78 million dollars. These include traditional research grants, program project grants, and conference grants. The 98 grants involve studies in the murine (78%), feline (10%), primate (6%), 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 biological activity of retroviruses and inhibition of viral replication and virus-induced cell transformation (20%). One research contract was active during FY 84, although funds for its support were not provided this fiscal year.

Studies in the RNA Virus Studies I area are concerned with experiments to elucidate molecular events associated with the conversion of a normal cell to the malignant phenotype utilizing retroviruses in animal model systems. 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 tumor 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 LTRs 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 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 elucidate our understanding of the transformation process.

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. Variants of the recently discovered human T-cell leukemia virus (HTLV), designated HTLV-III, also appear to have a similar immunosuppressive effect in man; this immunosuppression is most likely 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.

Studies conducted (24, 25) to determine if infection with HTLV was associated with immunosuppression in man involved both an analysis of the seroepidemiology of the agent in endemic areas of infection (especially southern Japan) and an evaluation of individuals with AIDS. It was found that individuals from endemic areas and from various risk groups for AIDS (homosexual men, Haitians, intravenous drug abusers, hemophiliacs, children of the latter groups, transfusion recipients of blood from members of the other risk groups) had much higher prevalence rates of antibody to HTLV. Conversely, control groups that consisted of healthy homosexual men, normal blood donors, kidney and heart transplant patients, and systemic lupus patients all had very low levels of exposure to HTLV. The only other groups that had elevated rates of exposure were healthy hemophiliacs and lovers of AIDS patients. It was also found that healthy hemophiliacs with exposure to HTLV had lower absolute levels of T4 cells than control hemophiliacs who had no exposure to HTLV. Moreover, when patients from the infectious disease wards in the endemic regions of infection (southern Japan) were examined, it was clear that they had a 3-fold increase in risk for infection with HTLV. These results suggest that different strains of HTLV may have a variety of immunosuppressive effects in man and that this spectrum of effects is similar in scope to those observed earlier in cats infected with FeLV.

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 were derived from normal cellular genes, 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 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 env gene recombinants. Although these viruses, in some cases, induce rapidly appearing lesions in infected animals, they do not appear to carry an oncogene of the classic type (i.e., a cell-derived oncogene). Rather, sequences located within the env region appear to be responsible for their pathogenic properties by some unknown mechanism. Extramural research involving all three types of viruses are being funded in the RNA Virus Studies I program component.

The genomes of highly oncogenic retroviruses, the sarcoma and acute leukemia viruses, contain specific genes responsible for oncogenicity which in many cases have partially replaced sequences necessary for normal viral replication, and thus are said to be 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.

The 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 which are not linked to viral DNA. Transcription of several of these genes has been detected in normal and neoplastic cells and, in some cases, normal cell proteins have been identified which are closely related to proteins encoded by the homologous viral transforming genes.

The highly oncogenic sarcoma and acute leukemia viruses thus appear to represent recombinants in which a transforming gene, derived from a homologous gene of normal cells, has been inserted into a retrovirus genome. The transforming genes of these viruses are expressed at high levels in virus-infected cells as a consequence of their association with viral transcriptional regulatory sequences. It is thus possible that transformation by these viruses is a consequence of abnormal expression of normal cell genes. Alternatively, transformation might result from structural differences between the viral and cellular proteins.

Cellular homologs of several known v-onc genes have been detected in human normal and tumor tissues, thus explaining and pinpointing their causal role in certain human cancers.

Oncogenes have been detected by transfection of DNAs from different human tumors. DNAs of colon, bladder, lung and pancreatic carcinomas, fibrosarcomas, leukemias, and even neuroblastomas have yielded potent transforming

sequences (15, 90). In those few cases that 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: that of a human bladder carcinoma oncogene and human lung carcinoma oncogene (90). 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 assay in 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 a 21 kD protein known as p21. The activating lesions of the cellular and virus-associated oncogenes studied to date affect the same site on the gene, altering in each case amino acid residue 12 (15, 40, 90). In each case, the activating lesions cause a replacement of glycine by a sterically more bulky amino acid, the replacements studied to date being valine, arginine, leucine, lysine and aspartate (4). Thus there are various allelic versions of the normal gene, each of which is sufficient to induce the transformed phenotype in NIH 3T3 cells. It has been found that, via unknown mechanisms, the amino acid substitutions affect the migration rate of the encoded p21 proteins (15). Analysis of transforming activity of ovarian carcinoma DNAs identified a primary serous cystadenocarcinoma which contained an activated ras^{KT} gene (15). DNA from normal cells of the same patient lacked transforming activity, indicating that ras^{KT} activation was a consequence of somatic mutation during development of the neoplasm. The protein encoded by this ras^{KT} gene differed in electrophoretic mobility from those found in two human lung and two human colon carcinomas, indicating that a different mutation was responsible for ras^{KT} activation in this neoplasm. The ras gene products from normal and tumor tissues were modified by the addition of lipid and were localized in the plasma membrane. The mutations of ras gene which activate the p21 into a transforming protein do not alter the protein's cellular localization or nucleotide binding properties. The identification of other cellular proteins which interact with p21 is critical to understanding the function of p21 in transformation.

The Ha-ras oncogene from the EJ human bladder carcinoma transforms NIH 3T3 cells with single hit kinetics (4, 90). Since carcinogenesis is known to be a multistep process, this raises questions about the status of NIH 3T3 mouse cells as representatives of truly normal cells. It was found that the same Ha-ras oncogene was able to stimulate soft agar growth, but failed to transform, rat embryo fibroblasts from Fischer rat embryos (4). In contrast, an established rat cell line, rat-1, could be morphologically transformed by the DNA transfection assay. These results raised the question of whether other oncogenes of cellular origin could collaborate with the ras oncogene to establish full transformation. Weinberg (4) found that certain human tumors carry two activated cellular oncogenes, N-ras, a member of the ras family, and a myc oncogene. This suggested a complementary function in carcinogenesis (cocarcinogenesis). It was further determined that whereas neither myc nor

ras oncogenes alone could transform rat fibroblasts, cotransfection with both oncogenes resulted in the transformed phenotype.

Harford (40), in collaboration with O'Brien (NCI), has localized the four c-ras genes on four different human chromosomes C-Ha-ras-1 on chromosome 11, C-Ha-ras-2 on the X chromosome, C-Ki-ras-1 on chromosome 6 and C-Ki-ras-2 on chromosome 12. In collaboration with Weinberg, she found that in the case of human bladder carcinoma EJ, the point mutation was a single nucleotide change of G to T affecting the codon for the 12th amino acid, thus changing glycine to valine (as mentioned above).

Haseltine (41) investigated the structure and function of the human homolog of the v-fes oncogene of ST and GA strains of feline sarcoma virus. A series of molecular clones were obtained which could be made to transform NIH 3T3 fibroblasts by recombining small portions of v-gag and v-fes sequences with large portions (80%) of human c-fes sequences. The oncogenic transformation was due to protein kinase coded by this recombined human fes oncogene.

Molecular clones of the retroviral oncogene fms from the Sarma-McDonough strain of FeSV were used to isolate recombinant viruses containing c-fms proto-oncogene sequences from a human placental library (79). Viral and cellular fms sequences were used in Southern blotting experiments with a panel of 32 human x mouse somatic cell hybrids to assign the human c-fms proto-oncogene to human chromosome 5.

In studies of a variety of human lymphoma-leukemia specimens (74), it was noted that c-erbB oncogene locus is associated with hematopoietic cell proliferation, and c-myc locus is transcribed at elevated levels in a majority of the chronic lymphocytic leukemia, B-cell type, cells.

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 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 the oncogene products. Recent evidence suggests that this model, termed the "insertional mutagenesis model" of oncogenesis, 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 (10) and mouse mammary tumor virus (MMTV) group (84). Studies of many investigators, including Casey (10), have provided compelling evidence that activation of the c-myc oncogene in cats by FeLV may be responsible for the induction of T-cell lymphomas in this species. In several cases, myc-related gene amplification in feline tumors was found. Molecular cloning of integrated FeLV resulted in the isolation of a new defective FeLV containing the oncogene myc. This is the first defective provirus found in an outbred mammalian species that induces lymphoid malignancy. As found by Weinberg (4), transfection of this c-myc alone into NIH 3T3 cells failed to elicit their transformation (10), presumably due to the fact that myc is a transcriptional activator of other oncogenic sequences (such as ras), the synergistic effect of both may be required for transformation.

Recent studies have also suggested that induction of mammary adenocarcinomas by mouse mammary tumor virus (MMTV) is strongly favored by MMTV insertions within a single locus, termed int-1 (84). This locus does not show DNA sequence homology to any of the cellular oncogenes so far described, but integration of the MMTV provirus within this chromosomal domain has been shown to induce the expression of a 2.6 kilobase cellular RNA transcript encoded by the int-1 locus. Increased expression of this cellular RNA transcript may be due to the enhancer sequences present in the MMTV long terminal repeat (LTR). This enhanced RNA expression is thought to be involved in the transformation event, but the function of this RNA transcript or its protein product is unknown. Recently, a second common integration site (int-2) distinct from int-1 was described (Peters, et al.), indicating that different chromosomal domains may be implicated in mammary tumorigenesis. Therefore, it may be that induction of adenocarcinomas by MMTV may also occur by promoter insertion.

While insertional mutagenesis has been convincingly shown for certain avian retroviruses and has been suggested for FeLV (10) and MMTV (84), the major question about induction of lymphomas by the slowly transforming mouse leukemia viruses is whether this mode of leukemogenesis is involved. Recent studies of Steffen (81) and Adams, et al. have provided preliminary evidence that activation of c-myc by adjacent proviral insertions may play a role in lymphomagenesis in the rat (81) and mouse (Adams, et al.). However, the failure to find c-myc activation in many tumors has suggested that as yet unidentified cellular oncogenes may be activated by insertional mutagenesis.

The oncogenic potential of acutely transforming viruses resides in the virally encoded oncogenes (v-onc) acquired by the viral genome through genetic recombination with certain normal cellular sequences, the c-onc. The latter, such as the c-ras and c-myc described above, are thought to encode proteins associated with cell growth, differentiation, and development. Although acutely transforming v-onc containing retroviruses occur in nature only rarely, characterization of their oncogenic sequences, their cellular homologs and the proteins encoded by these sequences have far-reaching implications for understanding oncogenesis mediated through the virus-mediated transduction process.

The v-mos oncogene of Moloney sarcoma virus is now being intensively studied. Transfection of v-mos into NIH 3T3 mouse cells led to increased expression of v-mos encoded RNA in some of the transfected lines, but no morphological transformation similar to that seen by v-ras transfection assay in this cell line was observed (52). The differential regulatory control of c-ras and v-mos oncogenes in 3T3 cells needs to be studied further.

The transforming gene, v-mos, encodes a 37kD phosphoprotein, p37mos, believed to be a protein kinase (46). In M-MSV 124-transformed mouse cells the bulk of this protein was found in association with the cytoplasmic membrane and was not involved in a specific association with other proteins. It will be necessary to identify and characterize the target proteins for tyrosine protein kinase activity of the p37mos protein, presumed to be the cause for oncogenic transformation.

The v-sis oncogene of simian sarcoma virus encodes a protein product which was recently shown to have a close structural relationship to the platelet-derived growth factor (PDGF) which is involved in promoting cell growth in the normal process of wound repair. Studies of Hunter (46) suggest that PDGF and epidermal growth factor (EGF) bring about a mitogenic response through transient tyrosine phosphorylation effect on a 42kD protein substrate. Thus viral tyrosine kinases of certain viral oncogenes appear to drive the cells to incessant growth by usurping the same growth control pathway.

Donoghue (19) constructed recombinants between the mos gene and nondefective Moloney murine leukemia virus to correct the transcriptional defects of M-MSV. The cells infected with recombinants contain mos protein which appears to be an envelope-oncogene fusion protein, i.e., the first five codons of the reading frame are donated by the M-MuLV env gene. The mos gene products were produced in very low amounts. To increase production of the mos gene product, recombinants with mos under the control of SV40 late promoter have been constructed. Studies are underway to characterize the mos protein whose functions are largely unknown. In a reverse experiment, the middle T gene of polyoma virus spliced into a MuLV derived retrovirus vector resulted in a replication defective transforming virus which, for the first time, allows efficient infection and transformation of murine cells with the transforming middle T gene of polyoma virus.

The protein product of another oncogene, v-fos of FBJ-murine osteosarcoma is a 55 kD phosphoprotein (85). The cellular homologs of v-fos gene in mice and human genomes have been identified, molecularly cloned and characterized. Another fos containing oncogenic virus, FBR-MSV was molecularly cloned and analyzed. It makes a gag-fos fusion protein of 75kD. Its nucleotide sequence was determined and compared to c-fos and FBJ-MSV sequence. Both v-fos and c-fos are localized in the nucleus. The v-fos sequences can transform rat 208 F cells while the c-fos gene is unable to do so. The c-fos gene can be made to transform cells by the addition of transcriptional enhancer elements and disruption of 3' interacting sequences. C-onc as well as c-fms (cellular homolog of v-fms of a feline sarcoma virus) are expressed in prenatal development in a stage and time-specific manner. This differential expression of c-onc genes during embryogenesis lends support to the widely held notion that c-onc genes play a role in normal metabolic processes.

In studies of the oncogenes of acutely transforming feline sarcoma viruses, Besmer (6) has found that HZ2-FeSV contains an oncogene homologous with that of Abelson MuLV. The HZ2-FeSV sequences were found to correspond to 5' A-MuLV v-abl sequences which are the tyrosine kinase domain of the A-MuLV v-abl gene. Another new FeSV isolate, HZ4-FeSV, contained an oncogene which was structurally unrelated to 19 other known oncogenes. This oncogene was named v-kit. Its cellular counterpart, c-kit, was found to be conserved in mammalian genomes. The oncogene of a third FeSV isolate, HZ5-FeSV, was found to be homologous to the fms sequences of the Sarma-McDonough FeSV (SM-FeSV).

Antisera specific for six regions of the v-abl protein were used to serologically characterize the A-MuLV tyrosine kinase (94). Two antisera were found to block the in vitro autophosphorylation of the v-abl protein as well as its ability to phosphorylate a peptide substrate. Examination of the sites

against which the kinase-blocking antisera were prepared revealed that both are in close proximity to the in vivo sites of tyrosine phosphorylation and thus fall within the region of high homology with v-src and other tyrosine kinases. Antisera directed against other regions of v-abl did not inhibit kinase activity.

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 host life cycle. Analysis of mutants and their gene products can directly determine the function of each protein.

Studies of a mutant of Moloney MuSV, the ts110 mutant of Mol-MuSV 124, were performed to characterize the oncogene product (1). A protein kinase activity previously unknown with a v-mos protein was ascribed to the mutant gene products, P85gag-mos and P100gag-mos, both of which are fusion proteins of gag and mos proteins. Genome structure studies of Mol-MuSV 124 suggested that the ts110 genome must have undergone deletion in order to generate a gag-mos hybrid protein.

The oncogene of an acutely transforming murine retrovirus Abelson MuLV-(A-MuLV) is a single fusion protein, P160gag-abl, which transforms cells via a tyrosine-specific protein kinase activity. Mutants involving the gag-abl-transforming proteins have been studied (71). These studies show that a mutant lacking most of the gag gene products of P30 and P12, but retaining a small portion of P15 was able to transform mouse fibroblasts in vitro, but failed to transform mouse lymphoid cells or induce tumors in mice (1). Two mutants lacking P12 only were efficient in transforming lymphoid cells. Mutants with deletions in region IV (extreme carboxy-terminal region of the protein) lose their ability to transform lymphoid cells unless they reacquire this region through recombination in vivo with endogenous viral sequences. Monoclonal and polyclonal antibodies against c-abl and v-abl proteins were used to determine the expression and subcellular localization of these proteins. Studies of Baltimore, Prywes and others (4) similarly show that the gag segment of the P160 gag-abl protein is not needed for fibroblast transformation, but is required for lymphoid cell transformation. Systematic deletion of v-abl sequences (4) showed that only 45kD of the 130kD molecular weight of the fusion protein is needed for fibroblast transformation and, at most, slightly more is needed for lymphoid cell transformation. It was further shown that insertion of four amino acid coding segments of DNA into the minimal transforming region can abolish the transforming activity. Small deletions at either end of this region have the same effect. It is precisely this region that has extensive amino acid homology to other viral oncogenes: v-src, v-yes, v-fps and v-fes. Studies are in progress to characterize the defective mutants and to define, by more precise deletion analysis, the region of gag required for lymphoid cell transformation. In studies of a rat sarcoma virus, Rasheed (69) has found that the gag-related portion of the oncogene is important for transformation and the transformed cells consistently express

the transforming protein, a 29kD fusion protein product of the gag and ras oncogenes.

Wong (95) found that a group of ts mutants of Mo-MuLV, instead of inducing T-cell lymphoma, induced hind-leg paralysis in newborn CFW/D mice similar to that reported by Gardner, et al. in Lake Casitas wild mice. The mutants share a common defective function, i.e., inefficiency in the processing of env precursor protein. Since body temperature of the adult mouse approaches restricted temperature for the ts mutants, accumulation of gp80 env on plasma membranes may interfere with neuronal function, e.g., neuromuscular transmission, leading to hind-leg paralysis.

Studies (38) using site specific mutagenesis of cloned DNA in the analysis of retrovirus replication provided several important results, including the findings that one of the products of the gag gene, gp80gag, is completely dispensible for virus replication and a p30 domain of the gag protein is required for assembly of virions. It was found that mutations could be repaired by recombination at high frequency with sequences endogenous to the host cell. In a similar study, Fan (28) also concluded that the glycosylated gag pathway involving gp80gag was not required for virus replication.

While participation of cellular and/or viral oncogenes explain a part of viral oncogenesis, another mode of cancer induction by retroviruses is the generation de novo of oncogenic recombinant retroviruses with greater potential for cancer induction than either parent.

High-leukemic inbred strains of mice inherit DNA copies of ecotropic type C viruses; expression of these loci results in a lifelong viremia. Despite compelling evidence that this viremia ultimately results in leukemia, a number of findings have suggested that the inherited ecotropic viruses themselves are not leukemogenic, but rather give rise, by genetic recombination, to novel type C viruses which are the proximal leukemogenic agents in these animals. One group of recombinant viruses which may serve as inducers of leukemia are the mink cell focus-forming (MCF) viruses. MCF viruses apparently arise during the lifetime of high-yield-ecotropic-virus mice by recombination between endogenous ecotropic and nonecotropic viruses and invariably appear in leukemias or at the sites of their imminent appearance. They possess novel gp70s and, as a result, a novel host range, being able to infect mouse cells like their ecotropic progenitors and mink cells like their putative xenotropic progenitors.

To determine which specific genome entity is required for oncogenesis in the mouse, Hopkins and Holland (45) used recombinant DNA technology to construct a series of recombinants between molecular clones of a nonleukemogenic MuLV, AKV virus, and a leukemogenic MuLV, MCF-247 (an isolate of a naturally-occurring recombinant virus of the AKR mouse). Studies done in collaboration with Drs. Hartley and Rowe at NIH support their earlier observation that gp70, p15E and the LTR are important in oncogenesis. The more genetic material the newly constructed recombinant virus derives from the leukemogenic parent (MCF 247), the more leukemogenic potential it has. When the nucleotide sequence of a DNA clone of MCF 247 virus was determined (4), it became apparent that MCF 247 arose by multiple recombinational events. Both the gp70 and p15E genes of

this virus are recombinant with the c-terminal portion of gp70 and the amino terminal portion of p15E contributed by the ecotropic parent, while the N terminal portion of gp70 and c-terminal portion of the p15E derive from (a) different endogenous virus(es). It remains to be seen why these particular patterns of recombination produce a leukemogenic virus.

In other studies, Hopkins and Chatis made the important discovery that the 3' end involving the U_3 region of the LTR of MuLV controls the type of cancer induced. Moloney MuLV induces T-cell lymphoma, whereas Friend MuLV induces erythroleukemias. Transposition of a small (621 base pair) fragment from the 3' end of Moloney genome into the corresponding segment of Friend virus produced a recombinant virus that induced T-cell lymphomas instead of erythroleukemias. When the Mo-MuLV segment was removed and replaced with the corresponding segment from Friend virus, the virus was again erythroleukemia-inducing. This fragment includes transcriptional sequences, in particular a so-called enhancer sequence. Other laboratories have recently confirmed the role of enhancer sequences in tissue specificity. The finding of Hopkins and Chatis may be a dramatic example of how such tissue specificity ("targeting") determines the type of disease caused.

In studies of mutants of nonleukemogenic spleen focus-forming virus (SSPV), Kabat (49) demonstrated that the env genes encoding membrane glycoprotein are oncogenes and are required for initiating the process of progressive erythro-leukemogenesis. The mutant SSPVs generate pathogenically active revertants when injected into newborn mice.

The mouse MCF virus has a dual host range (mink cell tropic as well as mouse cell tropic). The feline subgroup B FeLV has a wide host range (Sarma, et al.). Elder (23) made the interesting observation that GA-FeLV-B and Moloney MCF virus coded gp70-p15E polyproteins bore striking homologies, explaining, at least in part, the reason for the wide host range of these viruses.

In contrast to the studies implicating gp70, p15E and the U_3 region of LTR recombinant DNA, studies of Lenz and Haseltine (41) suggest that in another pathogenic variant of the AKR mouse leukemia virus designated SL3-3 (a nonMCF virus), the primary virulence determinant of this virus lies outside the region of the viral genome which encodes the envelope proteins, gp70 and p15E. Thus it is apparent that continued investigations are necessary to fully explain the role of different genome areas and molecular mechanisms in oncogenesis by MuLV.

Testing the oncogenicity of a feline recombinant virus PR8, which has the properties of the parental viruses, an exogenous FeLV (R-FeLV) and the endogenous feline RD-114 virus, Rojko (70), made the interesting observation that whereas the R-FeLV parent produces a thymic lymphoma and the RD114 parent virus is innocuous to cats, the recombinant virus produced a rare form of leukemia, eosinophilic leukemia (observed in 1 of 12 cats). Again, the roles, if any, of the gp70, p15E or U_3 regions of 3' LTR in the altered pathogenicity (targeting) of this virus needs to be investigated.

Expression of endogenous proviruses varies with the inbred strain and age of the mouse and appears to be under stringent control (16). Regulation of

provirus expression is thought to be at the transcriptional level and controlled by linked cis-acting inhibitory cellular DNA sequences, by DNA methylation, or both. To begin to differentiate between these different control mechanisms, three endogenous ecotropic proviruses, Emv-1, Emv-3 and Emv-13 were molecularly cloned. All three proviruses are poorly expressed in vivo, although they appear nondefective by restriction enzyme analysis. DNA transfection and marker rescue experiments using cloned DNAs showed that all three proviruses carry small mutations in their genomes that inhibit their expression in vitro as well as in vivo. Recombination between different defective proviruses also appears to occur in vivo, leading to the generation of nondefective recombinant viruses that spread throughout the animal. This phenomenon leads to viremia late in life. By Southern analysis and hybridization with an MCF- and xenotropic murine leukemia virus (MuLV)-related probe of DNAs from a number of recombinant inbred mouse strains, the chromosomal locations of many MCF- and xenotropic MuLV-related sequences in mouse chromosomes were mapped. Interestingly, many of these proviral sequences map near genes encoding mouse lymphocyte antigens, suggesting that they may be useful probes for molecular cloning and characterizing regions of the mouse genome important in lymphocyte development.

Studies of Schwartz (76) suggest that leukemogenic viruses in HRS/J mice arise as a result of a process of convergent evolution of recombinants. Recombination of the defective ecotropic proviruses Emv-1 and Emv-3 selects for infectious particles that can be spread throughout the animal. Further recombination with other endogenous viral sequences selects thymotropic agents, perhaps as a result of acquired p15E or U_3 genes, or both. A third recombination involving gp70 genes may confer leukemogenicity on the infectious particle. The role of gp70 in leukemogenicity thus seems of critical importance, but how this viral envelope glycoprotein confers oncogenic potential on the virus is unknown. Schwartz, et al. (76) have proposed that recombinant gp70 is recognized as an antigen within the thymus. The persistent antigenic stimulation produced by the thymotropic viruses may lead to a rare transforming event of T cells, in a manner analagous to the mechanism proposed for the oncogenic effect of Epstein-Barr virus on B lymphocytes.

Wilson (93) studied proviruses expressed by 129 mice. They were found to be related to oncogenic MCF leukemia viruses and probably represent the cellular parent for recombinant viruses which have recently lost the capacity to replicate as infectious virions. However, as found in infectious virions, these proviruses contain the same 200 nucleotide insertion within the U_3 region which may be involved in the unique regulation of these transcripts. Differences in cytosine methylation of promoter sequences of proviruses of different tumors suggest differential tissue-specific transcription.

The majority of leukemias and lymphomas of mice have been of T-cell origin, whereas spontaneous leukemias of the myeloid lineage are observed only rarely in inbred mouse strains. Bedigian (5) has 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. This virus is transmitted through mother's milk. Of the myeloid tumors induced in mice with this exogenous virus, 11 of 25 contained only a single detectable tumor-specific

provirus. It should be possible to evaluate the origin and structure of these proviruses and verify whether the promoter insertion model of oncogenesis is operative in these tumors.

Endogenous FeLV elements present in cat DNA, characterized by molecular cloning, are bounded by LTRs and contain major deletions in gag and pol genes (74). These results argue for their origin as a result of integration into the germ line of a complete virus followed by deletion; or by integration of a pre-existing, deleted variant at a single or limited number of chromosomal loci in a relatively recent ancestor of the cat, followed by gene duplication events and subsequent divergence. The detection of incomplete endogenous FeLV sequences in the cat genome may explain why the expression of the endogenous FeLV-related genes in domestic cat placental tissue is limited to RNA species which contain env gene-related sequences, but are deficient in gag and pol transcripts. In contrast to endogenous FeLV elements, a majority of the endogenous RD-114 sequences contain major deletions in the env region, and there appears to be a single, or at most two, complete RD-114 element(s) which probably are responsible for yielding an infectious virus under permissive conditions. All endogenous RD-114 sequences examined are 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 suggests that their expression is linked to growth and development as it varies with the gestational age of the fetus and with development from fetus to adult tissues (74). Enhanced levels of c-myc transcripts are detected in several feline neoplasms, including hematopoietic malignancies. The feline c-myc locus has been cloned and characterized. Polymorphisms at the locus including alleles differing at 3' coding sequences have been defined. Polymorphisms of the c-myc oncogene may affect the expression and structure of the oncogene and thereby influence its role in oncogenesis. Genetic rearrangement at the c-myc locus has been detected in four of ten cat neoplasms examined. While the presence of a c-myc, which occurs in higher frequency in tumor DNA can be demonstrated, a more extensive study will be required to reveal a correlation between allelic variation and susceptibility to DNA rearrangements or tumorigenesis.

The study of host genetic and epigenetic factors which can interfere with the development of a disease is a classical approach to the mechanism operating in its genesis. Through the work on California wild mice, Gardner (36) and his collaborators have discovered a powerful dominant gene, Akvr-1, which confers resistance to ecotropic type C retroviruses. This locus is either identical or highly linked to the Fv-4 locus which was described as an ecotropic resistance locus in Japanese wild mice. Akvr-1 resistance was shown to be due to the expression of an ecotropic gp70 presumably coded for by a defective provirus, which, by interference, blocks ecotropic virus. Through genetic crosses between Akv and Akv-1^R mice, Gardner also found that proviral DNA of virus-susceptible Akv mice is hypomethylated, whereas in nonpermissive Akvr-1^R mice proviral DNA is methylated. These results are interpreted to suggest an effect of the ARV-1 locus on methylation.

Duran-Reynals and Lilly (21) have described a maternal resistance factor (MRF) that females, but not males, of some mouse strains transmit to their progeny which confers relative resistance to spontaneous lymphoma and to the expression of endogenous ecotropic MuLV. This MRF is believed to be anti-E-MuLV antibodies transmitted in the mother's milk and perhaps transplacentally as well. Characterization of this factor has important implications for prevention of viral oncogenesis in mice.

Yang (97) found that embryo cell lines of RFM/Un mouse strain, when innoculated with B-tropic MuLV, resist virus infection by inhibiting linear viral DNA formation. This represents an extreme of $Fv\text{-}1^{n}$ host restriction of MuLV, namely, inhibiting the formation of not only viral form I supercoiled DNA, but also viral form III linear DNA duplexes. Similar Fv-1-related restriction has been observed in other mouse strains such as C57L and 129. This particular restriction mechanism was recognized originally by Pincus, Rowe and Hartley of NIH and was given the name $Fv\text{-}1^{nn}$ because of its apparent association with the Fv-1 locus. Yang (97) further found that endogenous N-tropic MuLV induced from the cell lines of RFM/Un are also severely restricted in these cells and show two-hit kinetics. It was shown that the Fv-1 host range property is due to nucleotide sequences coding for the 109th and 110th amino acid residues of the p30 gag protein.

The role of MMTV in mammary neoplasia of the mouse has not been precisely defined because of the number of potential interactions between endogenous and exogenous proviral genes and host DNA. In collaborative studies, Cohen (12) and Gardner (36) have developed a strain of wild mice devoid of any genetically transmitted MMTV sequences. Once these animals were identified by restriction endonuclease analysis of splenic DNA, appropriate selected breeding resulted in the first MMTV-free line of mice. Currently these mice are being inbred and experiments performed to determine the requirement for nonviral genes in tumorigenesis.

It has long been known that mouse mammary tumorigenesis by MMTV is strongly influenced by genetic and hormonal factors. Studying glucocorticoid stimulation of expression of endogenous proviral MMTV sequences, Peterson (63) and Yamamoto (96) have found that glucocorticoids selectively enhance the transcription of MMTV sequences that are hypomethylated, but not endogenous MMTV sequences which are methylated and transcriptionally inactive. Cohen (12) found tissue-specific variation in DNA methylation of endogenous proviral MMTV sequences and correlated these hypomethylated sites with the appearance of viral transcripts. These results confirm that methylation of endogenous proviral sequences may be an important parameter in programming these genes for transcriptional inactivity and conferring resistance to glucocorticoid-mediated promotion of viral transcription and mammary tumorigenesis.

Several investigators have reported retrovirus-related sequences in humans using low stringency hybridization procedures with nonhuman retrovirus sequences. Using high stringency hybridization, Craig (12) has identified two MMTV-related sequences in human cells. Not all human DNAs tested contained these sequences. This preliminary finding, similar to that reported by others

using low stringency hybridization, is being examined by studies of the structure and distribution of these sequences.

In recent studies of the bovine leukemia virus (BLV), Gupta and Ferrer (30) have shown that plasma from cows infected with BLV contains a nonantibody inhibitor of transcription of BLV genes. BLV exists in infected cattle in a noninfectious, quiescent state. The discovery of this plasma factor provides an explanation for the transcriptional repression of the BLV genome. The existence of a similar plasma inhibitory factor in humans against the evolutionarily-related human T-cell leukemia virus (HTLV) has been suggested since this virus is also repressed in chronically-infected humans.

Workshops, conferences and symposia supported by grants in FY 84 included the following: the Cold Spring Harbor Workshop on HTLV under the auspices of the Cold Spring Harbor Laboratory, September 13-15, 1983 (R13 CA36767); and a workshop on HTLV, supported by the Division of Cancer Etiology, held at the NIH on April 12-13, 1984 for the purpose of determining the state-of-the-art in research on this newly-discovered human cancer virus and identifying areas of extramural research that could be stimulated through NCI support by the grant mechanism. A new initiative on HTLV was based on input from this workshop.

RNA VIRUS STUDIES I

GRANTS ACTIVE DURING FY 84

	Investigator/Institution/Grant Number	Title
1.	ARLINGHAUS, Ralph B. Scripps Clinic and Res. Fdn. 7 R01 CA 36714-01	Biosynthesis and Characterization of Murine Oncornavirus
2.	AXEL, Richard Columbia University 2 P01 CA 23767-06	Molecular Virology
3.	BACHELER, Lee T. Temple University 5 R01 CA 29519-04	Organization and Expression of Leukemia Virus Genomes
4.	BALTIMORE, David Massachusetts Inst. of Tech. 5 P01 CA 26717-05	Molecular Analysis of Oncogenic Viruses
5.	BEDIGIAN, Hendrick G. Jackson Laboratory 5 R01 CA 31102-03	A New Murine Model for the Study of Nonthymic Leukemia
6.	BESMER, Peter Sloan-Kettering Inst. Ca. Res. 1 R01 CA 32926-01A	Oncogenes of New Sarcoma Virus Strains
7.	BHATT, Pravin N. Yale University 1 R13 RR 01549-01A	Infections of Laboratory Rodents: Effect on Research
8.	BURNS, William H. Johns Hopkins University 5 R01 CA 30090-03	Role of Thymic Epithelium in Viral Leukemogenesis
9.	CARDIFF, Robert D. Univ. of Calif. (Davis) 5 R01 CA 21454-06	MuMTV Gene Amplification and Expression
10.	CASEY, James W. Louisiana State Univ. Med. Ctr. 5 R01 CA 31702-03	Molecular Mechanisms of Retroviral Induced Leukemia
11.	COGGIN, Joseph H., Jr. University of South Alabama 5 R01 CA 23491-07	Etiology of a Lymphoma Epizootic in Hamsters

12. COHEN, J. Craig
Louisiana State Univ. Med. Ctr.
2 RO1 CA 34823-03
Role of Endogenous Viruses in Mammary Carcinogenesis

13. COHEN, J. Craig
Louisiana State Univ. Med. Ctr.
1 RO1 CA 35686-01
Retrovirus Sequence Specific Integration in Human Cells

14. COMPANS, Richard W.
Univ. of Alabama (Birmingham)
5 RO1 CA 18611-09
Molecular Studies of Oncorna and Arenaviruses

15. COOPER, Geoffrey M.
Dana-Farber Cancer Institute
2 RO1 CA 18689-08
Infectious DNA for Endogenous RNA Tumor Virus Genes

16. COPELAND, Neal G.
University of Cincinnati
5 RO1 CA 37283-02
Ecotropic MuLVs of Normal and Mutant Mouse Strains

17. DARNELL, James E., Jr.
Rockefeller University
5 PO1 CA 18213-08
Correlated Program in Viral Oncology

18. DATTA, Syamal K.
Tufts University
5 RO1 CA 31789-05
Genetic-Viral-Immunologic Studies in New Zealand Mice

19. DONOGHUE, Daniel J.
Univ. of Calif. (San Diego)
5 RO1 CA 34456-02
Expression of Retroviral Envelope Gene Fusion Proteins

20. DUDLEY, Jaquelin P.
University of Texas (Austin)
1 RO1 CA 34780-01A
Amplification of MMTV DNA in T-cell Lymphomas

21. DURAN-REYNALS, Maria L.
Yeshiva University
5 RO1 CA 07160-19
Possible Neoplastic Effects of Non-neoplastic Viruses

22. ECKNER, Robert J.
Boston University
2 RO1 CA 19562-09
Biological and Physical Properties of Friend Virus

23. ELDER, John H.
Scripps Clinic and Res. Fdn.
5 RO1 CA 25533-05
Structural Studies of Recombinant Retrovirus gp70s

24. ESSEX, Myron E.
Harvard University
5 RO1 CA 13885-10
Oncornavirus-Associated Cell Membrane Antigens

25. ESSEX, Myron E.
Harvard University
5 R01 CA 18216-08
Immune Response in Leukemia

26. ETKIND, Polly R.
Sloan-Kettering Inst. Ca. Res.
1 R01 CA 35643-01
Molecular Mechanism in C3hf Mammary Tumorigenesis

27. FAMULARI, Nancy G.
Sloan-Kettering Inst. Ca. Res.
1 R01 CA 36162-01
Stage Specific Events in Viral Leukemogenesis

28. FAN, Hung Y.
Univ. of Calif. (Irvine)
5 R01 CA 32454-03
Studies of Murine Leukemia Virus Integration

29. FAN, Hung Y.
Univ. of Calif. (Irvine)
5 R01 CA 32455-04
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.
Univ. of Calif. (Berkeley)
1 R01 CA 35547-01
Steroid Regulation of Protein Maturation

32. FLEISSNER, Erwin J.
Sloan-Kettering Inst. Ca. Res.
5 R01 CA 15297-10
Viral and Mouse Genes in Leukemia Virus Infection

33. FLEISSNER, Erwin J.
Sloan-Kettering Inst. Ca. Res.
2 P01 CA 16599-10
Hematopoietic Cell Transformation by Retroviruses

34. FRIEND, Charlotte
Mount Sinai School of Medicine
5 R01 CA 10000-18
Filterable Agents and Tumor Induction in Mice

35. GARDNER, Murray B.
Univ. of Calif. (Davis)
5 R01 CA 30912-03
Mammary Tumorigenesis in Hosts Lacking MuMTV DNA

36. GARDNER, Murray B.
Univ. of Calif. (Davis)
5 R01 CA 31619-02
Genetic Control of Ecotropic Retrovirus in Wild Mice

37. GIRARDI, Anthony J.
Institute for Medical Research
5 R01 CA 24940-05
Immunologic Studies in Mouse and Human Breast Cancer

38.	GOFF, Stephen P. Columbia University 5 R01 CA 30488-04	Construction and Analysis of Retrovirus Mutants
39.	HAAS, Martin Univ. of Calif. (San Diego) 2 R01 CA 34151-03A1	Viral Malignant Lymphomagenesis in X-irradiated Mice
40.	HARFORD, Esther C. U.S. Uniformed Serv. Univ. Hlth. Sci. 1 R01 CA 34582-01	Oncogenes (c-ras) in Human Cancer Induction
41.	HASELTINE, William A. Dana-Farber Cancer Institute 5 R01 CA 29294-03	Molecular Biology of Leukemia and Sarcoma Retroviruses
42.	HASELTINE, William A. Dana-Farber Cancer Institute 1 R01 CA 36974-01A	Study of Px Region of Human T-Cell Leukemia Virus
43.	HAYS, Esther F. Univ. of Calif. (Los Angeles) 2 R01 CA 12386-11A1	Development of Lymphoma in the Thymus
44.	HOOVER, Edward A. Colorado State University 5 R01 CA 32552-02	Pathogenesis of Animal Leukemia
45.	HOPKINS, Nancy H. Massachusetts Inst. of Tech. 5 R01 CA 19308-08	Studies on Endogenous C-type Viruses of Balb/c Mice
46.	HUNTER, Anthony R. Salk Inst. for Biol. Studies 2 RO1 CA 17096-09	Macromolecular Synthesis and Growth Control
47.	HUNTER, Eric Univ. of Alabama (Birmingham) 2 R01 CA 27834-04	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 Univ. 5 R01 CA 25810-05	Leukemogenic Membrane Glycoproteins: gp55s of SFFVs
50.	KAPLAN, Henry S. Stanford University 2 R01 CA 03352-28	Biological Aspects of Carcinogenesis by Radiation

51.	KAPLAN, Henry S. Stanford University 5 RO1 CA 29079-03	Studies of Retroviruses from Human Lymphoma Cells
52.	LERNER, Richard A. Scripps Clinic and Res. Fdn. 5 PO1 CA 27489-04	Consequences of Endogenous Retroviral Expression
53.	LEVY, Jay A. Univ. of Calif. (San Francisco) 5 RO1 CA 33137-02	Role of Endogenous Xenotropic Viruses
54.	LILLY, Frank Yeshiva University 2 RO1 CA 19931-07A	Mechanism of the H-2 Effect on Viral Leukemogenesis
55.	LUFTIG, Ronald B. Louisiana State Univ. Med. Ctr. 7 RO1 CA 37380-01	Assembly of Murine Leukemia Viruses
56.	MERUELO, Daniel New York University Med. Ctr. 2 RO1 CA 22247-07	Genetics of Resistance to Leukemia
57.	MERUELO, Daniel New York University Med. Ctr. 5 RO1 CA 31346-02	Loci Affecting Radiation/RadLV-induced Leukemogenesis
58.	MODAK, Mukund J. Sloan-Kettering Inst. Ca. Res. 5 RO1 CA 21404-06	Molecular Effectors of Enzymatic DNA Synthesis
59.	MURPHY, Edwin C., Jr. Univ. of Texas System Ca. Ctr. 1 RO1 CA 34734-01	MuSV ts110: A Potential ts Transcriptional Mutant of MuSV
60.	O'DONNELL, Paul V. Sloan-Kettering Inst. Ca. Res. 5 RO1 CA 31491-03	Kinetic Study of Virus-Accelerated Leukemia in AKR Mice
61.	OLSEN, Richard G. Ohio State University 5 RO1 CA 30338-03	FeLV Leukemogenesis and Preneoplastic Lesions
62.	OZANNE, Bradford W. Univ. of Texas Hlth. Sci. Ctr. (Dallas) 2 RO1 CA 23043-07	Peptide Transforming Factors from Transformed Cells
63.	PETERSON, David O. Texas A & M Research Foundation 5 RO1 CA 32695-02	Genetic and Molecular Analysis of Steroid Responsiveness

64. PIKO, Lajos
U.S. Veterans Admin. Hospital
5 R01 CA 24989-05
Gene Expression in Early Mouse Development

65. PINCUS, Theodore P.
Vanderbilt University
7 R01 CA 31920-01
Natural Host Control Mechanisms in Mouse Leukemia

66. PINTER, Abraham
Sloan-Kettering Inst. Ca. Res.
1 R01 CA 37107-01A
Biochemical and Genetic Studies of Mulv Env Proteins

67. PROFFITT, Max R.
Cleveland Clinic Foundation
5 R01 CA 20242-07
Autoimmunity and Virus-Induced Leukemia

68. RASCHKE, William C.
La Jolla Cancer Res. Fdn.
5 R01 CA 30903-03
Analysis of Transformed Cells of the Lymphoid System

69. RASHEED, Suraiya
University of Southern Calif.
2 R01 CA 27246-04A
Leukemia & Sarcoma Genes in Cellular Transformation

70. ROJKO, Jennifer L.
Ohio State University
1 R01 CA 35747-01
Pathobiology of Latent Lymphomagenic Feline Retroviruses

71. ROSENBERG, Naomi E.
Tufts University
5 R01 CA 24220-06
Abelson Leukemia Virus Transformation

72. ROSENBERG, Naomi E.
Tufts University
1 R01 CA 33771-01
RNA-Tumor Virus-Hematopoietic Cell Interaction

73. ROSNER, Marsha R.
Massachusetts Inst. of Tech.
5 R01 CA 32267-02
Isolation and Characterization of Retrovirus Receptors

74. ROY-BURMAN, Pradip
University of Southern Calif.
5 R01 CA 26809-03
Oncodevelopmental Gene Expression in Leukemia

75. SARKAR, Nurul H.
Sloan-Kettering Inst. Ca. Res.
2 R01 CA 17129-10
Components of the Murine Mammary Tumor Virus

76. SCHWARTZ, Robert S.
Tufts University
5 P01 CA 24530-05
Experimental Leukemogenesis

77. SCOLNICK, Edward M.
Gordon Research Conferences
1 R13 AI 20089-01
Gordon Conference on Animal Cells and Viruses

78. SHERR, Charles J.
St. Jude Child. Res. Hospital
1 RO1 CA 38187-01
The Fms Oncogene

79. SOMERS, Kenneth D.
Eastern Virginia Medical Sch.
5 RO1 CA 28474-03
Cellular Transformation by MSV

80. SORGE, Joseph A.
Scripps Clinic and Res. Fdn.
1 RO1 CA 36448-01
Gene Transfer and Expression Using Retroviruses

81. STEFFEN, David L.
Worcester Fdn. for Exper. Biol.
5 RO1 CA 30674-03
Mechanisms of Viral and Nonviral Leukemogenesis

82. THOMAS, Christopher Y.
Univ. of Virginia (Charlottesville)
5 RO1 CA 32995-02
Molecular Genetics of Leukemia Viruses of Inbred Mice

83. VAIDYA, Akhil B.
Hahnemann University Sch. of Med.
2 RO1 CA 22413-07A
Etiological Studies of Mammary Carcinoma

84. VARMUS, Harold E.
Univ. of Calif. (San Francisco)
5 RO1 CA 19287-08
Molecular Biology of Mouse Mammary Tumor Virus

85. VERMA, Inder M.
Salk Inst. for Biol. Studies
5 RO1 CA 16561-10
Viral and Cellular Oncogenes

86. VERMA, Inder M.
Salk Inst. for Biol. Studies
5 RO1 CA 21408-08
Genetic Organization of RNA Tumor Viruses

87. VOGT, Marguerite M.
Salk Inst. for Biol. Studies
5 RO1 CA 13608-11
Viral Gene Functions Involved in Transformation

88. WATSON, James D.
Cold Spring Harbor Laboratory
5 R13 CA 02809-28
Support for Symposia on Quantitative Biology

89. WATSON, James D.
Cold Spring Harbor Laboratory
1 R13 CA 36767-01
Cold Spring Harbor Workshop on HTLV

90.	WEINBERG, Robert A. Massachusetts Inst. of Tech. 5 RO1 CA 17537-09	Construction of Novel Sarcoma Virus Genomes
91.	WEISSMAN, Irving L. Stanford University 5 RO1 CA 32031-02	The Receptor-Mediated Leukemogenesis Hypothesis
92.	WHEELOCK, E. Frederick Hahnemann University 5 RO1 CA 32575-02	Role of Endogenous Virus in Tumor Dormant States
93.	WILSON, Michael C. Scripps Clinic and Res. Fdn. 1 RO1 CA 33730-01A	Regulation of Expression of the Gp70 Multigene Family
94.	WITTE, Owen N. Univ. of Calif. (Los Angeles) 2 RO1 CA 27507-04	Transformation by Abelson Murine Leukemia Virus
95.	WONG, Paul K. Univ. of Illinois (Urbana) 1 RO1 CA 36293-01	Molecular Basis of Pathogenesis Induced by MLV Mutants
96.	YAMAMOTO, Keith R. Univ. of Calif. (San Francisco) 5 RO1 CA 20535-08	Gene Regulation by Steroid Receptor Proteins
97.	YANG, Wen K. Oak Ridge National Laboratory 5 RO1 CA 30308-03	Mechanism of Fv-1 Restriction of Murine Leukemia Viruses
98.	YOSHIMURA, Fayth K. Fred Hutchinson Ca. Res. Ctr. 2 RO1 CA 25461-04A2	DNA Forms of Murine Leukemia Viruses

CONTRACT ACTIVE DURING FY 84

	Investigator/Institution/ Contract Number	Title
99.	KAPLAN, Henry Stanford University NO1-CP4-3228	Characterization of Hodgkin's Disease

SUMMARY REPORT

RNA VIRUS STUDIES II

The RNA Virus Studies II program involves primarily studies of avian tumor viruses and hepatitis B virus. This program consists of 93 research grants with an estimated total cost of $12.96 million dollars for FY 1984. Of these, approximately 80% are involved with studies of avian tumor viruses, and 14% concern hepatitis B virus (HBV) and its relationship to primary hepatocellular carcinoma (PHC). The remaining 6% touch on a variety of subjects which are more distantly related to human diseases. The majority of the studies funded by RNA Studies II area 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 of oncogenesis of viruses lacking oncogenes. Some selected examples of the types of studies accomplished within the last year follow.

Among the most significant scientific accomplishments of the area are the findings of a second kinase activity, specific for phosphatidylinositol, associated with the ros and src kinases of the UR2 and Rous sarcoma virus (RSV), respectively. These phosphatidylinositol kinases, acting through second messages, stimulate cell division in a manner similar to the phorbol esters. If confirmed, this would be the third and fourth examples (the others being the sis and erbB gene products) of an intersection of oncogene products with growth factor activity. In terms of oncogene structure, progress has been made in determining the way in which the viral oncogenes were derived from their cellular precursors. The nature and activity of oncogene products required for transformation continue to be elucidated, and the importance of tyrosine kinases in cell transformation seems to be less than originally thought. The finding that the v-erbB gene product is glycosylated makes it unique among avian retroviruses and may indicate that this product functions in oncogenesis in a different way as well, since it appears to lack protein kinase activity. Additionally, investigations on the hepatitis B virus and its role in primary hepatocellular carcinoma have resulted in the development of a rapid monoclonal radioimmunoassay for measurement of human alpha-fetoprotein (AFP) which may be useful in detection, early identification, and monitoring of AFP-producing tumors, such as liver cancer, in high risk populations.

A novel mechanism by which oncogenes might contribute to the malignant transformation of cells has recently been reported for the protein products of the ros oncogene of the UR2 virus (2) and src oncogene of RSV (23). The protein products of both of these genes are tyrosine kinases that can attach phosphate groups to tyrosine residues in proteins. The new work demonstrates that the enzymes can also phosphorylate phosphatidylinositol. Phosphorylation of phosphatidylinositol increases the formation of a polyphosphoinositide, which mediates signal transmission for several hormones, neurotransmitters, and growth factors.

The results of the two groups of investigators are similar. The RSV kinase phosphorylates phosphatidylinositol in vitro to produce phosphatidylinositol phosphate (PIP) which is the immediate precursor of phosphatidylinositol

4, 5 bis-phosphate (PIP_2). The purified src kinase phosphorylates both phosphatidylinositol and PIP. Upon activation of growth factor and other receptors, PIP is hydrolized, releasing diacylglycerol and inositol triphosphate, which serve as second messengers to evoke the cell's responses. By increasing the concentration of PIP_2, the ros and src products may mimic what happens during activation of the receptors. The src product also forms phosphatidic acid by attaching phosphate to diacylglycerol, a finding that suggests that the kinase may be involved in removing, as well as producing, this second messenger (2, 23).

Although investigators of the ros oncogene products (2) did not detect PIP_2 formation in their assay, they demonstrated that transformation of cells with UR2 virus results in increased incorporation of radioactively labeled phosphate into both PIP and PIP_2. This indicates that both compounds are being broken down and resynthesized more rapidly than in normal cells. The concentrations of the hydrolysis products of these compounds (inositol, bis, and trisphosphates) also were increased in transformed cells.

Using a temperature-sensitive mutant of RSV, it was shown (23) that increased turnover of PIP, PIP_2, and phosphatidic acid correlates with transformation. Cells infected by the mutant are transformed at 35°C, but not at 41°C. Within 20 minutes after the induction of transformation by lowering the temperature from 41° to 35°C, the incorporation of radioactive phosphate into the three compounds increases. Uninfected cells did not show these changes. These results suggested that the incorporation of phosphate was attributable either to the direct action of the src kinase or the stimulation of the activity of other enzymes by the src kinase.

The phosphorylation of phosphatidylinositol appears to be a specific effect of tyrosine kinases, since both groups of investigators find that another kinase which phosphorylates proteins on serine and threonine residues does not catalyze this reaction. Moreover, both groups of investigators have found that PIP_2 inhibits the ability of the oncogene products to phosphorylate phosphatidyl-inositol and also blocks their tyrosine kinase activity. This finding is consistent with the hypothesis that PIP_2 may help to regulate its own formation, a common mechanism in synthetic pathways.

Though the tyrosine kinase activity of the src gene product was discovered more than five years ago, investigators have had little success in determining which of the many proteins phosphorylated by the enzyme might produce the characteristic changes of transformation. The work described above suggests that the ros and src kinases may instead function by a different mechanism involving phosphorylation of phosphatidylinositol, which increases the availability of the PIP_2 and second messages, especially diacylglycerol. Diacylglycerol activates protein kinase C, an effect mimicked by the tumor-promoting phorbol esters which cause, among other things, a stimulation of cell division. The src and ros products are located at the inner cell membrane, the correct site for their proposed effects.

Two other oncogenes have already been linked to growth factors. Part of sis oncogene (from the wooley monkey and the cat) codes for a protein in

platelet-derived growth factor and the erbB gene (from the chicken) codes for a segment of the receptor for epidermal growth factor. If it is demonstrated that ros and src transform by virtue of their actions on the polyphosphoinositide system, it would be a third example of an intersection of oncogenes with growth factor activity. Further studies are needed to establish whether this model is correct. It would also be important to determine whether any of the other tyrosine kinases which include the products of several additional oncogenes, i.e., the receptor for epiderminal growth factor, the receptors for platelet-derived growth factor and for insulin can phosphorylate phosphatidylinositol. At present it is not possible to definitely state the roles of tyrosine kinase and phosphatidylinositol kinase involved in transformation.

Despite intensive investigation, the mechanisms by which viral oncogenes cause cells to be transformed from normal to malignant phenotypes remains largely unknown. A number of experiments have been undertaken to characterize the protein products of various viral oncogenes and to attempt to determine their biosynthetic, structural, and functional attributes. Oncogenic transformation by the Rous sarcoma virus (RSV) is mediated by a single viral gene product designated pp60src. To understand the event(s) involved in oncogenic transformation by RSV, it is important to identify cellular proteins which interact with pp60src. Since pp60src possesses a protein kinase activity specific for tyrosine residues, one approach used (11) has been to search for proteins which have enhanced levels of phosphotyrosine after RSV-induced transformation. The immunoprecipitation of RSV transformed cell lysates with antiserum to pp60src has resulted in the identification of several proteins which interact with pp60src. These phosphoproteins, with molecular weights 90kD and 50kD, are designated pp90 and pp50. They coprecipitate with pp60src because they are complexed with it. Both of these proteins are present in uninfected chicken cells. In uninfected cells, the pp50 protein is phosphorylated on serine residues; after RSV infection, two species of this protein are found, one phosphorylated on serine and one on both tyrosine and serine. The pp50 molecules, which are immunoprecipitated with antibody to pp60src, also contain both phosphotyrosine and phosphoserine, further suggesting that the interaction among pp60src, pp90, and pp50 might involve the phosphorylation of pp50 on tyrosine. The pp90 is phosphorylated on serine in both infected and uninfected cells. The pp90 protein is an abundant cellular protein whose levels are increased significantly after exposure of cells to various stress conditions including heat shock, arsenite or canavanine treatment and whose synthesis is decreased when cells are starved of glucose. The function of this highly regulated protein is as yet unknown.

The results of further experiments with this group of three proteins provide evidence that the interaction between pp60src, pp90, and pp50 involves a discrete population of pp60src molecules. That is, newly synthesized pp60src preferentially associates with pp90 and pp50. This bound form of pp60src is not phosphorylated on tyrosine. The complex formed between pp60src, pp50, and pp90 is unstable in cells transformed by nondefective strains of RSV, and complex formation takes place in a soluble fraction of the cell. Previous characterizations of pp60src indicated that this protein is translated on soluble polysomes and rapidly associates with the plasma membrane. The present study indicates that pp90 and pp50 might be involved in the processing of pp60src before its association with the plasma membrane. The exact nature of the

processing event is unclear. Formation of a complex between pp50, pp60src, and pp90 might have a structural role, i.e., in the transport or solublization of pp60src. Alternatively, perhaps the two cellular proteins may have a regulatory role affecting the functional activity of pp60src.

The comparison of the pp50, pp60src, pp90 complex with the complex formed between the 94kD simian virus 40 (SV40) tumor (T) antigen and the cellular protein of molecular weight 53 thousand (p53) provides an interesting contrast, since the formation of a stable complex between T antigen and p53 may be required for transformation in the SV40 system. It has also been shown that 94kD T antigen binding to p53 is reduced in mutants containing temperature-sensitive defects in the genes encoding T antigen. The polyoma middle T antigen also forms complexes with pp60src.

Although the functional nature of the interaction between pp60src, pp50, and pp90 is not clear, these investigations suggest that this association is highly specific and could involve events in the processing of newly synthesized molecules of pp60src. These events might directly or indirectly involve the phosphorylation of pp50. The finding that the transformation proteins of Fujinami and Yamaguchi viruses also interact with pp90 and pp50 strongly supports the possibility that pp50 and pp90 have a common functional interaction with viral transforming proteins having associated tyrosine phosphotransferase activity.

Monoclonal antibodies have been isolated that recognize the transforming proteins of of the Schmidt-Ruppin and Prague strains of RSV and the cellular homologue of pp60src. Using these antibodies, investigations have also been carried out on the catalytic domain of the phosphotransferase activity of two avian sarcoma virus-transforming proteins, the pp60src of RSV and pp90-yes of the Y73 sarcoma virus. The greatest degree of homology among all of the retroviral tyrosine kinases is present in a single domain in the carboxy-terminal half of these proteins; the only exception is the ABL gene product where the homologous domain is amino terminal. This highly conserved domain of these viral transforming proteins possesses the catalytic site of phosphotransferase activity.

It has been demonstrated that the carboxy-terminal 29,000 daltons of the pp60src can be released as an active phosphotransferase by limited proteolysis with trypsin. This fragment can phosphorylate IgG from the serum of animals bearing RSV-induced tumors as well as exogenous substrates such as angiotensin, tubulin, and casein. Proteolysis of pp60src does not alter the strict specificity of this enzyme for phosphorylation of tyrosine. It appears that the protease resistant fragment has a higher specific activity than the intact pp60src; this raises the possibility that structural constraints imposed on this domain by the amino terminal half of the pp60src could regulate the phosphotransferase activity of the carboxy domain.

It appears that the configuration within the carboxy half of the pp60src confers some protection against the limited proteolytic digestion undertaken in these experiments. Analysis of the amino acid sequence of this domain does not reveal any obvious structural features which would account for this behavior. The transforming protein of Y73 sarcoma virus was shown to contain a similar

protease resistant phosphotransferase domain, suggesting that this structural feature is conserved in other tyrosine kinases. This result agrees with the work of other investigators who have reported that limited proteolysis of the Fujinami sarcoma virus ppl40 gag-fps protein releases carboxy-terminal 29kD and 45kD molecular weight peptides which are phosphorylated upon addition of ATP and magnesium. Within this family of related viral tyrosine kinase transforming proteins, there is extensive homology in the amino acid sequences corresponding to the protease resistant domains of pp60src and pp90-yes. Sequences outside of this domain show little or no homology and have large differences in size. This suggests that the proteolysis resistant domains of these enzymes might have evolved as separate genetic entities which have subsequently become linked with different genetic elements. It is also possible that the non-protease resistant domains of these enzymes could regulate the functional activity of the phosphotransferase domain, possibly affecting the substrate specificity and/or the specific activity of the enzyme. The genetic linkage of the catalytic domain of tyrosine kinases with different regulatory elements would allow for functional diversification of tyrosine-specific protein kinases. For instance, sequences encoding the tyrosine kinase domain of cellular growth hormone receptors might have become genetically linked to sequences which can confer hormone responsiveness to the kinase domain. In any event, the isolation of this domain of kinase activity provides the means to investigate various aspects of the regulation of the enzymatic activity of the tyrosine kinase transforming proteins.

Although it is known that the protein product of the v-src gene is itself phosphorylated, the functions of this phosphorylation are unknown. Phosphorylation of serine is not required for kinase activity, since amino terminal deletion mutants lacking the phosphoserine and the carboxy-terminal half of the protein obtained from proteolytic digestion still possess kinase activity. Nothing is known about the role of phosphotyrosine, although temperature-sensitive mutants which lack tyrosine phosphorylation at the restrictive temperature also have reduced kinase activity. In an attempt to understand the effect of tyrosine phosphorylation on kinase activity and transforming properties of pp60src, a mutant in src, the RSV oncogene was constructed in which the major phosphorylated tyrosine (residue 416) located in the carboxy-terminal half of the protein was replaced by phenylalanine. Mouse cells transformed with this mutant of src formed foci and grew in soft agar, indicative of the transformed state. Also the mutant protein retained the wild type ability to phosphorylate proteins on tyrosine. Partial proteolysis revealed that the carboxy-terminal half of the mutant protein was still phosphorylated although apparently to a lesser extent. This residual phosphorylation was found to be on tyrosine. The results of these studies indicate that the major tyrosine phosphorylated in pp60v-src is not required for two of the proteins' notable properties; protein kinase activity and transformation of cultured cells.

Another interesting series of observations concerns the src gene product of the RSV. Two mutations in src responsible for phenotypic reversion and subsequent retransformation of a RSV-transformed rat cell line have been analyzed (5). Comparison of the nucleotide sequences of cloned proviral DNAs reveals that a single base pair insertion, approximately 438 base pairs from the 5-prime end of src, is responsible for the reversion from the transformed phenotype. This frameshift mutation accounts for an 18 kD protein as the prematurely terminated

product of src. The mutation is suppressed in a retransformed cell line by a 242 base pair duplication that corrects the reading frame to permit synthesis of a 68kD src protein. A 43kD src protein with a normal carboxy-terminus is also present in both cells. To make this protein, translation must begin at an internal AUG codon found just upstream from the frameshift mutation; in both cell lines, two src proteins appear to be initiated independently from the same mRNA species. The results imply that a protein of 7kD is synthesized from a second reading frame within a wild type src.

These results are interesting not only for their implications for the functioning of src gene products, but the results also indicate that src belongs to a small group of genes whose actions seem to confound the two generally held hypotheses regarding translation of eukaryotic genes. First, it is generally held that eukaryotic genes are expressed by translating a single cistron from each mRNA. However, the 43kD src protein initiated at an internal AUG codon appears to be translated from the same species of src mRNA as the 18kD and 68 kD src proteins that are initiated at the 5-prime end of the coding sequence; thus, two cistrons appear to be translated from one species of mRNA. There are few other examples of eukaryotic viral and cellular mRNAs that are translated in vitro or in vivo from more than one start codon. Perhaps the most provocative one is the case of the E1B genes of adenoviruses 5 and 12; here two proteins appear to be read from different reading frames using a single size class of mRNA. In addition, mutants of preproinsulin have been recently constructed that direct synthesis in vivo from two initiation sites in the same reading frame from within a single mRNA.

Translation of most mRNAs begins at the first AUG from the 5-prime end, prompting the second hypothesis that ribosomes bind at the 5-prime termini of mRNA, scan consecutively for the first AUG and initiate synthesis at that site. Translation of src violates the scanning hypothesis in at least two ways. The leader sequence of normal src mRNA contains four initiation codons 5-prime to the start of the src coding domain. All of the AUGs are derived from the 5-prime end of the RSV genome by splicing; they consist of three AUGs that are not thought to be used under any circumstances, plus the initiation codon for the gag polyprotein. The present work indicates that the 6th and 7th AUGs in src mRNA, as well as the 5th, can be used to initiate protein synthesis. Such observations, as well as results with several other viral and rare cellular translational units, suggest that ribosomal recognition and the initiation of translation may be a more complex process than implied by the original scanning hypothesis.

Investigations have continued to focus on possible mechanisms by which avian viruses transform various cells. The src gene product has been shown previously to phosphorylate vinculin, a cytoskeletal protein found in focal adhesion plaques; moreover, the phosphoprotein product of the src oncogene appears to be localized in adhesion plaques. Thus, it was thought that perhaps a phosphorylation of the cytoskeletal protein, vinculin, at tyrosine residues might be a mechanism of at least morphologic transformation by RSV. This could not be a universal mechanism, however, since other avian oncogenic viruses which produce similar tumors to RSV in vivo, but cause different changes in cell morphology in vitro, do not appear to phosphorylate vinculin. Independent investigations by several groups (34, 2) have not supported the hypothesis that

phosphorylation of tyrosine residues of vinculin has any relationship to the transformed morphology of the cells. In single fibroblasts transformed by Fujinami sarcoma virus, UR2, and RSV, no noticeable change in vinculin phosphotyrosine content was observed, thus demonstrating that phosphorylation of vinculin at tyrosine residues is neither a universal mechanism of transformation, nor is it an inevitable consequence of the neoplastic state. Indirect immunofluorescent staining of normal and transformed cells with a monoclonal antibody to vinculin revealed the presence of distinct adhesion plaques in all the cells studied, whereas staining with an anti-actin antibody showed a significant decrease in organized microfilament bundles in all of the transformed cells as compared to uninfected cells. Thus, tyrosine phosphorylation of vinculin is not necessary to promote the disappearance of microfilament bundles, nor is it a universal correlate of cytoskeletal disarray. The data suggest that alternations of cytoskeleton are most probably an effect rather than a cause of the neoplastic properties of transformed cells. In part, morphologic conversions may be related to altered levels of vinculin and/or cell-associated fibronectin rather than their phosphorylation.

Other investigators (5, 79) have studied the structural and sequence homology of the cellular gene (c-src) homologous to RSV src gene (v-src). The c-src gene was found to consist of 12 exons. The deduced amino acid sequence of pp60c-src was very similar to that of pp60v-SRC; however, the last 19 carboxy-terminal amino acids of pp60c-src were replaced by a new set of 12 amino acids in pp60v-src. The sequence encoding the carboxy terminal sequence of pp60v-src was found 900 base pairs downstream from the termination codon of the c-src gene. The splice receptor site used in the genesis of mRNA for v-src has been identified; it has been shown that an equivalent site is used for splicing the mRNA of c-src. The experimental data is generally consistent with the facts that (1) only a portion of c-src is represented within v-src, (2) the leftward recombination between the genome of the transducing virus and c-src occurred in an intron of the cellular gene, (3) v-src is in part a spliced version of a corresponding portion of c-src, and (4) nucleotide sequences represented once in the genome of the transducing virus have become duplicated to flank v-src. These findings indicate that the first step in transduction is probably recombination between DNA forms of the transducing viral genome and c-src and are in accordance with the prevailing model for transduction by retroviruses. As noted above, the carboxy-termini of the proteins encoded by v-src and c-src differ appreciably, and an unidentified domain of 127 or 128 nucleotides is located at different positions in the genome of two strains of RSV. This domain gives evidence of being a foreign element that entered the viral genomes by genetic transposition independent of the transduction of src.

These data are in accordance with recent findings for c-myb, the cellular gene that gave raise to the oncogene (v-myb) of avian myeloblastosis virus. This is an oncogenic retrovirus that rapidly causes myeloblastic leukemia in chickens and transforms myeloid cells in culture. It was found that the leftward recombination with c-myb occurred within an intron, that multiple introns within a transduced portion of c-myb are precisely spliced out of v-myb, and that the rightward recombination with c-myb occurred within an exon of c-myb and without benefit of extensive homology between c-myb and the genome of the transducing virus. In addition, a plasmid vector was constructed (5, 54) that allowed expression of a portion of the coding region for v-myb in a prokaryotic host,

E. coli. The v-myb encoded protein produced in the bacterium was used to immunize rabbits and the antiserum that was obtained was used to identify the proteins encoded by both v-myb and chicken c-myb. The protein encoded by the c-myb (75 kD) was larger than the protein encoded by v-myb (45kD). It is logical to assume that a substantial portion of c-myb was not transduced into the genome of AMV. Fusion of the exons of c-myb that are homologous to v-myb generates an open reading frame with a coding capacity of approximately 45 kD of protein. This reading frame apparently continues into one or more additional exons both upstream and downstream because the termination codons that potentially bound the coding frame are removed by splicing during the production of c-myb mRNA. The fraction of the untransduced portion of the c-myb protein which is encoded upstream or downstream of the transduced domain is unknown. These data are consistent with previous demonstrations that c-myb contains additional exons beyond both boundries of the transduced domain of the gene. In addition to scattered amino acid substitutions that distinguish the homologous regions of c-myb and v-myb, the proteins encoded by both genes differ significantly in size. V-myb is an extensively truncated version of c-myb. It is possible that this difference contributes to the oncogenicity of v-myb. To resolve this question, experiments are in progress to analyze and compare the properties of both proteins and to modify c-myb artificially by means of molecular cloning so that it resembles v-myb (5, 54).

Molecular biological techniques were also used to express a portion of the molecularly cloned v-erbB locus of the v-erbB oncogene of avian erythroblastosis virus (AEV) (5). Antisera derived by immunizing rabbits with the polypeptide expressed in E. coli, was reacted with the proteins obtained from virus-infected avian cells. By three criteria--tunicamycin inhibition, lectin binding, and metabolic labeling with radioactive sugar precursors--the product of the v-erbB gene appears to be a glycoprotein. After treatment with tunicamycin, the only v-erbB protein species synthesized is a 61 kD product, approximately equal in size to the in vitro translation product p61 of AEV v-erbB mRNA. In the absence of tunicamycin, glycoproteins of 65 and 68 kD are synthesized. This effect of tunicamycin strongly suggests that these polypeptides are glycosylated in AEV transformed cells. Secondly, in the absence of tunicamycin, the v-erbB protein could be metabolically labeled with tritiated mannose. Finally, the gp65 and gp68 species of the v-erbB polypeptide bound to a lectin column whereas the native p61 of v-erbB did not.

This glycosylation of the v-erbB protein appears to set it apart from the identified products of other avian retroviral oncogenes. However, precedence for this observation does exist in that two mammalian retroviruses, the McDonough strain of feline sarcoma virus and the spleen focus-forming viral component of the Friend virus complex have been reported to synthesize glycoproteins implicated in oncogenesis.

Other investigators (22, 49) have examined expression of the c-myc locus in four cell lines established from bursal lymphomas induced by MC-29 avian leukosis virus. In all four lines, levels of myc-related RNA were elevated and in three of the lines, a majority of myc-containing RNA lacked viral LTR-related sequences; this is in contrast to results obtained with primary tumors. These results suggest that LTR sequences are not required for maintenance of high levels of c-myc expression. Using hybrid selection of RNAs with myc DNA

followed by in vitro translation, myc-related proteins were translated in all four cell lines. The size of the proteins differed among the cell lines, with the major polypeptides being 64, 57, and 54 kD. These results suggest that events leading to elevation of c-myc transcription may be accompanied by alternations in mRNA initiation or processing which may generate different protein products.

An intriguing diversity has begun to emerge among avian retroviruses. RSV, MC-29 and AEV are all capable of transforming chicken fibroblasts, yet the oncogene proteins encoded by these viruses appear to have quite different properties. The p110 gag-myc protein encoded by MC-29 is found in the nucleus of infected cells and has been reported to be a DNA binding protein. In contrast, the pp60src protein of RSV is found to be associated with the inner surface of the plasma membrane of transformed cells and possesses protein kinase activity. The v-erbB protein is glycosylated, and in infected cells the majority of this peptide is associated with particulate membrane fractions within the cells; and there is no detectable protein kinase activity in the v-erbB protein preparations. Further study should reveal whether the mechanisms by which these viruses transform cells are as distinct as the oncogene proteins they encode (5).

Thus, while considerable progress has been made in the avian retrovirus area in terms of continuing identification of oncogenes and continued studies of oncogene products, the basic mechanisms through which oncogenes initiate and maintain the transformed state are still obscure. In some instances, tyrosine-specific protein kinases, phosphatidylinositol kinases and other mechanisms appear to be involved. Identification of precise cellular targets for such enzymes and an appreciation of how the proteins encoded by oncogenes perform their functions are the key areas to which further research must be directed in order to understand the mechanism of oncogenesis by retroviruses.

Under the auspices of a request for grant application (RFA), the Branch has been able to fund eight new grants involving studies of the relationship of hepatitis B virus (HBV) to primary hepatocellular carcinoma (PHC). These are supported at a level of approximately one million dollars. Several animal viruses are currently being explored to evaluate their relation to the hepatitis B virus of man and their utility as models for human hepatocellular carcinoma. These are: the woodchuck hepatitis virus, which seems to cause a hepatitis-like condition and liver cancer in woodchucks; the ground squirrel hepatitis virus, which has never been associated with malignancies in its native host; and the duck hepatitis virus which may produce liver cancer in aging ducks.

The ongoing work in this area has resulted in the development of a human hepatocellular carcinoma cell line designated Focus, derived from a patient with PHC. The karyotype of these cells is human in origin, and the morphological and ultrastructural features are consistent with neoplastic hepatocellular origin. These cells do not show evidence of density-dependent inhibition of growth under confluent conditions; their malignant potential is confirmed by the production of gross tumors after subcutaneous injection into nude mice. This cell line represents an additional model for further investigations of tumor-specific antigens and the relationship between HBV and hepatocellular carcinoma. It does contain integrated HBV sequences.

In additional work (89), a rapid multisite monoclonal radioimmunoassay for measurement of human alpha-fetoprotein (AFP), using two high affinity monoclonal antibodies directed against distinct determinants of the protein, has been developed. The test may be useful in the early identification, diagnosis, and monitoring of patients with primary hepatocellular carcinoma and other AFP-producing tumors in high risk populations. The sensitivity of the system is approximately 0.5 nanograms of antigen per milliliter of serum after one hour of incubation. Serum AFP levels have been measured in 1,747 individuals with PHC, acute and chronic hepatitis B infection, chronic hepatitis B infection, chronic hepatitis B surface antigen carrier states, cirrhosis, other malignant tumors as well as normal individuals and heterologous disease controls to determine specificity of the assay. Eighty percent (68 of 85) of the patients with hepatitis B surface antigen-positive PHC had AFP levels of greater than 200 nanograms per ml. In contrast, all 400 normal subjects and 477 chronic hepatitis B surface antigen-positive carriers had levels of less than 20 nanograms per ml. More importantly, in acute and chronic hepatitis B, cirrhosis, other malignant tumors, and the remaining disease controls, AFP levels were below 20 nanograms per ml in 99.3% of the subjects, the great majority being below 5 nanograms. Only two of 1,635 individuals, one with acute hepatitis and the other with carcinoma of the esophagus, had AFP levels greater than 100 nanograms per ml. These observations are at variance with previous studies which found AFP levels above 20 nanograms per ml in approximately 40% of acute and chronic hepatitis and 30% of cirrhosis using conventional polyvalent radioimmunoassays. This striking specificity of the monoclonal RIA is probably due in part to recognition of epitopes unique to AFP.

Other recent studies (71) indicate that viral replication is suppressed or inactive in many PHC patients and that HBV potential infectivity is presumably very low or absent in these individuals. However, when viral replication is present in hepatitis B surface antigen ($HBsAg)^+$/anti-hepatitis Be antigen $(HBe)^+$ carriers as demonstrated by serum HBV DNA and/or nuclear HBc antigen, active liver disease is often found. In these individuals, active chronic liver disease appears to be related to continued replication and secretion of HBV and may occur in a much higher proportion of $HBsAg^+$/anti-HBe^+ carriers than was previously suspected.

A final set of observations on the HBV-PHC research area (71) concerns the role of interferon and natural killer cells in nude mice in inhibiting tumorigenicity of human hepatocellular carcinoma cells. In these studies the human hepatoma cell line, PLC-PRF-5, was used. This line, which is persistently infected with HBV, has integrated HBV DNA, secretes hepatitis B surface antigen and does not grow readily on congenitally athymic nude mice, was studied to determine whether the low tumorigenicity of this cell line was governed by host immune responses and/or was related to expression of hepatitis B surface antigen. Subcutaneous injection of PLC-PRF-5 cells at 4 to $5X10^6$ cells into BALB/c nude mice produced localized encapsulated tumors with morophologic features of PHC in 25% of the animals within 29 to 40 days. No tumor growth was observed at lower cell number inoculations. In contrast, SK-HEP-1, an HBV negative human hepatoma cell line produced tumors in 66% of the animals when 1 to $5X10^6$ cells were inoculated. Immunosuppression of mice with antilymphocyte serum or radiation increased tumor incidence to almost 100% in mice inoculated with $1X10^6$ PLC-PRF-5 cells. Immuno-suppression also reduced the latency time, produced local invasiveness and

increased mean tumor weight. These results suggest that tumorigenicity was limited by the host immune response.

The nature of the response was further delineated by treating nude mice with sheep anti-mouse interferon immunoglobulin followed by challenge with tumor cells. When $2X10^6$ cells were injected, tumor growth occurred in 75% of the anti-interferon treated mice, whereas controls injected with the same number of cells but not receiving the anti-interferon failed to develop tumors. The tumors in the anti-interferon-treated mice were highly invasive, and the latency period for tumor appearance was reduced to 3 to 5 days. An inverse correlation was found between susceptibility of hepatoma cells to natural killer (NK) activity in vitro and resistance to tumor growth in vivo. In vitro cytotoxicity for PLC-PRF-5 cells was eliminated by anti NK 1.1 and complement, establishing the effector cell as an NK cell. Treatment of mice with anti-lymphocyte serum, irradiation or with anti-interferon abolished NK activity against PLC-PRF-5 cells. These results suggest that the interferon/NK cell system may play a role in limiting tumorigenicity and invasiveness of HBV-infected human hepatocellular carcinoma cells by mechanism(s) similar to those found for other cells persistently infected with viruses.

RNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY 84

Investigator/Institution/Grant Number	Title
1. ACS, George Mount Sinai School of Medicine 1 RO1 CA 34818-01A1	Studies on the Replication and Oncogenicity of HBV
2. BALDUZZI, Piero C. University of Rochester 1 RO1 CA 32310-01A1	The Transforming Genes of Avian Sarcoma Viruses
3. BALUDA, Marcel A. Univ. of Calif. (Los Angeles) 5 RO1 CA 10197-17	Tumor Induction by Avian Myeloblastosis Virus
4. BEEMON, Karen L. John Hopkins University 5 RO1 CA 33199-02	Location and Function of M6A in Retrovirus RNAs
5. BISHOP, J. Michael Univ. of Calif. (San Francisco) 5 RO1 CA 12705-13	Rous Sarcoma Virus: Replication & Cell Transformation
6. BOETTIGER, David E. University of Pennsylvania 5 RO1 CA 16502-10	Genetic Analysis of RNA Tumor Viruses
7. BOETTIGER, David E. University of Pennsylvania 5 RO1 CA 30383-03	Virus-Induced Myeloid Leukemia
8. BOSE, Henry R., Jr. Univ. of Texas (Austin) 1 RO1 CA 33192-01A1	Transformation by Avian Reticuloendotheliosis Virus
9. BOYD, Juanell N. Cornell University 1 R23 CA 36160-01	Dietary Choline, Aflatoxin, and Carcinogenesis
10. BREWER, John I. Northwestern University 5 RO1 CA 29461-03	Trophoblastic Tumors: New Organism/Immunology/Therapy
11. BRUGGE, Joan S. SUNY (Stony Brook) 2 RO1 CA 27951-04	The Association of Two Cellular Proteins with pp60SRC

12. BUTEL, Janet S.
Baylor College of Medicine
1 RO1 CA 37257-01
Hepatitis B Virus and Human Liver Cancer

13. CARBON, John A.
Univ. of Calif. (Santa Barbara)
5 RO1 CA 11034-16
Studies on Gene Organization and Expression

14. CASPAR, Donald L.
Brandeis University
5 RO1 CA 15468-11
Assembly of Viruses, Membranes and Tissue

15. CHIRIKJIAN, Jack G.
Georgetown University
5 RO1 CA 16914-07
RNA Modifying Enzymes in Normal and Neoplastic Cells

16. CHRISTENSEN, James R.
University of Rochester
1 RO1 CA 36312-01
Oncogenes and Neoplastic Progression

17. COFFIN, John M.
Tufts University
2 RO1 CA 17659-09
Relationship of Avian Tumor Virus RNA and Host Genome

18. COFFIN, John M.
Tufts University
5 RO1 CA 27108-04
Mechanisms of Variability of Tumor Virus RNA

19. COLLETT, Marc S.
Univ. of Minn. (Minneapolis)
5 RO1 CA 29041-03
Structure and Function of the ASV Transforming Protein

20. CRITTENDEN, Lyman B.
U.S. Regional Poultry Research Lab
5 RO1 CA 29656-03
Avian Endogenous Retroviral Gene Expression

21. DUESBERG, Peter H.
Univ. of Calif. (Berkeley)
5 RO1 CA 11426-15
Molecular and Genetic Analyses of RNA Tumor Viruses

22. EISENMAN, Robert N.
Fred Hutchinson Ca. Res. Ctr.
5 RO1 CA 20525-08
Control Mechanisms in Avian Oncornavirus Replication

23. ERIKSON, Raymond L.
Harvard University
5 RO1 CA 34943-03
Biosynthesis of Viral RNA

24. FARAS, Anthony J.
Univ. of Minn. (Minneapolis)
5 RO1 CA 18303-09
RNA-directed DNA Polymerase and 70S RNA of Oncornaviruses

25. GOLDBERG, Allan R.
Rockefeller University
5 R01 CA 13362-12
RSV Functions Involved in Transformation

26. GORDON, Julius A.
University of Colorado
1 R01 CA 35378-01
Studies of pp60SRC Activity and Substrate Phosphorylation

27. GOULIAN, Mehran
Univ. of Calif. (San Diego)
5 R01 CA 11705-14
DNA Synthesis Studies

28. GRANDGENETT, Duane P.
St. Louis University
5 R01 CA 16312-10
Avian Retrovirus DNA Synthesis and Its Regulations

29. GRANOFF, Allan
St. Jude Children's Research Hospital
2 R01 CA 07055-22
Studies on Lucke Tumor Associated Viruses

30. GRAY, Horace B., Jr.
University of Houston
5 R01 CA 11761-12
Hydrodynamics of Circular DNA Forms

31. GUNTAKA, Ramareddy V.
Univ. of Mo. (Columbia)
7 R01 CA 36790-01
Synthesis, Structure and Function of Avian Tumor Virus D

32. HAGER, Lowell P.
Univ. of Ill. (Urbana)
41 R13 CA 38747-01
Plasmids in Bacteria Conference

33. HALPERN, Michael S.
Wistar Institute
5 R01 CA 31514-03
Oncornavirus-Induced Immunosuppression

34. HANAFUSA, Hidesaburo
Rockefeller University
5 R01 CA 14935-11
Cellular Alteration Induced by Rous Sarcoma Virus

35. HARRISON, Stephen C.
Harvard University
2 R01 CA 13202-13
Virus Structure and Assembly

36. HAYWARD, William S.
Sloan-Kettering Inst. Ca. Res.
5 R01 CA 34502-03
RNA Tumor Virus Gene Expression

37. HOLOWCZAK, John A.
Rutgers Medical School
5 R01 CA 11027-16
Transcription and Translation in Pox Virus-Infected Cells

38. HOLTZER, Howard
University of Pennsylvania
5 RO1 CA 18194-08
Conversion of Embryonic Cells Into Transformed Cells

39. HUMPHRIES, Eric H.
Univ. of Texas (Dallas)
1 RO1 CA 32295-01A1
Endogenous Avian Retroviruses in Non-Permissive Cells

40. HUNTER, Eric
Univ. of Ala. (Birmingham)
2 RO1 CA 29884-04
Site-Specific Mutagenesis of the ENV Gene of RSV

41. JOKLIK, Wolfgang K.
Duke University
2 PO1 CA 30246-04
Regulatory Functions of Protein-Nucleic Acid Interaction

42. KAJI, Akira
University of Pennsylvania
5 RO1 CA 19497-06
Replication of RNA Tumor Virus

43. KNOWLES, Barbara B.
Wistar Institute
1 RO1 CA 37225-01
Hepatitis B Virus and Primary Hepatocellular Carcinoma

44. KOPROWSKI, Hilary
Wistar Institute
5 PO1 CA 21124-07
Genetics and Virology of Cancer

45. KUNG, Hsing-Jien
Michigan State University
5 RO1 CA 33158-02
Erythroleukemia: Oncogene Activation by Retrovirus

46. LAI, Michael M.
Univ. of Southern Calif.
5 RO1 CA 16113-09
Structure and Replication of RNA Tumor Viruses

47. LAU, Alan F.
Univ. of Hawaii (Manoa)
1 RO1 CA 35578-01
Cellular Substrates of pp60SRC in ASV-Infected Cells

48. LEIS, Jonathan P.
Case Western Reserve University
1 RO1 CA 38046-01
Studies on Retroviral Proteins

49. LINIAL, Maxine L.
Fred Hutchinson Ca. Res. Ctr.
5 RO1 CA 18282-09
Viral Coded Functions in Rous Sarcoma Virus

50. MARCUS, Philip I.
Univ. of Conn. (Storrs)
5 PO1 CA 14733-10
Gene Expression, Virus Replication and Cell Growth

51.	MARTIN, G. Steven Univ. of Calif. (Berkeley) 5 RO1 CA 17542-09	Genetics of RNA Tumor Viruses
52.	MENKO, Sue University of Pennsylvania 7 R23 CA 36749-01	The Effect of SRC on Cytoskeletal Functions
53.	MONTELARO, Ronald C. Louisiana State University 9 RO1 CA 38851-04	EIAV: Antigenic Variation and Retrovirus Persistence
54.	MOSCOVICI, Carlo University of Florida 5 RO1 CA 10697-18	Avian Leukemia Viruses and Cell Differentiation
55.	NEIMAN, Paul E. Fred Hutchinson Ca. Res. Ctr. 5 RO1 CA 20068-09	Molecular Mechanisms in Neoplasia
56.	NEIMAN, Paul E. Fred Hutchinson Ca. Res. Ctr. 5 PO1 CA 28151-04	Retroviruses and Cancer
57.	OGSTON, Charles W. Rush-Pres.-St. Luke's Med. Ctr. 1 RO1 CA 37276-01	Molecular Biology of Woodchuck Hepatitis Virus In Vivo
58.	PARSONS, John T. Univ. of Va. (Charlottesville) 2 RO1 CA 29243-04	Avian Sarcoma Virus-Specific Tumor Antigens
59.	PARSONS, John T. Univ. of Va. (Charlottesville) 2 RO1 CA 27578-04	Expression of Avian Retrovirus Transforming Genes
60.	POGO, Beatriz G. Mount Sinai School of Medicine 5 RO1 CA 29262-03	The Expression of Oncogenicity of Shope Fibroma Virus
61.	RHODE, Solon L., III Inst. of Medical Res. of Bennington 5 RO1 CA 26801-05	Replicon Control in Normal and Transforming Cells
62.	ROBINSON, Harriet L. Worcester Fdn for Exper. Bio. 5 RO1 CA 27223-04	Avian Leukosis Viruses and Cancer
63.	ROBINSON, Harriet L. Worcester Fdn for Exper. Bio. 2 RO1 CA 23086-08	Inheritance and Expression of Avian C-Type Viruses

64. ROBINSON, William S.
Stanford University
1 RO1 CA 34514-01
Hepatitis B Virus: Infection and Disease

65. ROGLER, Charles
Albert Einstein College of Medicine
1 RO1 CA 37232-01
Molecular Aspects of WHV-Induced Persistent Infection

66. ROHRSCHNEIDER, Larry R.
Fred Hutchinson Ca. Res. Ctr.
2 RO1 20551-08
Mechanisms of Oncornavirus-Induced Transformation

67. RUECKERT, Roland R.
Univ. of Wis. (Madison)
5 RO1 CA 08662-18
Structure and Synthesis of Retro- and Nodaviruses

68. SCHUBACH, William H.
Univ. of Minn. (Minneapolis)
1 R23 CA 36977-01
Structure and Expression of the Endogenous MYC Region

69. SCOTT, June R.
Emory University
5 RO1 CA 11673-15
Lysogeny and Bacteriophage P1

70. SEFTON, Bartholomew M.
Salk Institute
5 RO1 CA 17289-09
Viral Membranes and Viral Transformation

71. SHAFRITZ, David A.
Yeshiva University
5 RO1 CA 32605-03
Hepatitis B Virus - Chronic Hepatitis - Liver Cancer

72. SHALLOWAY, David I.
Penn. State University
5 RO1 CA 32317-02
Role of pp60c-SRC, Homolog of the RSV Oncogenic Protein

73. SHANK, Peter R.
Brown University
5 RO1 CA 32980-02
Stability and Disease Tropisms of Proviral DNAs

74. SIDDIQUI, Aleem
University of Colorado
5 RO1 CA 33135-02
Expression of Hepatitis B Virus Genes and Hepatoma

75. SMITH, Ralph E.
Colorado State University
5 RO1 CA 35984-02
Biochemistry of RNA Tumor Virus Replication

76. STAVNEZER, Edward
Sloan-Kettering Inst. Ca. Res.
5 RO1 CA 32817-02
The Origin, Structure, and Biological Activity of SKVS

77. STOLTZFUS, Conrad M.
University of Iowa
5 RO1 CA 28051-05

Retrovirus RNA Metabolism

78. SUMMERTON, James E.
Anti-Gene Development Group
1 R43 CA 37441-01

Development of Broad-Spectrum Antiviral Agents

79. SWANSTROM, Ronald I.
Univ. of N.C. (Chapel Hill)
1 RO1 CA 33147-01A

Retrovirus Replication: Interaction With Host Genome

80. TATTERSALL, Peter J.
Yale University
2 RO1 CA 29303-04

Molecular Basis of Parvovirus Target Cell Specificity

81. TAYLOR, John M.
Institute for Cancer Research
5 RO1 CA 22651-06

Early Events in Avian Retrovirus Replication

82. TEMIN, Howard M.
Univ. of Wis. (Madison)
2 PO1 CA 22443-06

Molecular Biology and Genetics of Tumor Viruses

83. TENNANT, Bud C.
Cornell University
1 RO1 CA 37264-01

Woodchuck Hepatitis, Aflatoxin, and Hepatocarcinogenesis

84. TIOLLAIS, Pierre
Institut Pasteur
1 RO1 CA 37300-01

Hepatitis B Virus DNA Oncogenes and Liver Cancer

85. VARMUS, Harold E.
Univ. of Calif. (San Francisco)
1 RO1 CA 37281-01

Oncogenic Potential of the Hepatitis B-Type Viruses

86. VOGT, Peter K.
Univ. of Southern Calif.
2 RO1 CA 13213-13

Interactions Between Avian Tumor Viruses and Their Hosts

87. VOGT, Peter K.
Univ. of Southern Calif.
5 RO1 CA 29777-03

Avian Oncoviruses: Transforming Genes and Proteins

88. VOGT, Volker M.
Cornell University
2 RO1 CA 20081-07

Avian Retrovirus Structure and Assembly

89. WANDS, Jack R.
Massachusetts General Hospital
1 RO1 CA 35711-01

Pathogenesis, Immunodiagnosis, and Therapy of Carcinoma

90. WANG, Lu-Hai
Rockefeller University
2 R01 CA 29339-04

Transforming Genes of Avian Sarcoma Viruses

91. WEBER, Michael J.
Univ. of Ill. (Urbana)
5 R01 CA 12467-12

Early Cellular Changes in Viral Oncogenesis

92. WEINTRAUB, Harold M.
Fred Hutchinson Ca. Res. Ctr.
5 R01 CA 26663-05

Cell Transformation by RSV

93. WEISS, Gary B.
Univ, of Texas (Galveston)
5 R01 CA 31800-03

Infidelity of Human RNA -Directed DNA Polymerase

SUMMARY REPORT

RESEARCH RESOURCES

The Research Resources component of the Biological Carcinogenesis Branch (BCB) is responsible for planning, initiating and maintaining a coordinated program of research material support to meet the needs of extramural investigators funded by the Branch as well as other investigators in cancer research. This coordinated program includes initiation, development, maintenance and management of resource contracts and the responsibility for the day-to-day general management and direction of all resources distribution.

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 purified viruses and viral reagents; activities concerned with animal resources, including production of pathogen-free species of animals, breeding of cotton-topped marmosets, 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.

In addition, the research resources component of the Branch has coordinated the distribution of a variety of resources to Russian, French and Japanese scientists in keeping with formal international agreements with these countries covering the exchange of cancer research materials.

The viral reagents produced during this period to meet program needs were avian myeloblastosis virus (AMV), reverse transcriptase and Epstein-Barr virus (EBV). A consistently active supply of AMV reverse transcriptase is vital to biological carcinogenesis studies involving the interactions of cDNA copies of retrovirus genomes with cellular protein synthesis. To meet these needs, more than 2,100,000 units of AMV reverse transcriptase were produced and distributed to research laboratories. While the amount of transcriptase shipped during this period was significantly less than the previous year, shipments have remained fairly constant during recent years with approximately 500 shipments going to laboratories in the United States and about 100 shipments to foreign laboratories. The decreased demand is the result of a change in purchase requirements from multiples of 5,000 units to multiples of 2,000 units. While grantees were generally satisfied with the payback system, often the previous purchase requirement was for more material than an investigator needed and grant funds were wasted. This policy of smaller minimum purchases, instituted in response to requests from grantee purchasers, will be evaluated over the remaining year of the contract. Consumer demand has dropped considerably for characterized EBV DNA during this period. Distributions of this material will be terminated during FY 1984 (4, 7).

Animals have an important role in the biological carcinogenesis research program. Experimentation for the biological activity of candidate human viruses must not

be carried out on 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 68 cotton-topped marmosets consisting of 47 adults, 19 juveniles, and two experimental animals is being maintained. The two experimental animals are assigned to Dr. Boris Lapin as part of the scientific bilateral agreement between the United States and Russia. 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, because of its small size, the marmoset is economical to house, yet it is large enough for routine surgical procedures and serological monitoring (3).

Several research laboratories use eggs to study viral effects on cell differentiation. More than 1,200 fertile or embryonated eggs produced by an outbred specific pathogen-free, leukosis-free flock of Japanese quail were distributed to nine laboratories for these and other cancer research projects. The quail are maintained under environmentally controlled conditions which preclude intercurrent infection by pathogenic microorganisms or infestation by parasites (5).

Genetic control of susceptibility to spontaneous and viral-induced leukemia in mice has been well documented. However, the mechanism of control by the several loci involved has only recently received attention. Therefore, a breeding program was designed to assign specific functions to each controlling genetic allele. Identification of genetic control mechanisms in murine strains should form the groundwork for identifying similar controlling factors in other species, including man. At present, there are 15 strains of congenic mice in various stages of development and two Gix-gp 70 mutant strains. The gene substitutions involved include Akvp, Fv-1, Gv-1, Gv-2, H-2, Pca-1 and Tla. In several cases, reciprocal substitutions of alleles have been effected between inbred strains that differ categorically in one or more characteristics pertaining to leukemia providing a quartet of inbred strains, two standard and two congenic with switched alleles, for each gene system. A total of 376 congenic mice were shipped to 12 recipients during the past year (1).

A transfer of funds in the amount of $247,425 was made to the National Institute for Allergy and Infectious Diseases to support production of captive-born woodchucks and evaluation of their suitability 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. An experimental vaccine appears to be both immunogenic and protective against antigenemia. Also, it appears that chronic carriers can be established in the woodchuck. This animal may furnish a valuable model for studies on the development of hepatocellular carcinoma in humans and it is apparently superior to the duck in that histopathologic characteristics of hepatoma have been demonstrated in the woodchuck. The breeding effort to produce seronegative woodchucks will continue and the animals will be used in experimental studies (9).

In the search for oncogenic viruses, many cell cultures from the same or different species are used concurrently, which offer frequent opportunities for cross contamination. In cross-species tumor transplantations, the derivation of induced tumors sometimes comes into question. 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 for approximately 270 cultures. Three assays were utilized in these procedures: immunofluorescence staining for species-specific surface antigens, isoenzyme analysis, and cytological analysis by means of chromosome banding (8).

In addition, during this period, more than 5,600 human specimens and over 2,200 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 and monkey sera obtained from normal animals and from animals inoculated with viruses. Data relevant to 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, 6).

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 on 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.

There are two general modes under which the payback system is being implemented in the Branch. The first approach involves the immediate and full implementation of the system. This is most appropriate to 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, the payback system is being phased in over a period of time in such a way that investigators will not have to unduly curtail their research activities. In either case, as a general rule, when the resources payback system is fully implemented, all grantees, contractors, and intramural scientists will pay for the resources which they receive. The only exception anticipated to a general implementation will be 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.

Six of the nine resource contracts active during this period are now payback projects. These six include two for the production and distribution of viruses and viral reagents, one for specialized testing services and three for animal resources. 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 in FY82, FY83 and FY84 (4).

Consumer demand has dropped for congenic mice, quail eggs and EBV since the implementation of the payback system. Distribution of quail eggs and EBV will be terminated during this period. While the demand for congenic mice has decreased, several laboratories continue using this resource and the payback system is functioning well. 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 (1, 3, 5, 7, 8).

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 or termination of several resource support contracts.

RESEARCH RESOURCES

CONTRACTS ACTIVE DURING FY84

Investigator/Institution/Contract Number	Title
1. BOYSE, Edward A. Sloan-Kettering Institute for Cancer Research NO1 CP 71003	Influence of Virus-Related Genes on Susceptibility to Cancer
2. BURTON, Robert Information Management Services, Inc. NO1 CP 11014	Computer Support for Resources Management
3. CLAPP, Neal K. Oak Ridge Associated Universities NO1 CP 21004	Operation of a Marmoset Colony for Cancer Research
4. ELLIS, Carmen Life Sciences, Inc. NO1 CP 11013	Production and Distribution of Avian Myeloblastosis Virus and AMV Reverse Transcriptase
5. FARROW, Wendall M. Life Sciences, Inc. NO1 CP 61005	Germfree and SPF Quail Production
6. McKINNEY, Cynthia E. Microbiological Associates NO1 CP 11000	Repository for Storage and Distribution of Viruses, Sera, Reagents and Tissue Specimens
7. NONOYAMA, Meihan Showa University Res. Institute for Biomedicine in Florida NO1 CP 11012	Production, Purification and Concentration of Potentially Oncogenic DNA Viruses
8. PETERSON, Ward D. Children's Hospital of Michigan NO1 CP 21017	Inter-and Intraspecies Identification of Cell Cultures
9. TENNANT, B. C. Cornell University NO1 AI 02651	Breeding Facility for Woodchucks (Marmota Munax)

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