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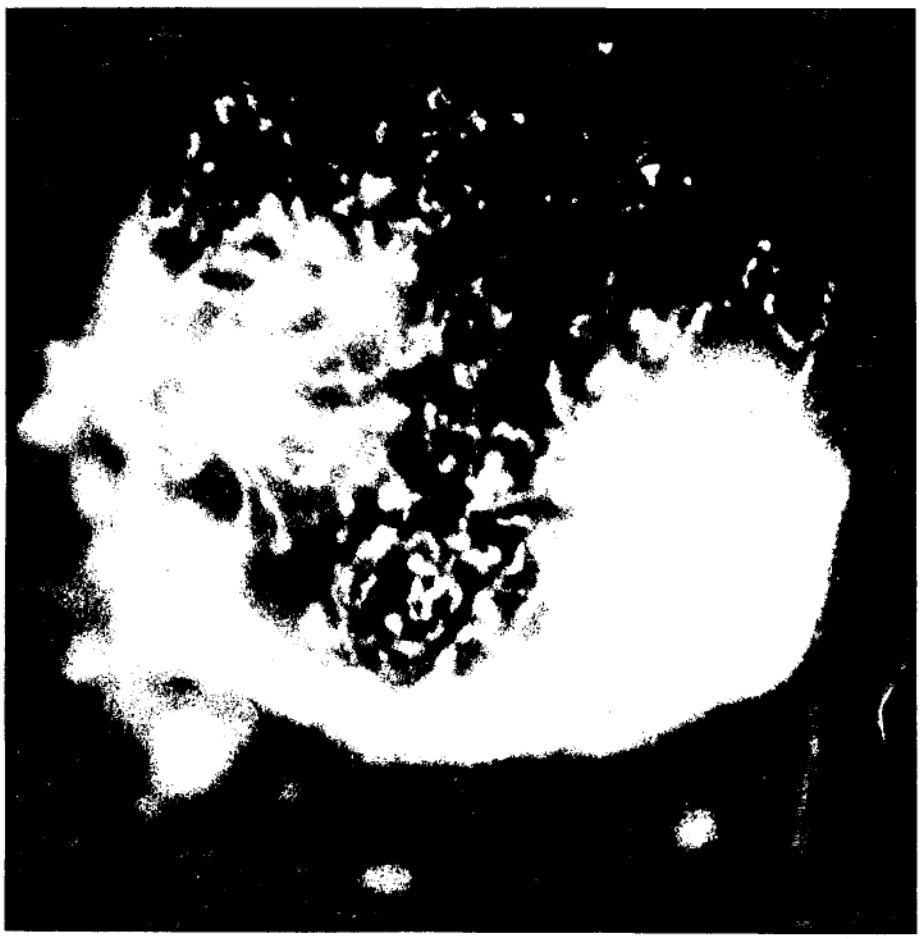

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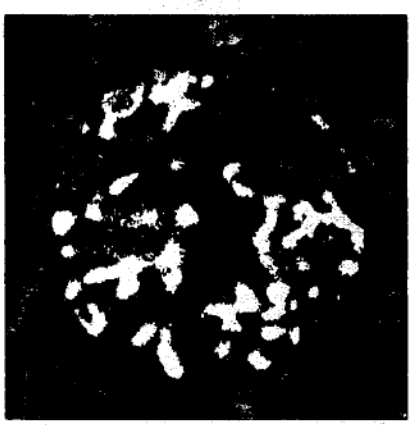

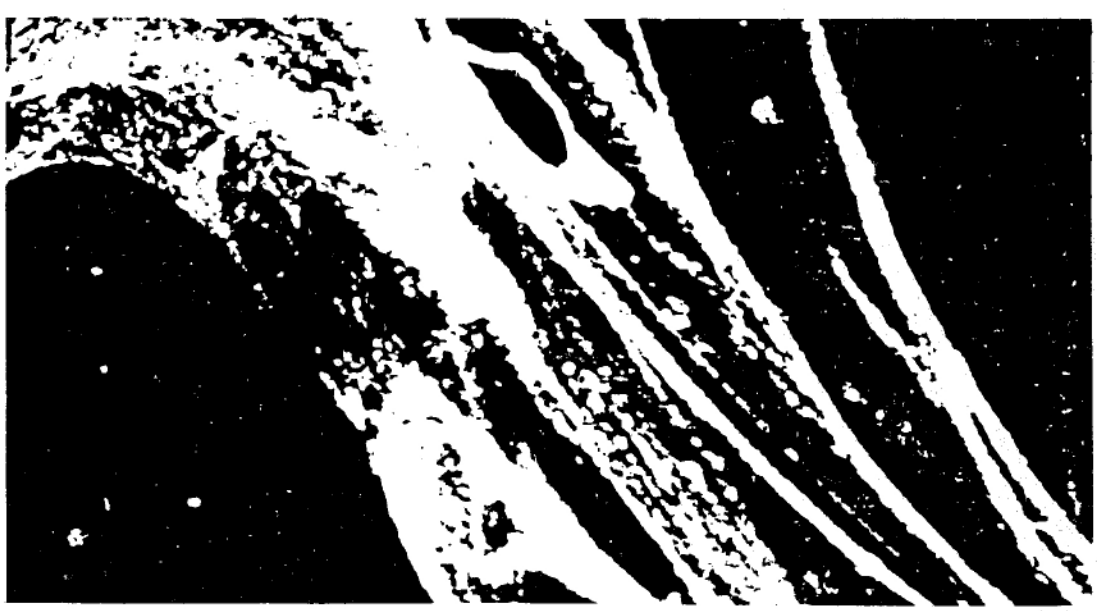

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In this laboratory, we use two intriguing and medically- important classes of animal viruses---the retroviruses and hepatitis B-type viruses---as points of departure for studying various aspects of the behaviour of eukaryotic cells at the molecular level. Several properties of these viruses extend our concerns beyond the usual confines of virology: the oncogenes of retroviruses are derived from normal cellular genes that are themselves targets for mutations implicated in many types of cancers; the DNA form of the retroviral genome (the provirus) is structurally similar to transposable elements from all types of living organisms; RNA-directed DNA synthesis is central to the life cycles of both classes of virus and now appears to be a mechanism used in the molding of the eukaryotic genome; the complex pathological consequences of persistant infections by both virus classes involve a wide range of interactions between viral and host macromolecules; and the regulation of viral gene expression displays many features characteristic of cellular genes, but more conveniently addressed with viral reagents.

I. RETROVIRUSES AS TRANSPOSABLE GENETIC ELEMENTS

A. The Mechanism of Proviral Integration

The central event in the retrovirus life cycle is the covalent integration of a DNA copy of the RNA genome into host chromosomes. Although a great deal is known about the synthesis of unintegrated double stranded viral DNA by the virus-coded enzyme, reverse transcriptase, we know only the structure features of integrated (proviral) DNA and none of the functional properties of the integrative mechanism. Like many transposable elements from plants, bacteria, yeast, and insects, proviruses can be found at many different sites in host genomes but are always joined to host DNA at the same sites in viral DNA. The provirus contains viral genes arranged as they are in viral RNA (most commonly: 5'-*gag*-*pol*-*env*-3'), flanked by long terminal repeats (LTRs) that are generated during reverse transcription and used for regulation of transcription. The LTRs terminate with short inverted repeats, and the entire provirus is flanked by short direct repeats of cellular origin generated during the integration step.

Several enzymatic activities are likely to be required to make the appropriate scissions and joinings of viral and host DNA during the integrative process. Larry Donehower has been testing the role of a DNA endonuclease activity present in the products of the viral *pol* gene. He has used techniques for *in vitro* mutagenesis to produce murine leukemia virus (MLV) *pol* mutants that retain reverse transcriptase activity but have lesions in the endonuclease domain. These mutants appear to affect the integrative mechanism since mutant virus particles can enter cells and make full-sized unintegrated DNA, both linear and circular forms, but no virus is produced by the infected cells.

B. Proviral Signals for Gene Expression

Proviruses are templates for synthesis of full-length viral RNA by host RNA polymerase II. Sequencing of viral LTRs---in our laboratory by Ron Swanstrom and John Majors, and elsewhere---has revealed customary signals for initiation and polyadenylation of transcripts. We have also determined the sequences that are joined post-transcriptionally to generate subgenomic, spliced messenger RNAs for env and certain oncogenes. Perry Hackett, Ron Swanstrom, and Richard Parker have used conventional S-1 mapping procedures to determine splice sites for Rous sarcoma virus (RSV). Two particularly interesting features emerged: (i) the leader sequence at the 5' end of subgenomic mRNAs contains a functional translational start (from the gag gene) that is not used for synthesis of the product of the viral oncogene, src; and (ii) the splice acceptor site for src mRNA is derived from the cellular progenitor, c-src, indicating that transduction of the cellular gene involved recombination within an intron of c-src. John Majors has determined the splice sites for the mouse mammary tumor virus (MMTV) by sequencing a fortuitously encountered provirus copied from env mRNA. As in the RSV genome, the splice donor and acceptor sites conform to deduced consensus sequences for cellular genes; in addition, no translational start sites are found 5' to the splice junction. One proposed splicing event, that required to produce an mRNA for pol, remains uncharacterized because it occurs infrequently and removes few sequences; Mike Scott has devised a sensitive method of cloning cDNA transcribed from pol mRNA in hopes of defining the sites involved.

We have been interested in a number of regulatory mechanisms that appear to depend upon sequences in retroviral LTRs and govern the transcription of viral or linked cellular genes:

(i) An enhancer function, capable of augmenting the transcription of an adjacent cellular oncogene in a manner independent of orientation and position, was assigned to the DNA of avian leukosis virus (ALV) in the light of Greg Payne's study of ALV-induced B cell lymphomas (see below, B.(2)(a)). Paul Luciw has mapped and characterized this function more systematically, using an assay in which plasmids containing a herpes simplex virus thymidine kinase gene (HSV tk) are microinjected into thymidine kinase (TK^-) mouse cells (performed in collaboration with Mario Capecchi). A region of viral DNA that includes about 100 bp from the 5' end of the LTR, in any of the four possible arrangements with HSV tk, is sufficient to increase the efficiency of TK transformation about 20-40 fold; the mechanism augments the likelihood of achieving adequate levels of expression of HSV tk rather than the chances of stabilizing the microinjected plasmid by integration.

(ii) Synthesis of viral RNA from the MMTV LTR is known to be regulated by glucocorticoid hormones. John Majors has constructed deletion mutants of a sequenced, steroid responsive MMTV LTR linked to HSV tk or RSV DNA and has defined a region 120 to 170 bp on the 5' side of the initiation site that is required for the response. Regulation does not depend upon a

strict distance between the components of a responsive domain, and responsiveness can be conferred upon a heterologous (RSV) promoter. Roel Nusse's studies of MMTV-induced carcinomas (see below, B.(2)(b)) indicate that the MMTV provirus also has an enhancer function that acts upon a cellular gene called int-1 in mammary tumors; we do not yet know whether this type of enhancement is hormone-dependent.

(iii) The function of LTRs seems to be dependent upon context since identical units function as promoters at the 5' ends of proviruses and as polyadenylation sites at the 3' ends. We have been examining this problem with materials derived from MLV insertion mutants of an RSV provirus, in which the functions of MLV LTRs appear to suppressed by surrounding RSV LTRs (Cell 25:23, 1981). Suzanne Ortiz has cloned one of the inactive MLV LTRs and shown that it can function as a promoter when freed of adjacent RSV LTRs and reintroduced into cells.

(iv) Expression of RSV proviruses in mammalian cells appears to be governed by a trans active, negative regulator that extinguishes transcription in a significant proportion of RSV-infected cells. We have identified an RSV-transformed rat cell in which the regulatory mechanism does not function, and we are attempting to define components of the regulator. For example, virus recovered from this cell line is subject to regulation when introduced into new cells, but superinfection of a non-transformed src mutant of the original line does not establish a regulated provirus. This suggests that the cell is a regulatory mutant.

C. Retroviruses as Genetic Vectors

Retroviruses have demonstrated their ability to transduce cellular genes and ferry them as part of viral genomes into new cells, where they are efficiently expressed. We and many others are exploiting this property of retroviruses to study genes in hand and isolate new ones. Mike Scott has engineered a series of MLV vectors suitable for expressing a variety of cloned genes (or cDNA copies of mRNAs) in the form of gag fusion proteins or as independent proteins.

D. Transposable Elements as Analogues of Proviruses

Kevin Mossie has been assessing the possibility that transposable elements constructed like proviruses in yeast (e.g., Ty-1) or Drosophila (e.g., copia) might transpose via RNA intermediates. As a first step, he has sought unintegrated DNA intermediates that resemble unintegrated forms of retroviral DNA. Though this search has been unrewarding in yeast, he has found that many copia-like elements are present as closed circular molcules both in cultured Drosophila cells and in embryos. The number of unintegrated molecules varies greatly among the several elements and does not correlate with the number of integrated copies or the abundance of transcriptional products.

II. RETROVIRUSES AS ONCOGENIC AGENTS

Retroviruses competent to induce tumors are conveniently grouped in

two categories: those highly oncogenic agents that carry oncogenes transduced from normal cells and those less efficient agents that lack their own oncogenes. Among the first group are viruses employing about twenty distinctive oncogenes (v-onc's); our studies have been confined almost exclusively to the most intensively examined member, v-src, the oncogene of Rous sarcoma virus (RSV). v-src encodes a phosphoprotein of 60,000 daltons ($pp60^{v\text{-}src}$) that displays protein kinase activity in vitro and induces phosphorylation of tyrosine residues in several putative target proteins in vivo. In the second group are a large number of viruses producing of wide spectrum of diseases; we have focused principally upon the avian leukosis virus (ALV) and myeloblastosis-associated virus (MAV), which induce B cell lymphomas and nephroblastomas, and the mouse mammary tumor virus (MMTV), which induces mammary carcinomas. Viruses of this second type appear to act as insertional mutagens, enhancing the expression of adjacent cellular oncogenes as an initial step in tumorigeneis. We are also interested in other kinds of mutations (e.g., amplifications and translocations) that affect cellular oncogenes in various tumors, including those in man. These studies have been performed principally in collaboration with Mike Bishop and are described in his report.

A. The Function of the src Gene of RSV.

(1) Analysis of mutants of src.

We have recently isolated a large group of non-conditional src mutants arising spontaneously in an RSV provirus integrated in the genome of an infected rat cell. These mutants make full sized products without protein kinase activity (presumably due to missense mutations) or truncated proteins (presumably due to nonsense or frameshift mutations). Graeme Mardon has cloned and sequenced two of the mutant alleles that encode truncated proteins and has identified frameshift mutations. In one case, the position of the lesion allowed detection of an unexpected initiation site for translation in a second src reading frame, implying that the wild-type gene encodes a 7,000 dalton protein as well as $pp60^{v\text{-}src}$. Both frameshift mutants have undergone secondary changes that at least partially restore the wild type phenotype. In one instance, the genetic revertant exhibits a duplicated domain of src and encodes an atypically large protein. Mary Anne Schofield has shown that this protein has about 50% of the normal src kinase activity and induces a partial phenotype: the cells are transformed by some criteria but do not form colonies in agar or tumors in animals. A number of the mutant proteins display different characteristics when expressed in rat or chicken cells; host dependent mutants of this type are likely to help identify host functions required for the action of $pp60^{v\text{-}src}$.

(2) Effect of altered doses of v-src and c-src. To examine the level of $pp60^{v\text{-}src}$ required to elicit components of the transformed phenotype, Eddy Jacobovits and John Majors have placed v-src under the control of the MMTV LTR. The phenotype of rat cells containing such hybrid genes displays a dramatic dependence upon glucocorticoid hormones: a five-fold induction of the expression of src permits cells to form colonies

in soft agar, an attribute that generally correlates with tumorigenicity. Could these differences in dose explain the non-oncogenic behaviour of normal cells, in which c-src is expressed at very low levels? Richard Parker has expressed the cloned chicken c-src gene in rat cells under control of an SV-40 promoter at levels higher than those required to achieve complete transformation by v-src. Under these conditions, the cells manifest no evidence of transformation, indicating that the c-src gene product is qualitatively different from $pp60^{v\text{-}src}$.

B. Oncogenesis by retroviruses without viral oncogenes.

(1) ALV induced B cell lymphomas. During his graduate training, Greg Payne showed that ALV proviruses could initiate tumorigenesis by enhancing RNA synthesis from the cellular oncogene, c-myc, in any of three arrangements: with the provirus on the 5' side of the coding region for c-myc in the same transcriptional orientation, on the 5' side in the opposite orientation, and on the 3' side in the same orientation. He and Dave Westaway have cloned mutant alleles displaying each of these arrangements, subjected them to further analysis, and found additional mutations in the regions of the insertion mutations. In one case, homologous recombination has occurred between the ALV LTRs, leaving a single LTR positioned to act as a promoter for c-myc. In a second case, a provirus on the 5' side of the c-myc coding domain in the opposite orientation has suffered an internal deletion that prevents viral gene expression; moreover, at least three base substitutions appear to have occurred in one of the c-myc exons. This suggests that somatic mutations may render oncogenes more potent at activated loci.

We still have an incomplete picture of the structure of the chicken c-myc gene and its products. Carol Nottenberg has constructed a cDNA library from a tumor with a proviral insertion on the 3' side of the gene; analysis of isolated c-myc-specific clones should indicate the boundaries of c-myc exons with precision.

(2) MMTV-induced mammary carcinomas. Roel Nusse has used the technique known as "transposon tagging" to identify a novel cellular gene (called int-1 that serves as a target for insertion mutation during tumor induction by MMTV. A provirus cloned from a tumor with a single insertion of viral DNA was shown to be positioned in a domain of about 20 kb that harbored proviruses in about 75% of tumors in C3H mice. The proviruses are present on both sides of a transcriptional unit; they are generally pointed away from the transcribed region and hence act as enhancers rather than promoters of int-1. Roel and Dave Cox have located int-1 on chromosome 15 of the mouse, Teddy Fung has shown it is highly conserved and has cloned its homologue from a Drosophila DNA library. Int-1 is unrelated to available retroviral oncogenes, and its function is unknown; Teddy Fung has recently isolated many cDNA clones of int-1 made from tumor RNA, in hopes of generating reagents for production of antisera.

(3) MAV-induced nephroblastoma. On the assumption that MAV induces renal tumors by activating cellular genes, Dave Westaway has sought the target for insertion mutation. However, none of the cellular homologues of retroviral oncogenes appear to be rearranged by proviruses in these

tumors, and the cellular sequences linked to a solitary MAV provirus in one tumor are not interrupted in 15 other tumors. The search continues.

III. HEPATITIS B VIRUSES AS ANALOGUES OF RETROVIRUSES

The hepatitis B viruses of man and other animals---woodchucks, ground squirrels, and ducks---have some striking resemblances to retroviruses. Recent reports from Summers and Mason at the Institute for Cancer Research indicate that these viruses replicate their DNA genomes through RNA intermediates, using a viral enzyme to synthesize viral DNA from an RNA template in the final phase of the life cycle. Secondly, the hepatitis B viruses frequently establish a chronic infection and their genomes are sometimes integrated into the host genome, though not apparently with the specificity manifest by retroviruses. In addition, the human and woodchuck viruses seem to have a role in the generation of primary hepatocellular carcinoma (hepatoma), a tumor arising after long latency and often carrying integrated viral DNA.

A. Replication of a Hepatitis B Type Virus of Ground Squirrels (GSHV).

Since the hepatitis B viruses do not replicate in cultured cells and have a narrow host range (infecting specific cell types in selected species), it is usually necessary to study their replication in infected livers of their natural hosts. To this end, Don Ganem, later joined by Barbara Weiser, established a colony of Beechey ground squirrels at the University of San Francisco, with the help of Dr. James Brown and several pre-medical students. Our colony, approximately 160 animals strong, was trapped in Palo Alto and consists mainly of uninfected animals that are susceptible to virus strains obtained from other animals in the same field. Don and Barbara have shown that they can transmit these virus strains serially in our animals; about two-thirds of the animals produce virus about six weeks after infection and about 15% remain infected chronically (6 months to 2 years or more). However, we find very little or no evidence of inflammatory change and have observed no hepatomas; hence GSHV appears to be non-pathogenic.

Don Ganem and Linda Greenbaum (a visiting medical student from Columbia University) obtained several molecular clones of the genomes of two strains of GSHV and generated physical maps with restriction enzymes. Christoph Seeger has recently shown that one of these cloned genomes is infectious when introduced directly into the liver in the form of dimers or re-circularized monomers. Virus can be recovered from the serum approximately 3 months after intra-hepatic injection of DNA, opening the first prospects for a genetic analysis of this virus. Christoph has determined the complete nucleotide sequence of the infectious genome: the arrangement of coding domains is very similar to that observed in the genomes of the woodchuck and human hepatitis B viruses, with extensive use of overlapping reading frames. In addition, the GSHV genome is strikingly similar to that of the (pathogenic) woodchuck virus, with sufficient conservation of restriction sites to permit the construction of recombinant genomes in vitro. These recombinants are being tested in animals to seek the determinants of host range and pathogenicity in the two genomes.

Sequential liver biopsies from animals infected in captivity provide a rich source of GSHV-specific nucleic acids. Barbara Weiser has identified

closed circular viral DNA and a heterogeneous collection of partially duplex molecules in which one strand (that complementary to viral RNA) is about ten fold more abundant than the other. This strand, like one of the strands in virion DNA, is tightly bound to an unidentified protein that is likely to serve as a primer in DNA synthesis. Our results are consistent with the model proposed by Summers and Mason, and we have proposed that the circular DNA is transcribed into RNA that serves as a template for reverse transcription. GSHV RNA in infected liver is complex; Greg Enders and Don Ganem have found at least two species of polyadenylated RNA, genomic and subgenomic in length. Further definition is in progress.

B. The Oncogenic Role of Human Hepatitis B Virus (HBV).

We are attempting to decipher the role of HBV in human hepatomas using a series of tumors sent to us by colleagues at the Queen Mary Hospital in Hong Kong. Tumors from HBV-infected patients generally contain integrated HBV DNA; in one case Teddy Fung found a single insertion of HBV DNA. This viral insert was cloned in lambda phage and flanking cellular DNA was obtained for use as probes for insertions within the same locus in other tumors. To date we have obtained no evidence for a common target that would suggest insertion mutations of cellular oncogenes by HBV DNA. Similarly, DNA from our tumors fails to transform cultured mouse 3T3 cells, in experiments performed in collaboration with Robert Weinberg's laboratory at MIT. Since we lack evidence for viral oncogenes, insertion mutations, or transforming genes in HBV-induced tumors, the role of the virus remains uncertain.

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MAJOR RESEARCH SUPPORT

Source:	American Cancer Society (MV48I)
Title:	Biochemical aspects of Rous sarcoma virus.
Total period of award:	January 1, 1983 to December 31, 1983.
Source:	National Institutes of Health (CA 19287-07)
Title:	Molecular biology of mouse mammary tumor virus.
Total period of award:	July 1, 1976 to June 30, 1986.
Source:	National Institutes of Health
Title:	The molecular biology of hepatitis B-type viruses.
Total period of award:	September 1, 1982 to August 31, 1985.

PERSONNEL

Delicia Caballero	Lab Helper
Lawrence Donehower	Postdoctoral Fellow
Gregory Enders	Graduate Student
Yuen Kai Fung	Postdoctoral Fellow
Edward Jakobovits	Postdoctoral Fellow
Graeme Mardon	Staff Research Associate
Janine Marinos	Administrative Assistant
Kevin Mossie	Graduate Student
Carol Nottenberg	Postdoctoral Fellow
Suzanne Ortiz	Staff Research Associate
David Persing	Graduate Student
Mary Anne Schofield	Graduate Student
Michael Scott	Postdoctoral Fellow
Raymond Scott	Lab Helper
Christoph Seeger	Postdoctoral Fellow
Barbara Weiser	Postdoctoral Fellow
David Westaway	Postdoctoral Fellow

PERSONNEL WHO LEFT THE LABORATORY 1982-1983

Donald Ganem	Assistant Professor of Medicine and Microbiology and Immunology, UCSF
Paul Luciw	Staff Scientist, Chiron Corporation Emeryville, California

John Majors	Assistant Professor of Biochemistry, Washington University
Richard Parker	Assistant Professor of Microbiology, Columbia University
Gregory Payne	Postdoctoral Fellow, UCB
Roel Nusse	Staff Member, Antoni van Leeuwenhoekhuis, Department of Virology, Amsterdam