ACTIVATION OF THE c-Ha-ras ONCOGENE

by

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ABSTRACT

The oncogene of the human EJ bladder carcinoma cell line arose via alteration of a cellular proto-oncogene, c-Ha-*ras*. Experiments are presented that localize the genetic lesion that led to activation of the oncogene. The lesion has no affect on levels of expression of the oncogene. Instead, it affects the structure of the oncogene-encoded protein.

To further investigate the activation and biological properties of the c-Ha-*ras* oncogene, an efficient means was required for introducing the gene into Mammalian cells in culture and in animals. The feasibility of using retroviruses as vectors for transferring DNA sequences into animal cells was investigated. The thymidine kinase (*tk*) gene of herpes simplex virus was chosen as a convenient model. The internal *Bam*HI fragments of a DNA clone of Moloney leukemia virus (MLV) were replaced with a purified *Bam*HI DNA segment containing the *tk* gene. In all respects studied, the chimeric MLV-*tk* virus behaved like known replication-defective retroviruses. These experiments suggest great general applicability of retroviruses as eucaryotic vectors.

Using this vector system, the activation of the cHa-*ras* oncogene in the EJ/T24 bladder carcinoma cell line was compared with the activation of the same gene in the rat-derived Harvey murine sarcoma virus (HaSV). The results indicate that, like the human oncogene, the HaSV-borne *ras* gene owes its oncogenic capacity to point mutations in coding sequences rather than to the alteration in transcriptional control that occurred when the formerly cellular *ras* sequences were acquired by the virus. In addition to their mode of activation, the biological properties of the EJ/T24 and HaSV oncogenes were compared by infecting newborn mice with chimeric retroviruses bearing each form. The two alleles acted equivalently, causing erythroleukemias and sarcomas with similar kinetics.

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TO MY BOSTON AND CHICAGO FAMILIES

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On the fifth floor of the Cancer Center practical lab knowledge does not come from professors but from postdocs and fellow students. That creates a great variability in the quality of ones training, depending on whom one hooks up with. I was extraordinately fortunate in that David Steffen (before my time off) and Steve Goff (after my return) acted as my

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There are many, many other people on the fifth floor of the Cancer Center who have enriched my graduate experience scientifically and personally. In spite of the complaints we all have, and the reputation we have acquired outside of the floor, the truth is that for the most part this is as helpful, interactive and friendly a place to do science as can be imagined and it will be very difficult for future labs to match it.

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CHAPTER 1

INTRODUCTION

1.1 DNA Damage in Carcinogenesis

The early days of tumor biology were fraught with contravening hypotheses attempting to explain the origins of cancer. One which seems now to be most accurate was the "mutation theory of cancer" (Boveri, 1914). The mutation theory was proposed to explain the observed stable inheritance of the transformed phenotype through successive divisions of cancer cells. Ofcourse, epigenetic models could also account for this observation.

Stronger indications of a genetic nature of cancer were provided by the discovery of lines of laboratory animals which inherited in a Mendelian manner strong predispositions to develop particular cancers. For example, C3H mice have a 90% incidence of mammary cancer (Strong, 1935) and Ak mice have an 85% incidence of leukemia (Furth et al., 1933). These animal cancers turned out to be based upon the inheritance of retroviruses through the germline. Viral involvement was demonstrated by transmitting the cancers with cell-free material to animals which had not inherited them (Gross, 1951).

Subsequently, dominantly inherited susceptibilities to non-viral cancers were identified in humans. While a very small fraction (1-2%) of human malignancy is genetically determined, a high proportion of certain rare tumor types is. For example, 40% of all human retinoblastomas, 30% of Wilm's tumors, and 30% of all human neuroblastomas are inherited. Such alleles can have extremely high penetrance. Colon carcinoma, for example, develops in 100% of the people who inherit Gardner's syndrome (Knudson, 1977).

This evidence shows that genetic information can be involved in allowing tumors to develop. However, it does not demonstrate that further genetic damage is involved in the creation of a specific tumor cell from a normal progenitor cell. The inherited defect could even exert its effect in a totally different cell from that which becomes malignant; for instance, specifically preventing cells of the immune system from detecting and/or killing retinoblastomas.

Another inherited disorder does give an indication that continued DNA damage is a factor in the development of tumors. Xeroderma pigmentosum is a recessive disease in which patients are defective in repair of ultraviolet damage to DNA, usually in the excision repair pathway. The defect varies in severity in different cohorts of patients. Individuals with xeroderma pigmentosum almost all develop some form of skin cancer and the kinetics of their tumorigenesis is well correlated with their degree of repair deficiency (Setlow, 1978). This suggests that DNA damage is involved in carcinogenesis. Yet this could take the form of a stably altered epigenetic state created in response to the UV damage rather than a state induced by specific changes in the DNA itself.

DNA alterations themselves have been correlated with cancer at the cytogenetic level. The first chromosomal abnormality identified in a particular cancer was the "Philadelphia chromosome" in human chronic myelogenous leukemia (CML) (Nowell and Hungerford, 1960). This consists of a translocation of a fragment of chromosome 22 to chromosome 9 (Rowley, 1978). Over 80% of all patients with CML carry this chromosomal marker and it is only found in their leukemic tissues. In the case of a pair of identical twins discordant for CML, only the afflicted individual had the Philadelphia chromosome in his bone marrow (Sandberg and Hossfeld, 1973).

Other characteristic chromosomal abnormalities have now been identified for a wide variety of cancers (Mitelman and Levan, 1976). Unfortunately, these cannot be taken as definitive evidence for the importance of DNA alterations. In many cases (especially with solid tumors) in addition to the characteristic translocation, tumor cells carry other karyotypic abnormalities. Moreover, the degree of aneuploidy can vary from cell to cell within the same tumor (Sandberg and Hossfeld, 1973). In other cases patients with acute leukemias showed no chromomosal abnormality at diagnosis and only later in the development of the disease developed altered karyotypes (Sandberg and Hossfeld, 1973). Thus chromosomal alterations could be related to tumor progressions instead of initiation; or they may reflect a karyotypic instability that is a result of, rather than the cause of, tumorigenesis.

As a way of investigating those cellular lesions which are involved in the initial creation of tumor cells, other investigators turned their attention to agents which were known to experimentally induce tumors: carcinogens. Many carcinogens are electrophiles. These were soon shown to interact chemically with a variety of cellular nucleophilic targets. Hepatic proteins were shown to covalently bind a metabolite of N,N-dimethyl-4-aminobenzene, a dye causing liver tumors when administered to rats. The protein binding was considered to be of possible significance because those carcinogen binding proteins were absent in subsequent tumors (Miller and Miller, 1947). Another carcinogen which causes liver tumors is dimethylnitrosamine. When that potent carcinogen was injected into various rodents, it was found that cellular RNA was methylated in several different organs. The greatest methylation occurred in the liver which was the primary oncogenic target (Lee et al., 1964). Further evidence that the RNA

damage might be of significance came from an observed physiological alteration. When the dimethylnitrosamine-induced, methylated ribosomal RNA was observed more closely, it was shown to have a shorter half-life than unmethylated rRNA (Muramatsu et al., 1972). Finally, carcinogens were found to form adducts with DNA as well (Miller and Miller, 1966). Of the various nucleophilic substrates that carcinogens react with, this last has been viewed as the most important: chemicals reactive with DNA are, in general, mutagenic, providing a parsimonious explanation for the stably inherited tumor phenotype in terms of the mutational hypothesis.

A number of screens for carcinogens have been developed based on this utilizing mutational selections in microorganisms. The best known, the Ames test in the his^- bacterium Salmonella typhimurium has been used to demonstrate that approximately 90% of all carcinogens are mutagenic (McCann et al., 1975). Moreover, very few noncarcinogens showed a positive result in the test.

Radiation is also known to be mutagenic and, as the DNA target model would predict, it has been found to be carcinogenic as well. For example, a variety of skin cancers are associated with ultraviolet radiation exposure (Scott and Straf, 1977). Again, radiation can also be shown to have an effect on RNA. While suggestive, none of these experiments demonstrate that DNA is the true target of carcinogenic action while excluding other cellular effects of the same agents.

In summary, by the late 1970's, the evidence was mounting that DNA damage is of major importance in carcinogenesis but it had not been directly demonstrated.

1.2 The Discovery and Early Characterization of Oncogenes

If genetic lesions were at the core of carcinogenesis, they could take one of two forms: recessive or dominant. After being damaged by a carcinogen, the DNA of a cell could have lost information that was needed to maintain a normal phenotype, or conversely, the altered DNA could exist in a new form which actively participated in oncogenically transforming the cell. A strong prediction would be that if this latter, dominant case were correct, then there would exist a segment of DNA in a tumor cell which, if placed into a normal cell, could oncogenically alter it. If DNA lesions in cancer were strictly recessive, then no DNA segment with such a potential would exist in a tumor cell.

M.P. Goldfarb in R.A. Weinberg's laboratory began a series of experiments both to investigate this question and, in the process, to directly demonstrate for the first time the importance of DNA damage in carcinogenesis. The experiment consisted of preparing DNA from tumor cell lines, transferring it into normal cells, and observing whether this resulted in the conversion of the normal cells into tumor cells. The work depended on two key technical abilities: a way to transfer DNA into cells and a way to observe a cell converted to a tumor cell among a vast excess of other cells which remained phenotypically unaltered.

Fortunately, techniques for both of these existed. A procedure known as DNA transfection had been developed in which cells are induced to take up and then stably maintain DNA applied to them in a calcium-phosphate matrix (Graham and van der Eb, 1973). The answer to the second problem came from the nature of the cells themselves. Normal fibroblasts, when properly maintained in culture, stop growing when they touch each other. This property of "contact inhibition" means that cells grow until confluent

on the dish and then stop in a single-cell monolayer. When oncogenically transformed, for example by a tumor virus, these cells are no longer contact inhibited. Thus a single transformed cell will continue to grow even when surrounded by normal fibroblasts until it is macroscopically visible as a "focus". Under the microscope the transformed cells in the focus appear more refractile (i.e., shiny) and rounder than the normal fibroblasts.

A non-tumorigenic morphologically normal murine fibroblast cell line (termed NIH3T3) had been previously identified as being quite efficient at incorporating transfected DNA and also as being strongly contact-inhibited. It had been used in earlier experiments in the laboratory in which tumor virus DNA was transfected and transformed foci were detected (Smotkin et al., 1975, Anderson et al., 1979). Utilizing this methodology, DNA was prepared from 3-methylcholanthrene (3MC) in vitro transformed mouse fibroblasts and transfected into the NIH3T3 cells. Two weeks later, transformed foci appeared on the dishes of these cells, while none arose on plates of cells transfected with DNA prepared from nontransformed fibroblasts!

This work was extended by C. Shih. His experiments suggested that the kinetics of transfection indicated a single segment of transfected DNA was involved in transformation of recipient cells. He also showed that if the transformed foci were single-cell cloned, allowed to expand, and DNA was prepared from those cells, that this DNA would, in turn, elicit foci after transfection. This "serial passage" could be continued, with foci being obtained from tranfection of secondary focus DNA and so on. After carefuly excluding the possibility of tumor virus contamination, it was concluded that, in at least this specific case, carcinogenesis involved DNA

alterations, and these alterations were dominant in their mode of action (C. Shih et al., 1979).

To provide a biochemical verification that the foci obtained were indeed due to the presence of transfected DNA within the recipient cells, one needed a molecular "tag" in the donor DNA, the presence of which could be assayed in the resulting transfected cells. Since the identity of the activated DNA segment was unknown, and the donor and recipient genomes were otherwise similar, it was necessary to add such a tag DNA sequence to the transfection. Thus when many copies of the bacterial plasmid pBR322 were added to tumor DNA in a transfection, those pBR322 sequences could be detected not only in the initially transfected cells, but in subsequently derived secondary foci as well (Shilo et al., 1980).

A major question which still remained was whether or not the dominantly acting DNA consisted of a single discrete unit (a gene or linked genes). The serial passage suggested that unlinked cooperating segments of DNA were not required for the observed transformation. Unlinked genetic elements would become dissociated during the transfection process. However, it has been proposed that DNA might be globally altered in carcinogenesis (Humphries, 1981). Thus it was possible that generally damaged DNA rather than specifically damaged gene(s) was being detected by the development of transformed foci.

To address this question, DNA was prepared from 3MC transformed cells and then cut with various restriction enzymes before transfection. Certain enzymes reproducibly inactivated the transforming activity of the DNA, while others spared it. This indicated that, indeed, discrete genetic elements were involved (Shilo and Weinberg, 1981a). Since restriction sites occur randomly throughout the genome, the identities of the enzymes

that cut any one gene is a matter of chance. A pattern of restriction enzyme specificities can therefore be used to identify a particular locus involved in transformed cells. On this basis it was determined that the DNA from four independently derived chemically transformed cell lines all owed their transforming capabilities to the oncogenic activation of the same genetic region (Shilo and Weinberg, 1981a).

As more tumor lines were tested in a number of laboratories, other patterns of sensitivity to restriction enzymes were discovered, indicating the involvement of other distinct genetic segments. It was soon found that activated DNA capable of transforming fibroblasts exists in tumor cell lines from a variety of tissues. Moreover, tumor cell lines from many different species scored positively in the NIH3T3 mouse cell assay (Cooper and Neiman, 1980; Krontiris and Cooper, 1981; Lane et al., 1981; Perucho et al., 1981; Shih et al., 1981). The potency of these activated segments ranged from 1-100 foci per 75 ug of transfected DNA. Thus, the existence of dominantly transforming "oncogenes" was established for cell lines derived from sarcomas, carcinomas, neuroblastomas, and leukemias.

A major criticism of this early work on oncogenes revolved around the fact that the first reported transfections were done with DNA prepared from tumor cell lines, not fresh tumor samples. It was therefore possible that the observed transforming activity of these DNAs was an artifact of passaging the cells in tissue culture and of no relevance to the original cancer. This possibility was eliminated when it was reported that the same results could be achieved using DNA directly prepared from biopsies of malignant tissue (Pulciani et al., 1982; Lane et al., 1982; McCoy et al., 1983; Murray et al., 1983a).

Thus oncogenes can be found in both tumor cell lines and in tumors. However, not all tumor cell lines or tumors contain detectable oncogenes. The transfection protocol succeeds with only approximately 10-15% of the tumor DNA's tested regardless of source (Krontiris and Cooper, 1981; Perucho et al., 1981; Shih et al., 1981, Pulciani et al., 1982). Yet oncogenes do seem to have importance in a wide range of tumor types from many species including our own, so they remain an attractive probe for investigating the molecular basis of cancer.

The fact that human tumor DNA was active in NIH3T3 mouse fibroblast assay (Shih et al., 1981, Krontiris and Cooper, 1981) provided a technical advantage in pursuing these investigations further. The human genome contains a number of scattered highly repeated DNA sequences (Schmidt and Deininger, 1975, Jelinek et al., 1980, Tashima et al., 1981). The most abundant of these familes of sequences is the Alu family (so named because of a diagnostic AluI restriction enzyme site contained in each repeat unit) (Houck et al., 1979). The Alu repeat is 300 bases long and is reiterated 600,000 times in the human haploid genome (Rinehart, et al., 1981). Being randomly dispersed, Alu repeats are found on the average of every few kilobases throughout the genome. Mouse DNA contains families of repetitive DNA sequences related to the Alu sequences. However, when small amounts of human DNA are mixed with an excess of mouse DNA, the Alu family had been found to be specifically detectable by DNA hybridization above the background homologies in the mouse genome (Guesella, et al. 1980).

The first use of the Alu sequences in studying transfected oncogenes was simply as a sequence tag analogous to the pBR322 tag discussed earlier. As expected, Alu hybridizing sequences were present in the DNA of transformed primary or secondary foci derived from transfection of human

tumor DNA and not in the untransfected NIH3T3 cells. This verified once again that the foci were directly attributable to cells which had taken up the human DNA (Murray, et al., 1981). This verification was especially useful in assuring the authenticity of foci arising in experiments that suffered from low transfection efficiencies. When there are only a few foci on a dish, they could otherwise be argued to represent rare spontaneous overgrowth of NIH3T3 cells.

When primary focus DNA was cut with a restriction enzyme, separated by electrophoresis through an agarose gel, transferred to a piece of nitrocellulose filter paper and hybridized with a radioactively labeled Alu sequence probe, a black smear was seen in the lane following autoradiography. The smear represented a large number of Alu-containing fragments of different sizes, all of which had been acquired in the primary transfection. They were not, however, all required for transformation. This was demonstrated when a similar "Southern blot" analysis was carried out on DNA from secondary foci. Secondary foci result from the transfection of DNA prepared from mouse cells which were themselves transformed by DNA transfection. Therefore, most of the DNA fragments in the secondary transfection are of mouse origin. Thus secondary foci are derived from cells which which have taken up just as much DNA as the cells which gave rise to primary foci, but in secondary foci most of the newly acquired DNA is murine. The only human DNA likely to be present is therefore the transforming DNA whose presence was selected for by the NIH3T3 cell assay. As expected, after hybridization to an Alu probe, the secondary foci DNA was found to contain only a few bands in a Southern blot.

There were two further conclusions which could be made on the basis of these experiments. When a number of secondary foci derived from transfer

of a common transforming DNA were simultaneously analyzed, certain bands were found to be shared in common. These "conserved bands" represented Alu sequences closely linked to the transforming oncongene(s), since they were observed every time the oncongenes were selected. Adding the sizes of the conserved fragments gave a minimal estimate of the size of the transforming sequences (Murray et al., 1981).

A second conclusion which could be obtained from the Alu Southern blot analysis stemmed from the fact that different segments of the human genome are associated with different arrangements and numbers of Alu sequences. Thus the same oncogene(s) independently activated in separate human tumors would be expected to give the same Alu pattern when cut with the same restriction endonuclease. Conversely, oncogenes associated with different DNA sequences should yield different Alu "signatures". On this basis it was possible to identify three different oncogenes in foci derived from the transfection of human DNA from colon and bladder carcinoma cell lines and a promyelocytic leukemia cell line (Murray et al., 1981). It should be noted that the same conclusion could have been drawn using the above-described restriction enzyme sensitivity assay which is somewhat more laborious. Indeed, other investigators have reported a number of distinct human oncogenes on the basis of differing restriction sensitivity patterns (Lane et al., 1982).

The presence of Alu hybridizing sequences tightly linked to transforming sequences provided a convenient way of isolating molecular clones of such sequences. The first transforming sequence to be cloned had been identified in the human EJ/T4 bladder carcinoma cell line. A phage carrying a 16 kb insert was isolated which contained both Alu homology and in addition transformed NIH3T3 cells in transfection at a very high

efficiency (5×10^4 foci/ug). The transforming activity was also present in a 6.6 kb subclone into pBR322 which did not contain any Alu-related sequences (Pulciani et al., 1982; Shih and Weinberg, 1982).

Alu sequence tags have thus been of great use in isolating human transforming sequences. However, other laboratories have cloned oncogenes using other strategies (Goldfarb et al., 1982; Goubin et al., 1983).

The isolation of molecular clones of oncogenes derived from the DNA of tumor cells finally directly demonstrated the existence of discrete DNA fragments in cancer cells capable of transforming normal cells in a dominant manner. The assumption throughout the proceeding work had been that the detected transforming sequences were of cellular origin. However the existence of such DNA segments could have been explained formally either by an alteration of some pre-existing DNA sequences in the afflicted cell or by a segment of DNA which had been exogenously introduced into the cell, for instance by a virus. The molecular isolation of the transforming sequences allowed this question to be addressed. When the cloned EJ/T24 oncogene was used as probe in a Southern blot analysis, it was found to hybridize strongly with DNA from normal human cells as well as DNA from the EJ/T24 bladder carcinoma cell line. The normal and EJ/T24 DNA hybridization patterns were indistinguishable after a number of different restriction enzyme digestions (Pulciani et al., 1982; Goldfarb et al., 1982; Shih and Weinberg, 1982). This demonstrated that, as had been assumed, the oncogene was created by an alteration of sequences pre-existing in the target cell. This provided a final proof of the validity of the DNA mutational theory of carcinogenesis. Moreover, the mutations in this instance were subtle, since no indication of structural change could be found by comparing the Southern blot analyses of the oncogene DNA and DNA of its normal homologous segment.

The next question which investigators addressed was the nature of the activating alterations that created the oncogene. Perhaps the most important aspect of the question was whether the mutation altered the regulation (timing or amount of production) of an otherwise normal gene product or whether a normally regulated gene was structurally altered to produce an aberrant product. In addition, of course, it was important to know the exact molecular basis of the change, since point mutations, deletions, insertions, and rearrangements would each have implications for how carcinogenesis proceeds. Finally, the molecular consequences of the change had to be explored: if there was an altered gene product, how did its function change, and if there was a regulatory change, why did over-or under-expression have effects on the cell. To even begin this sort of inquiry requires a great amount of knowledge about the structure of the gene being studied and the properties of its RNA and protein products. In addition, a number of reagents, such as antiserum prepared against the gene product, are required to carry out the investigation. The study of the activation of cellular oncogenes was greatly aided by the discovery that such knowledge and reagents had already been generated in studies of these same genes in another guise: as the cellular homologues of oncogenes transduced by retroviruses. This connection was made when various radioactively labeled retroviral probes were hybridized to DNAs prepared from different tumor transfectants (Der et al., 1982; Parada et al., 1982; Santos et al., 1982).

To understand both why these experiments were done and the significance of the results, we must now discuss retroviruses and their place in cancer research.

1.3 Retroviruses

The study of tumor viruses developed from early investigations into the mechanisms of cancer. The first tumor viruses to be studied caused sarcomas (Rous, 1911) and leukemias (Ellermann and Bang, 1908) in chickens. In mammals, tumor viruses were first identified in studies of inherited forms of cancer in inbred mouse strains. By studying patterns of inheritance, a virus causing mammary tumors was identified which was transmitted only through milk from mother to offspring (Bittner, 1942; Andervout and Bryan, 1944). Inherited leukemias in mice were also found to be due to the presence of a virus (Gross, 1951). All these viruses proved subsequently to have a similar chemical composition, organization, and life cycle, and have been therefore classified as members of a single group: the retroviruses.

Tumor viruses of other types were also subsequently discovered. Almost every class of DNA virus has members which are implicated in some way as tumor viruses (Tooze, 1980). However, these are usually classed as tumorigenic agents either on correlative epidemiological grounds (e.g., herpesvirus), or because of their action in heterologous hosts other than those which they normally productively infect (e.g., adenoviruses and papilloma viruses). In contrast, among RNA viruses only the retroviruses cause tumors (Weiss et al., 1982). These, on the other hand, cause a vast array of different specific neoplasias in their natural hosts. For this reason they have come under intense study in attempts to learn about the process of oncogenesis.

Retroviruses are characterized by their unique life cycle. They are RNA viruses which replicate by making a DNA copy from their virion RNA using the viral enzyme reverse transcriptase. This viral DNA integrates

into the host cell's chromosomal DNA. It can then replicate as a DNA genome as part of the replication of the host DNA. By and large, retroviral infections are not cytotoxic. Consequently, the viral genome can be perpetuated in all of the clonal descendants of the initially infected cell. The integrated genome can also be transcribed to make new copies of the virion RNA and to make mRNA. The viral transcription is driven by promotor and enhancer regulatory signals contained within viral sequences termed the "LTR" (Varmus, 1982). In addition, the proviral expression is modulated by the cell depending upon the site of integration (Hayward and Hanafusa, 1976; Ringold et al., 1979; Quintrell et al., 1980) and upon the degree of methylation imposed upon the proviral DNA (Stuhlmann et al., 1981; Hoffmann et al., 1982).

When a germ cell is infected by a retrovirus it can lead to the introduction of the retroviral genome into the animal's germline (Jaenisch, 1976). Proviruses in the germline are termed "endogenous proviruses". They are inherited as normal Mendelian genetic loci (Payne and Chubb, 1968; Weiss and Payne, 1971a).

Activated endogenous proviruses are responsible for the inherited high incidence of leukemias in some strains of animals (Lilly et al., 1964). It was this connection to leukemia that first brought retroviruses into the focus of scientific research (Ellerman and Bang, 1908). Retroviruses have been studied for nearly three quarters of a century since then, but it has only been in the last few years that the retroviral induction of leukemia has been even partially understood on a mechanistic level.

1.4 Retroviral Oncogenes

Oncogenic retroviruses may be divided into two classes: those which cause disease with a very short latency period following infection, the "acute transforming viruses", and those which cause neoplasms only after protracted latency periods, the "slow leukemia viruses". This terminology is a bit confusing in two respects. First, some of the acute transforming viruses cause leukemias in addition to solid tumors. Leukemic disease is thus not an exclusive property of the slow leukemia viruses. Second, while the latency period can be very long and hence the disease "slow" in its development, tumors caused by the slow viruses can be as malignant and rapidly growing as those of the acute viruses once these tumors have begun to grow.

The transforming ability of the acute viruses is attributable to specific transforming genes called onc genes (Baltimore, 1975). With the major exceptions of the oncogene carried by several forms of Rous sarcoma virus (RSV), the oncogene sequences replace some or all of the viral sequences coding for the gag, pol, and env genes. Since most acute transforming viruses lack the ability to make a full complement of viral proteins, they are "replication-defective" (Huebner et al., 1966).

Defective viruses can therefore only replicate in cells also infected by complementing non-defective "helper" retroviruses which are able to supply missing viral functions in trans. A defective virus particle thus has the glycoproteins of its helper virus and therefore the host range of that helper virus. When a cell is infected by a defective virus alone, it is called a "non-producer" cell (Aaronson and Rowe, 1970). A non-producer cell is characterized by the presence of a proviral genome integrated into the cellular DNA (Karpas and Milstein, 1973) and genomic RNA in the

cytoplasm (Benveniste and Scolnick, 1973; Green and Tsuchida, 1974), but no virus particles being produced. The defective virus can then be "rescued" by "superinfection" with a helper virus (Aaronson and Rowe, 1970).

As mentioned above, some strains of the acute avian sarcoma virus RSV are not defective. In those strains the retrovirus still carries the full gag, pol, and env sequences, and in addition has an onc gene called src (Lai et al., 1973). It was studies of src that first led to the discovery of the origins of retroviral onc sequences.

When they were first identified by genetic criteria, the origins of the onc genes were as obscure as the tumorigenic sequences of DNA viruses, or as any other viral genes. However, the existence of quiescent proviruses in most vertebrate genomes gave rise to an interesting hypothesis. Non-virally induced cancers could, in fact, be due to the activation of silent proviral onc genes (Huebner and Todaro, 1969; Todaro and Huebner, 1972). Some additional evidence for this idea came from the fact that carcinogenic agents such as ionizing radiation and chemical carcinogenes are able to induce the expression of otherwise cryptic proviruses (Lowy et al., 1971; Weiss et al., 1971b). As we shall see, the model was close to the mark; retroviral and non-viral cancers can involved the participation of homologous onc genes. However, the original proposal portrayed the origins of these sequences reversed. Studies of RSV first demonstrated that sequences closely related to src are present in the DNA of normal avian and mammalian cells (Stehelin et al., 1976, Spector et al., 1978). As the onc genes carried by other acute retroviruses were investigated, it was found that, with a single exception, each was homologous to sequences present in uninfected cells (Bishop, 1981). The exception is the oncogene of spleen focus-forming virus, which is an

altered form of the purely viral env gene (Linemeyer et al., 1982).

Three pieces of evidence together show that the cellular onc-related sequences are of cellular and not viral origin. First, the genetic loci of cellular onc genes are invariant among members of the same species as opposed to great polymorphism in location of endogenous proviruses among individuals (Hughes et al., 1979). Second, cellular onc sequences contain intervening sequences which are typical of cellular genes, and are not found within the coding region of any retrovirus gene (Weiss et al., 1982). Finally, no cellular onc gene has been found to be closely linked to even a partial proviral genome (Hughes et al., 1979).

There is, of course, no reason why only transforming sequences should be acquired by retroviruses. Other cellular sequences have not been observed transduced in naturally arising retroviruses largely because there is no selection for observing them. However, at least 17 distinct onc genes have been found to be transduced by retroviruses of a variety of host species (Weiss et al., 1982). Each onc gene has been given its own three-letter name (e.g., src, ras, myc, abl). The virally transduced form of a gene is designated by "v"-onc and the corresponding cellular homolog by "c"-onc. Cellular sequences homologous to each v-onc have been found in all vertebrate species screened and some have been found in as distantly related taxa as Drosophila melanogaster (Shilo and Weinberg, 1981b) and Saccharomyces cerevisiae (DeFeo-Jones et al., 1983; Gallwitz et al, 1983).

While the number of cellular genes which can be activated as v-oncs is unknown, the present list of 17 may be nearing the total, since several have been isolated more than once. For example, the most frequently isolated viral oncogene (called fps in chickens and fes in cats) has been found in seven independent transductions (Shibuya et al., 1980; Shibuya et

al., 1982; Frankel and Fischinger, 1977).

The oncogenic action of the acute transforming viruses is attributable to these genetically identifiable oncongenes. Conversely, no oncogenic sequences have been identified in the genomes of the slow leukemia viruses. Rather, evidence indicates that infections by the slow viruses activate cellular oncogenes (Neel et al., 1981; Payne et al., 1981). These cellular oncogenes have turned out in some cases to be homologous with transforming genes transduced by acute viruses (Hayward et al., 1981).

1.5 Viral and Cellular Oncogenes

Cellular oncogenes have thus been implicated in the development of both viral and non-viral neoplasia. In retrovirus induced diseases the oncogenes were activated either by infection with a "slow virus" or in the process of being transduced by an "acute virus". In non-viral cancer the cellular oncogenes were activated in some ill-defined carcinogenic act. Since both types of oncogenes were of cellular origin, shared certain *in vitro* properties such as the ability to transform NIH3T3 fibroblasts in transfection, and were implicated in the pathogenesis of similar diseases, it seemed that regardless of route of activation the two classes might well overlap. This was tested by nucleic acid hybridization experiments. DNA of secondary transfectants derived from various non-viral tumors were subjected to Southern blot analysis using radioactive probes made from various v-*onc*s. The results demonstrated that the two classes of oncogenes did indeed overlap. The EJ human bladder carcinoma oncogene is homologous with the oncogene transduced by Harvey sarcoma virus (HaSV) called v-Ha-*ras*. Another oncogene found in one human colon (SK-CO-1) and two human lung carcinomas (SK-LU-1 and Calu-1) are homologous with the viral

oncogene transduced in Kirsten sarcoma virus (KiSV) called v-Ki-ras (Parada et al., 1982; Santos et al., 1982; Der et al., 1982). These two oncogenes, Ki-ras and Ha-ras are evolutionarily related to one another as demonstrated by serological cross-reactivity of the encoded proteins (Young et al., 1979) and weak homology detected in hybridization of their nucleic acids under conditions which allow stabilization of partially homologous sequences (Young et al., 1980). A third cellular oncogene present in a human neuroblastoma (SK-N-SH), a human promyelocytic leukemia (HL-60), and two human sarcomas (HT1080 and RD) has been found to be weakly homologous to both Ki-ras and Ha-ras by both serological and sequence homology criteria (Shimizu et al., 1983; Murray et al., 1983a; Hall et al., 1983). This onc gene has been named N-ras and represents the third identified member of the ras "gene family".

As further comparisons have been made, by far the majority of all transfected cellular oncogenes, from every tumor type which has been investigated at any depth, have turned out to be homologous to one of these three ras genes. It was, therefore, of great interest to learn how the ras genes were activated in non-viral carcinogenesis. As discussed above, the information gained in earlier studies of the viral ras genes greatly accelerated these studies.

1.6 Ras Oncogenes

There have been four independently isolated defective retroviruses which carry ras oncogenes. The first acute retrovirus isolated from mammals was Harvey sarcoma virus (HaSV). It was discovered when a stock of leukemia virus taken from an infected rat was found to cause splenomegaly and sarcomas in newborn mice (Harvey, 1964). The virus also caused an

erythroblastosis similar to that caused by the Friend virus complex (Chesterman et al., 1966). The same diseases were obtained regardless of the helper virus used to rescue and passage HaSV, showing that the pathology was attributable to the HaSV genome (Sher et al., 1975).

Kirsten sarcoma virus (KiSV) was isolated in attempts to recapitulate the genesis of HaSV by infecting rats with leukemia virus and then passaging virus prepared from them into newborn mice (Kirsten et al., 1967a). This virus also caused sarcomas, splenomegaly, and an erthryoblastosis; although this last was less malignant than that caused by HaSV (Kirsten and Meyer, 1967b). Rasheed sarcoma virus (RaSV) was isolated by co-cultivating rat cells infected with and producing leukemia virus with chemically transformed rat cells (Rasheed et al., 1978). Balb sarcoma virus (BaSV) was isolated by passaging filtered plasma from a Balb/c mouse with a spontaneous solid leukemia. After four rounds of infecting newborn mice, the preparation induced acute sarcomas (Peters et al., 1974).

The transduced sequences of the four viruses were compared by nucleic acid hybridization under stringent conditions. BaSv and RaSV were found to carry sequences homologous with those of HaSV (DeFeo et al., 1981). Thus there are three known viruses which transduce v-Ha-ras and one which transduces v-Ki-ras.

The first knowledge of the ras-encoded transforming proteins came from in vitro translation of HaSV and KiSV viral RNA. Both RNAs, when translated in a nuclease-digested reticulocyte lysate yielded a 21,000 dalton protein and the KiSV RNA in addition yielded a 50,000 dalton protein (Parks and Scolnick, 1977; Shih et al., 1978). In order to identify the ras gene products in vivo, antisera was prepared in rats to syngeneic non-producer cells transformed by KiSV and HaSV. Such sera identified a

21,000 dalton protein (called p21) in cells infected with KiSV or HaSV (T. Shih et al., 1979).

A large amount of p21 was found in all cells transformed by transfected subgenomic fragments of HaSV or by infection with deletion mutants of HaSV (Chang et al., 1980a, Chang et al., 1980b, Wei et al., 1980). In addition, a temperature-sensitive KiSV was found to produce a thermolabile p21 (T. Shih et al., 1979). These results together established that the onc protein of HaSV and KiSV was the ras p21.

BaSV also encodes a p21. RaSV encodes a relaed p29, that is longer because it is a fusion protein containing both viral gag- and ras-encoded peptides. The p29 can, therefore, also be immunoprecipitated by antisera raised against viral gag determinants.

Besides ras-transformed cells containing high levels of p21, a variety of normal cells display low levels of p21 (Langbeheim et al., 1980). A potentially important difference, however, is the fact that phosphorylated forms of KiSV, HaSV, and RaSV p21s could be detected, whereas no phosphorylated form was found for p21 from normal cells (T. Shih et al, 1979b; Young et al., 1979). Similarly, biochemical studies showed that HaSV p21, unlike normal cellular p21, has an autophosphorylating activity in which the terminal phosphate of GTP is transferred to a threonine in p21 (Shih et al., 1980). This difference was subsequently shown to be due to the presence of a threonine at amino acid position 59 in the viral p21s which serves as an accepter of the phosphate of GTP. The cellular forms of p21 have alanine at that position (Dhar et al., 1982).

Other properties discovered for p21 are shared by the viral and normal cellular forms. Biochemical studies have shown that all known forms of p21 have a high affinity for binding GTP, GDP, and to a lesser extent other

guanine-containing nucleotides (Scolnick et al., 1979). Also, both the transforming and non-transforming variants of p21 are found associated with the inner surface of the plasma membrane (Willingham et al., 1980). Further studies on ras gene products have been facilitated by the development of monoclonal antibodies raised against p21 (Furth et al., 1982).

Cellular DNA homologues have been identified for both v-Ki-ras and v-Ha-ras. Two distinct Ha-ras-homologous genes were found in both rat (DeFeo et al., 1981) and human genomic DNA (Chang et al., 1982a). Both of the rat genes can be oncogenically activated by addition of a high level retroviral LTR promoter in vitro (DeFeo et al., 1981). However, one of these, termed c-Ha-ras1, is a typical eukaryotic gene including intervening sequences and 5' promoter sequences. The other gene, c-Ha-ras2, appears to be a "pseudogene" by virtue of the fact that it has lost its introns and has become detached from cellular 5' regulatory signals. There are, similarly, two identified c-Ki-ras loci in the human genome. Again one, c-Ki-ras2, contains several introns and the other, c-Ki-ras1, seems to be a pseudogene (Chang et al., 1982a, McGrath et al., 1983).

The c-Ha-ras1 gene consists of four coding exons of similar size in both rats and humans. The human c-Ha-ras1 exons are spread over approximately 3 kb (Chang et al., 1982a). Two species of RNA transcribed from the gene have been detected in normal human cells. One transcript is 1.2 kb and the other is 5.1 kb. Both can be translated in vitro to yield p21 proteins (R. Ellis, personal communication).

The study of the viral ras genes had thus provided knowledge of the c-Ha-ras gene organization, its transcription and its gene product, p21. Moreover, the viral investigations had generated molecular clones of the

normal cellular Ha-*ras* allele and monoclonal antibodies against p21. Previous work in our own laboratory had produced a biologically active clone of a transforming variant of c-Ha-*ras*1 from the EJ/T24 bladder carcinoma cell line. Thus armed, I was in a position to investigate the mechanism of activation of the c-Ha-*ras*1 oncogene.

CHAPTER 2

ACTIVATION OF THE EJ/T24 HUMAN ONCOGENE

2.1 Activity of the c-Ha-ras1 Gene in Normal and Transformed Cells

As discussed above, there are two general models for the way an oncogene could be created. A proto-oncogene could undergo an alteration which involved a change in sequences regulating the expression of the gene; alternatively, the transformed phenotype could be due to changes in the protein-encoding portion of the gene. The first hypothesis would argue for up-regulation of transcription or translation of the gene, yielding high levels of an otherwise normal protein product, while the second model would suggest synthesis of an altered protein. Both types of alteration could also act in concert to create the observed difference in function.

If the significant difference between the oncogene and proto-oncogene were one of regulation, one would expect to see differences in levels of RNAs and proteins specified by these genes. To address this question, EJ bladder carcinoma cells and their counterpart normal cells were examined. Normal bladder epithelial cells were cultured from a specimen of normal human bladder as described (Wu et al., 1982) and were found to synthesize keratins, require a fibroblast feeder layer for support, and possess a limited lifespan in culture, as would be expected for normal epithelial cells (T. O'Connell and J.G. Rheinwald, personal communication). The cultures were free of human stromal fibroblasts, and were freed of irradiated NIH3T3 feeder cells before nucleic acid extraction. These cultured cells therefore provided a source of genetic material and RNA transcripts that were normal counterparts of the bladder carcinoma genes

and transcripts.

Total cellular RNA was prepared from both normal and transformed bladder cells. Transcripts were analyzed by running the RNA on a formaldehyde gel, transferring it to a nitrocellulose filter, and probing the filter with nick-translated EJ oncogene clone. Figure 1a shows that similar levels of RNA were detected in the two cultures. The transcripts were of a size (1.2 kb) previously identified from c-ras genes (Parada et al., 1982).

The only known products of the ras genes are proteins of molecular weight about 21,000 referred to as p21. Monoclonal antisera against the v-Ha-ras p21 protein (Furth et al., 1982) were used to precipitate metabolically labeled protein lysates from both EJ and normal bladder cells. Control experiments ensured that the amounts of antibody used in this and subsequent experiments were in excess of that required to immunoprecipitate the amount of antigen present. The data shown in Figure 1b indicate that at least two bands of radio-labeled protein were specifically precipitated by the anti-p21 sera from normal bladder cells. Detailed examination of the protein pattern of the bladder carcinoma seen in this and other gels reveals a more complex array of bands. These could represent altered forms of the c-Ha-ras p21 or cross-reactive p21s specified by other ras genes in the EJ cells. Without being sure of their origin, the intensity of individual bands could not be accurately compared with those immunoprecipitated from normal bladder cells. However, it is apparent that the total amount of p21 proteins of the normal and the tumor cells differed by less than three-fold.

It thus seems that increased steady state levels of transcription are not responsible for the novel activity exhibited by the EJ oncogene since

Figure 1

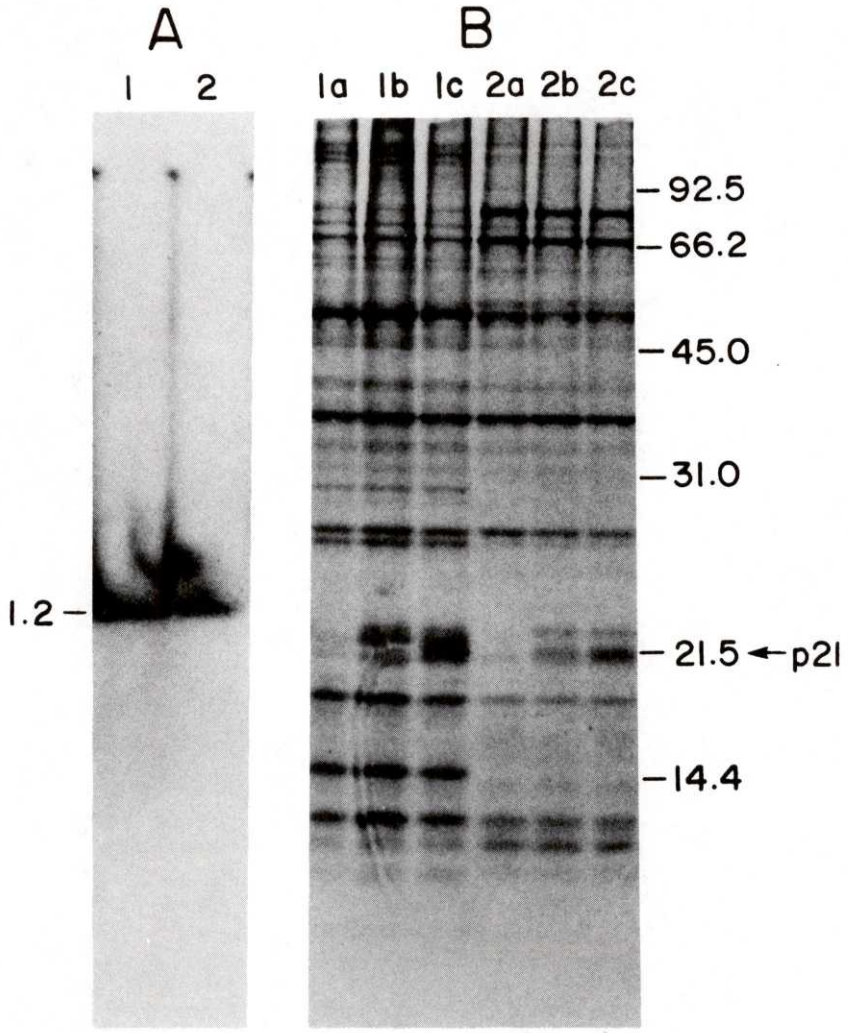


FIGURE 1

Comparison of expression of the c-Ha-ras gene in normal human bladder epithelial cells with that in the EJ transformed bladder cell line. The normal bladder epithelial cells, strain HB1-5, were secondary and tertiary passage cultures derived from explants of a 5-month fetal human bladder. (a) The relative levels of c-Ha-ras specific RNA in the two cell types: lane 1, RNA from EJ cells; lane 2, RNA from HB1-5 cells. 4 ug of RNA was fractionated by electrophoresis through formaldehyde-containing 2% agarose gels and transferred to nitrocellulose. Bands hybridizing to a ras specific probe were visualized by autoradiography. (b) A comparison of p21 proteins immunoprecipitated from cell lysates of EJ cells (lanes 1, a-c) and HB1-5 cells (lanes 2, a-c). Cultures were labelled with ^{35}S-methionine for 12 h. Lysates were then prepared and immunoprecipitated with non-immune serum (lanes 1a and 2a), a monoclonal antiserum (Y13-238) which precipitates the p21 encoded by HaSV but not the p21 encoded by KiSV lanes 1b and 2b) or a monoclonal antiserum (Y13-259) which detects both the HaSV and the KiSV p21s (lanes 1c and 2c.) 2.0 x 10^6 c.p.m. of lysate per sample were resolved by electrophoresis through a 12.5% SDS-polyacrylamide gel.

the EJ and normal bladder cells contained equivalent levels of RNA hybridizing to the Ha-ras probe. This conclusion rests in part on the fact that under the hybridization conditions used here, the oncogene probe reacts exclusively with transcripts of the human c-Ha-ras1 gene. Interpretation of the protein data is less clear. It is apparent that both cells have comparable levels of proteins that are reactive with the monoclonal antibody raised against the Ha-ras encoded p21. However, this antibody could be cross-reactive with other p21s, which would obscure the analysis.

Another potential problem with this analysis was due to the fact that it was not proven that the bladder epithelial cells were, as believed, representatives of the normal precursors of the bladder carcinoma cells. If they were not the true precursers then the interpretations would be clouded, since a ras gene could be expressed at a high level in one cell type without affecting the phenotype while the same level in another cell type might transform it. It was important, therefore, to measure the levels of transcription and translation of the two genes in the same cellular background.

To that end, molecular clones of both genes were introduced into NIH3T3 cells. Colonies acquiring the EJ oncogene could be identified by their transformed morphology. However, cells acquiring clones of the normal allele were not identifiable by any obvious change in behavior. Because of this, NIH3T3 cells were cotransfected with a clone of the selectable Ecogpt gene which provides resistance to mycophenolic acid (Mulligan and Berg, 1981) together with a 10-fold excess of either the cloned EJ oncogene (pEJ) or the cloned proto-oncogene (pEC). In each case, colonies were selected for resistance to mycophenolic acid. This strategy

was used because the introduction of a non-selected DNA segment can be ensured by co-transfection with a selectable gene (Wigler et al., 1979). Of the mycophenolic acid-resistant colonies deriving from co-transfection of Ecogpt and pEJ 75% were seen to be morphologically transformed, whereas none of the colonies emerging after co-transfection with pEC was transformed. The cellular DNA of both classes of colonies was analyzed for the presence of pEC or pEJ sequences. DNA was cleaved with the restriction enzyme BamHI, which would be expected to liberate a 6.6 kb fragment from each intact copy of the cloned oncogene or proto-oncogene. The normal mouse homologue of the ras gene hybridizes only weakly to the pEJ probe (Parada et al., 1982), and so its presence does not obscure the analysis. To ensure that transfected pBR322 sequences would not interfere with interpretation of the data, ras-specific sequences were prepared from pEJ and used as probe. When blotted, 75% of non-transformed, oncogene-transfected colonies showed the presence of pEJ-homologous sequences migrating at 6.6 kb (data not shown).

Two cell lines containing intact copies of the oncogene and two lines containing an approximately equal number of intact copes of the proto-oncogene were selected for further analysis. Photographs of these cell lines are shown in Figure 2. NSF mice were injected with 4×10^6 cells of each of these lines. Each of the pEJ-transfected lines elicited tumors in 5/5 mice, while none of the mice injected in parallel with pEC transfectants developed tumors. The difference between the two transferred ras alleles thus not only affects cell morphology but also tumorigenicity.

Total cellular RNA was prepared from all four transfected cell lines. The RNA preparations were then run on a formaldehyde gel, transferred to nitrocellulose filters, and probed with the ras-specific DNA. As shown in

Figure 2

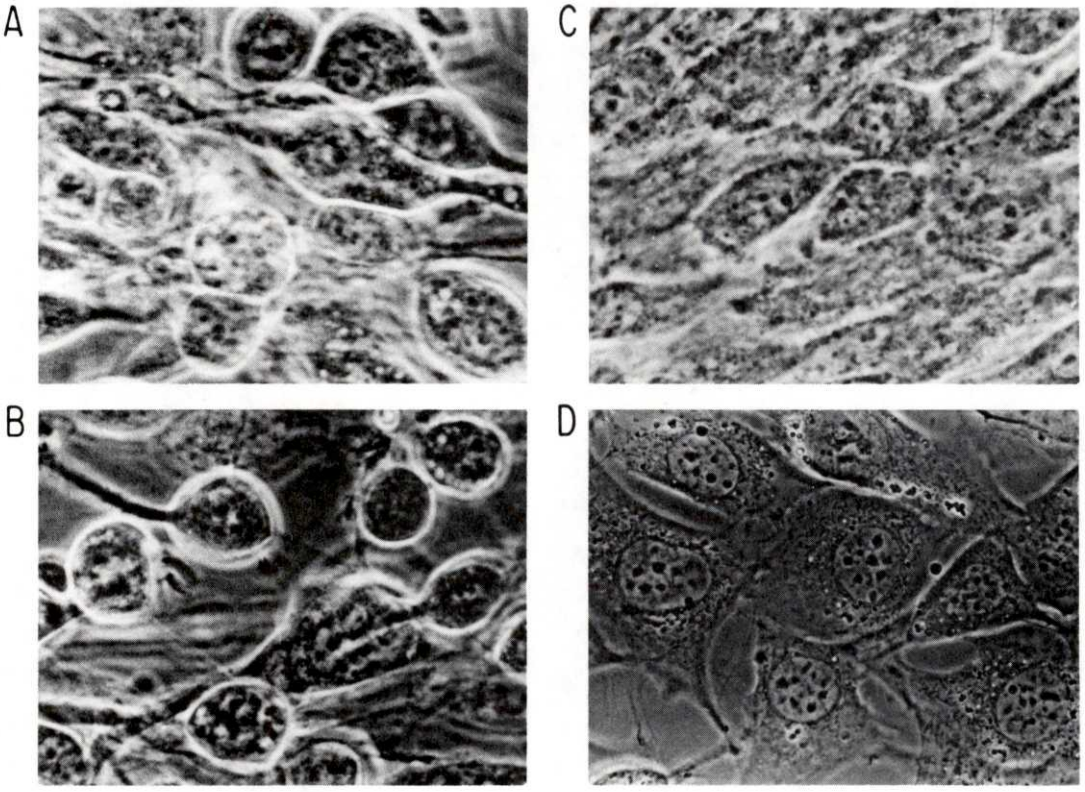


FIGURE 2

Photographs of cell lines transfected with pEJ or pEC through a phase contrast microscope (x300). Cell lines were derived as described in Fig. 2 legend. Cell lines transfected with pEJ are shown at confluence (EJ/Gpt-2, a) and subconfluence (EJ/GPT-3, b). Cell lines transfected with pEC are also shown at confluence (EC/Gpt-5, c) and subconfluence (EC/Gpt-1, d).

Figure 3a, the level of ras-specific RNA in cells containing the oncogene was comparable with those carrying the transfected proto-oncogene. Levels of p21 were also examined in these cell lines. Figure 3b shows that monoclonal antibody against p21 precipitates similar amounts of p21 protein in pEC and pEJ transfected cells.

These data do not address the question of whether the two cloned genes are transcribed at the same rate in these cells, as it remains formally possible that few of the acquired copies of the pEJ are active in these

those carrying the transfected proto-oncogene. Levels
of p21 were also examined in these cell lines. Figure 3b shows that
monoclonal antibody against p21 precipitates similar amounts of p21 protein
in pEC and pEJ transfected cells.

These data do not address the question of whether the two cloned genes
are transcribed at the same rate in these cells, as it remains formally
possible that few of the acquired copies of the pEJ are active in these
particular pEJ-transfected cells, while all copies of the transfected pEC gene in the other cells might be active. In such a case, the comparable levels of protein and RNA observed would not accurately reflect the intrinsic transcriptional activites of the two genes.

However, it is clear that a certain level of EJ-specified p21 induces transformation, while a comparable if not higher steady state level of the proto-oncogene-specified p21 has no effect on cellular phenotype. This suggests that the difference in function between the EJ oncogene and the proto-oncogene might derive from structural alterations in the p21 protein and that regulatory alterations might not be critical to the transforming activity of the oncogene.

Figure 3

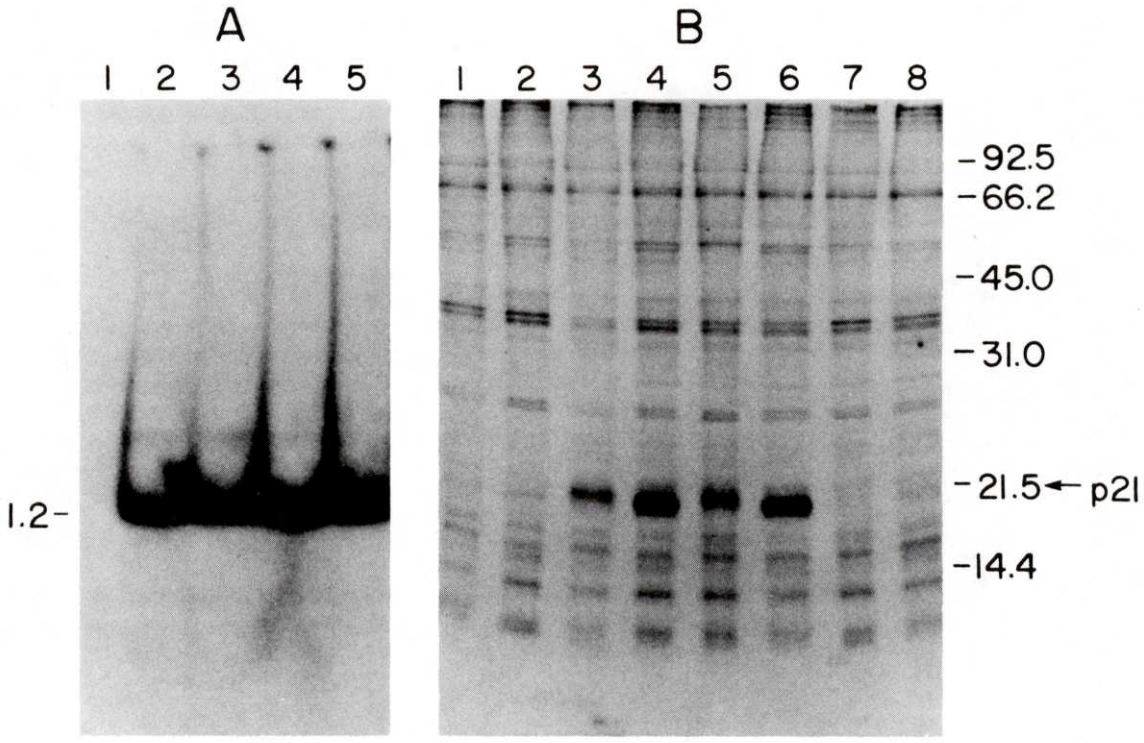


FIGURE 3

Analysis of cells transfected with molecular clones of the EJ transforming gene (pEJ) or its normal cellular homologue (pEC). Transfections were carried out using 75 ug NIH 3T3 carrier DNA, 500 ng pEJ or pEC DNA, and 50 ng pEcogpt DNA per $2x10^6$ cells. Colonies resistant to mycophenolic acid were selected. (a) Analysis of total polyadenylated RNA from the four transfected cell lines. RNAs were analysed as described in Fig. 1. Lane 1, RNA from NIH 3T3 cells; lane 2, EJ/Gpt-2 cells; lane 3, EJ/Gpt-3 cells; lane 4, EC/Gpt-1 cells; lane 5, EC/Gpt-5 cells. (b) A comparison of p21 proteins immunoprecipitated from cell lysates of the four lines transfected with pEJ or pEC. Cell lysates were prepared, immunoprecipitated and analysed as described in Fig. 1 legend. Immunoprecipitations were performed with non-immune serum (lanes 1,2,7,8) or the monoclonal antiserum (Y13-238) which precipitates the HaSV p21 (lanes 3-6). Cell lysates were prepared from EJ/Gpt-2 (lanes 2, 4); EJ/Gpt-3 (lanes 5, 7) and EC/Gpt-5 (lanes 6,8).

2.2 Detection of Differences in the Size of the EJ Oncogene Product

In view of the above conclusion, new importance was attached to the previously detected slight variations in migration rates of the p21 proteins from different cells (Figures 1b and 3b). The p21 protein of v-Ha-ras had previously been shown to have a larger precursor form that is postranslationally cleaved to generate the mature protein (T. Shih, 1982). Thus, if the cellular p21 had a similar metabolic precusor it was possible that it too would exhibit a different mobility in the EJ and EC transfected cells. Alternatively, the detected shift in mobility might reflect a difference in processing of identically sized precursors. To test this the p21 proteins were reanalyzed after shorter periods of metabolic labelling. Data shown in Figure 4a indicate that the pEJ and pEC transfectants each exhibit two bands of p21. Kinetic labeling experiments indicate that in each case the more slowly migrating band behaves as a kinetic precursor to the more rapidly migrating band (S.M. Bradley, personal communication).

Further examination of Figure 4a reveals that the higher molecular weight p21 protein of the pEJ transfectant migrates more slowly than the higher molecular weight protein of the pEC transfectant, and also that the lower molecular weight p21 of the pEJ transectant migrates more slowly than the lower molecular weight p21 of the pEC transfectant. The relative migration rates of these proteins, and the relationship we impute to them from kinetic data and previously published experiments (T. Shih, 1982) are indicated schematically in Figure 4a. As none of the p21 proteins seems to be phosphorylated to any extent (data not shown), the differences in migration between the pEC and pEJ proteins are most readily attributed to alterations in the number of amino acids or to changes in conformation.

Figure 4

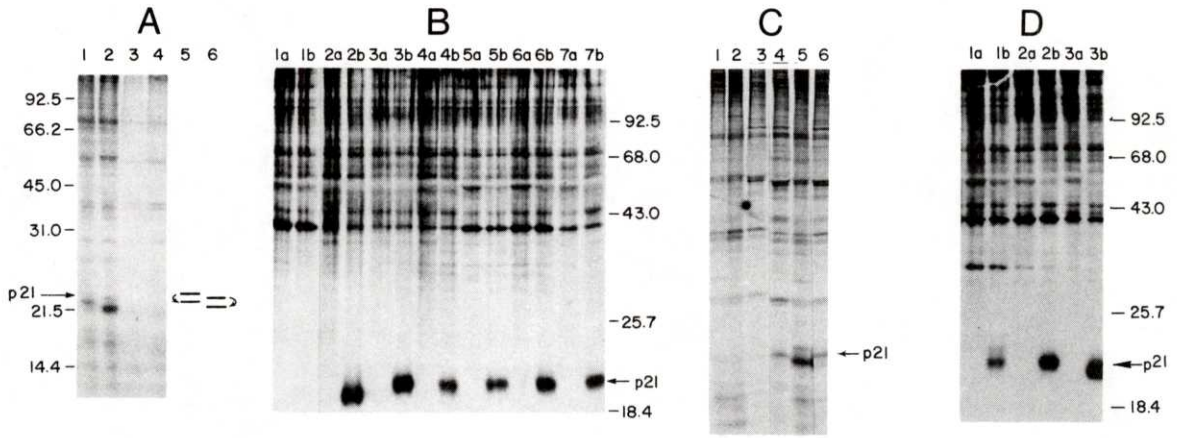

A
1 2 3 4 5 6
92.5
66.2
45.0
31.0
p21
21.5
14.4
B
1a 1b 2a 2b 3a 3b 4a 4b 5a 5b 6a 6b 7a 7b
92.5
68.0
43.0
25.7
p21
18.4
C
1 2 3 4 5 6
p21
D
1a 1b 2a 2b 3a 3b
92.5
68.0
43.0
25.7
p21
18.4

FIGURE 4

Comparison of the mobility of p21 proteins encoded by the EJ bladder carcinoma transforming gene and its normal homologue. (a) Immunoprecipitations from cells transfected with pEJ or pEC. Cells were metabolically labelled with ^{35}S-methionine for 3 h; Lysates from the cell line EJ/Gpt-3 (lanes 1,3) and from the cell line EC/Gpt-1 (lanes 2,4) were precipitated with the monoclonal anti-p21 antiserum Y13-238 (lanes 1,2) or with non-immune serum (lanes 3,4). Schematic diagrams (lane 5, EJ/Gpt-3; lane 6, EC/Gpt-1) show both the relative poaitions of the detected p21 bands and the relationships of those bands (arrows) based on kinetic data (not shown). (b) Analysis of the migration of p21 proteins immunoprecipated from cells transfected with <u>in vitro</u> recombinants of the EJ oncogene (pEJ) and its homologous proto-oncogene (pEC). NIH 3T3 cells transformed with the EJ bladder tumor oncogene, its normal proto-oncogene, or recombinants between the two genes were first biologically cloned in 0.35% agar and then metabolically labelled with ^{35}S-methionine for 18h. Lanes 1a-7a, no antibody; lanes 16-76 anti-p21 monoclonal antibody Y13-172. Cell lysates were from: NIH 3T3 cells (lane 1); cells transformed with the proto-oncogene (the 3-kb SacI fragment of the pEC proto-oncogene that has been activated by fusion with a retrovirus LTR transcriptional promoter) (lane 2); cells transformed with the EJ oncogene (6.6-kb fragments in pBR) (lane 3); cells transformed with the ligation of proto-oncogene 1-kb SacI fragment to EJ oncogene 3-kb SacI fragment (construction b2, Fig. 5) (lane 4); cells transformed with ligations of a fragment of the EJ oncogene extending from the XhoI site to the second BstEII site to a clone of the proto-oncogene from which the homologous fragment had been removed

(construction e2, Fig. 5)(lanes 5 and 6); and cells transformed with the ligation of the EJ oncogene to the left of the KpnI site to the proto-oncogene to the right of this site (construction a2, Fig. 5) (lane 7). (c) Analysis of the migration of the p21 proteins immunoprecipitated from cells transfected with the in vitro recombinant in which the 350-bp XmaI-KpnI fragment of the pEJ oncogene clone was inserted into the corresponding region of the pEC proto-oncogene clone. (construction h2, Fig. 5) Immunoprecipitations were performed with non-immune serum (lanes 1-3) or with the monoclonal antiserum (Y13-238) which precipitates the HaSV p21 (lanes 4-6). Cell lysates were prepared from EJ/Gpt-3 (lanes 1 and 4); EC/Gpt-1 (lanes 2 and 5); and the XmaI-KpnI recombinant transfected cell line (lanes 3 and 6). (d) Analysis of the migration of the p21 proteins immunoprecipitated from cells transfected with the in vitro recombinant in which the 274-bp NarI-KpnI fragment of the pEJ oncogene was inserted into the corresponding region of the pEC protooncogene clone (construction i2, Fig. 5) cells were metabolically labelled with ^{35}S-methionine for 18 h. Immunoprecipitations were performed with nonimmune serum (lanes 1a-3a) or with the monoclonal antiserum (Y13-238) (lanes 1b-3b) cell lysates were prepared from cells transfected with the NarI-KpnI recombinant (lane 1); EJ/GPT3 (lane 2); and EC/GPT-1 (lane 3.)

2.3 Genetic Mapping of the Functionally Altered Region of the EJ Oncogene

The observed differences in the migration of the EJ and normal c-Ha-*ras*1 gene products implied that there was a difference in the coding sequences of the two genes. The lack of detectable regulatory differences between the two alleles suggested that a coding alteration might be responsible for the differences in their oncogenic activity. However, it was apparent that definition of these differences could not be achieved by simple comparison of the sequences of the two genes. As the oncogene and its normal proto-oncogene counterpart sequence were derived from the DNAs of separate individuals, most sequence differences might reflect naturally occurring, silent polymorphism at this locus. Other sequence differences might be consequences of mutational insults suffered during carcinogenesis, which were silent functionally and thus of no importance to the activation process. Therefore, before any comparisons were made, it was was important to narrow the area of functional importance to a segment so small that it would contain few if any silent mutations.

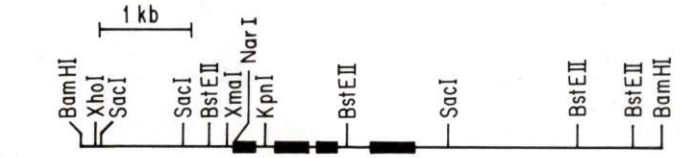
In order to genetically localize the regions of the oncogene that specify the altered migration rate of the protein and the change in gene function, several *in vitro* homologous recombinations were made between clones of the two genes. In each case a restriction fragment was excised from the oncogene and used to replace the homologous piece of the proto-oncogene, or conversely a segment of the proto-oncogene was used to substitute for a piece of the oncogene. In the simplest case this could be achieved by using two restriction enzymes, XhoI and KpnI, each of which cut the gene a single time. As progressively smaller fragments were exchanged the constructions became somewhat more difficult. Partial digests were required to isolate fragments cut in the appropriate places by SacI,

BstEII, XmaI and NarI. Very small products of partial restriction enzyme digestions could always be purified by electrophoresis through an appropriate gel. However, large products that had only lost a short sequence were impossible to separate from similarly sized DNA segments which had not lost the short sequence. Therefore, in some of the constructions it was necessary to reconstitute the clones from three or four fragments which could be conveniently isolated, even though only one of them was being interchanged between the two ras clones. When three or four segments of DNA were needed to produce a full clone, these were joined in a single ligation reaction. This could be accomplished because the restriction enzymes which generated the fragments were chosen to leave different single-stranded sequences at the ends of each purified piece of DNA. The T4 DNA ligase used to join the segments will not attach non-homologous ends. Following in vitro ligation the recombinant clones were amplified and mapped to confirm their structure. The constructions are shown schematically in Figure 5.

These recombinant constructs were then tested for transforming ability in the transfection assay. The ability of a fragment of the oncogene to impart transforming activity when placed in the midst of a proto-oncogene clone was tested, and conversely, in the reciprocal recombination, the loss of activity was assayed when the corresponding proto-oncogene fragment was inserted into the oncogene clone.

It was vital to verify that the transforming clones were indeed chimeras of mixed pEJ and pEC origin, rather than contaminants of one origin or the other. This was done in three ways. In the simpler construction, involving ligations of two fragments at a time, results obtained with amplified recombinant clones were verified by directly

Figure 5



		Average Foci per 20ng DNA	Number of Independently Transfected Clones
a	1	0	2
	2	910	2
b	1	0	2
	2	780	2
c	1	0	2
	2	720	1
d	1	0	3
	2	1030	3
e	1	0	3
	2	1070	3
f	1	590	3
	2	0	2
g	1	730	5
	2	0	5
h	1	0	5
	2	800	5
i	1	0	2
	2	830	2

FIGURE 5

Transfection data and structures of in vitro recombinants between the molecular clones of the EJ transforming gene (pEJ) and its normal cellular homologue (pEC). The restriction map shows the cleavage sites for various enzymes within the 6.6 kb BamHI insert in pBR322. All sites specific for the enzymes are shown except for XmaI and NarI which cut in several other places which have not been well characterized. The sites shown are the only XmaI and NarI sites between the first BstEII site and the KpnI site. The solid boxes on the map show the locations of coding exons. pEJ/pEC chimaeras are shown, with segments derived from pEJ shown as solid bars and segments from pEC shown as open bars. pEJ and pEC were cleaved with the indicated enzymes either to completion or in a partial digest as required to obtain each indicated fragment. The products were separated by electrophoresis through 1.2% agarose and eluted by melting in NaI and absorbing to glass beads. The fragment containing pBR322 was then treated with calf intestional phosphatase. The indicated fragments were joined either with the enzyme T4 DNA ligase or in a mock ligation without enzyme. Constructs a-e were made in bimolecular ligations. Constructs in f were made by mixing the three fragments simultaneously and in g, h, and i by mixing the four fragments simultaneously. The ligation mixtures were directly transformed into the HB101 strain of Escherichia coli. Only when colonies from mock ligations were less than 2% of the ligations were colonies analyzed for the presence of clones with appropriate restriction maps; 20 ng of each clone were transfected to NIH 3T3 cells and then carried without selection until foci were visualized in 10-14 days. Results of the transfections are shown in the first column. The second column shows the number of independent bacterial colonies screened and then transfected into NIH3T3 cells.

transfecting the unamplified products of ligation reactions and of mock ligations containing isolated fragments not treated with the ligase. A single amplified clone might be a non-recombinant contaminant in a reaction mixture. This could not be true of the bulk of the products of a ligation when the mock ligation failed to transform cells and thus did not contain an appreciable amount of non-recombinant transforming clone DNA. A second confirmation of the origins of the recombinant clones came from certain diagnostic restriction digests. The plasmids pEJ and pEC had their respective ras genes inserted in the pBR322 plasmid vector in opposite orientations. Thus, the origin of one parent of a recombinant could be learned by diagnostic restriction digests of the flanking plasmid regions (Figure 6). There were also known sequence polymorphisms at the extreme 5' (MstII) and 3' (SphI) ends of the 6.6 kb clones which could be used in a similar manner (Figure 7). Since contaminating pEC could itself not give a false positive result, as it is not transforming, any active clone carrying proto-oncogene flanking sequences must have arisen as a consequence of acquisition of portions of the transforming gene. Finally, we confirmed all of our results with several independent clones obtained from a ligation reaction. The products of a ligation were used only if mock ligation of the same fragments gave no more than 2% of the bacterial colonies seen on use of the DNA ligase.

The results of transfecting the recombinant clones are summarized in Figure 5. As can be seen, ultimately a genetic region 274 nucleotides long was identified which, when transferred from the oncogene to a corresponding region in the proto-oncogene, is able to impart activity to the latter. This region extends from the first NarI endonuclease site to the KpnI site (Figure 5i). A verification of the construction of the NarI/KpnI

Figure 6

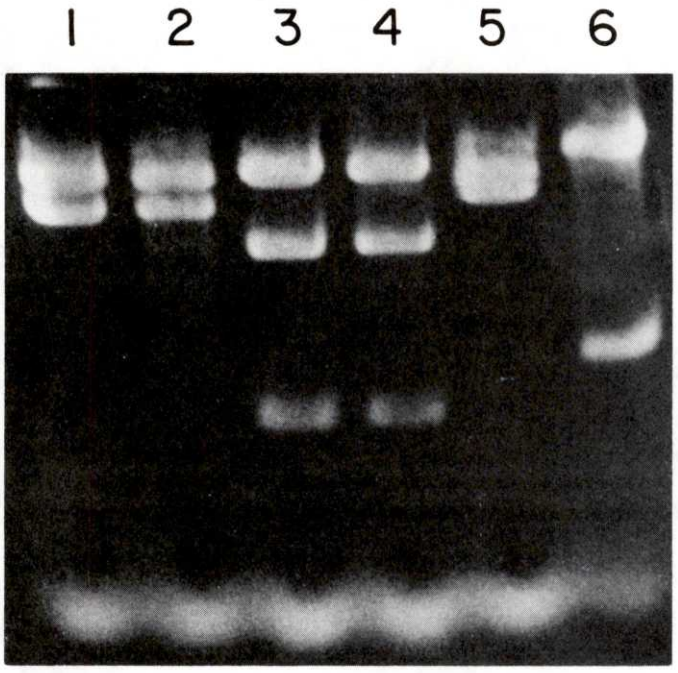


FIGURE 6

Determination of the relative orientations of the 6.6 kb EJ and EC inserts in pBR322. pEJ 6.6 (lanes 1,3,and 5) and pEC 6.6 (lanes 2, 4, and 6) were cut with the restriction enzymes BamHI (lanes 1 and 2), SacI (lanes 3 and 4), and ClaI (lanes 5 and 6.)

Figure 7

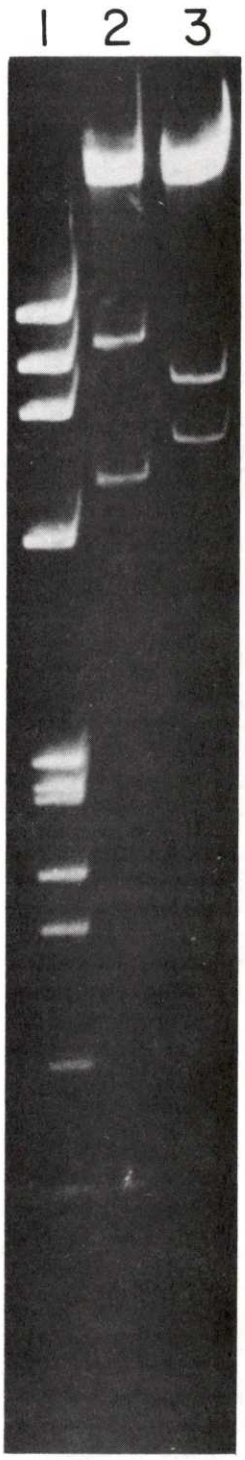


FIGURE 7

Identification of a 3' polymorphism between the EJ and EC c-Ha-*ras* alleles. pEC6.6 (lane 2) and pEJ6.6 (lane 3) were cut in a double digest with the enzymes (ClaI and SphI, separated by electrophoresis through a 12.5% polyacrylamide gel to resolve low molecular weight bands and compared to ØX-HaeIII size markers (lane 1). The lowest band in each digestion (lanes 2 and 3) reflects the different relative orientations of the clones in pBR322, the second fastest migrating band in each lanes corresponds to the ClaI-SphI fragment internal to the c-Ha-*ras* sequence.

recombinant clones is shown (Figure 8, lanes 1-4). This region consists of most of the first exon and part of the first intron. Since there were no 5' flanking sequences in the fragment identified as containing the transforming lesion, it was again suggestive that the EJ/T24 c-Ha-ras oncogene might be activated by alterations in its coding sequences rather than by regulatory changes.

2.4 Detection of Difference in Size of the Gene Product from the In Vitro Recombinants

In experiments described above, a difference in the migration of the p21 protein encoded by the oncogene and the proto-oncogene had been identified. Having determined a short region of the gene which contained the transforming lesion, it became important to ascertain whether the region also encoded the altered protein size. Therefore cells transfected with the products of the in vitro recombinations were immunoprecipitated with the monoclonal antibodies directed against p21. As seen in Figure 4b, the protein brought down in lysates from XhoI-second BstII site, SacI-SacI, and XhoI-KpnI recombinant transfectants all comigrate with the the EJ protein and have a mobility which differs from that of the EC protein. Similar results were obtained for the XmaI-KpnI and NarI-KpnI recombinants, Figure 4c and Figure 4d respectively. The lysates analzyed in Panels 4b and 4d were from cultures that were labelled for 18 hours, resulting in high levels of label in the lower molecular weight forms of the p21, and undetectable amounts of label in the kinetically unstable higher molecular weight forms. Thus, the phenotypes of oncogenic transformation and altered electrophoretic migration cosegregate, and the two are encoded by the same 274 bp segment of DNA.

Figure 8

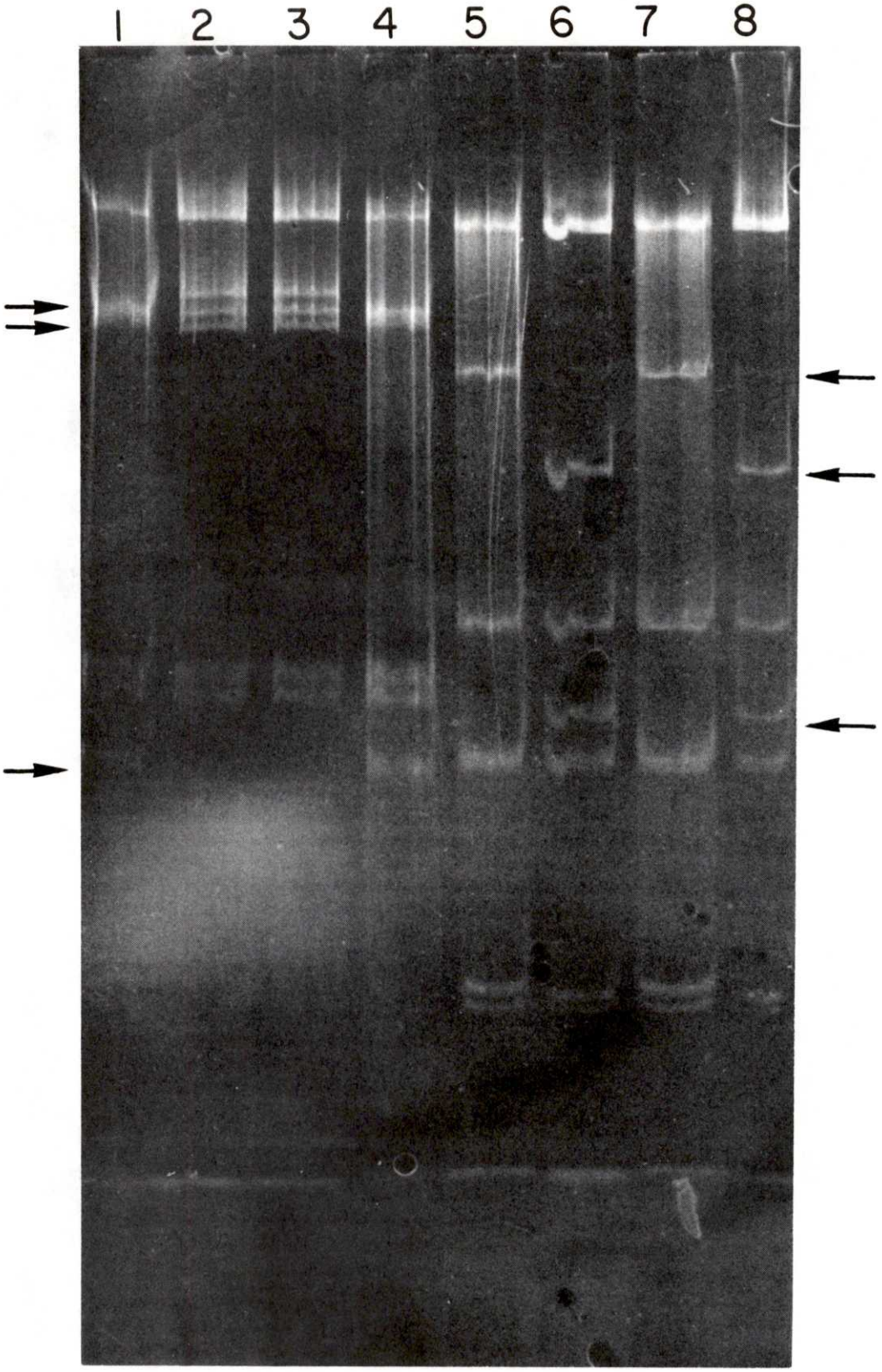


FIGURE 8

Verification of the construction of *in vitro* recombinants containing interchanged 274-bp NarI-KpnI fragments as diagramed in Fig. 5. pEJ 6.6 (lanes 1 and 5), pEC 6.6 (lanes 2 and 6), the oncogenically active clone shown in diagram i2, Fig. 5 (lanes 3 and 7) and the inactive clone shown in diagram i1, Fig. 5 (lanes 4 and 8) were cut either with the enzyme MstII (lanes 1-4) which has a polymorphic site 5' of the *ras* coding region in pEJ 6.6, but absent from pEC 6.6 (and from constructs containing pEC 6.6 flanking sequences); or were cut with the enzyme NaeI (lanes 5-8) which cuts at the coden coding for the twelfth aminoacid in pEC 6.6 but not in pEJ 6.6. Arrows mark the migration of the polymorphic bands when uncut (uppermost arrows) or cut (two lower arrows) by each enzyme.

2.5 Comparison of Nucleotide Sequence

Having identified a short fragment of biological significance, the reported sequences in this segment of the two genes were compared (Tabin et al., 1982). This comparison is shown in Figure 9.

The only difference between the two segments is in the p21 encoding region of the first known exon, specifically 6 nucleotides from the NarI cleavage site. It occurs in a triplet that encodes glycine in the normal rat (M. Ruta et al., unpublished data) and human c-Ha-*ras* genes. The sequences observed in the EJ oncogene causes conversion to a valine. This alteration is thus responsible for the alteration in function of the p21 protein, and for the oncogenic activation of the c-Ha-*ras* gene that occurred in the EJ bladder carcinoma.

A second independent consequence of of the single base change was the alteration in the cleavage sites of two different site-specific endonucleases. The sequence GCCGGC occurs in the proto-oncogene, and thus represents the recognition site of the endonuclease NaeI. It also contains the CCGG recognition site of the endonuclease HpaI. Both of these are changed in the oncogene, whose equivalent sequence reads GCCGTC. The NaeI endonuclease is the more useful of the two, since it cleaves DNA less frequently. This was used to independently verify the two sequences. As expected, the pEC clone exhibited one more NarI cleavage site in its inserts than did its pEJ counterpart (Figure 8, lanes 5 and 6). Perhaps more useful was the retrospective verification of the *in vitro* recombinant clones. The allele specifying transformation and abnormal p21 migration rate was seen to precisely cosegregate with the allele disallowing NaeI cleavage at this site (Figure 8, lanes 7 and 8).

Figure 9

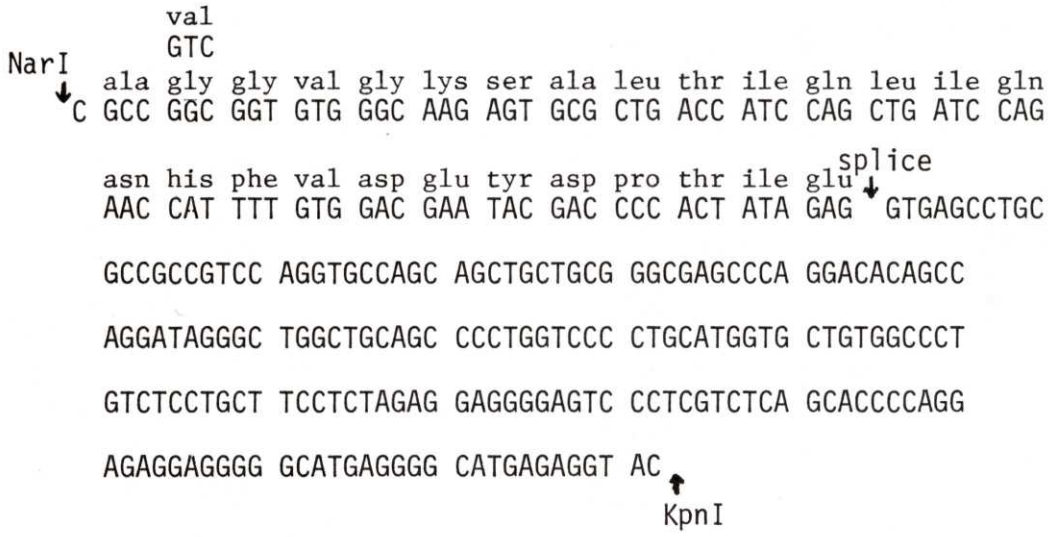

val
GTC
NarI
ala gly gly val gly lys ser ala leu thr ile gln leu ile gln
C GCC GGC GGT GTG GGC AAG AGT GCG CTG ACC ATC CAG CTG ATC CAG
splice
asn his phe val asp glu tyr asp pro thr ile glu
AAC CAT TTT GTG GAC GAA TAC GAC CCC ACT ATA GAG GTGAGCCTGC
GCCGCCGTCC AGGTGCCAGC AGCTGCTGCG GGCGAGCCCA GGACACAGCC
AGGATAGGGC TGGCTGCAGC CCCTGGTCCC CTGCATGGTG CTGTGGCCCT
GTCTCCTGCT TCCTCTAGAG GAGGGGAGTC CCTCGTCTCA GCACCCCAGG
AGAGGAGGGG GCATGAGGGG CATGAGAGGT AC
KpnI

FIGURE 9

Comparison of the DNA sequence of the molecular clone of the EJ transforming gene (pEJ) and its non-transforming cellular homologue (pEC). Sequence data between the restriction enzyme sites NarI and KpnI are shown (See Fig. 5). The coding DNA strand is printed as is the inferred amino acid sequences. For coding regions where pEJ and pEC differ, both sequences are indicated, the pEJ sequence above.

The demonstration that the point mutation was functionally significant came from the transfection experiments described above. To directly demonstrate that the point mutation we had identified was involved in the development of cancer, as opposed to *in vitro* cell transformation, we needed a vehicle for delivering the cloned c-Ha-*ras* sequences into cells in a living animal. Retroviruses, by virtue of their ability to infect many cell types and their capacity for stably introducing genetic information into the chromosomal DNA, seemed a promising choice for such a vehicle or "vector". Creating a chimeric retrovirus carrying the EJ-c-Ha-*ras*1 allele would have the additional advantage of allowing us to directly compare that allele with the homologous gene already transduced by HaSV.

CHAPTER 3

ADAPTATION OF A RETROVIRUS AS A EURARYOTIC VECTOR

3.1 Construction of MLV-tk Recombinants

Retroviruses seemed to be likely to be adaptable for use as vectors since the naturally arising acute transforming viruses are examples of retroviruses transducing cellular sequences. However, no one had yet developed retrovirus vectors and it was possible that the naturally arising transduced segments were specially selected or modified to allow their passage. Moreover, those segments differed from the c-Ha-*ras* gene we wished to mobilize in two further respects: the c-Ha-*ras* gene contains intervening sequences which could not be easily removed and it also carries its own transcriptional promotor which could be exploited; the *onc* genes transduced by the acute transforming viruses have neither of these features. In order to investigate the utility of the retrovirus vector system one step at a time, the first gene which was inserted into a retrovirus genome was the herpes virus thymidine kinase gene (*tk*). The *tk* gene carries its own transcriptional promotor but contains no introns.

For convenience of further manipulation, a derivative of pBR322 was made which lacks the unique BamHI site normally present in the plasmid sequence. pBR322 DNA was linearized with BamHI endonuclease followed by DNA PolI treatment to fill in overhanging single-stranded DNA ends. The plasmid DNA was then recircularizd by T4 DNA ligase. After bacterial transformation, colonies were identified which carried plasmids resistant to further BamHI treatment.

Moloney MLV was chosen for construction of a mammalian vector. A clone of the virus, termed lambda 21, was derived from infected normal rat kidney cell DNA (Hoffmann et al., 1982). The cloned sequence, which contained the provirus flanked on both sides by rat cellular DNA, had been inserted into the lambda phage vector Charon 4A via EcoRI sites. The 10.9 kb cloned insert was subsequently transferred to the EcoRI site of the modified pBR322 described above. This plasmid has been termed pZIP (Shoemaker et al., 1981).

The thymidine kinase (tk) gene of herpesvirus was inserted into this plasmid-borne MLV provirus. A tk clone, pTK3, was a gift of Saul Silverstein. The 3.4 kb BamHI fragment containing the tk gene was purified by fractionation through an agarose gel. To prepare the MLV vector to receive the tk insert, the MLV-carrying plasmid was digested with BamHI (Figure 10). Because the BamHI site of the pBR322 plasmid had been removed and since the flanking rat cellular DNAs carried no BamHI sites, the only BamHI cleavage sites remaining were located within the MLV provirus. The fragments resulting from BamHI treatment were two MLV-specific fragments 3.05 and 0.40 kb in length and pBR322 MLV proviral chimeric fragment. This mixture of DNA fragments was treated with bacterial alkaline phosphatase and ligated to the purified tk fragment. By coincidence, the insertion of the 3.4 kb tk gene almost precisely reconstituted the full length of the original clone before BamHI endonuclease digestion.

After bacterial transformation, clones were identified with both orientations of the tk gene relative to the MLV genome. An example of each, termed pMLVTK and pMLVKT, was selected for further study. The configuration of these is diagrammed (Figure 10).

Figure 10

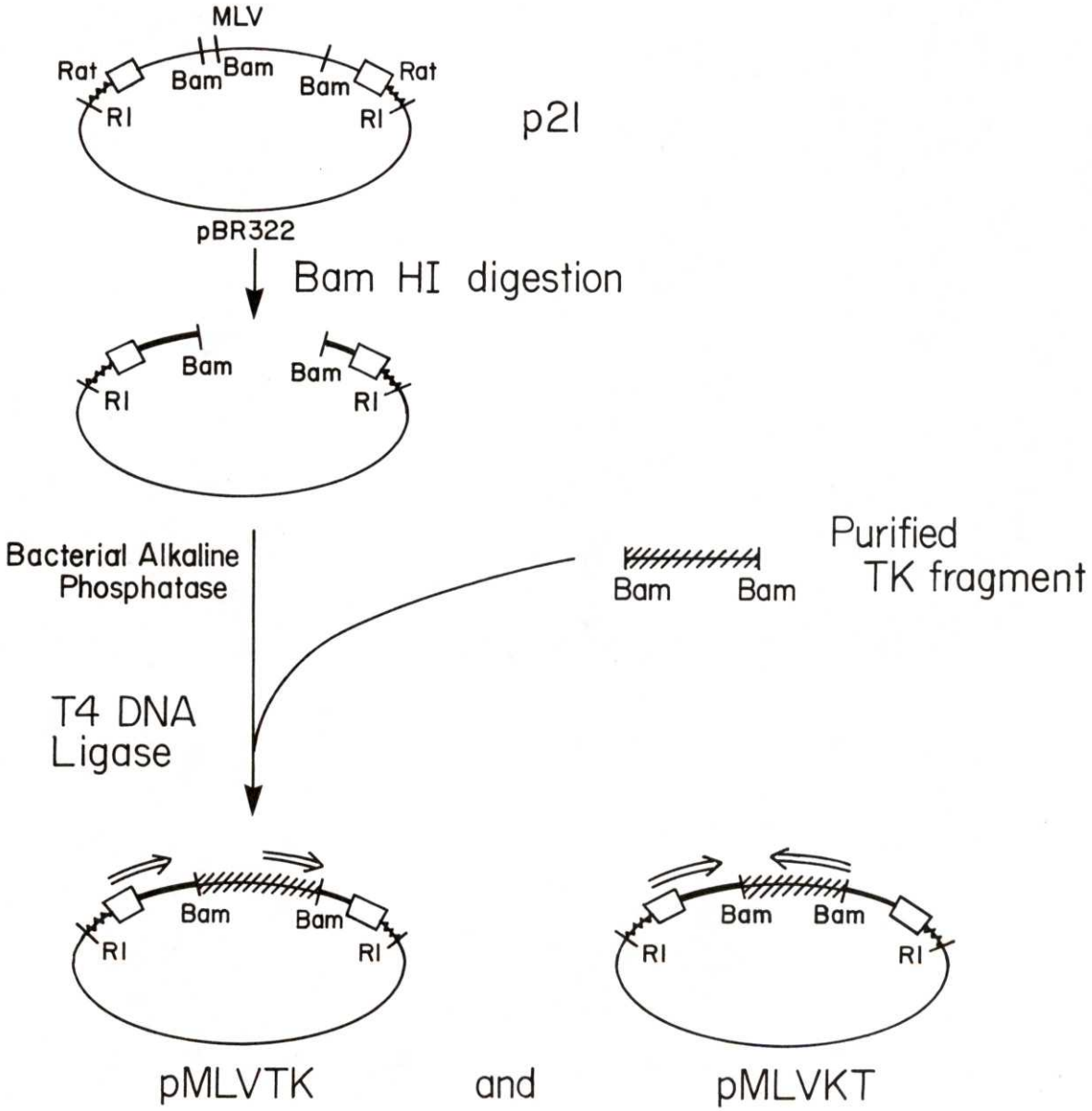

MLV
Rat
Bam
Bam
Bam
Rat
RI
RI
p21
pBR322
Bam HI digestion
Bam
Bam
RI
RI
Bacterial Alkaline Phosphatase
Purified TK fragment
Bam
Bam
T4 DNA Ligase
Bam
Bam
RI
RI
Bam
Bam
RI
RI
pMLVTK
and
pMLVKT

FIGURE 10

Construction of chimeric plasmids carrying the tk gene inserted into MLV. BamHI and EcoRI sites are shown. pBR322 sequences are represented by thin lines, MLV sequences are represented by hatched lines. The boxed regions at the termini of the MLV sequences indicate the long terminal repeats. The arrows represent the directions of transcription initiated within the MLV long terminal repeat and in the tk gene.

3.2 Transfection of Cells with Cloned Chimeric tk/MLV DNAs

The first step in creating "producer cells" secreting the chimeric virus was to isolate cells containing the above described DNA constructs. The HAT selection media of Littlefield (Littlefield, 1964) can be used to select those members of a cell population that produce the enzyme thymidine kinase. In the present case, this selection was exploited to identify those cells in a thymidine kinase-deficient (TK^-) population that had acquired the TK^+ phenotype as a consequence of transfection with the chimeric tk-containing DNAs.

The two chimeric plasmid clones were applied to cultures of TK^- L cells (Ltk^-) as calcium phosphate precipitates. The transfected cells were then cultured in HAT medium. We observed that both molecular clones yielded TK^+ colonies with the same efficiency: 1×10^5 to 2×10^5 colonies per microgram of tk insert per 2×10^6 transfected cells. This efficiency was identical to that which we observed when using pTK3, the plasmid which carries the tk gene inserted directly into pBR322. NIH3T3 (TK^-) cells provided by E. Scolnick were also transfected. TK^+ colonies were again obtained, but at an efficiency of 10^4 colonies per microgram of insert. Thus, the biological activity of the tk gene is not impaired by its being physically juxtaposed to the MLV sequences.

The orientation of the tk insert in the MLV vector was not verified in the transfected cells because typically in a transfection many partial and intact copies of a introduced sequence are acquired which would obscure the analysis. However, the structures of the chimeric clones were checked before transfection and no trace of cross contamination was observed. It can therefore be estimated that at least 95% of the transfected DNA had the expected orientation. Since pMLVtk and pMLVkt gave rise to TK^+ colonies at

identical efficiencies, there is a high probability that both orientations were active in conveying the TK^+ phenotype to recipient cells.

3.3 Rescue of TK^+ Transducing Virus

The structures of the chimeric DNA genomes were formally analogous to the DNA genome of a sarcoma virus in which portions of sequences responsible for replication competence have been replaced by foreign oncogenic sequences. The resulting virus genomes thus lack some or all of the information necessary to produce new virus particles. They may be complemented by coinfected helper virus, so that the genomic RNA of the defective sarcoma virus is rescued in pseudotyped particles specified by the helper virus.

To obtain lines of cells producing pseudotyped chimeric MLV-*tk* virus, three strategies were employed which are diagrammed in Figure 11. In the first strategy TK^+ colonies derived by transfecting NIH3T3 (TK^-) cells with pMLVTK or pMLVKT were infected with Moloney MLV stocks. The spread of the MLV was verified by a reverse transcriptase (RT) spot assay (Goff et al., 1981b). This assay is a screen for the presence of virion-bound RT in the culture medium.

A second approach used for the introduction of helper MLV and chimeric virus into the same cell was the application of pMLVKT DNA to a population of NIH3T3 (TK^-) cells which had previously been infected with MLV. As in earlier transfections, TK^+ colonies were selected with HAT medium. Out of 10 TK^+ colonies so obtained, 10 were positive in the RT spot assay.

These strategies would not allow similar lines of mouse L cells to be obtained. The Ltk^- cells used for these experiments already contained an expressed MLV. This endogenous virus is poorly infectious and therefore

Figure 11

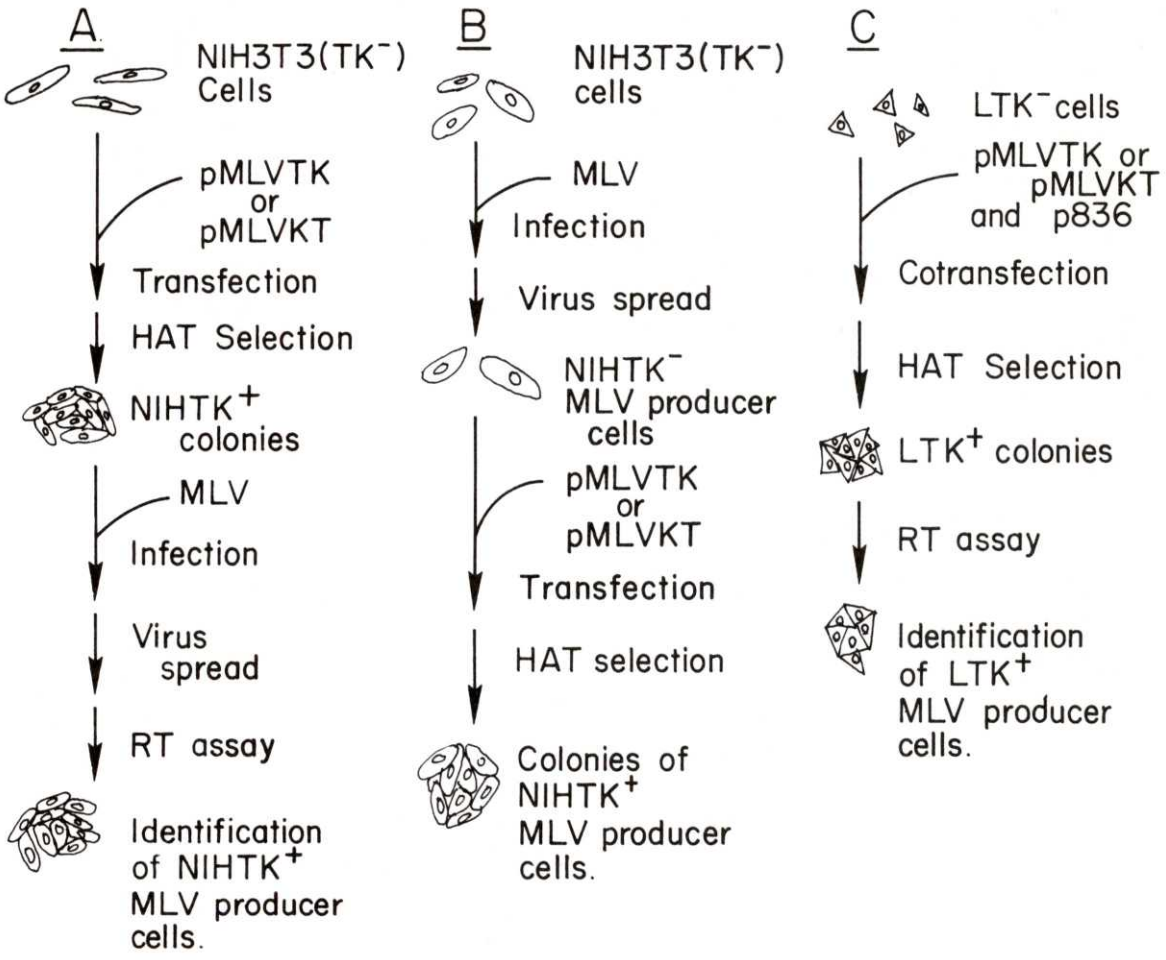

A
NIH3T3(TK⁻) Cells
pMLVTK or pMLVKT
Transfection
HAT Selection
NIHTK⁺ colonies
MLV
Infection
Virus spread
RT assay
Identification of NIHTK⁺ MLV producer cells.
B
NIH3T3(TK⁻) cells
MLV
Infection
Virus spread
NIHTK⁻ MLV producer cells
pMLVTK or pMLVKT
Transfection
HAT selection
Colonies of NIHTK⁺ MLV producer cells.
C
LTK⁻cells
pMLVTK or pMLVKT and p836
Cotransfection
HAT Selection
LTK⁺ colonies
RT assay
Identification of LTK⁺ MLV producer cells.

FIGURE 11

Strategies employed to obtain lines of cells producing MLV and containing MLVTK or MLVKT chimeric constructs. pMLVTK and pMLVKT are the cloned MLV-HSV tk gene recombinant plasmids in opposite orientations. p836 is a biologically active MLV provirus clone.

incapable of acting as an efficient helper virus. On the other hand, the presence of this virus creates an interference barrier which effectively prevents superinfection by added MLV particles. Since this interference barrier has no effect on the introduction of DNA via transfection, calcium phosphate precipitates of either pMLVTK or pMLVKT DNA were applied together with an infectious MLV DNA clone (p836, also called pZAP) to Ltk$^-$ cells. The p836 MLV DNA was present at a five-fold molar excess to ensure that any cells which had taken up the chimeric cloned DNA would be likely as well to have received p836 MLV DNA (Wigler et al., 1979). Colonies were again obtained with HAT selection. Out of 22 TK$^+$ colonies examined, 20 were strong producers of MLV as shown by an RT spot assay.

Media from cultures of cells derived in these three ways were harvested to obtain virus stocks. The filtered stocks were then tested for the ability to transmit both the MLV-positive and the TK$^+$ phenotypes. MLV production was assayed by the standard XC plaque assay, in which NIH3T3 cells are infected, overlaid with XC cells, and scored for syncytia of fused XC cells (Rowe et al., 1970). (It should be noted that the weakly produced L cell endogenous virus is RT positive but does not register in the XC plaque assay.) Passage of the TK$^+$ phenotype via viral particles was assayed by HAT selection of infected NIH3T3 (TK$^-$) cells.

The results of these assays are shown in Table 1. All of the tested stocks were XC positive, indicating that they contained non-defective MLV. In addition, they were all positive when tested for their ability to convert TK$^-$ cells to TK$^+$ phenotype, implying that they contained particles carrying chimeric MLV-*tk* genomes. Similar titers were observed with virus stocks whose chimeric genomes carried *tk* in both orientations relative to MLV (Table 1). While the orientation of viral genomes was not directly

TABLE 1. Assay of clonal cell lines for production of XC^+ and TK^+ transducing virus[a]

Method of obtaining cell line	Assayed clone	TK^+ CFU/ml[b]	XC PFU/ml[c]
A. MLV infection of pMLVTK-transfected NIH3T3 (TK^-) cells	A-1	4×10^4	6×10^5
	A-2	1×10^4	2×10^5
	A-3	1×10^4	1×10^5
B. MuLV infection of pMLVKT-transfected NIH3T3 (TK^-) cells	B-1	1×10^4	3×10^5
	B-2	2×10^4	3×10^5
	B-3	2×10^4	5×10^5
C. pMLVKT transfection of MuLV-infected NIH3T3 (TK^-) cells	C-1	4×10^4	7×10^5
	C-2	3×10^4	7×10^5
	C-3	4×10^4	6×10^5
D. pMLVTK and p836 cotransfection of Ltk^- cells	D-1	2×10^3	1×10^4
	D-2	2×10^3	1×10^4
	D-3	1×10^3	4×10^4
E. pMLVKT and p836 cotransfection of Ltk^- cells	E-1	2×10^3	3×10^4
	E-2	7×10^2	9×10^3

TABLE 1

(a) Media containing virus were harvested from confluent 10-cm plates 12 h after feeding.

(b) TK^+ colony-forming units (CFU)/ml were determined by titering colony formation on 10^6 NIH 3T3 (TK^-) cells infected with 1 ml of virus-containing medium. Infections were for 2 h. At 24 h cells were placed in HAT selective medium. Colonies were scored after 2 weeks.

(c) Standard XC assays were performed, using NIH 3T3 (TK^-) cells as indicator cells.

verified, the stocks were generated from cell lines which, as discussed above, were believed to contain the appropriate DNA sequences.

When a sarcoma virus infects a cell without helper virus co-infection, the cell may become oncogenically transformed, but no progeny virus particles are produced. Such a nonproducer cell can subsequently be infected with helper virus to allow rescue of the sarcoma virus. It should have been possible to create analogous non-producer cells harboring the chimeric MLV-tk viruses.

To test this, colonies were obtained by HAT selection of cells infected with viral stocks at low dilution. Upon analysis, these TK^+ colonies fell into two classes. The culture media of one group of colonies were RT and XC positive and, in addition, induced TK^+ colonies in HAT medium. These colonies were therefore active producers. The culture media of a second class of TK^+ colonies were negative in both the XC and RT tests and also failed to pass the HAT-resistant phenotype to other cells.

Examples of the later class of cells were chosen as putative nonproducer cell lines. These cells were infected with MLV helper stocks. After allowing for the spread of the helper virus, cultures were recloned in microtiter dishes. The clones that were grown from the microtiter wells were assayed in parallel with known producer cells. As expected, chimeric tk containing genomes of these cells were rescued by superinfection with MLV, thus proving their nonproducer status.

These data also show that only one chimeric MLV-TK-containing particle is necessary for the conversion to a TK^+ phenotype. The requirement for a single infectious event can be seen by plotting TK^+ colony-forming units per milliliter versus log of dilution of virus. The plot demonstrates a linear, single-hit kinetic response to dilution (Figure 12).

Figure 12

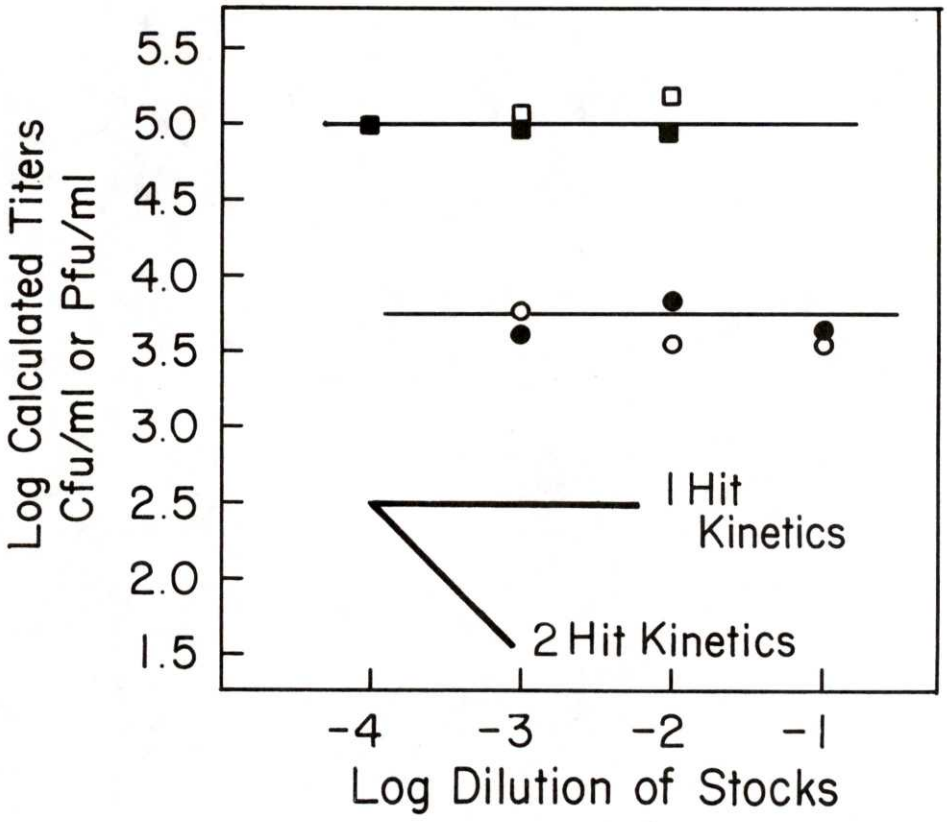
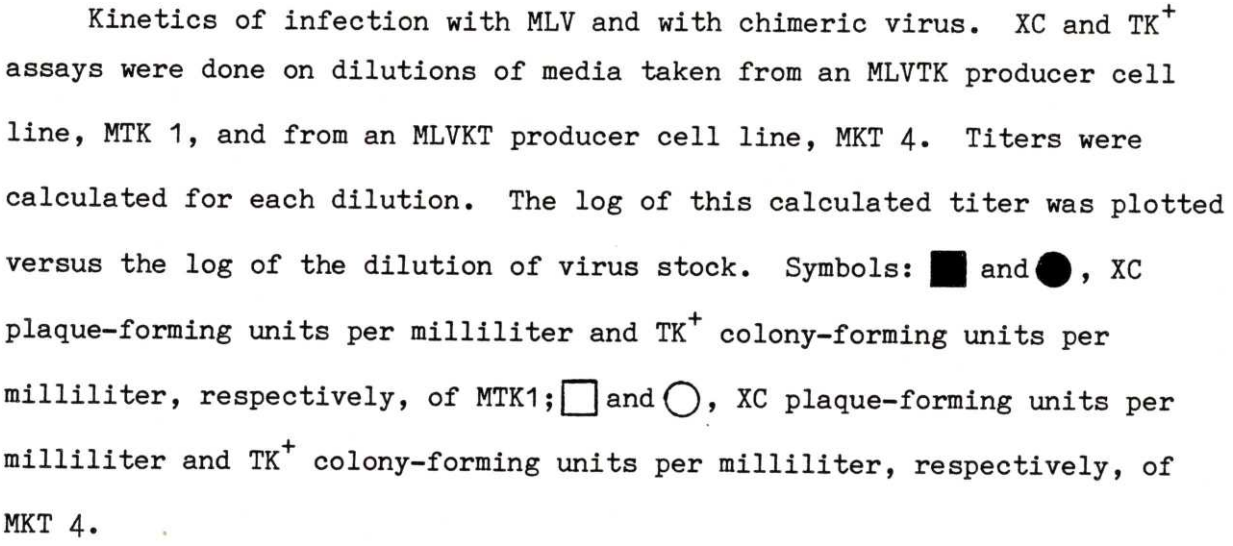

Log Calculated Titers
Cfu/ml or Pfu/ml
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1 Hit
Kinetics
2 Hit Kinetics
-4
-3
-2
-1
Log Dilution of Stocks

FIGURE 12



Kinetics of infection with MLV and with chimeric virus. XC and TK^+ assays were done on dilutions of media taken from an MLVTK producer cell line, MTK 1, and from an MLVKT producer cell line, MKT 4. Titers were calculated for each dilution. The log of this calculated titer was plotted versus the log of the dilution of virus stock. Symbols: ■ and ●, XC plaque-forming units per milliliter and TK^+ colony-forming units per milliliter, respectively, of MTK1; □ and ○, XC plaque-forming units per milliliter and TK^+ colony-forming units per milliliter, respectively, of MKT 4.

3.4 Analysis of Virion RNA

Since it was presumed that the TK^+ phenotype was passed by retrovirus particles, *tk*-specified RNA should be identifiable in virions. Because the recombinant genome was so close in size to that of the parental Moloney MLV genome, the molecular weight measurements of these RNAs would not have been a sensitive indicator for distinguishing chimeric *tk*-carrying RNA from helper MLV RNA. Therefore, these RNAs were analyzed by a filter hybridization procedure which yields information on both the size and the sequence homologies of RNAs of interest (Alwine et al., 1977).

Particles were prepared from cells producing MLVTK and MLV, MLVKT and MLV, MLV alone, and from uninfected cells. These RNAs were run on glyoxal gels and transferred to diazophenylthio paper (McMaster and Carmichael, 1977). These were then hybridized wtih ^{32}P-labeled probe specific for either MLV or *tk* sequences. Bands representing sequences homologous to the probe were visualized by autoradiography.

When an MLV probe was used, a band 8.2 kb in length was seen in lanes where RNA from $MLVTK^-$, $MLVKT^-$, and MLV-producing cells was run. This indicates the presence of MLV-hybridizing RNA of full viral genome length in particles produced by these cells. The *tk*-specific probe hybridized to bands also 8.2 kb in length, only in lanes with RNA from $MLVTK^-$ or $MLVKT^-$ producing cells. From this it was concluded that transmission of the TK^+ phenotype was, indeed, mediated by virus particles containing chimeric MLV-*tk* RNAs whose size was, as predicted, very similar to that of MLV (Figure 13).

Figure 13

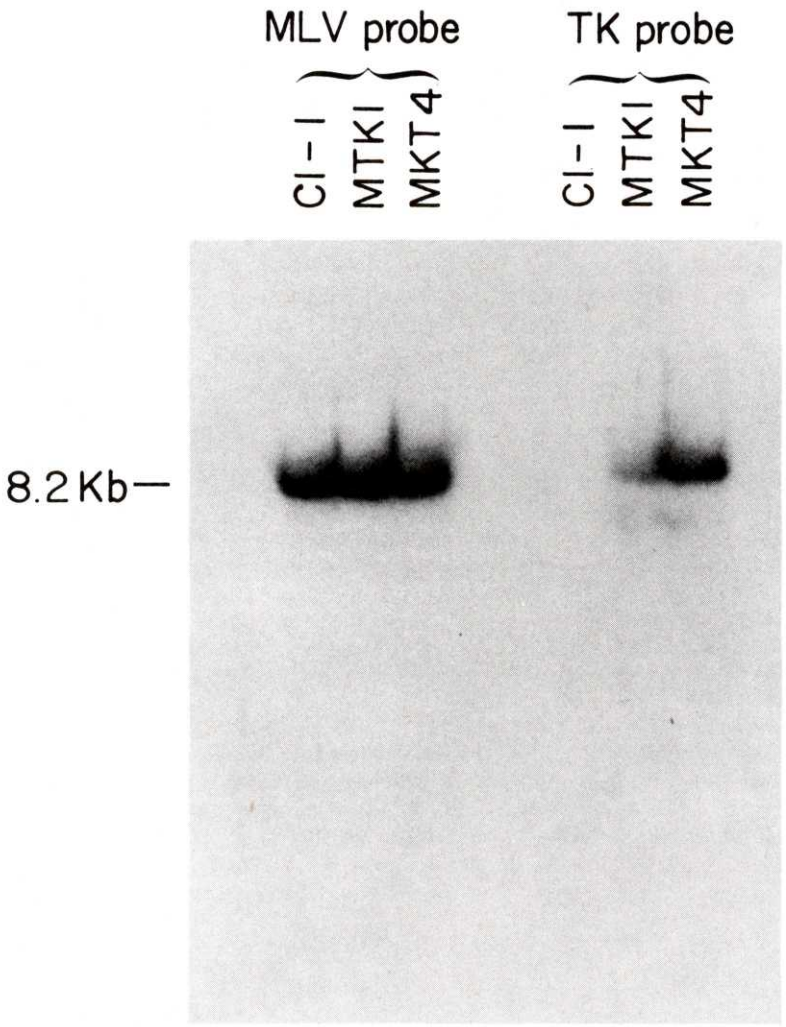


FIGURE 13

Analysis of RNAs in virions. Virus was harvested after 2.5 h and purified on a sucrose step gradient. Extracted genomic RNA was fractionated through a 1% agarose gel. The RNA was transferred to diazophenylthio paper and hybridized to a nick-translated plasmid carrying either an MLV provirus (left lanes) or the HSV tk gene (right lanes). Cl-1 is a cell line known to produce MLV alone. MTK1 and MKT4 are cell lines producing the chimeric viruses MLVTK and MLVKT, repectively, as well as MLV helper virus.

3.5 Analysis of DNAs of TK^+ Cells

It was assumed that transmission of chimeric genomes depended upon intermediates similar to those seen in a normal retrovirus replication cycle. This implies that the chimeric DNA genomes should be integrated in an intact form into cellular chromosomes rather than in the fragmented configuration of transfected DNA. Moreover, such integrations should occur in a manner colinear with the genomic RNA of the virus, in contrast to the permuted representations of the SV40 genome typically seen in the integrated state. In addition, like retrovirus genomes, these chimeric DNAs should be able to integrate randomly into sites in the cellular genome.

Cellular DNA prepared from colonies infected with chimeric virius was examined to determine whether they would indeed yield restriction fragments consistent with a retrovirus-mediated passage of the TK^+ phenotype. DNA was purified from TK^+ colonies derived from infection with MLVTK or MLVKT stocks. These DNAs were digested with diagnostic restriction endonucleases. These DNAs were then run on an agarose gel, blotted onto nitrocellulose paper, and annealed with a *tk*-specific ^{32}P-labeled probe. Annealed probe was detected by autoradiography.

The enzyme HindIII does not have any recognition sites in the cloned DNA genome of the chimeric virus. If the integration events through which the chimeric genomes became fixed in the cells were indeed random in their location in the cellular DNA, then cutting with HindIII would cause each provirus to be carried in a uniquely sized restriction fragment. Each fragment would be expected to be larger than the viral genome.

HindIII digestions of DNAs from independent colonies of chimeric genome-infected cells yielded a number of DNA fragments with a *tk* probe.

All of these were larger than 8.8 kb (Figure 14). The number of bands is a reflection of the number of chimeric proviruses in the cell. The results were the same for both orientations of the tk sequences relative to the MLV genome in infecting chimeric virus. The results showed that these integrations occurred at random cellular loci. They were also consistent with the integration of intact genomes.

Upon integration, retroviruses form terminally redundant sequences which flank the viral genome. An enzyme which cuts the viral DNA only within the redundant sequence will, therefore, release a virus-specific fragment of DNA the same length as the RNA of the virus. Such a digestion will leave no other major DNA fragments of viral origin.

The restriction endonuclease XbaI cuts only in the redundant termini of the cloned chimera. With all colonies, cutting with XbaI resulted in a single fragment 8.2 kb in length. This comigrated with a fragment resulting from the digestion of pMLVTK DNA mixed with an appropriate amount of uninfected NIH3T3 DNA (Figure 14). These data showed directly that the integration of the chimeric genomes left the sequence intact and unpermuted with respect to the original clone.

The chimeric clones were constructed by ligating a BamHI fragment containing the HSV tk gene into MLV DNA which had been cut with BamHI. Therefore, cutting the DNA from infected clones of NIH3T3 (TK^-) cells with BamHI would be expected to liberate the original HSV tk gene fragment from the integrated chimeric virus, were it still intact. BamHI digestion produced the expected 3.4 kb tk fragment in every case. This fragment ran alongside a marker derived from the BamHI digestion of pMLVTK DNA mixed with uninfected NIH3T3 DNA (Figure 14).

Figure 14

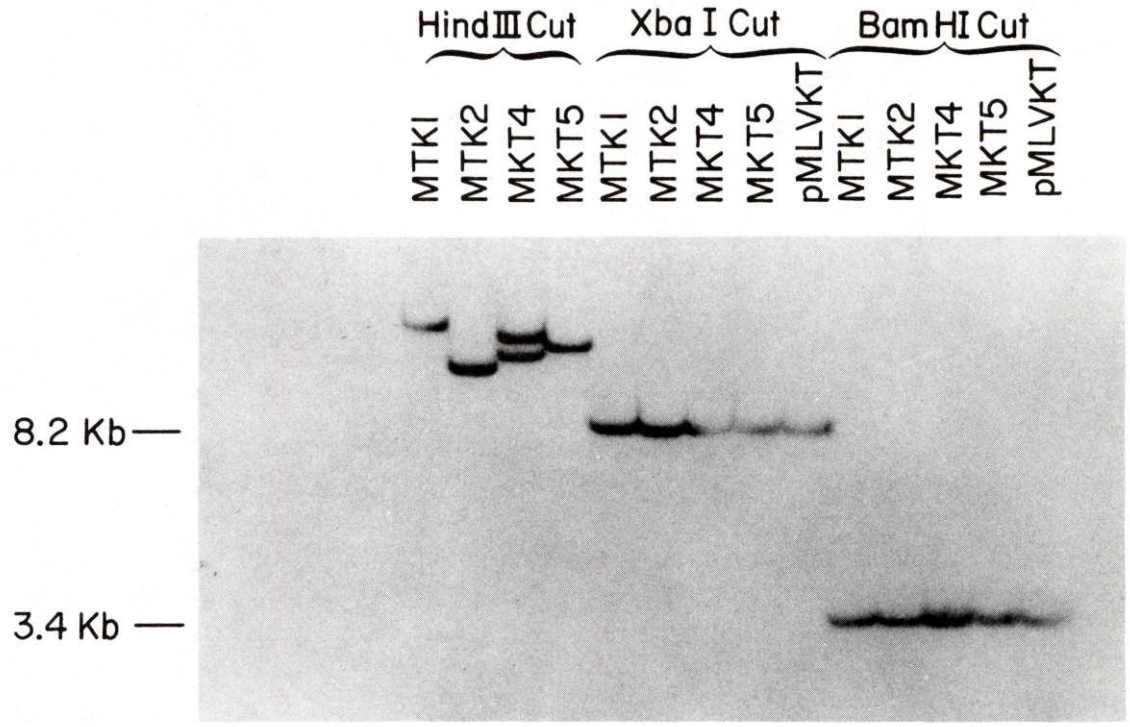

Hind III Cut
Xba I Cut
Bam HI Cut
MTK1
MTK2
MKT4
MKT5
MTK1
MTK2
MKT4
MKT5
pMLVKT
MTK1
MTK2
MKT4
MKT5
pMLVKT
8.2 Kb
3.4 Kb

FIGURE 14

Analysis of proviral DNA in cell lines infected with rescued chimeric virus. Total cellular DNAs from four cell lines producing chimeric virus were analyzed. Cell lines MTK4 and MKT5 are producers of the chimera MLVKT, and cell lines MTK1 and MTK2 are producers of the chimera MLVTK. DNA (10 ug) from each line was cleaved with the indicated restricted enzyme. Plasmid pMLVKT (50 pg) was mixed with 10 ug of NIH3T3 DNA and then cleaved with two of the enzymes in a reconstruction experiment where indicated.

The chimeric virus was thus shown to integrate into infected cell DNA in a manner consistent with replication as a retrovirus but not, as might be expected, in other kinds of genetic transfer seen after transfection or papovavirus infection.

3.6 Analysis of Cellular RNA of TK^+ Cells

In previous sections, it was shown that an 8.2 kb RNA species containing both MLV- and *tk*-specific sequences is present in virions isolated from cultures of MLV-*tk* chimeric virus-infected cells. Given the structures of these chimeric viruses, including two transcriptional promoters and two polyadenylation sequences, it was possible that other RNAs might be found within the cells but not packaged and exported in virions. RNA from infected cells was, therefore, analyzed by the filter hybridization procedure (Alwine et al., 1977).

Polyadenylated RNA was prepared from MLVTK nonproducer cells, MLVKT non-producer cells, and MLV producer cells. The RNAs were run on gels containing formaldehyde and transferred to nitrocellulose paper. These immobilized RNAs were then incubated with ^{32}P-labeled probe specific for either MLV or *tk* sequences. As before, bands representing sequences homologous to the probes were visualized by autoradiography.

When RNA from MLV producer cells was hybridized with an MLV-specific probe, 8.2 and 4 kb bands were seen on the autoradiographs. No bands were seen when the same RNA was probed with *tk*-specific sequences (Figure 15). This was expected, as MLV proviruses are known to direct the synthesis of two RNA classes: an 8.2 kb unspliced full-genome-length RNA and a spliced glycoprotein message of 4 kb (Weiss et al., 1982).

Figure 15

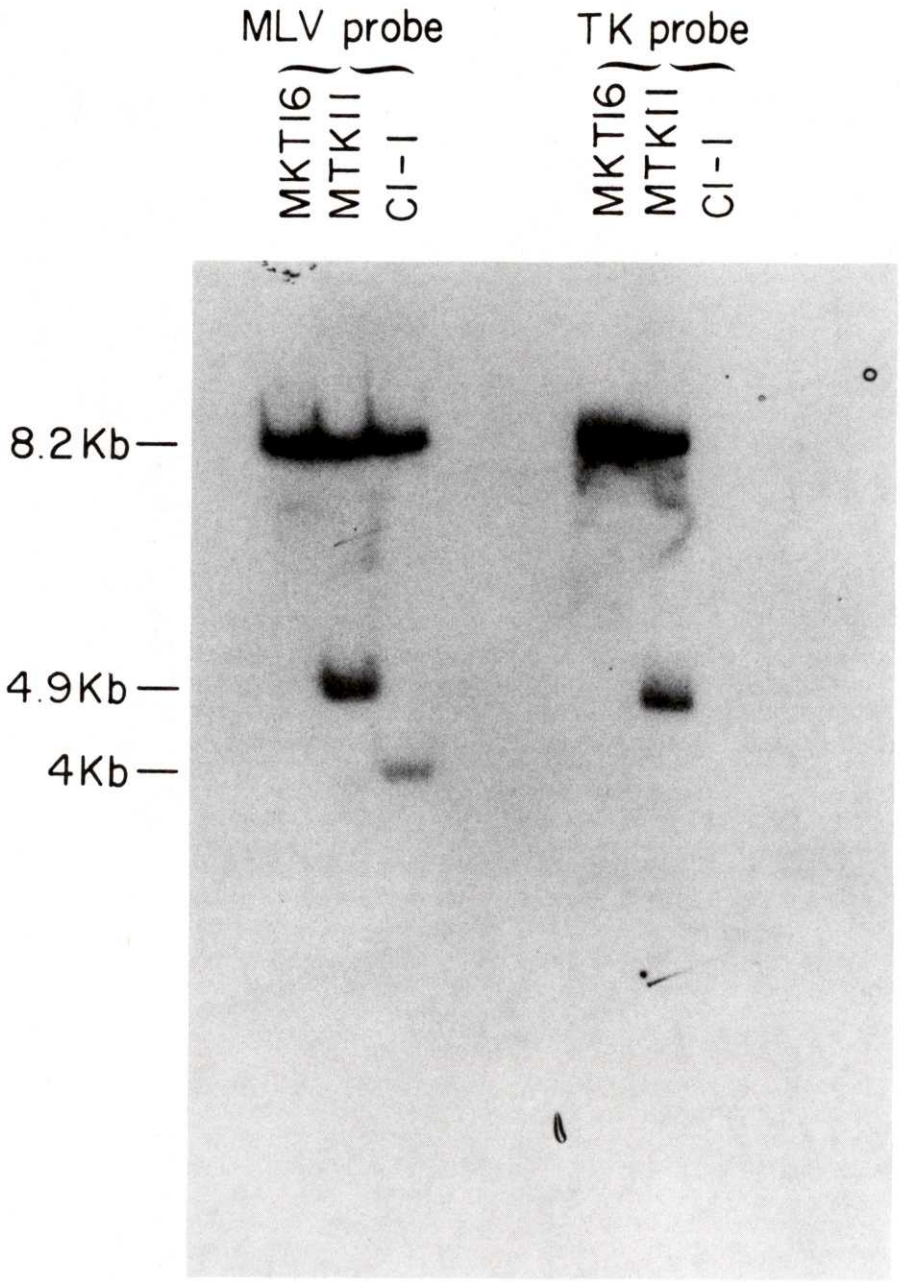

MLV probe
TK probe
MKT16
MTK11
CI-1
MKT16
MTK11
CI-1
8.2Kb
4.9Kb
4Kb

FIGURE 15

Analysis of polyadenylated RNA in cells infected with rescued chimeric virus. Polyadenylated RNA was isolated from non-producer cell lines infected with MLVTK (MLVTK11) and MLVKT (MLVKT16) chimeric virus and from Cl-1 cells, which are producers of MLV. RNA was fractionated through formadelhyde containing agarose gels and transferred to nitrocellulose. The gels were hybridized to either MLV or tk-specific probes made by nick translating plasmid DNA carying either an MLV provirus or the HSV tk gene as shown.

RNA from cells infected with MLVTK was analyzed in a similar manner. The chimeric provirus of these cells is arranged such that the MLV and tk promoters are both oriented in the same direction. Thus transcripts initiate at the MLV promoter would have to pass through the tk promoter and the tk polyadenylation sequences before they could reach the MLV-encoded polyadenylation site. When RNA isolated from cells infected with this MLVTK chimera was hybridized with either MLV- or TK-specific probes, two bands were seen, one 8.2 and the other 4.9 kb (Figure 15).

The 8.2 kb band represents the genome-length message, which was also seen packaged in virions in experiments discussed above. The structure of the 4.9 kb transcript is most consistent with an RNA resulting from an aberrant splicing event. The length of this molecule is compatible with a splice extending from the MLV splice donor site to a site 0.4 kb into the tk insert and then continuing to the MLV termination sequences. The MLV splice donor sequence was left intact in the chimeric genomes, whereas the corresponding MLV splice acceptor sequences were deleted. It is possible that the MLV donor finds a compatible splice acceptor within the introduced tk sequences. The structure of this RNA was not pursued further.

RNA from nonproducer cells of the MLVKT chimera was also examined. This recombinant virus was constructed such that the MLV and tk promoters initiated RNA transcripts on opposite strands. RNA from cells harboring this chimera yielded only a single 8.2 kb band on autoradiographs when hybridized with either the MLV- or tk-specific probes (Figure 15). This band represents the full-length transcript made from the chimeric provirus.

RNA transcripts initiated by the tk promoter could not be detected in either of the chimeric nonproducer lines. When filter-bound RNA was hybridized with tk-specific probe and then exposed for long times, any

small tk-promoted bands which may have been present were obscured by degradation products of the MLV-promoted species. The blots suggest that any transcripts arising from the tk promoter must be present within the cells in molar amounts at least 50-fold below those of the MLV-promoted RNA transcripts. Nonetheless, such tk-initiated transcripts should exist in these cells and are likely to be the functional templates for thymidine kinase synthesis. This is especially true in the case of cells harboring the MLVKT chimera, since in that construct the sense of tk is opposite that of MLV whose 8.2 kb transcript cannot, therefore, encode the thymidine kinase in infected cells.

3.7 Construction of MLV - c-Ha-ras1 Chimeric Virus

The results described above with the MLVKT and MLVTK viruses indicate that foreign DNA can be inserted into retroviral genomes in a way which allows the resultant viral genome to function and passage in a manner analogous to that of the natural sarcoma viruses. This was true in spite of the fact that the inserted tk gene contained its own transcriptional promoter and polyadenylation signals unlike the natural v-onc sequences. It thus seemed likely that the c-Ha-ras1 gene could be similarly passaged when placed in the pZIP derived MLV vector.

The promoter and coding regions of the EJ allele of c-ha-ras1 were isolated as a single 6.6 kb BamHI fragment. This fragment was inserted into the pZIP retroviral vector from the 5' PstI site to the 3' BamHI site using BamHI linkers. Both orientations of insertion were identified. These chimeras, termed pEJV (ras and LTR in the same transcriptional polarity) and pJEV (opposite polarity) were transfected into NIH3T3 cells. Both gave rise to transformed foci which were single-cell cloned.

After superinfection, transfectants of both clones were found to passage transforming virus at low titers: 10^3 ffu/ml for EJV and 5 x 10^3 ffu/ml for JEV virus. Cells transformed by infection with JEV and EJV were picked, expanded and used to prepare secondary viral stocks. When these stocks were used to infect NIH3T3 cells, much higher titers were observed (5 x 10^6 ffu/ml for both EJV and JEV).

3.8 Analysis of DNAs from Transformed Cells

As discussed, a potentially important difference between the tk gene used above as a model insert and the c-Ha-ras gene is that the latter contains intervening sequences. This, along with the observed increase in focus-forming titer that accompanied EJV and JEV passage, made it important to ascertain whether there were structural changes in the chimeric viral genomes acquired during passage. The proviral DNA in cells transformed by infection with "high titer" JEV and EJV was therefore examined.

Cellular DNA was prepared from eight transformants of each class and cut with the restriction endonuclease EcoRI, which does not cut the chimeric viral genomes. These were subjected to Southern blot analysis using a ^{32}P-labelled ras-specific probe. In this way two independent lines which contained single copies of the EJV provirus and two lines with single copies of JEV were identified for further study (data not shown). These four lines were used to prepare more DNA for detailed analysis.

The DNAs were first cut with the enzyme BamHI, which cuts the EJ insert out of the MLV vector. Thus an unspliced, undeleted genome would generate a 6.6 kb band when probed with nick-translated ras DNA. A spliced genome would be predicted to give a 5.5 kb band. In fact, all four DNAs examined produced smaller bands: 4.8 and 3.2 kb for EJV infected cells and

4.4 and 4.6 kb for JEV infected cells (Figure 16). Thus all four genomes had undergone rearrangements. Splicing could not explain the patterns observed, but the sizes were consistent with splicing having occurred in addition to other deletion events.

To find out whether intronic sequences had been lost, the DNAs were next cut in a double digest of BamHI and KpnI. KpnI cuts once in the first intron of c-Ha-ras. Thus an unspliced, undeleted pattern would be two bands, a 2.2 kb band from the 5' end of the gene and a 4.4 kb band from the 3 end. A spliced genome would generate a single 5.5 kb band. The blot shows single bands of 4.8 and 3.2 kb for the EJV infected DNA indicating that they had lost the KpnI sits in their first introns. In contrast, the JEV-infected DNA produced two bands 2.2 and 2.2 kb (a doublet) in one case and 2.2 and 2.4 kb in the other, showing that they had not lost their KpnI sites (Figure 16). The fact that the two JEV viruses retained their 2.2 kb bands, originating from the 5' end of the genome, and had diminished bands mapping to their 3' ends indicating that in the reverse orientation, it was 3' sequences which were lost.

To further determine the regions being deleted in these viruses, another double digest was carried out using the enzymes BamHI and BstEII. BstEII cuts the BamHI fragment 5 times; once 5' of the coding region, once in the intron between the third and fourth exons, and three times at the 3' end of the sequence, well past the coding exons. Thus the double digest of the intact clone yields (from 5' to 3') fragments of 1.3, 1.5, 2.9, 0.6, 0.3 and 0.05 kb. A spliced version of the gene would give fragments 1.3, 3.3 (the combined 1.5 and 2.9 fragments minus the intron sequences), 0.6, 0.3 and 0.05 kb. The .05 kb fragments were not detected in the control or experimental lanes; and the 0.3 kb fragments were not detected in the

Figure 16

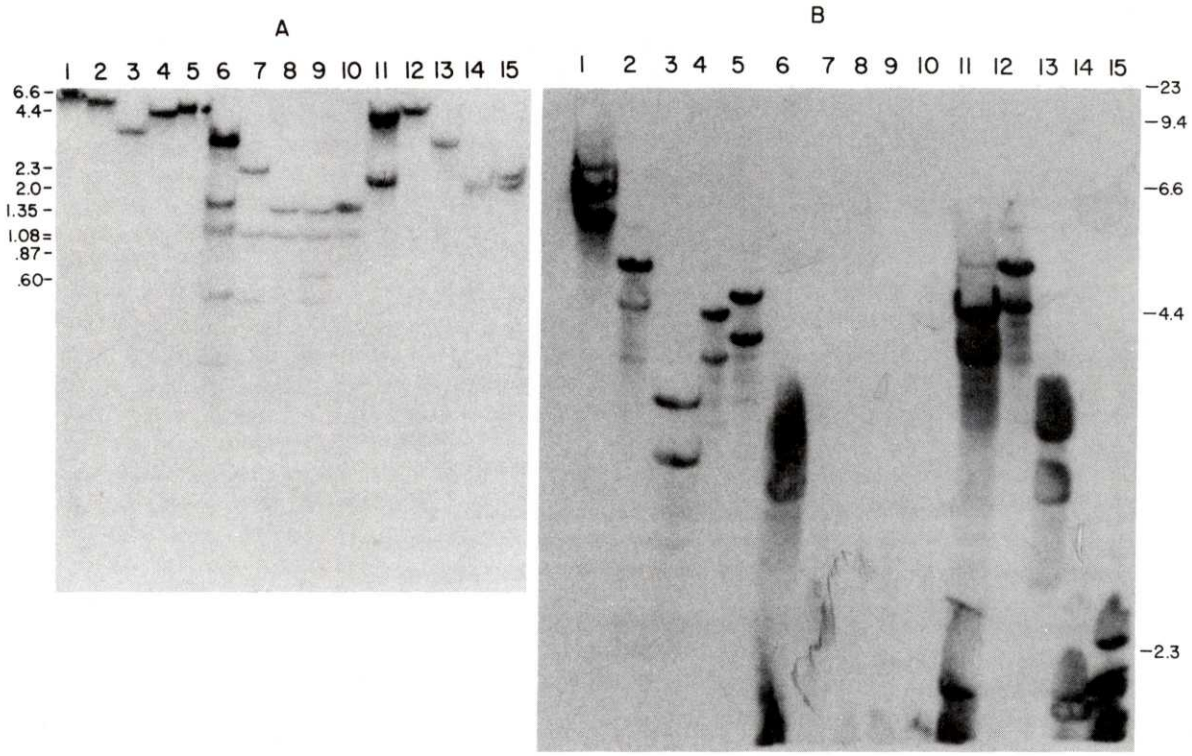

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
6.6
4.4
2.3
2.0
1.35
1.08
.87
.60
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
23
9.4
6.6
4.4
2.3

FIGURE 16

Analysis of proviral DNA in high ffu titer-producing cell lines created by infection with EJV and JEV chimeric virus. Total cellular DNAs from four cell lines producing chimeric virus and which were known to contain single proviruses were analyzed. Cell lines EJV-1 (lanes 2,7 and 12) and EJV-2 (lanes 3,8, and 13) are producers of the chimeric virus EJV. Cell lines JEV-1 (lanes 4,9 and 14) and JEV-2 (lanes 5,10 and 15) JEV-2 are producers of the chimeric virus JEV. DNA (10 ug) from each line was cleaved with the restriction enzymes BamH1 (lanes 1-5); BamH1 and BstEII (lanes 6-10); and BamH1 and KpnI (lanes 11-15). Plasmid pEJ6.6 (50 pg) was mixed with 10 ug of NIH 3T3 DNA and then cleaved with the appropriate enzymes as markers (lanes 1,6 and 11). The endogenous murine fragments were not detected under the stringency used with a human ras specific probe. The fragments were analyzed by electrophoresis through both 2.4% agarose (panel A) and .8% agerose (panel B) to resolve different sized fragments. The digestions in Panel B were mistakenly done under low salt conditions resulting in BamHI* activity which created additional fragments. These obscure the identification of low molecular weight bands, however the sizes of the largest band can be determined.

experimental lanes in this blot. BamHI/BSTEII double digests yielded bands of 1.3, 2.6, and 0.6 kb for one EJV-infected DNA and 1.3 and 1.5 kb for the other. These were consistent with a loss of intervening sequences along with deletions at the 3' end of the sequence. These extra deletions could include the polyadenylation sites, but that need not be the case. The JEV-infected DNAs gave patterns of 1.3, 1.5, 0.7, and 0.6 kb; and 1.3, 1.5, and 1.5 (a doublet) kb (Figure 16). These, as before, indicated a deletion toward the 3' end of the gene. The first JEV provirus could have deleted the polyadenylation sequences, but the second provirus can only be interpreted in that way if one postulates that two independent 3' deletions occurred. There is no obvious reason why a reverse orientation insert should be more viable without a polyadenylation site, and this may well not be the case here. Both JEV proviruses maintained the third intron as well as the first intron assayed in the KpnI digest. The postulated deletions of the four proviruses are shown schematically (Figure 17). While the exact end points of the deletions have not been identified, the data shows that such deletions, and not splicing events alone, occurred in the regions shown.

3.9 Insertion of a Smaller ras Fragment into the Vector

The fact that a particular region of the ras insert was deleted in independent viruses might indicate that those sequences were deleterious to the virus. Alternatively, the deletions could have been selected purely on the basis of genomic size limitations. To test this, two more EJ c-Ha-ras/pZIP chimeras were constructed (Figure 18c and 18d). A 1 kb fragment from the extreme 3' end of the 6.6 kb EJ clone was removed in vitro before introduction into the pZIP retroviral vertor. The modified

Figure 17

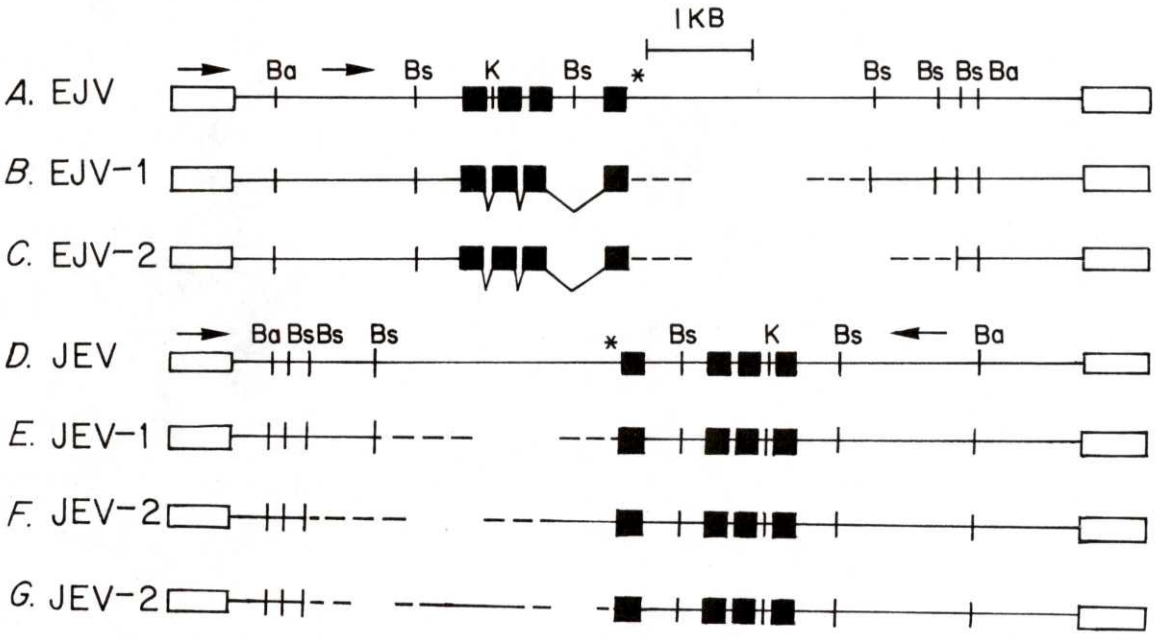

1KB
A. EJV
Ba
Bs
K
Bs
*
Bs
Bs
Bs
Ba
B. EJV-1
C. EJV-2
D. JEV
Ba
Bs
Bs
Bs
*
Bs
K
Bs
Ba
E. JEV-1
F. JEV-2
G. JEV-2

FIGURE 17

Deduced structure of EJV and JEV proviruses in high ffu titer-producing cell lines based on restriction analysis in Fig. 16. (a) The structure of the chimeric virus EJV. (b and c) The hypothesized structure of proviruses contained in the cell lines EJV-1 and EJV-2 infected with EJV. (d) The structure of the chimeric virus JEV. (e) Structure of the provirus contained in the cell line JEV-1. (f and g) Two alternative structures for the provirus contained in the cell line JEV-2. JEV-1 and JEV-2 were derived by infection with JEV. Open boxes represent the retroviral LTR sequences; closed boxes represent ras gene coding exons; arrows are used to indicate the approximate location of the relevant transcriptional regulatory regions and the direction of transcription; and the stars mark the location of the cellular polyadenylation signal sequences. These polyadenylation sites may have been deleted in all four cellular proviruses (b,c,e and g) although for this to occur in the JEV-2 provirus would have required two independent deletions. A simple derivation of a provirus consistent with the data for JEV-2 is also shown (f), but the size of the restriction fragments indicate that in this case the polyadenylation signal was retained. Hypothesized deletions resulting from "splicing" of RNA transcripts are diagramed by jagged lines, and other deletions by broken lines. Restriction sites are indicated wherever they were shown to exist. The following abbreviations are used for restriction sites: Ba (BamHI), Bs (BstEII), and K (KpnI).

Figure 18

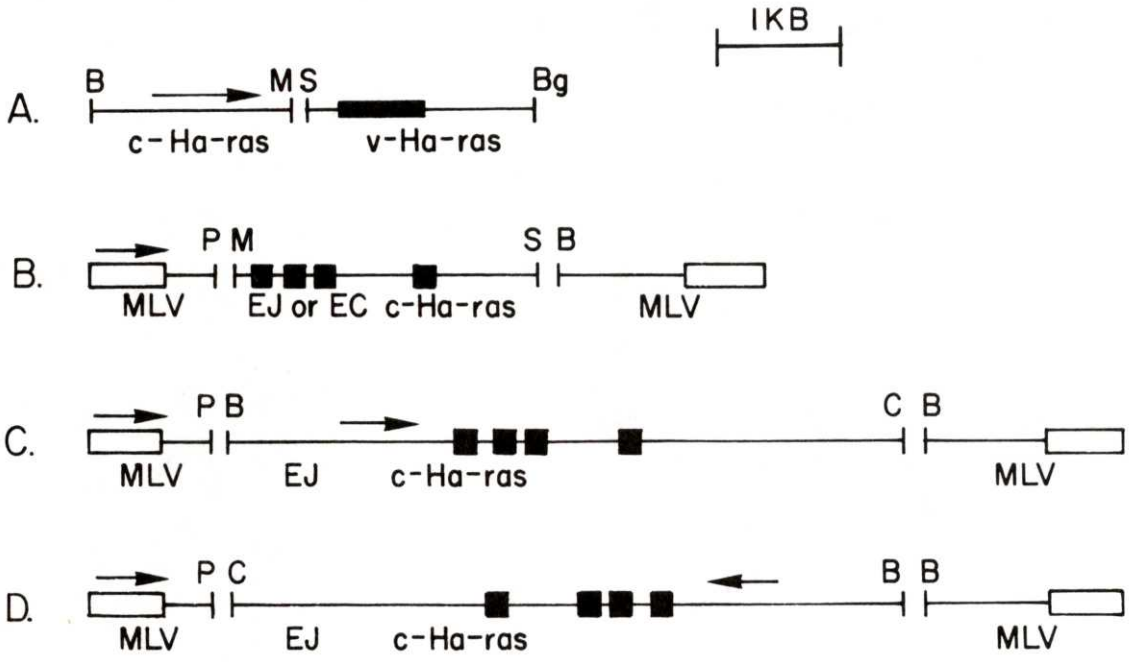

IKB
A.
B
M S
Bg
c-Ha-ras
v-Ha-ras
B.
P M
S B
MLV
EJ or EC
c-Ha-ras
MLV
C.
P B
C B
MLV
EJ
c-Ha-ras
MLV
D.
P C
B B
MLV
EJ
c-Ha-ras
MLV

FIGURE 18

Structure of constructs containing Ha-ras coding sequences. (a) The c-Ha-ras regulatory sequences used to drive the transcription of the HaSV-borne v-Ha-ras gene. (b) A transmissible retroviral vector carrying either the transforming (EJ) or normal (EC) c-Ha-ras coding sequences inserted so that the transcription of the ras genes is controlled by the high level retroviral LTR promoter. (c) The transforming (EJ) c-Ha-ras gene including the c-Ha-ras transcriptional promoter and coding sequences inserted into a transmissible retroviral vector in an orientation such that the cellular and viral LTR promoters initiate transcription in the same direction. (d) The same as in (c) except that the insert is in the opposite orientation relative to the retroviral sequences. The following abbreviations are used for restriction sites: B (BamHI), M (MstII), S (SacI), Bg (BglII), P (PstI), and C (ClaI). Nonhomologous ends were joined using DNA linkers. Open boxes represent the retroviral LTR sequences, closed boxes represent ras gene coding exons, and arrows are used to indicate the approximate location of relevant transcriptional regulatory regions and the direction of transcription.

insert, while considerably smaller, still contained the c-Ha-*ras* polyadenylation signals.

These clones were transfected into NIH3T3 cells and transformants were superinfected with MLV helper. Once again high titer stocks could only be obtained after passage, and the resultant proviruses contained deletions (data not shown). This suggests that specific sequences not in the c-Ha-*ras* coding or 5' regulatory regions are selected against in the production of virus.

While the deletions which appeared in the c-Ha-*ras*/MLV chimeras in the generation of highly transmissible varients were not fully characterized, the missing sequences appeared to lie outside of the p21 *ras* coding and 5' regulatory regions. The transduced c-Ha-*ras* constructs could, therefore, be used to further analyze the activation and biological potential of the oncogene.

CHAPTER 4

VIRAL AND SOMATIC c-Ha-ras ACTIVATIONS

4.1 Mechanism of Activation of the HaSV-Associated Oncogene

One of the motivations for the development of a retrovirus transducing the EJ c-Ha-ras gene stemmed from its use as a reagent to compare the EJ activated allele of c-Ha-ras with the v-Ha-ras oncongene transduced by Harvey sarcoma virus (HaSV). The activation of the EJ oncogene was shown to result from changes in the sequence of the gene, while the creation of the HaSV ras oncogene involved both recombinational events with a retroviral genome (Scolnick and Parks, 1974b; Stehelin et al., 1976), and sequence alterations within the ras protein-encoding region (Dhar et al., 1982).

Although arising in distinct fashions, the Ha-ras oncogenes of HaSV and EJ/T24 origin share a property in common: each carries a mutation in the codon specifying amino acid residue 12 of the 21,000 dalton protein known as p21 (Chapter 2; Reddy et al., 1982; Taparowsky et al., 1982, Capon et al., 1983a). These mutations cause the glycine 12 specified by the Ha-ras proto-oncogenes to be replaced by either arginine or valine. In the case of the EJ/T24 allele, the amino acid 12 substitution has been shown to be sufficient to activate the gene (Chapter 2; Reddy et al., 1982; Taparowsky et al., 1982; Capon et al., 1983a).

The above results might suggest that the activity of the two oncogenes derived ultimately from the same type of molecular event. However, the HaSV oncogene differs from its EJ/T24 homolog in two other, potentially important respects. First, the viral oncogene encodes an additional amino

acid substitution at residue 59 of the p21, causing the normally present alanine to be replaced by a threonine (Dhar et al., 1982). This replacement appears to be significant as it can be found in the p21 encoded by the independently arising Kirsten sarcoma virus (Tsuchida et al, 1982). Second, the expression of the HaSV p21 is driven by the high-efficiency transcriptional promoter present in the LTR (long terminal repeat) of the viral genome (Hager et al., 1979). This contrasts with the regulation of the bladder carcinoma oncogene, which continues to be controlled by the relatively weak promoter that regulates expression of the normal proto-oncogene. The high level expression of the viral gene assumes some importance, since others have reported that the association of a non-mutated p21-encoding DNA segment with the LTR promoter is sufficient, on its own, to create an oncogene (Blair et al., 1981; DeFeo et al., 1981; Chang et al., 1982).

To investigate whether the coding alterations in the HaSV-encoded p21 were sufficient to transform cells independent of changes in regulation of the gene, the HaSV-borne oncogene was attached to the cellular c-Ha-ras promoter (Figure 18a). This recombinant was transfected into NIH3T3 cells. An unaltered clone of HaSV (pH-1, a kind gift from Dr. E. Scolnick) (Ellis et al., 1980), as well as the clones of the EJ bladder carcinoma transforming allele (pEJ6.6), and the normal non-transforming cellular Ha-ras allele (pEC6.6) were transfected in parallel. While the normal c-Ha-ras clone did not produce any foci upon transfection, all of the remaining three constructs gave rise to morphologically indistinguishable foci at an efficiency of 5×10^4 ffu/ug DNA. Thus, the HSV-encoded ras gene is able to transform cells without relying on any additional regulatory advantages provided by the strong retroviral promoter. This

suggested that HaSV might transform by the same mechanisms as the cellular transforming allele in the EJ/T24 cell line.

These results bring into question the role of the highly efficient viral transcriptional promoter in the activation of the viral oncogene. Others had shown that this LTR promoter can activate an unmuted c-Ha-ras gene (DeFeo et al., 1981; Chang et al. 1982). The resulting transforming activity was demonstrated in a transfection assay that delivers a high copy number of the transfected cloned gene into a recipient cell. To test whether a single copy of the LTR-driven gene, as would be delivered by a virus, would also be adequate to transform a cell, we prepared a chimeric virus transducing the normal allele of c-Ha-ras such that its transcription was regulated by the viral LTR. Clones of both the normal and transforming alleles of c-Ha-ras were cut at an MstII restriction site that is located 9 bases 5' of the translational initiation codon AUG. These Ha-ras segments were then inserted into the DNA clone of MLV previously used as a retroviral vector (pZIP). The Ha-ras segments were inserted in such a way that we would expect transcription to be driven by the viral LTR (Figure 18b). For convenience, we used a normal c-Ha-ras clone, pP3, kindly provided by Drs. M. Goldfarb and M. Wigler (Taparowsky et al., 1982). This clone caried a sequence polymorphism that created a convenient restriction enzyme site. The resulting plasmids were referred to as pMLV(LTR)/EJ and pMLV(LTR)/EC.

Both the MLV(LTR)EJ and MLV(LTR)/EC chimeras were cotransfected into NIH3T3 cells with an SV40 linked neo gene which confers resistance to the drug G418 (Southern and Berg, 1982). Drug-resistant colonies were then selected. All of the MLV(LTR)/EJ transfected colonies contained cells that showed strong morphological transformation. Some of the MLV(LTR)/EC

colonies carried transformed cells while others did not. ^{35}S-methionine-labeled whole cell lysates were prepared from representative colonies of each type as well as from NIH3T3 cells transfected with the SV40-neo marker alone. These lysates were analyzed by immunoprecipitation with monoclonal antibodies directed against p21 and electrophoresis through a 15% polyacrylamide gel (Figure 19). Levels of p21 were found to be present above the background level of NIH3T3 cells (lane A) in those colonies transfected with MLV(LTR)/EJ (lane D), and in those non-transformed colonies transfected with MLV(LTR)/EC (lane C). Very high levels of p21 were present in morphologically transformed cells transfected with MLV(LTR)/EC (lane B). The p21 encoded by the EJ allele has been previously shown (above) to migrate with a slightly slower mobility than the normal variant. Thus the two constructs induce synthesis of biologically active proteins which to some extent are capable of transforming cells in transfection.

In order to passage the MLV(LTR)/EC and MLV(LTR)/EJ constructs as viruses, transfected cells were superinfected with MLV. Seven out of eight superinfected MLV(LTR)/EJ colonies produced focus-forming supernates with an average titer of 2×10^3. In contrast, no foci were observed in passage of virus rescued from 20 independent MLV(LTR)/EC lines. To determine whether those foci which arose during passage of MLV(LTR)/EJ stocks represented the transmission of the expected virus, the presence of an altered form of p21 was assayed in infected cells. In order to visualize the p21 produced in picked foci of MLV(LTR)/EJ infected cells, it was necessary to immunoprecipitate protein from isolated membranes rather than whole cell lysates. These membranes were prepared from approximately eight times as many cells as were used in analysis of the lysates of transfected cells. This alternative procedure was required because less protein is

Figure 19

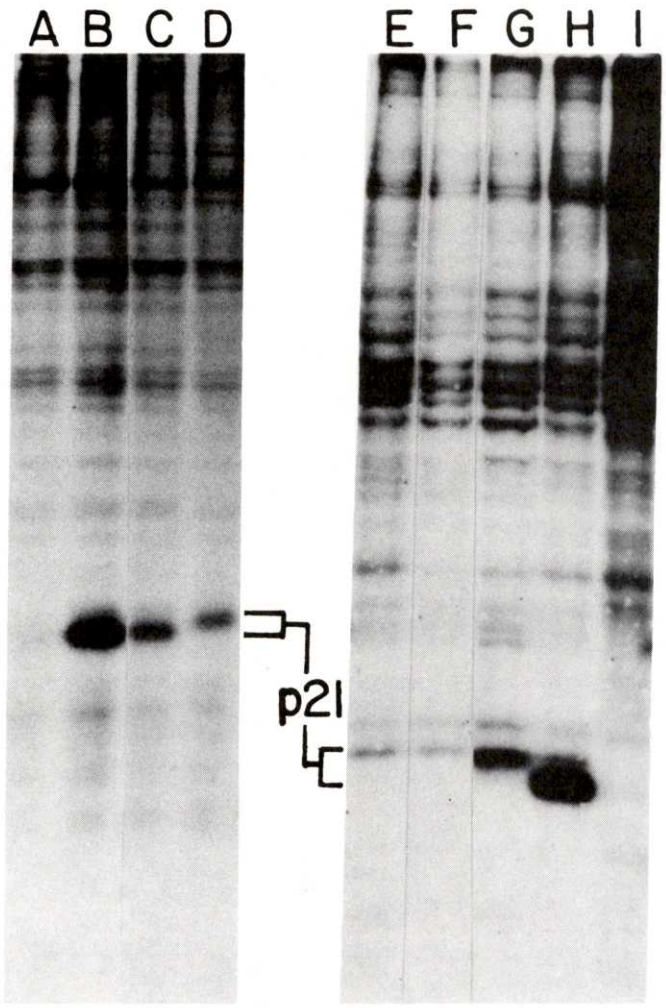


FIGURE 19

Immunoprecipitation of ^{35}S-labelled p21 from cell lines transfected or infected with various constructs. Lanes A-D are from whole cell lysates, lanes E-I are from partially purified membrane preparations consisting of 8X the number of cells used in the first four lanes; thus intensity of bands in the two panels can not be directly compared. The brackets indicate the location of the EJ transforming variant of p21 (upper line) and the normal cellular p21 variant (lower line) in each lane. (a) NIH 3T3 cells. (b) NIH 3T3 cells transfected with MLV(LTR)/EC: the normal allele of c-Ha-ras in a retroviral vector utilizing the viral LTR promoter. These cells were morphologically transformed after transfection. (c) NIH 3T3 cells transfected with the same construct, but not transformed by it. (d) NIH 3T3 cells transfected with MLV(LTR)/EJ: the transforming EJ allele of c-Ha-ras in a retroviral vector utilizing the viral LTR promoter. (e) NIH 3T3 cells infected with EJV, a chimeric retrovirus carrying the EJ c-Ha-ras coding sequences expressed off of the viral LTR promoter. (h) NIH 3T3 cells transfected with MLV(LTR)/EC and described in (c) which had been subsequently infected with JEV. (i) NIH 3T3 cells. Immunoprecipitations were performed with an anti-p21 monoclonal antibody, Y13-259, and resolved by 15% polyacryalmide gel electrophoresis.

produced in an infected cell containing only a single copy of the introduced ras gene than in a transfected cell which carries many acquired copies. This allowed p21 to be observed in EJ(LTR)/MLV cells (Figure 19, lane G) in amounts well above the level seen in NIH3T3 cells (Figure 19, lane I). Since no equivalent foci developed when infected with the virus transducing the normal c-Ha-ras allele, it can be concluded that the LTR-driven normal c-Ha-ras allele is unable to transform cells when it is present in the low copy number resulting from viral infectious events.

The number of acquired Ha-ras sequences in the lines was investigated by preparing cellular DNA from morphologically transformed cells cotransfected with pMLV(LTR)/EJ, morphologically transformed cells cotransfected with pMLV(LTR)/EC, morphologically normal cells cotransfected with pMLV(LTR)/EC and transformed cells resulting from infection with MLV(LTR)/EJ stocks. These DNAs were analyzed by restriction enzyme cleavage, electrophoresis through a 0.75% agarose gel and, after transfer to nitrocellulose filter, by hybridization to a Ha-ras-specific DNA probe (Figure 20). As expected, the DNA from transfectants contained a large number of Ha-ras hybridizing sequences (lanes A-C). There was no obvious difference in amount of Ha-ras hybridizating sequences in transformed (lane B) versus non-transformed (lane C) MLV(LTR)/EC transfected cells; however, as shown above, the transformed MLV(LTR)/EC cells contained a greater amount of p21 protein product than their non-transformed counterparts. In contrast to the transfected DNA, the infected DNAs of infected cells contained only a few acquired copies of the Ha-ras sequence (lane D). (The endogenous cellular Ha-ras sequences of mouse origin were not detected at this stringency of the hybridization with the human probe.)

Figure 20

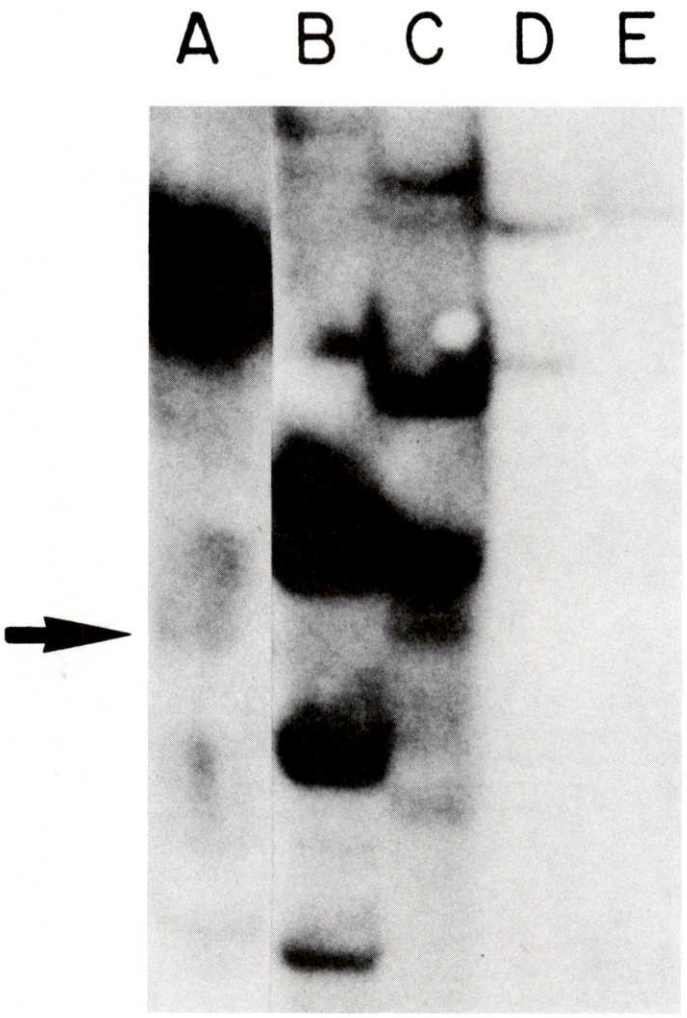


FIGURE 20

Analysis of Ha-ras sequences present in cellular DNA following infection or transfection of various constructs. (a) NIH 3T3 cells which were morphologically transformed by transfection of pMLV(LTR)/EJ. (b) NIH 3T3 cells which were transformed by transfection with pMLV(LTR)/EC. (c) NIH 3T3 cells which were not morphologically transformed following transfections with MLV(LTR)/EC. (d) NIH 3T3 cells transformed by infection with MLV(LTR)/EJ. (e) NIH 3T3 cells transformed by infection with JEV. DNAs were cut with the enzyme EcoRI which does not cut the MLV and ras sequences being analyzed and following electrophoresis through .75% agarose and transfer to a nitrocellulose filter, the DNA was hybridized to a nick translated human c-Ha-ras probe. Under the hybridization conditions used the mouse homologous sequences were not visible. The arrow indicates the approximate mobility of an 8.4 kb fragment, which would be the intact size of the infected and transfected constructs. Thus bands below the arrow mark reflect fragmentary sequences.

These results suggest several conclusions. First, the removal of the normal Ha-ras gene from its chromosomal environment and its affiliation with a viral genome is not sufficient to impart oncogenicity. Second, LTR-controlled transcription is not per se sufficient to impart oncogenicity to a normal Ha-ras gene. Instead, the normal Ha-ras sequence can elicit transformation only when it is expressed in extremely high amounts, much higher than those created by a single copy of an LTR-driven gene. From the immunoprecipitations shown in Figure 19 it can be estimated that morphologically normal cells can contain as much as fifty fold more of the normal cellular p21 than is required of the EJ specified p21 to transform cells. Cells transformed by the normal Ha-ras allele contain at least two hundred times that level found in some EJ p21 transformed cells. In the present case, such high amounts were achieved in the cells transfected with LTR driven Ha-ras genes. These transformed cells acquired multiple copies of the viral genome as a consequence of the transfection process. Infected cells, which acquired only one or several copies of the normal gene and linked LTR, were morphologically normal.

4.2 Mechanism of activation of the Cellular Oncogene

Because LTR-mediated regulation was not sufficient, on its own, to impart oncogenicity to the normal c-Ha-ras allele, the role of transcriptional regulation in the activation of the EJ c-Ha-ras oncogene required further exploration. The cloned EJ oncogene was able to transform NIH3T3 cells in transfection, but it was not clear whether it would be able to transform cells at the much lower level of expression achieved when driven by the weak cellular Ha-ras promoter and also delivered to cells in single copy. This was investigated using the EJV and JEV viruses described

above in which the mutated c-Ha-ras oncogene remained under the control of the normal cellular c-Ha-ras transcriptional promotor.

The transformants created by infection with the EJV and JEV viruses were indistinguishable morphologically. DNA was prepared from cells transformed by JEV infection. This DNA was analyzed by restriction enzyme cleavage, electrophoresis through a 0.75% agarose gel and, after transfer to a nitrocellulose filter, by hybridization to a Ha-ras specific DNA probe. As expected, the DNA of infected cells contained only single acquired copies of the Ha-ras sequences (Figure 20, lane E).

When membranes made from the EJV and JEV transformants were analyzed, they showed similar levels of p21 (Figure 19, lanes E and F), much below the level found in cells infected with the MLV(LTR)/EJ virus (lane G), although clearly detectable above the p21 level immunoprecipitated from membranes of uninfected NIH3T3 cells (lane I). The level is also below that of the p21 in the EJ/T24 bladder carcinoma cells, in which the Ha-ras encoded protein can be easily visualized from precipitates of whole cell lysates (see Chapter 2). Thus, linkage of the complete EJ/T24 oncogene with a viral LTR did not significantly increase its level of expression. This was true in both transcriptional orientations. In spite of the low level of p21 induced by these constructed viruses, strong morphological transformation was observed. Therefore, the point mutation carried by an Ha-ras oncogene appears sufficient to impart oncogenicity, even when the oncogene is expressed from a weak transcriptional promoter and is introduced into the cell in low copy number.

4.3 Interaction of the Oncogenic and Normal Ha-ras Alleles

These results showed that a low level of the EJ Ha-ras oncogene encoded protein could transform NIH3T3 cells. Because these cells express their own, endogenous normal Ha-ras alleles, this suggested that the introduced oncogenes behaved in a dominant fashion. The reagents provided by the above-mentioned experiments allowed this dominance to be directly tested by seeing whether the oncogene's dominance would still be displayed in the presence of a large excess in the normal, non-transforming gene and protein product. Cells which had previously been transfected with the normal c-Ha-ras clone driven off the retroviral LTR promoter, and which appeared morphologically untransformed, were infected with JEV virus. After infection, a great many transformed foci were observed. Such foci were picked and expanded.

When lysates of these transformed cells were analyzed for immunoprecipitable p21 in their membranes (Fig. 19, lane H), a great excess of the normal p21 protein was seen relative to the more slowly migrating transforming protein (> 20:1 ratio). This demonstrates the strength of the dominance of the oncogenic allele over its normal counterpart.

4.4 Comparison of the Biological Effects of the Viral and Cellular Oncogenes

While EJV, JEV, and HaSV were equally able to induce morphological transformation, it remained possible that other, more subtle phenotypic manifestations were affected by the remaining difference between the oncogenes: the altered residue 59 specified by HaSV. We thus assayed other biological effects of these various viruses by injecting them into 1 to 2 day-old Balb/c mice. A titer of HaSV comparable to the titers of JEV and

Figure 21

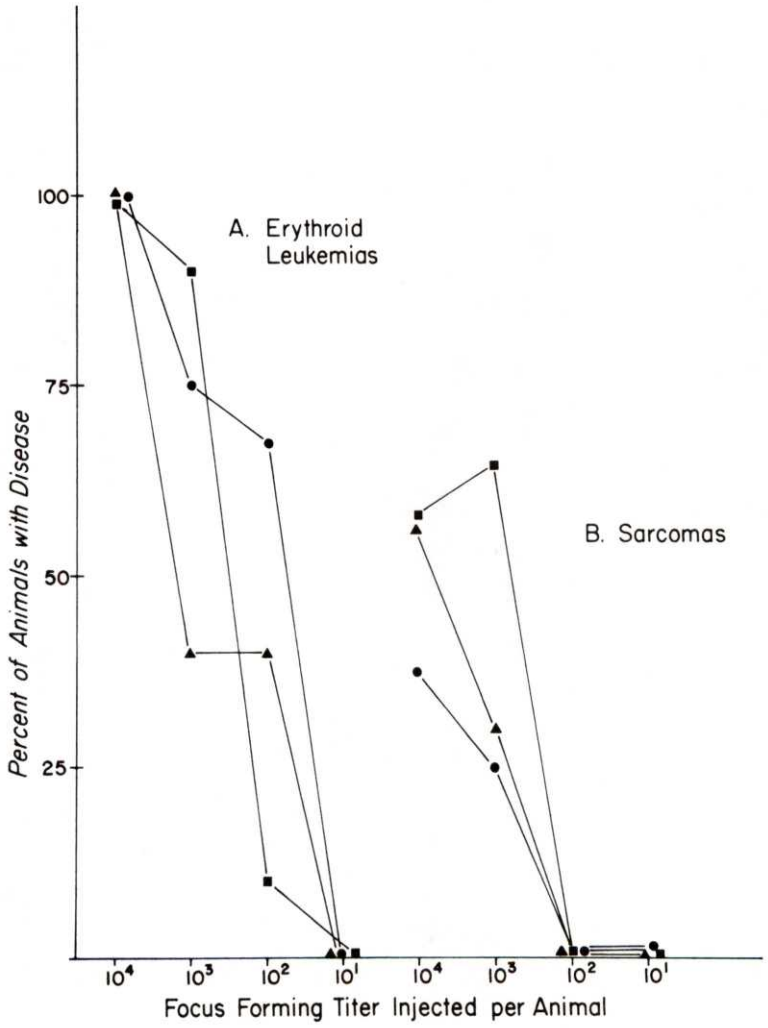

100
75
50
25
Percent of Animals with Disease
A. Erythroid Leukemias
B. Sarcomas
10^4 10^3 10^2 10^1 10^4 10^3 10^2 10^1
Focus Forming Titer Injected per Animal

FIGURE 21

Ability of Ha-ras viruses to cause malignant disease in newborn Balb/c mice. Approximately 10 mice were innoculaed with .1 ml of each dilution of .45 micron filtered virus stock. Undiluted titers were 5×10^5 ffu/ml in a focus assay on NIH3T3 cells and 10^6 pfu/ml in an XC assay for titer of helper virus (MLV). Mice were autopsied at death or after 4 weeks if they still survived. (a) erythroid leukemias. (b) sarcomas. ● HaSV. ■ EJV. ▲ JEV.

EJV was obtained by dilution. All of the viral stocks exhibited an MLV helper virus titer of 10^6 in an XC assay.

Various dilutions (see Figure 21) of each virus stock (EJV, JEV, HaSV) were filtered through a 0.45 micon filter and innoculated intraperitoneally. Approximately 20 animals received each dilution of virus. Animals began to die after 3 weeks and were autopsied. All remaining animals were sacrificed at 4 weeks. As shown (Figure 21A), all three transforming virus stocks (HSV, EJV and JEV) gave rise to an erythroleukemic disease that was manifested by a grossly enlarged spleen which was readily palpable 3 weeks after innoculation. In a number of animals, the enlarged spleen ruptured causing death. This rupturing was observed in animals infected by any of the three viruses. The spleens contained both dark colored nodules full of blood, and pale ill-defined foci previously described by others as being erythroblastic foci (Harvey, 1964; Chesterman et al., 1966). The foci induced by all 3 viruses were of similar appearance macroscopically and microscopically. All three transforming viruses also gave rise to small solid tumors (sarcomas) at a lower incidence than leukemia (Figure 21B).

Similar titers of EJV, JEV and HSV were required to observe each disease. When the helper virus, MLV, was injected by itself, no pathology was observed during the time of the experiment. Tumors caused by each of the three transforming viruses were minced and applied to dishes with fresh medium. After 24 hrs, media were harvested, filtered, and used to infect NIH3T3 cells. All three stocks gave rise to foci of the transformed cells, indicating that transforming virus was indeed present in each tumor.

Thus, the two Ha-ras oncogenes behaved similarly in this test of biological activity in spite of their different modes of regulation, levels of expression, and encoded p21 proteins.

CHAPTER 5

DISCUSSION

5.1 The Activation of c-Ha-ras in the EJ Bladder Carcinoma Cell Line

Use of gene transfer and molecular cloning had previously made it possible to localize an important lesion of the EJ bladder carcinoma genome to a 6.6 kb DNA segment constituting only 10^{-6} of the genetic content of the tumor cell. The segment appears to contain the entire transforming activity previously associated with the whole tumor cell DNA. This oncogene is an important, although likely not the sole, determinant of the transformed phenotype of these carcinoma cells. Sequence hybridization showed that this oncogene was related in structure to a similar sequence existing in the normal cellular genome (Goldfarb et al., 1982; Shih and Weinberg, 1982; Pulciani et al., 1982, Der et al., 1982). It appeared that the oncogene arose from the activation of these normal sequences, and this activation occurred during the process of carcinogenesis which led to formation of the original EJ carcinoma. Experiments described in this thesis were designed to resolve and define the critical differences that distinguish the oncogene from its proto-oncogne precursor.

Since it was not possible to obtain normal tissue from the patient who gave rise to the EJ bladder carcinoma cell line, comparison of a full nucleotide sequence of the EJ c-Ha-*ras* oncogene with that of a normal human c-Ha-*ras* allele would be likely to unveil a number of polymorphic differences. Most of these would be expected to be functionally silent and not involved in the activation of the oncogene. For this reason a series of *in vitro* recombinants were generated to localize the region in which the

activating lesion had occurred. Only after the functional segment was narrowed were the sequences of the two alleles compared.

The sequence difference observed in this region was a single point mutation which could have only one consequence for the structure of the p21 protein - a simple amino acid substitution leading to the replacement of a glycine by a valine residue at position 12.

There are three nucleotides which encode the altered glycine at amino acid position 12. The last codon position is totally degenerate; however, any of the possible six nucleotide substitutions at the first two codon positions change the encoded amino acid. All of these substitutions at position 12 (ser, arg, cys, asp, ala, and val) have been shown to be oncogenically activating (A. Levinson, unpublished results). It is perhaps not coincidental that the glycine to valine change observed in the bladder carcinoma oncogene was caused by a G-T transversion, precisely that mutation favored by many suspected bladder carcinogens (Swenson and Kadlubar, 1981).

This substitution appears to represent the critical agent of the conversion of the proto-oncogene into an active oncogene. Such a simple substitution might not be expected to effect a profound change in the function of the p21 protein. However, identities of the particular amino acids involved may shed some light on this problem. Glycine is replaced by an amino acid having a relatively bulky side chain. Glycine is an anomaly among the 20 amino acids because it lacks a side chain. It is therefore able to participate in extremes of bending and folding of the polypeptide backbone and is the strongest breaker of alpha-helices of any amino acid (Cantor and Schimmel, 1980). Thus, replacement of glycine by valine represents an abrupt change in the local stereochemistry of the protein.

The loss of glycine at residue 12 may thus cause a significant change in an essential domain of the p21 protein. One consequence of this change may be a conformational shift of the protein, leading in turn to the observed aberrant electrophoretic migration. Processing of the p21 proteins could also be affected by the amino acid replacement and hence be responsible for the altered migration, although no such change in processing has been discovered. A second, more important consequence of the substitution is a profound effect on the function of the p21 protein. This alteration probably affects interaction of the p21 with cellular targets. Precedents exist for the single amino acid changes having profound effects on cellular and organismic physiology. The most well known of these is the sickle-cell syndrome, in which a glutamine to valine conversion affects the solubility of hemoglobin within erythrocytes.

In the case of sickle-cell anemia, the molecular consequences of the nucleic acid change are well understood at the level of the functioning of the encoded protein. This is far less true for p21. The physiological role of p21 is not yet clear. The discovery of homologous genes first in Drosophila (Shilo and Weinberg, 1981b) and then in yeast (DeFeo-Jones et al., 1983; Gallwitz et al., 1983) opened up the possibility of genetic analysis, which may soon shed some light on the protein's function. While the function of p21 is not known, a number of its biochemical properties have been identified.

As discussed earlier, monoclonal antibodies have been raised against mammalian ras p21 (Furth et al., 1982). These were used to study the properties of the protein. Mature p21 is located on the inner surface of the plasma membrane (Willingham et al., 1980). The protein is acylated post-translationally, which is hypothesized to serve as an anchor to the

membrane (Sefton et al., 1982). Finally, there are guanine nucleotide binding and autophosphorylating activities associated with the HaSV virally encoded p21 (Shih et al., 1980).

The autophosphorylating activity is not present in the c-Ha-ras p21s, as discussed earlier, because the cellular p21s lack the threonine that serves as the phosphate acceptor in the virally encoded protein. The donor of the phosphate in the phosphorylation reaction is GTP. The observed reaction could either represent a fortuitous substrate utilization by a p21 kinase activity which normally acts on other proteins, or a trapping of an intermediate of a p21 GTPase enzymatic activity. In either case, the presumption is that the enzymatic activity is present in the cellular p21 proteins even though it cannot be monitored in the absence of threonine 59. Unpublished reports indicate that this is the case, since in vitro mutation of the 59th amino acid of c-Ha-ras to threonine results in a p21 which undergoes autophosphorylation (E.M. Scolnick, unpublished result). This activity has been tied to the transforming activity of the oncogene because an amino acid 59 threonine mutation is sufficient to activate the c-Ha-ra proto-oncogene (D. Lowy, personal commuciation) and because other activating lesions, at amino acid position 12, have been shown to up-modulate the autophosphorylation activity at position 59 (Gibbs et al. 1984).

Unlike autophosphorylation, GTP binding is a property of both viral and cellular p21. This has been used as an assay in the purification of the proteins (Papageorge et al., 1982). With the purified proteins a p21 GTPase activity can be directly detected (E.M. Scolnick, unpublished result).

When the activating lesions in p21 were known, there were immediate attempts to relate the activating nucleotide changes to the known biochemical properties of p21. Amino acid 12, the site of the first identified p21 activations, lies in a putative GTP binding site (G. Otto, unpublished analysis; Wierenga and Hol 1983). Three-dimensional models were developed which explained how an amino acid 12 alteration would change nucleotide binding. However, as will be discussed below, substitutions of amino acids 61 or 59 cause functionally identical oncogenic activations of p21. These activations could not be explained by the binding site models as originally proposed. The possibility of a second GTP binding site on p21 was also suggested (G. Otto, unpublished analysis). However, biochemical studies have shown that the transforming and normal cellular p21 varients have similar GTP binding affinities and total GTP binding capacities (C. Tabin, unpublished results; Finkel et al. 1984a). This, along with the modulation of the autophosphorylation discussed above, might suggest that it is a GTP hydrolysis step which is altered in oncogenic activation. This speculation is substantiated by very recent reports that the GTPase activity which can be detected in purified normal p21 is reduced more than five-fold in transforming varients of p21 (A. Levinson, unpublished result; E.M. Scolnick, unpublished result).

5.2 Other ras Activations

Since the identification of the activating mutuation in the EJ/T24 c-Ha-*ras*1 allele, a number of other naturally arising activations have been identified for Ha-*ras* , Ki-*ras* , and N-*ras*. By far the majority of these involve a similar mechanism, that is a point mutation in a coding region. These have not, however, all occured at amino acid 12.

The first example outside of that site was of a human lung carcinoma derived c-Ha-ras activation at the codon specifying amino acid 61 (Yuasa et al., 1983). Examples of amino acid 61 alterations activating N-ras and Ki-ras soon followed (Taparowsky et al., 1983; Nakano et al., 1984). Substitutions at these two sites (12 and 61) are the most prevalent ras gene activations. However, other amino acid changes can also activate the genes. For example, amino acid 59 changed from alanine to threonine is as effective a way to form an Ha-ras oncogene as changing glycine to valine at position 12 (D. Lowy, personal communication). The relative number of activations at different sites which are observed may in part reflect the comparative target sizes rather than differences in effect. It has been reported that researchers at Genentech have succeeded in activating the Ha-ras gene by at least 18 different amino acid 12 substitutions. It may be that at position 59 only a threonine substitution will succeed in activating the gene. This explanation can be rationalized on the basis that amino acid 12 changes likely act by conformation shifts, while the substitution at 59 may activate by trapping an intermediate phosphate which can only occur when threonine is introduced at this position.

Other kinds of mutations, such as small deletions and insertions around amino acid 12 introduced in vitro can oncogenically activate the ras genes (R. Chipperfield, personal communication). The absence of such examples from naturally occurring tumors is likely a reflection of the relative likelihoods of point mutations as compared to in-frame insertions and deletions.

The rate at which point mutations occur spontaneously is in fact, quite high. The frequency of base substitutions in preproinsulin has been calculated at 7×10^{-9} substitutions per nucleotide site per year (Perler

et al. 1980) and for globin a very similar 6 x 10^{-9} per nucleotide per year (Miyata and Yasunaga, 1981). Thus a healthy individual with 10^{12}-10^{13} cells would be expected to carry activated oncogenes in thousands or tens of thousands of cells yet no tumors are apparent. One explanation for this paradox could be that only a small subset of an individual's cells are actually susceptible to the action of oncogenes. Other possible resolutions could lie in the requirement for several "hits" to create a full tumor cell phenotype, and in an organism's responses (e.g., immune surveillance) which prevent or limit tumor growth.

While almost all ras activations observed in vivo involve point mutations, it was noted earlier that ras gene overexpression is an alternative mechanism which can also act to transform cells (DeFeo et al. 1981). This mechanism is implicated in the instance of at least one tumor: the mouse adrenocortical tumor cell line Y1. In that line, the c-Ki-ras gene is amplified, being present both in double minute chromosomes and in a homogenously staining region (Schwab et al. 1983). These cells exhibit a corresponding elevation in Ki-ras specific RNA and protein. The fact that this mechanism is less frequently observed may come from either of two causes. It may be that such activations involving amplification are quite prevalent. However, since multiple copies on independent double minute chromosomes or even existing in vast tandem arrays are unlikely to be co-transfected, such examples have gone undetected. Alternatively, it may again just be easier to create a point mutant then a substantial amplification, and so overexpression activation may in fact be quite rare.

The fact that two different mechanisms, mutation and amplification, can both activate the same gene must be taken into account in attempting to sort out the function of the oncogene protein. As discussed above, the

point mutations that activate the ras genes may act to decrease the rate of hydrolysis of GTP bound to p21. Thus, a cell carrying a mutated ras gene would contain more GTP-p21 and less hydrolyzed-GTP-p21. An amplified ras gene would not perturb the rate of hydrolysis of the encoded p21. However, a cell with such a lesion would, like a cell with a ras gene activated by point mutation, contain more GTP-p21, although, in this case, more hydrolyzed-GTP-p21 as well. If the transforming effect derives from a critical level of GTP-p21, then the equivalance of the two modes of oncoganic activation becomes understandable.

5.3 Activation of the v-Ha-ras Oncogne

A survey of the genome of HaSV might suggest that the Ha-ras oncogene it carries was activated by both of these mechanisms: overexpression and mutation. However, experiments presented above demonstrated that the level of overexpression provided by the viral transcriptional control is insufficient to transform a cell. Moreover, the coding alterations contained within the ras gene were shown to be capable of oncogenically activating the gene without overexpression.

The HaSV v-Ha-ras oncogene differs from its normal cellular homologue at two coding positions, specifying amino acids 12 and 59. Since the EJ c-Ha-ras oncogene also carries an amino acid 12 substitution, one might speculate that the mutation affecting residue 12 of the v-Ha-ras encoded protein may have been already sufficient to impart oncogenicity to HaSV. The other changes suffered by the viral Ha-ras, association with an LTR and mutation at residue 59, would then appear to be of secondary importance.

However, work in other laboratories, while confirming that the residue 12 valine substitution can potentiate both sarcomas and erythroleukemias,

has indicated that the amino acid 12 arginine substitution found in the HaSV is only able to cause sarcomas in the absence of an additional alteration at residue 59 (D. Lowy, personal communication; E. Chang, personal communication). The arginine mutation would appear to be a weaker oncogenic allele in their hands. The arginine alteration is also less potent then the valine change in recently reported biochemical experiments where the valine substitution shows greater up modulation of viral p21 phosphorylation (Gibbs et al., 1984).

The results shown above nonetheless demonstrate the functional equivalence of c-Ha-ras gene altered by the single mutation found in the EJ/T24 bladder carcinoma cell line and the double mutation present in the homologous gene of Harvey sarcoma virus.

The HaSV borne v-Ha-ras is not the only case of the accumulation of multiple oncogenic alterations in the activations of viral ras oncogenes. The ras genes transduced by HaSV and KiSV both contain activating mutations at amino acid 12. As discussed, at least the arginine substitution borne by HaSV is less than optimally activating. The HaSV and KiSV oncogenes both also carry the strongly activating threonine 59 substitution. One could postulate a sequence of events in the generation of these viruses in which first the proto-oncogenes were brought under the control of the viral LTR promoter. This would lead to an overexpression of p21 which could itself be very weakly transforming. The growth enhancing capacity of the viruses would be furthered by the subsequent acquisition of the lesion at amino acid 12. When that activation was less than optimal there would be a still greater selective advantage for a variant sarcoma virus which acquired the threonine 59 change. Other ras transducing viruses (BaSV and RaSV) only have substitutions at amino acid 12. This may

indicate that the substitutions they carry at that position are stronger activators of the gene, and thus there is no selection for further alterations. The progression of the viruses would be facilitated by the extraordinarily high mutation rates observed for retroviruses (Goff et al. 1981b; Darlix and Spahr 1983). It is possible that multiple "hits" could occur within a single ras oncogene in the progression of nonviral cancer. The likelihood of such an occurance would be increased when the first hit involved amplification such as that observed for K-ras in the Y-1 cell line discussed above. Amplification would lead to over expression of p21, but also increase the target size for subsequent point mutations.

5.4 Activation of ras Oncogenes during Tumorigenesis

Before localizing the ras lesions to any particular step of carcinogenesis one must address the question of whether or not ras activations are peculiar to cancer cells at all. The supposition throughout in this thesis has been that ras activations represent somatic mutational events. However, formally the activated oncogenes could pre-exist in the germline of some individuals, perhaps indicating a hereditary predisposition to cancer, or that oncogenes are not involved in carcinogenesis at all. The facts that DNA prepared from normal cells does not give rise to foci in transfection assays, and that in some systems normal cells can be chemically transformed in vitro concomitant with oncogene activation, have indicated that oncogenes are indeed transformation-specific. However, direct proof of somatic mutation required comparison or normal and tumor tissue from the same patient with a naturally arising cancer. Such evidence now exists for all three ras oncogenes. Ki-ras activation in lung carcinoma (Santos et al. 1984) and

ovarian carcinoma (Fieg et al. 1984); N-ras activation in an acute myeloblastic leukemia (Gambke et al. 1984) and Ha-ras activation in bladder and renal carcinomas (Fujita et al. 1984) all occurred in malignant but not normal tissue of the same patient.

There is very little data concerning the stage in which the oncogene activations occur. Part of the difficulty in obtaining such information lies in the fact that the earliest stages of carcinogenesis are not observable, and even truly malignant growths are not usually detected until they have progressed to a palpable size. For this reason, animal models would seem preferable in probing the timing of ras hits. One group has investigated ras activation in a two step, carcinogen-tumor promoter system. External treatment of either of two different mouse strains with the carcinogen dimethylbenzathracene (DMBA) induces benign skin papillomas. These papillomas can then be induced to form carcinomas by treatment with TPA. Both the carcinomas and the papillomas contained Ha-ras oncogenes which were active in DNA transfections (the normal tissues of these mice, again, did not contain such transforming activity) (Balmain et al., 1984). The transforming efficiencies of all papilloma DNA samples were at least as high as those of the primary carcinomas, indicating that the ras genes were fully activated at that early step. Thus ras activation can perhaps act as the initial event in carcinogenesis.

On the other hand, investigators studying a human metastatic melanoma compared the transforming activity of DNAs prepared from five independent metastases from the same patient. Comparison of marker chromosomes demonstrated that the five samples were clonal in origin. Yet only one of these contained an activated N-ras allele (Albino et al., 1984). Their interpretation was that this indicated that ras activation was neither

involved in initiation nor in maintenance of the tumor but instead was a manifestation of tumor heterogeneity. They point to the fact that cancer cells have high genetic instability and extensive chromosomal arrangements and aneuploidies as causes of the activation. Thus oncogenes would be results rather than causes of transformation.

I would propose two alternative explanations, more consistent with the weight of the evidence presented throughout this thesis. First, N-ras activation could have been a vital early step in the genesis of this melanoma. Later additional alterations may have rendered the ras oncogene superfluous and it may then have been lost in four out of the five lines in the processes of chromosomal rearrangement which they cite. Second, and perhaps more likely, I would suggest that the N-ras activation did indeed occur in just one metastic line. However, far from being insignificant, I believe that the activation would have given that metastasis a further growth advantage had it been allowed to continue in vivo . Indeed it may have been key to that clone's progression to a metastatic state. The other four metastases could have acquired other genetic alterations which allowed them to progress similarly, but those other four genetic lesions may not have involved the activation of detectable oncogenes. This is not unreasonable, since only one out of ten tumors is found to possess oncogenes that can be assayed in DNA transfection, although all tumors presumably contain mutations which participated in their development.

Taking the melanoma and papilloma studies together it would appear that ras oncogenes are not specific for any one stage of carcinogenesis. Rather they seem to be one of a number of ways of relaxing growth control which accumulate during the progression of a tumor. Whenever they arise they give a subclone of a tumor a selective advantage. The timing of their

activation may be influenced in part by the particular carcinogenic agents a target tissue or tumor encounters and the time at which such an encounter occurs.

In this regard, there is considerable evidence that a particular oncogene can be repeatedly activated by a given carcinogen. In experimental systems Ki-*ras* is always the oncogene activated when 3-methylcholanthrene is used *in vitro* to transform mouse fibroblasts (Parada and Weinberg, 1983; Eva and Aaronson, 1983b). Ha-*ras* is activated by 3-methylcholenthrene, benzo[a]pyrene and N-methyl-N-nitro-N-nitrosoguanidine in *in vitro* transformation and by diethylnitrosamine in *in vivo* transformation of guinea pig fibroblasts (Sukumar et al., 1984). Ha-*ras* is also activated in mouse skin carcinomas induced *in vivo* with dimethylbenzathracene (Balmain and Pragnell, 1983). Induction of rat mammary carcinomas by nitrosomethyl urea is still another example of reproducible Ha-*ras* activation. That these regularities are dependent upon the activating agent is most clearly shown in the case of *in vivo* induced mouse lymphomas where tumors caused by injection of the carcinogen N-nitrosomethylurea contained activated N-*ras* oncogenes, and lymphomas of identical phenotype induced by gamma radiation had activated Ki-*ras* genes (Guerrero et al., 1984).

5.5 Retroviral Vectors

In the course of investigating the activation of *ras* oncogenes, it was useful to develop a mammalian vector based on retroviruses. Since the pZIP vector was developed for a particular purpose, all of the potential uses of retroviral vectors were not explored. Nonetheless, certain advantages and disadvantages of this system became apparent.

Techniques which allow the transfer of DNA into higher cells are the key to many studies of the function and regulation of eukaryotic genes. A number of methods have been employed to deliver DNA into cells, such as the uptake of calcium phosphate precipitates of naked DNA (Graham and van der Eb, 1973), the transfer of metaphase chromosomes (Miller and Ruddle, 1978), and the fusing of DNA-carrying liposomes with cells (Dimitriadis, 1978). These techniques suffer from several weaknesses, including the lack of retrievability of transferred DNAs, low efficiencies of transfer, and an uncertainty concerning the physical state of the DNA which has entered the cells.

An alternate approach which overcomes many of these problems has been the use of animal viruses as vectors to mediate the transfer of DNA sequences into cells (Ganem, et al., 1976, Goff and Berg, 1976). A segment of DNA is inserted into a viral genome in the place of certain viral sequences. As a consequence, certain virus-encoded functions are lost, and the chimeric virus is rendered replication-defective. Such chimeric genomes can be rescued from cells by co-infection of the cells with a competent virus. The competent helper virus can supply, via complementation, the gene products not made by the chimera. Using such a strategy, many cells can then be infected with the chimeric transducing virus. In principle, the chimeric genome may become established in the cell in a molecular configuration which reflects closely that seen when studying cells infected by replication competent, helper virus. In addition, viral genomes may provide transcriptional promoters, splice sequences, and other regulatory sequences which can be used to modulate the expression of associated genes (Hamer and Leder, 1979; Mulligan et al., 1979; Mulligan and Berg, 1980).

The viral vectors which utilize the genomes of simian virus 40 (SV40) (Goff and Berg, 1976) and bovine papilloma virus (Sarrer et al., 1981) share a disadvantage with other DNA transfer techniques that is especially relevant to the oncogene studies reported here: namely, they are not well suited for use in most laboratory animals, such as mice. In addition, the replication-competent helper viruses that are required in these cases may oncogenically transform the cells which they infect, thus limiting the general utility of these viruses as vectors. Finally, the use of SV40 vectors in cells permissive for SV40 replication culminates in the death of the host cells.

Murine retrovirus vectors represent an attractive alternative to papovavirus vectors. They are readily introduced into tissue cultures and laboratory animals. Disease induced by the replication-competent helper virus generally has a long latency period. This allows ample time to study the effects of transduced genes before any helper-induced disease intervenes. The virus grows to a high titer within the animal and infects a variety of cell types. Productive retrovirus infections do not result in the death of infected cells. Moreover, once cells are infected by retroviruses, the viral genome is efficiently inserted into a chromosome of the host cell. Such integration is important, since it assures the retention of the introduced sequences in the recipient cell and its descendents. Finally, up to 7.5 kb of non-retroviral coding information can be packaged (Goff et al., 1981a), allowing room for more than one gene to be inserted into a given vector.

To demonstrate the utility of the retrovirus vector system, the herpes thymidine kinase gene (tk) was first inserted into Moloney leukemia virus (MLV). The tk gene carries its own transcriptional promoter. This

leaves the MLV promoter free for subsequent use with a second gene.

The site of insertion of the tk gene was arbitrarily chosen based upon the presence of convenient of restriction sites. The only retroviral genetic elements which were intentionally preserved were the LTR regions at either end, which are required for initiation and termination of genomic RNA transcripts as well as for reverse transcription, and the psi sequences, which are cis acting packaging signals encoded near the 5' end of the viral genome (Linial et al., 1978; Watanabe and Temin, 1982; Mann et al., 1983).

Others have also created a chimeric retrovirus transducing the HSV tk gene (Wei et al., 1981). Their method of construction involved linking the tk gene to the 5' end of murine sarcoma virus and then relying on in vivo recombination with wild-type virus to restore the 3' end. Retrovirus transcripts missing 3' sequences are known to be packaged in particles, and such recombination is known to occur, likely during reverse transcription in cells co-infected with helper virus (Goldfarb and Weinberg, 1981; Linemeyer et al., 1980). Such a strategy may prove useful in transducing genes which can be selected, but whose structure is ill-defined. The construction described here did not depend upon this form of recombination because the MLV molecular clone from which our chimeras were derived included both 5' and 3' sequences.

The inserted gene, the HSV tk gene, was chosen for the ease of its selection in recipient cells. The successful transmission of tk indicates that MLV can serve as a flexible vector for the transfer of a wide variety of sequences.

To further test the flexibility of retrovirus vectors, chimeras were constructed carrying the insert in two opposite orientations relative to

the MLV vector. While the structures of the resultant viruses were not directly verified, both orientations appeared to be equally active in both transfections and infections of cells. Thus, the retrovirus vector system has an additional degree of freedom, since some foreign DNAs may be inserted in different orientations without preventing the expression of the vector or of the inserted gene.

The work with the tk gene did not take full advantage of all of the MLV sequences which could be exploited in chimeric genomes. The HSV tk fragment introduced into the MLV vector contained its own transcriptional promoter. Some of the Ha-ras chimeras, however, were constructed such that the ras sequences were detached from their own promoters and depended upon the promoter of the vector. Other work has shown that the retrovirus promoters can be used in conjunction with many exogenous cloned genes. For example, the MLV promoter (Murray et al., 1983b) and the mouse mammary tumor virus promoter (Lee et al., 1981) have been utilized to drive the transcription of a cloned cDNA, dihydrofolate reductase; and others have used MLV promoters to transcribe clones of cellular DNA sequences homologous to those picked up by sarcoma viruses (Blair et al., 1981). MLV also contains a splice donor site near its 5' end. In principle, this can be exploited in conjunction with the MLV promoter to direct the synthesis of a spliced subgenomic RNA whose sequences include those of an inserted gene. This could be used to force the MLV promoter to drive the transcriptions of two independently inserted foreign genes. Retroviral vectors making use of these properties have been constructed in other laboratories (Cepko et al., in press).

Another aspect of the flexibility of retrovirus vectors derives from the range of different-sized RNAs which can be accommodated by the virions.

This size flexibility was not tested here as the inserted tk fragment was, by coincidence, of the same length as viral DNA segments which it replaced and the ras chimeras spontaneously deleted sequences for unknown reasons. The limits on packageable size are not known. Studies in this laboratory, however, have shown that RNA genomes as small as 3.4 kb (Goldfarb and Weinberg, 1981) and as long as 9.8 kb (Goff et al., 1981a) are packageable and transmissible.

Some of the deletions observed with the ras-MLV chimeras could have been generated by processing of the full length RNA transcripts using the normal ras splice signals. Others have shown that introns are lost in passage of retroviral vectors containing cellular inserts (Shimotohono and Temin, 1982). However, this alone could not explain the deletions observed with the Ha-ras insert.

Experience of others with recombinant retrovirus constructions has indicated that success in passaging a given chimeric virus intact cannot be predicted from the information we now possess. For example, others have found that the HSV tk gene cannot be successfully passaged in a particular retroviral vector unless the tk polyadenylation signals are deleted either in vitro or in vivo (Shimotohono and Temin, 1981). The retroviral vector they used was based upon the avian non-defective spleen necrosis virus (SNV). When a second tk gene, this one from chickens, was placed in the same vector, the polyadenylation sequences of that gene did not inhibit virus production. However, when the promoters of the LTR and chicken tk gene were in the same orientation, deletions were observed in both the SNV sequences and the insert. A high titer of virus was produced, but it had a low tk^+ enzymatic activity. In the reverse transcriptional orientation the viral tk^+ activity was high, but the virus production was considerably

lower. This low level of virus production could be increased by removing the chicken tk promoter. As expected, no loss of introns was seen in the construct where the tk gene was in the opposite orientation from the viral sequences (Bandyopadhyay and Temin, 1984).

From the reported work to date on retroviruses used as vectors, it can be concluded that a number of problems may arise depending on the insert, vector, and site of insertion. These include: (in the same transcriptional orientation) (a) interference by inserts containing polyadenylation signals which may cause premature termination of chimeric-virus genomic RNA transcripts; (b) splicing of introns in the insert with varying kinetics (Shimotohono and Temin, 1982; Cepko et al., in press); (c) splicing to and from cryptic sites not normally used by the genes in the construct (Tabin and Weinberg, unpublished data; R. Prywes, personal communication; C. Cepko, personal communication); and (in the reverse transcriptional orientation) (d) interference between RNA synthetic machinery operating on the same DNA template in the opposite directions and (e) again cryptic splice signal utilization. Whether or not these problems will arise in any given case will depend on the existence of cryptic regulatory signals such as splice sites in the inserted sequences, the relative strengths of the viral and inserted regulatory signals and the level of successful transcripts required to observe whatever phenotype is being monitored.

In spite of the problems listed above, many of which can be overcome by utilizing alternative constructions, retroviral vectors are powerful tools for the insertion of genes into mammalian cells or into laboratory animals. In the present case they were used to help to elucidate the activation and range of action of the Ha-ras oncogene. Variants of this oncogene have been important determinants in the development of both viral and non-viral neoplasia. The point mutation implicated as the central

event in the creation of the oncogene represents the first demonstration of a lesion in cellular DNA whose occurrence is directly related to the carcinogenic process.

CHAPTER 6

MATERIALS AND METHODS

6.1 Cells and Viruses

NIH3T3 fibroblasts were maintained in Dulbecco modified Eagle medium containing 10% calf serum as described (Andersson et al., 1979). Normal bladder epithelial cells, strain HB1-5, were secondary and tertiary passage cultures derived from explants of a 5-month fetal human bladder. The cells were cultured as described (Wu et al., 1982). Ltk^- $aprt^-$ cells were a gift from S. Silverstein. These cells were grown in Dulbecco modified Eagle medium containing 10% calf serum and 100 ug of bromodeoxyuridine per ml. Moloney MLV clone 1 was the source of infectious MLV virus preparations (Fan and Paskind, 1974).

Virus stocks were harvested from producer cells 12 h after feeding. Infection of cells with MLV or MLV pseudotyped virus was carried out in the presence of 8 ug of Polybrene per ml for 2 h at 37°C, after filtering through a 0.45 micron syringe filter. MLV titers were determined by XC plaque assay, using NIH3T3 (TK^-) cells or NIH3T3 cells as indicator cells (Rowe, et al., 1970). Rapid assays for MLV production were carried out by a reverse transcriptase spot assay (Goff et al., 1981a). TK^+ transducing virus was assayed by a colony formation assay. NIH3T3 (TK^-)(5×10^5) cells were plated 24 h before the experimention on 10-cm plates. These were infected for 2 h and then fed with Dulbecco modified Eagle medium containing 10% fetal calf serum. After 24 h the growth medium was changed to selective HAT medium, consisting of Dulbecco modified Eagle medium containing 10% fetal calf serum, 15 ug of hypoxanthine per ml, 0.2 ug of

aminopterin per ml, and 5.0 ug of thymidine per ml (Littlefield, 1964). The selective medium was changed after 3, 6, and 9 days, and colonies were scored after 2 weeks upon staining with 1% crystal violet in 20% ethanol. Titers of transforming virus were determined by infecting NIH3T3 cells with various dilutions of virus and observing the number of foci arising after 10-14 days.

6.2 DNA Transfections

Transfections of cloned DNAs were essentially as described for tumor DNAs (Graham and van der Eb, 1973; Andersson et al., 1979). With transforming clones 10 ng of cloned DNA was used with 75 ug carrier NIH3T3 cell DNA. Foci were observed macroscopically in 10-15 days. Clones were also co-transfected into cells with various selectable markers. An SV40 linked *neo* gene confers resistance to the drug G418 (Southern and Berg, 1982). Neo cotransfections were performed by precipitating 100 ng of non-selected DNA in a 20:1 molar ratio with pSV2Neo and 75 ug NIH3T3 DNA. One day after transfection the drug G418 was added at a concentration of 1 mg/ml. The selective media was changed after 3 days. Colonies of resistant cells were observed macroscopically in 10-15 days.

The bacterial *gpt* gene can be selected in mammalian cells with the drug mycophenolic acid (Mulligan and Berg, 1981). 75 ug NIH3T3 DNA, 500 ng nonselected DNA and 50 ng pEcogpt were used per transfection. The HSV tk gene can be selected in HAT medium (Littlefield, 1964). Transfections of clones physically linked to the gene were introduced into either NIH3T3 (TK^-) cells or MLV infected NIH3T3(TK^-) cells in coprecipitates of 75 ug of NIH3T3 DNA and 100 ng of cloned DNA. To cointroduce nonselected and tk-carrying clones into Ltk^- cells coprecipitates of 75 ug NIH3T3 DNA, 100 ng cloned tk DNA and 800 ng of nonselected cloned DNA were used.

6.3 Cloned DNAs

pEJ6.6 is a previously described clone of the transforming allele of c-Ha-ras isolated from the EJ bladder carcinoma cell line (Shih and Weinberg, 1982). pEC6.6 is a 6.6 tk BamHI fragment in pBR322 containing a nontransforming allele of c-Ha-ras isolated initially as a λ-phage clone as described (Chang, et al., 1982b). pP3 is a similar normal cHa-ras clone obtained from Drs. M. Wigler and M. Goldfarb which contains an MstII site missing in the pEC6.6 clone (Taparowsky, et al., 1982). pSV2gpt, a gift of Dr. R.C. Mulligan, is a clone of the Ecogpt gene which is active in mammalian cells (Mulligan and Berg, 1981). pTK3, a gift of Dr. S. Silverstein is a plasmid containing the HSV tk gene. pZAP (also called p836) and pZIP (also called p21) are proviral clones of MLV described previously (Hoffmann, et al., 1982). pH-1, a gift from Dr. E.M. Scolnick, is a biologically active, circularly permuted form of the Harvey sarcoma virus genome cloned in the EcoRI site in pBR322 (Ellis, et al., 1980). pSV2Neo, a clone confering resistance to G418 in mammalian cells, was a gift of Dr. R.C. Mulligan (Southern and Berg, 1982).

6.4 Molecular Cloning

Detailed protocols have recently been published for most of the techniques used in this thesis (Maniatis et al., 1982). Plasmid preparations were done using the SDS lysis, CsCl/Eth Br gradient method (Godson and Vapnek, 1973). Phage DNA was prepared by PEG precipitation of clarified, lysed bacterial cultures followed by CsCl step gradients (Yamamoto et al., 1970). Restriction enzyme digestions, as with all enzymatic reactions in this work, were carried out using commercially available enzymes under buffer conditions recommended by the suppliers.

Restriction sites were removed from clones by restriction enzyme digestion, filling in overhanging 5' ends with E.coli DNA polymerase I, and treatment with T_4 DNA ligase. DNA fragments for cloning were purified by (depending on the size of the fragment) preparative electrophoresis through 1% agarose followed by NaI-glass bead elution or electrophoresis through 12.5% polyacrylamide gels followed by electroelution in dialysis bags. In both cases the bands were visualized in the gels by staining in 1 mg/ml Ethidium bromide and sizes were determined by comparison with commercially available size markers (usually HindIII digests of λ DNA, HaeIII digest of ØX DNA, or a synthetic 1 kb "ladder"). In order to join fragments with nonhomologous ends, purchased synthetic DNA linkers were attached to the fragments. Linkers were treated with polynucleotide Kinase. When necessary the fragment to be modified was treated with the Klenow fragment of E.coli DNA polymerase I to generate flush ends. The products of the reaction were cut with the appropriate restriction enzyme and repurified through a gel. Vector plasmid was treated with calf intestinal phosphatase to prevent recirculation and joined to the prepared insert with T_4 DNA ligase. In some cases, a number of fragments with differing terminal sequences were joined in a single ligation step.

Bacteria (E.coli HB101, C600 or X1776) were transformed with the products of ligation reactions by the calcium chloride procedure (Mandel and Higa, 1970) and the bacteria were plated on selective plates containing 50 mg/ml Ampicillan, 50 mg/ml Kanamycin or 10 mg/ml Tetracycline depending upon the markers contained in the transformed clone. Colonies were screened using a replica colony hybridization protocol (Grunstein and Hogness, 1975) or by restriction enzyme digestion of small quantities of plasmid DNA prepared by the alkaline lysis procedure (Ish-Horowicz and Burke, 1981) or by a triton lysis procedure (Rambach and Hogness, 1977).

6.5 Analysis of Virion RNA, Cellular RNA, and Cellular DNA

Viron RNA was prepared by extraction from virus particles banded in sucrose step gradients (Shields et al., 1978). DNA was phenol extracted from cells lysed in 1% sodium dodecyl sulfate and 0.2 mg of proteinase K per ml. Polyadenylic acid-containing RNA was prepared by a technique involving batch-binding of RNA to oligo (dt)-cellulose (Varmus et al., 1981). In brief, cells were lysed in 0.5% sodium dodecyl sulfate and 0.2 mg of proteinase K per ml. The lysate was disrupted for 20 s with a probe sonicator. Oligodeoxythmidylic acid [oligo(dT)]-cellulose was then added, the salt concentration was adjusted to 0.5 M, and the mixture was agitated with a stir bar for 1 h. The olido(dT)-cellulose was then poured into a column, and the polyadenylated RNA was eluted.

DNA was analyzed by restriction enzyme digestions, gel electrophoresis, and transfer to nitrocellulose (Southern, 1975; Goff et al., 1981c). Virion RNA was analyzed by electrophoresis through agarose gels after denaturation with glyoxal (McMaster and Carmichael, 1977). The virion RNA was transfered to diazophenylthio paper (Wahl et al., 1979). Cellular RNA was analyzed by electrophoresis through gels containing formaledhyde and then transferred to nitrocellulose (Maniatis, et al., 1982). ^{32}P-labeled DNA probes were prepared by nick translation (Rigby et al., 1977) and were hybridized to the immobilized RNAs and DNAS in the presence of formamide and dextran sulfate (Alwine et al., 1977; Wahl et al., 1979).

6.6 Mice

Untimed pregnant Balb/c mice were purchaed from Charles River Biological Laboratories. Each female, and subsequent litter, was maintained in a separate cage throughout the experiments.

1-2 days after birth, mice were injected intraperitoneally with 0.1 ml of virus which had been filtered through a 0.45 micron filter. Each litter group received the same virus dilution. Litters ranged from 3 to 14 animals. 1-3 litters were injected with each dilution to a total of 8-14 mice. Animals were autopsied when they died beginning 3 weeks post-injection and remaining animals were sacrified at 4 weeks. Mice were carefully dissected. Air-dried smears of blood and dissociated spleen cells were stained with hematoxylin, eosin, and Giemsa stains.

APPENDIX

A.1 Introduction

This thesis has addressed the nature of the lesions which activate oncogenes. Such an approach to understanding cancer can be characterized as an extreme form of reductionism. Reductionist studies can provide unique insight into a problem. Yet, it should be obvious that as complex a phenomenon as cancer can not be fully explicable on such a basis. Rather a variety of approaches on different levels ranging from molecular to epidemiological ultimately will be required. The significance of a contribution on any one level can only be fully ascertained by viewing it in perspective as it relates to the general problem on all levels. The conclusions of this thesis concern DNA alterations. To learn what they mean for tumorigenesis, they must be considered in terms of the circumstances in which the mutations arise, and in the context of what they mean for the functioning of the encoded proteins, for the metabolism of the cell, and for the physiology of the organism.

Such a discussion is relevant to defining the meaning of results reported in chapters II-IV. However, since these issues are only peripherally related to the experimental work, their consideration has been postponed until this point. After first discussing in more depth the place of reductionism in a multileveled view of cancer, I will attempt to put the work presented in this thesis in perspective on a variety of levels. I will begin on the molecular level on which the studies were performed and work up toward levels of increasing complexity. It will be easiest to interpret the place of this work, therefore, at the beginning and harder to sort out as increasing levels of complexity are addressed.

A.2 Reductionism and All That

In 1983 a debate appeared in the correspondence section of the journal Nature, a debate which at first glance seemed hardly worth serious thought. However, the core of the argument revolved around the place of the type of work that is presented here.

The debate was spurred by two "News and Views" editorials. The first, inspired in part by the work described in chapter II, supported the relevance of the reductionist methodology to understanding the phenomena of cancer (Oncogenes, Reductionism and All That, Nature, 1983.) The authors defined a reductionist as "one whose objective is to account for even the most complicated phenomena in terms of elementary interactions between more or less elementary entities... in biology reductionists appear as molecular biologists." The second editorial extended the premise further to argue that reductionism is a superior approach; "the truth is that microscopic explanations are in principle simpler and often more stimulating as well, than those which are essentially correlations between one set of phenomena (effects) and another (causes)" (Maddox, 1983). Most of the research on cancer to date has been carried out by clinicians, developmental biologists, cell biologists, geneticists, immunologists, and epidemiologists, not molecular biologists. As one would therefore expect, there was a swift reply from those of more "holistic" ilks who held "the view that currently the oncogene hypothesis is naively reductionist" (Habeshaw, 1983).

One would be tempted to dismiss the entire discussion on the grounds that the truth is obviously in between the two extremes, if not for the facts that the attitudes they reflect are fairly pervasive in molecular biology and that similar debates are currently raging in other far-flung

areas such as physics (Capra, 1982), learning theory (Fox, 1983), and evolutionary biology (Gould and Lewontin, 1979). Moreover, the reductionist-holist controversy, or by its earlier name, the mechanist-vitalist controversy, is not a new one raised in response to scientific advance. Rather, it existed much in its present form in the early 19th century and has roots traceable to the rise of Cartesian thought 400 years ago (Capra, 1982). The reason that the debate has such an extended history is that its nature is more philosophic than scientific and, as such, is essentially irresolvable. In biological terms, the vitalist position was that a vital principle, a spark of life, separates all living things from all other material forms. Today's holists would rephrase it to say that life is too complex to be understood in any significant way by viewing it through its minute constitutive parts. However, the almost mystical and reverant quality of the holist's view of life remains. At the opposite extreme is reductionist philosophy, holding that the best way to view any aspect of the universe, including living forms, is from the bottom up. Understanding life's most basic structure will give way to divining its higher principles. The rationalist prescription for knowing is equally a matter of faith: from the parts you can (not "might") know the whole.

As people whose profession is the pursuit of knowledge, we gain a measure of confidence and security from such a perspective. As a child I was given a book entitled All About Dinosaurs (Andrews, 1953). It recounts an episode involving the great French anatomist Georges Cuvier. He is described as having had the reputation of being able to reconstruct the entire anatomy of any animal, living or extinct, from a single tooth or wristbone. I was more inspired by that single statement than by all the fantastic monsters described in the rest of the book. How wonderful to

know that much about anything--the reductionist dream: that basic laws governing the parts of an animal can be used to predict the whole. I could not even imagine the detailed knowledge required for such a prediction. The truth, of course, is that no one, Cuvier not excepted, has yet developed such knowledge.

For Cuvier, a classic reductionist, such a dream was far from an implausible goal. The father of comparative anatomy, Cuvier developed a principle which he called the "correlation of parts". This predecessor of modern allometric analysis recognizes the integrated nature of an organism such that "none of the separate parts can change their forms without a corresponding change in the other parts of the same animal" (Cuvier, 1812). Cuvier knew he had not yet discovered a full set of laws of predictive morphology. Yet he labored making empirical associations in the faith that such laws could be found so that someday "by a careful survey of any one bone by itself, a person who is sufficiently master of the laws of organic structure, may, as it were, reconstruct the whole animal to which that bone had belonged", (Cuvier, 1812).

Today's molecular reductionists do not seriously believe in such a miracle of knowledge. No one pretends that someday scientists by reworking equations of statistical and quantum mechanics will understand the intricacies of predator/prey population fluctuations or of tumor progression. And the molecular biologist would rightfully dismiss such a characterization as an absurdity.

Equally fallacious, however, is the quite prevalent softer form of reductionist thought which holds that while we will never be able to acquire such knowledge, nonetheless a hypothetical all-knowing being <u>would</u> be able to explain an organism fully on the basis of its molecules. The

implication of such a belief is that, even if we will never know all the answers, the molecular scale is indeed the correct place to look. It is more basic. It is somehow purer. And, because isolating a small aspect of a system makes it possible to investigate it with more rigor, it is a harder form of science. Each of these statements has its implied inverse, with the derogatory connotations inherent in the words "less basic," "impure," and "softer" science.

The flaw in this modifed reductionist point of view is that such an omniscient being would not understand an organism from having knowledge of its molecular structure alone because new properties--with new laws governing them--develop as a result of higher levels of organization. There is a hierarchy of levels in nature, each based on the others, but independent enough that they can only be approached on their own plane.

To borrow an example from Steven J. Gould (Smith, 1984), evolution is most often approached by population biologists, following Darwin, in terms of natural selection favoring some individuals at the expense of others. Molecular biologists have now demonstrated that genes themselves undergo events (gene conversion, transposition, etc.) that allow them to evolve independent of their effects on the organism's phenotype. Thus processes at the molecular level can provide more than random variation for natural selection to act upon; whether "molecular drive," or molecular random walk, they can have great impact on higher order evolution. Moving up a level of organization, paleontologists contribute a third, again separate, level of evolution: species selection. This is not an extended individual selection, such as individual snails with thicker shells do better, so species with thicker shells do better. Rather, the concept applies to taxons which replace others on the basis of traits with no analogous

advantage on the individual level. For example, in the Cretaceous period, species of Volute gastropods and their competitors had similar average species lifespans (equivalent exstinction rates). However, the Volutes did not have a free-floating planktonic larval stage, whereas their competitors did. This meant that the Volutes faced greater geologic barriers to gene flow. Thus new variants had a greater chance to establish themselves. They speciated more frequently. So as both groups died off at the same rate, the Volutes, in effect, "overgrew" their competitors, for reasons with no advantageous explanation on the level of individuals.

From this hierarchical evolutionary perspective, selection and random drifts occur on levels of genes, individuals, and species. You can never learn about one level just from the study of another. In the absence of molecular biology and modern paleontology, all of evolution seemed to be adequately explained by models based on individual selection. Only by studying a phenamonon on many levels can one be aware of which levels are significant to a complex problem. Moreover, there can be apparent contradictions between levels. An individual bower bird can increase its reproductive success by developing more elaborate ornamentation and displays. In the process, however, a population can become so specialized that the species is at a disadvantage when faced with a dramatic environmental change. To understand the outcome of such a situation requires understanding the relative effectiveness of selection at different levels.

Almost every complex phenomenon in the natural and human realms needs to be viewed from such a multileveled perspective. A professional football league is made up of teams and teams consist of players, but no matter how

detailed your knowledge of the players, there are things you will never intuit about the league. Herschel Walker became a rich man by accepting a 1.5 million dollar per year contract and the New Jersey Generals became a viable organization by signing him (Associated Press, 1983), but by initiating the recruitment of college underclassmen and by overextending the league's economic potential, they may have condemned the USFL in the process. Or, closer to the topic of this thesis, a cancer cell's growth advantage is quite obviously to the detriment of the organism in which it formed. Each level must be studied on its own. It is not due to the mere limit of human intellect that we are biologists instead of all theoretical physicists. It is not because complexity is impenetrable, but rather because complexity leads inevitably to higher grades of organization not wholly explanable on the basis of their lower level constituents alone.

The implication of this is that there is no such thing as a most basic level. No ultimate reference frame exists. Thus, no level of inquiry is purer than any other. The nature of a given level will define the problems one investigates on that plane and the approaches that are appropriate to understanding them. The most rational approach to a given problem cannot be denigrated, the form of the problem is constrained by the character of the level being investigated, and all levels of nature are equally worth probing. Thus the claim, that reductionism is most basic, is flawed.

The antireductionist prejudice that microscopic levels are irrelevant to complex phenomenon is equally flawed. If you can not fathom successively higher levels, then you certainly can not comprehend connections between levels by studying any single one alone. The different levels in nature are interdependent and interlinked. The interdependence is obvious: one cannot have a cell without a membrane, nor a membrane

without a lipid, and so on. The interlinkings are less predictable, although one is aware that they must exist. No one a priori could have expected that studying the mechanism of drug resistance in bacterially mediated disease would lead to profound revelations at the level of the fluidity of the genome; nor that these, in turn, would give insight into the mechanisms of a key stage (integration) of a mammalian viral life cycle. The insights into the connections only came from simultaneous studies at physiological and genomic levels. Such serendipitous rewards, when they occur, are both fun and important. But, since we can never predict which lines of inquiry will give understanding at a higher or lower level or organization, all we can do is attack problems which we find intriguing in a manner appropriate to those questions. When a topic is as complex as tumor biology--with many planes of explanation--it must be approached on just as many levels.

Indeed, cancer has been investigated on many levels. To disparage one approach as too superficial and descriptive or another as naively reductionistic is foolishness. Each is appropriate to understanding on a particular level of explanation. Since we can never know before hand what the connections between the levels will be, we should not be terribly surprised if someday one or another tack leads to therapeutic breakthroughs. But if the study of oncogenes were to lead directly to a cure for cancer (in the commonly used sense of "cure"), it would no more be a triumph of reductionism than the lack of such a miracle would be a reductionist failure. The connections between levels currently hidden by nature are not a reflection of the validity of the approaches used in the study of each level.

A.3 Tissue Specificity?

The nature of the lesions which activate oncogenes was discussed at the DNA level in Chapter V. To understand the significance of these lesions the context in which they occur must be examined.

Tissue specificity in various guises has long had a place in tumor biology. Inherited predispositions to cancer in human families involve risks of specific tumor types (Knudson, 1977). In experimental systems, different chemical carcinogenes reproducibly induce specific tumors when delivered in the same manner to the same inbred animal line (Becker, 1975). Finally, most significant in thinking about cellular oncogenes, retroviruses which carry specific oncogenes themselves cause very specific neoplasms (Weiss et al., 1982). Thus it was logical that the possibility of tissue specific action shoud be raised when discussing cellular oncogenes.

Tissue specificity can be thought of from two sides. Each oncogene could be activated only in a single or a few tissues. This could perhaps be the case if a developmentally regulated function were important in oncogenesis. This would not exclude the possibiliy of several different oncogenes functioning in the same tissue at different times. Conversely, tissue specificity could be thought of as a given tissue being specifically transformed by one and only one oncogene. A given oncogene could then be active, however, in several different tumor types. Finally, both definitions of tissue specificity could be correct and there could be a one-to-one correspondence of oncogenes and tumor types.

In the early days of oncogene study, investigators reported that indeed there was such a rigidly defined, one-to-one mapping of tissue-specific oncogenes onto tumor types (Shih et al., 1983). The strongest

case of this sort of tissue specificity involves stage-specific transforming genes from human and mouse lymphocytic tumors (Lane et al., 1982). Unfortunately, these conclusions were in retrospect based on too little data. Later work has shown that neither of the two definitions of tissue specificity in fact holds for lymphocytic tumors: different oncogenes (N-ras and Ki-ras) can be active in the same stage lymphocytic tumors, and the same oncogene (Ki-ras) can be active in different stage lymphocytic tumors (Eva et al., 1983a). No strict tissue specificity has been found to hold for any pair of oncogenes and tumor types to date.

While a given tumor type can be created by different oncogenes under different circumstances, it is possible that a particular sequence of events might reproducibly activate the same oncogene in the same tissue. This could occur because of the nature of the lesions inflicted by a given carcinogen. For example, many known bladder carcinogens favor G to T transversions such as that which activated the EJ allele of c-Ha-ras in a bladder carcinoma (Swenson and Kadiubar, 1981). An oncogene which, due to its primary nucleotide sequence, could not be activated by a transversion, would not be subject to the action of those bladder carcinogenes. Other less obvious mechanisms may also direct the influences of certain agents to particular oncogenes in particular cells.

No matter what the explanation, there is considerable evidence that this form of tissue specificity exists. As detailed above, all three cellular ras genes can be reproducibilly activated by treatment with particular forms of radiation or chemical carcinogen, both in vitro and in vivo.

Other than carcinogen-correlated tissue specificities there is as of yet no evidence for tissue specificity for oncogenes. Nonetheless, we are

still dealing with a limited data base for many specific tumor types. We have to withhold a final judgment on the question, especially since two things we do know about oncogenes would actually suggest that some specificities should exist. First, the point mutation model for activation (as opposed to regulatory change models) presupposes that the proto-oncogene is being transcribed in the target cell prior to activation. A transcriptionally silent sequence cannot cause transformation no matter what it encodes unless a second "hit" in the same gene transcriptionally activates it. Thus, while oncogenes might not be specific to certain tumor types, there might well be oncogenes which are specifically absent from given tumor types.

Recent work bearing on this type of oncogene specificity studied levels of expression of different proto-oncogenes in a number of tumor types. An average of seven independent tumors were investigated in each of eight categories of malignancy for expression of 15 different proto-oncogenes (Slamon et al., 1984). The investigators attempted to connect the varying levels of expression to the development of the neoplasms. These arguments were flawed as follows. To ascribe meaning to the levels they observed, they tried to compare the expression of five of the proto-oncogenes in 14 tumors to RNA levels in normal tissue taken from the same patients. The first problem is that the normal tissue (from the same organ as the tumor) is a heterologous mixture of different cell types, and the RNA levels measured are therefore a weighted average of these cell types biased by the proportion of each cell type in the tissue. The tumor, conversely, is of clonal origin and therefore the RNA levels measured in the tumor sample can only rightly be compared with those in a single progenitor cell type. In short, there was no possible adequate control for

normal expression levels. Moreover, the two genes which appeared to have significant changes in expression were c-myc and c-Ha-ras. These two genes are, however, known to be regulated through the cell cycle (Campisi et al., 1984). Thus the fact that the tumor cells were growing while the surrounding tissue was not could explain the differential levels observed, independent of activation of those oncogenes.

Nonetheless, the paper does show that there are clear tissue specificities of the proto-oncogene expression. For example, c-myb is active only in malignancies of hematopoietic origin; Ha-ras and Ki-ras are active in all varieties of tumors; and erbA, erbB, mos, rel, sis, and yes are silent in all tumors investigated. Following the reasoning presented above, one would argue that (whether or not they actually were activated in the cases looked at in the study) myb could be activated in hematopoietic tumors, ras genes could be activated in any of the tumor types investigated and erbA, etc. could not be activated in any of these tumor types; provided, of course, that we limit our reasoning to those oncogenes exclusively activated by point mutations.

The second indication that some tissue specificities might be real comes from our understanding of the function of particular oncogenes. One of the viral oncogenes, v-sis, has now been shown to be an altered form of an intercellular growth factor PDGF (Waterfield et al. 1983; Doolittle et al., 1983; see below). No cellular oncogenes have yet turned out to be derived from growth factor genes, although at least one codes for a growth factor receptor. If altered growth factors are implicated as oncogenes in nonviral tumors, one could predict that they would show specificity to cell types which produce the appropriate growth factor receptor.

Obviously our ability to understand specificities on this level is somewhat constrained by our limited knowledge of the function of oncogenes. Understanding what oncogene products do in a cell would not only help to clarify any specificities that oncogenes may have, but in addition such knowledge would allow us to comprehend why the specific amino acide changes we observe are, in fact, oncogenically activating. We therefore must move up a level from nucleic acids to protein functions.

A.4 Oncogene Protein Function

The experiments reported above studied a particular oncogene: c-Ha-*ras*1. The function of its encoded protein, p21, is not known. However, since the cellular phenotypic effects of different oncogenes are similar, it is clear that the function of p21 must converge directly or indirectly with those of other oncogene products. For that reason this discussion will not be limited solely to *ras* genes.

Since heritable predispositions to cancer involve risk of tumors in particular cell lineages (Knudson, 1977), an obvious idea is that oncogenes themselves are involved in control of differentiation of cell lineages. This would lead to a model of cancer as a breakdown of developmental control. If this were the case, then oncogenes, whose importance would lie in control of developoment regulation, would be expected to vary in expression in differing tissues and in different embryological stages.

Studies have, therefore, been conducted, monitoring c-*onc* expression among various tissues (e.g., Gonda, 1982) and through pre- and postnatal development (e.g., Muller et al., 1982). Variations in expression have been observed for some oncogenes (for example, c-*abl* and c-*fos* are active only at certain times in embryonic mouse development), while other

oncogenes seem to ubiquitously transcribed (including Ha-ras) (Muller et al., 1982). I will not go into greater detail concerning these investigations because not much functional significance has been gleaned from them. That is not to say that the observed patterns of expression are not important; ultimately we will surely comprehend why proto-oncogenes are regulated in the way that they are. However, experiments designed to detect differential gene expression can not differentiate genes expressed to direct the course of development from genes expressed in response to development. Thus our initial knowledge of oncogene function must come from another direction.

One very promising direction is the use of genetics. The vertebrate species in which viral and cellular oncogenes were first identified are clearly not amenable to easy genetic manipulation. However, the discovery of homologous genes first in Drosophila (Shilo and Weinberg, 1981b) and then in yeast (DeFeo-Jones et al.; 1983, Gallwitz et al., 1983) opened up the possibility of genetic analysis.

The original reports of yeast ras genes was on the basis of nucleic acid hybridization under low stringency conditions. However, further analysis showed that the sequences identified contained open reading frames with predicted amino acide sequences that are greater than 90% homologous with the first two exons of the mammalian ras genes (Dhar et al., 1984; Powers et al., 1984). Moreover, the proteins encoded by the yeast ras genes cross-hybridized with monoclonal antibodies raised against the mammalian p21 protein (Papageorge et al., 1984). With that great conservation of sequence, it seemed that the functions of the yeast and mammalian proteins were likely to be homologous as well. Genetic studies in yeast could, therefore, give powerful clues to mammalian gene function.

Such studies are just beginning, but it has already been shown that there are two members of a yeast ras gene family. These two genes can complement each other and one can be removed without loss of viability. However, a double mutant spore, possssing neither ras gene, is not viable and fails to undergo a single round of cell division or even to form buds (which occurs early in S phase of the cell cycle) (Tatchell et al., 1984). The homology of the yeast and mammalian ras genes is verified by the observation that c-Ha-ras driven off of a yeast promoter can rescue sporulation in such double mutants (M. Wigler, unpublished result). Other workers have reported as of yet unpublished studies in which the yeast ras amino acid position 19 (homologous to the mammalian position 12) has been mutated from glycine to valine, the analogous change to that which dominantly activated the EJ c-ha-ras oncogene. Such a mutant yeast ras also dominantly affects cell growth, giving a phenotype down 500-1000 fold in sporulation (M. Wigler, unpublished observations). Perhaps the most promising genetic result to date is the reported isolation of four second-site mutants (not in the yeast ras genes), which can act to rescue sporulation in yeast also carrying ras mutations. Characterization of these mutants is underway (M. Wigler, unpublished results).

Besides genetic studies, workers have also taken a biochemical approach to studying ras oncogene function. As discussed above, p21 is a GTP binding protein on the inner surface of the cell membrane. The protein also possesses a GTPase activity which is reported to be greater in the normal variants of p21 than in the transforming proteins.

On the basis of the few known properties of p21 one could propose a model for its function based on an analogy to the G proteins of the adenyl cyclase system. Adenyl cyclase is an enzyme in the plasma membrane which

catalyzes the conversion of ATP to cAMP. The activity of the enzyme is modulated in response to the binding of particular hormones to their receptors. Depending on the hormone, the bound receptor will then interact with a stimulatory G protein (Gs) or an inhibitory G protein (Gi). In either case the interaction results in the removal of GDP formerly bound to the G protein and the binding of GTP to the G protein. The GTP-bound G proteins have a high affinity for the adenyl cyclase enzyme, which is either activated or repressed from its basal level of activity by binding Gs or Gi, respectively. The stimulatory or inhibitory response continues until an associated GTPase activity hydrolyzes the G protein-bound GTP to GDP and Pi. The GTP, in conjunction with the associated GTPase thus serves as a kind of a clock, timing the length of the response to the initial hormone receptor induced adenyl cyclase activity. Besides the Gi and Gs forms of G protein in the adenyl cyclase system, a related GTP-binding protein, transducin, activates a phosphodiesterase in response to the activation of a receptor, rhodopsin (in this case responding to light rather than hormone). The family of G proteins could be larger. (For a review of the adenyl cyclase system, see Ross and Gilman, 1980.)

The idea that the ras-encoded p21s could be members of the family originally came from the facts that they are also membrane-bound, GTP-binding, and GTP-hydrolyzing proteins. Since p21 is also approximately half the size of the GTP binding subunit of G protein and since p21, like the GTP binding subunit of G protein, can be ADP-ribosylated in the presence of cholera toxin (A. Schechter, unpublished result), it seemed conceivable that p21 and G protein would turn out to be distantly homologous. However, the model that p21 acts in an analogous role modulating and transducing signals across the plasma membrane does not depend upon homologous origins.

This model is consistent with both known mechanisms of ras activation. If p21 activates (or inhibits) an activity of another protein as long as p21 is bound to GTP, then there would be several ways to increase the effect of p21. One would be to alter p21 so that it hydrolyzes GTP less frequently. This is what Levinson and Scolnick have reported occurs with the oncogenically mutated ras gene products. A second way to overstimulate the downstream protein would be to have an excess of GTP bound p21 in the membrane. Then, when one p21 molecule hydrolyzed its GTP and dissociated from the protein it was influencing, a second p21 would immediately replace it. Indeed, overexpression of ras genes is known to be an alternate means of transforming a cell. This would also suggest that nonhydrolyzable analogues of GTP should phenocopy the effects of transforming variants of p21 when added to normal p21 in an in vitro assay for the function of the downstream protein. To test this, however, one first has to identify the downstream protein.

Others have also noted the similarities between p21 and the G proteins, but to date no one has been able to demonstrate a G-protein-like role for p21 in any known system. There are, however, reports circulating that S29 cells, which are unresponsive to hormonal stimulation of adenyl cyclase because they fail to make G protein, can be reconstituted to hormone responsiveness by transfection of high levels of Ha-ras. If true, it would not necessarily implicate p21 as a normal participant in the adenyl cyclase system, but it would mean that there is sufficient homology that at very high levels p21 can be forced to act in that capacity. Such a result would certainly be a great impetus for further experiments in this direction.

Meanwhile, we may finally have a clue for what system p21 works in, even if we are not yet certain of its role. Investigators have immunoprecipitated p21 with monoclonal antibodies under conditions which might not interfere with pre-existing protein complexes in the hope of identifying coprecipitating proteins whih might interact wtih p21. Such experiments have coprecipitated a 90,000 dalton protein with p21 from three human tumor cell lines. Two of these contain activated ras oncogenes, the third does not give any foci in DNA transfection experiments. The 90 kd protein has been identified as the transferin receptor (Finkel and Cooper, 1984b). There are potential problems with these experiments. The stoichiometry of transferrin receptor to p21 is hard to interpret. The low and variable ratios may, however, just reflect the nature of the interaction between the two proteins. A second problem is that they are unable to identify the complex in normal or ras-transfected NIH cells. This could be due to a higher background which they observe in their immunoprecipitations from murine as opposed to human cells. Others have postulated artifactual explanations for the p21-transferrin results.

If transferrin does not turn out to interact with p21, an obvious alternative hypothesis is that p21 mediates growth factor signals. While any involvement of p21 with growth factor messages remains highly speculative, the relationships between growth factors and other oncongenes are a lot clearer. One oncogene transduced by both simian and feline acute sarcoma viruses, called v-sis, is derived from a cellular gene encoding the growth factor PDGF (Waterfield et al., 1983; Doolittle et al., 1983). PDGF consists of two polypeptide chains, A and B, in a heterodimer (Johnson et al., 1982). The sis gene product exists as a structurally similar homodimer of two B chains (Robbins et al., 1983).

The sis gene product has been identified as a cytosolic protein rather than a secreted protein (Devel et al., 1983). The PDGF receptor's binding domain is on the outside of the plasma membrane; however, the sis product may be able to interact with the receptor in an intracellular compartment during synthesis and transport. Such an internal interaction may, in fact, be more powerful than an externally delivered signal. Cell cultures tested with exogenous PDGF roundup and overgrow somewhat resembling properties of transformed cells, but not to the same extent as sis-transformed cells (Westermark et al., 1983).

Oncogenes can also consist of inappropriately expressed or altered growth factor receptors. The erbB oncogene of avian erythroblastosis virus is homologous to a portion of the EGF receptor (Downward et al., 1984). The erbB product corresponds to a truncated EGF receptor containing only the inner domain. The lack of the EGF-binding may cause a constitutive activation of the effector region of the molecule (Downward et al. 1984). A second example of a receptor acting as an oncogene comes from a cellular oncogene activated in a series of rat neuroblastomas. Its gene product, p185, is distantly related to the EGF receptor and may be homologous to the PDGF receptor (Vaidyanathan and Schechter, unpublished result). In this instance the activation did not involve the loss of the extracellular domain, and the level of the membrane bound p185 is decreased by the addition of PDGF (Stern and Heffernan, unpublished result). Such down modulation is a typical response of receptors in response to the binding of growth factors (Heldin et al., 1981). The activating lesion in the oncogene encoding p185 has not yet been determined, but it should be shortly forthcoming. Since the function of the encoded protein is well characterized, it should soon be possible to relate the DNA alternations to

the functional changes in the protein. In this instance we should, thus, be close to connecting the levels of DNA damage and mechanisms of transformation.

The biggest difficulty in making such a connection in the near future is that the mechanisms by which growth factor receptors influence cell metabolism are still poorly understood. Whatever the mode of action it must ultimately provide for the large number of parameters that are all affected by either growth factor binding or transformation including changes in cell cycle, glucose transport, adhesiveness, cell shape and intracellular architecture, ion transport, etc. These coordinate changes could be carried out by a single receptor/transforming protein with pleiotropy achieved by multiple targets of action or by a single initial site of action feeding into a branching cascade of secondary events. The truth likely lies between these two extreme models.

A candidate for a growth factor receptor enzymatic activity which could have such multiple targets has been discovered. A tyrosine-specific kinase activity has been associated with the PDGF receptor (Ek et al., 1982), the EGF receptor (Buhrow et al., 1982), and the insulin receptor (Roth and Cassel, 1983a), among others. Tyrosine kinase activity is uncommon in the cell. Phosphotyrosine accounts for less than 0.1% of the protein-bound phosphate in a normal cell (Sefton et al., 1980). It is probably highly significant, therefore, that several oncogene products are also equipped with tyrosine kinase activities including src (Gilmer and Erikson, 1981); yes (Kitamura et al., 1982); fes/fps (Hampe et al., 1982, Feldman et al., 1983); ros (Feldman et al., 1982); and abl (Witte et al., 1980). There is also strong sequence homology between these oncogenes in the regions encoding the kinase domains (Czernilofsky et al., 1981; Reddy

et al., 1983). This homology has been extended to several other oncogenes which have not yet been shown to contain kinase activity; mos (Van Beveren et al., 1981), rel (Stephens et al., 1983), and erbB (Yamamoto The amino acid homology also extends to the EGF receptor (Downward et al., 1984) and to a receptor for the tumor promoter TPA which has a serine-kinase activity: the cAMP-dependent protein kinase (or kinase C) (Varker and Dayhoff, 1982). Most of the tyrosine kinase oncogenes are situated in the cell membrane, like the growth factor receptors.

The tyrosine kinases associated with growth factor receptors and oncogenes work on a broad set of substrates. Some differences exist between the substrates utilized by different kinases while many substrates are the same for all of them. This could reflect the differences in phenotypic effects caused by various growth factors as well as indicating common metabolic pathways in growth stimulation and oncogenic transformation. To date, however, none of the substrates that have been identified seem to be of biological significance (J.A. Cooper et al., 1982). This suggests that the important substrates may exist at low levels. Candidates for such low-level substrates would be enzymatic proteins, including other kinases.

Whether or not the tyrosine kinases directly phosphorylate other kinases, a serine kinase is at least indirectly activated by the binding of growth factors such as EGF (Hunter and Cooper, 1981), PDGF (Nishimura and Deuel, 1981), and insulin (Kasuga et al., 1982) to their receptors. Elevated phosphoserine levels are also seen in cells transformed by tyrosine kinase oncogenes such as src (Cooper and Hunter, 1981) and abl (Decker, 1981). These results may be an indication of a complex set of enzymes which form a regulatory network controlling cell growth.

Such a regulatory network might be expected to have feedback mechanisms as well. There are indications that this could be the case. Protein kinase C is a serine kinase which can be activated by the binding of the tumor promoter TPA (Castagna et al., 1982). (This may be how tumor promoters promote oncogenesis, by stimulating the actions of some physiological agonist of C kinase--see below.) The binding of TPA to kinase C leads to a decrease in the ability of EGF to bind to its receptor (Magun et al., 1980). This may be directly mediated by kinase-C-induced serine phosphorylation of the EGF receptor since TPA is known to induce the serine phosphorylation of other receptors including those for insulin and somatomedin (Jacobs et al., 1983). Although this response has not yet been reported for the EGF receptor, the EGF receptor is known to be phosphorylated on serine (Hapgood et al., 1983).

Oncogene tyrosine kinases are also known to contain phosphoserine (Collet et al., 1979; Weinmaster al., 1983). These may also be due to the action of protein kinase C, since kinase C is activated by cAMP, and increases in the cellular concentration of cAMP led to serine phosphorylation of the src gene product (Roth et al., 1983b). This again has a regulatory effect as the associated tyrosine kinase activity increases (Roth et al., 1983b).

I speculated above that p21 may modulate enzymatic proteins such as one or several of the growth factor receptors. There are reports that the insulin receptor is regulated by a GTP binding "G-protein-like" molecule, but it has not yet been identified (M. Housely, unpublished results). Alternatively, if p21 turns out to function as another member of the G protein family in regulating adenyl cyclase it could still feed to the regulatory network described above by controlling cAMP levels which, in turn, help to regulate the C kinase.

In spite of the evidence presented above, some investigators have suggested that the tyrosine kinase activities of oncogenes and growth hormone receptors may be a false lead. This belief is suggested by several considerations. First, there is the failure thus far to identify a physiologically significant tyrosine kinase substrate. Also, the turnover rates for some of these enzymes, such as src, have been measured and are slower than would be expected for an important enzymatic activity (on the order of nanomoles per minute per milligram enzyme). This could, of course, be explained by the investigators measuring the turnover with the wrong substrates. Most important, however, there is an alternative enzymatic activity for these proteins, one for which there is a potentially significant substrate. Several oncogene-specified "tyrosine kinases" (src and ros) have now been demonstrated to phosphorylate lipids as well as protein (Sugimoto et al., 1984; Macara et al., 1984). The particular lipids which are substrates for these enzymes are phosphatidylinositol (Ptd Ins), phosphatidylinositol-4-phosphate (Ptd Ins 4P), and 1,2-diacylglycerol (1,2-DG). The first two are precursors in inositiol lipid biosynthesis. thus Ptd Ins + Pi→Ptd Ins 4P, and Ptd Ins 4P + Pi→Ptd Ins 4,5P_2, which is the final product. When appropriate receptors are activated, this Ptd Ins 4,5P_2 product can be hydrolyzed to yield diacylglycerol (DG) and inositol triphosphate (InsP_3) (Michell et al., 1981; Creba et al., 1983; Berridge et al., 1983). These two breakdown products are significant because DG is an important activator of the protein kinase C (Nishizuka, 1984) and InsP_3 causes the release of intracellular calcium ion (Streb, 1983). Calcium ions are important intercellular messengers and act in many cases in synergy with the protein kinase C (Kaibuchi et al., 1982; Kajikawa et al., 1983).

The "tyrosine kinases" also act to phosphylate DG to create phosphatidic acid which can no longer stimulate the C kinase. This apparent paradox of the same enzyme acting to create and metabolyze the same signal could be resolved by determining the relative utilizations of the various lipid substrates, and hence finding which is the rate limiting step.

A final touch to this model could be provided by the fact that mobilization of calcium in the cell has been shown to be regulated by a GTP-binding protein (Gomperts, 1983). Since $InsP_3$ induced release of calcium is a postulated result of the action of the "tyrosine kinases" on lipids, this could again provide for a convergence of ras p21 with the products of other oncogenes.

Militating against the inositol lipid hypothesis is the fact that some members of the tyrosine kinase family such as the growth hormone receptor and the abl oncogene product do not phosphorylate lipids (G. Foulkes, personal communication). Moreover, these protein kinases have a much higher tyrosine kinase activity than that reported for src, on the order of micromoles per minute per milligram, which is the same order of activity as exhibited by protein kinase C, for example.

It is hard at present to determine which phosphorylating activity is the physiologically significant one. It is possible that they both are. For example, an ancestral tyrosine kinase may have duplicated and diverged to evolve one class of tyrosine kinases such as abl and a second class modified to phosphorylate lipids but retaining a residual phosphotyrosine activity such as src. Or the evolutionary divergence could have proceeded in the opposite order, with an ancient lipid phosphorylating enzyme, which also had a low level of tyrosine kinase activity, giving rise to a set of enzymes which used tyrosine containing proteins as their primary or only

substrates. In evolutionary terms one would say that the low level tyrosine kinase activity "preadapted" the common ancestral protein to take on such a new role.

In any event, the evidence for interactions and feedback mechanisms operating between the various growth regulating proteins is clearly mounting. These networks are unfortunately often referred to in terms of a "cascade". While the metaphor is apt in the sense of an inevitable waterfall of effects resulting from an initial signal, the term has already become common in biochemistry in a slightly different sense. The prototype biochemical cascade is that which translates a 10^{-12} molar hormone signal into an enormous mobilization of glycogen. Thus cascades have come to stand for enzymatic amplifications of initial messages. While some amplification may be associated with the growth control pathways, many of the reactions function more as adaptors and branch points. I submit that "cascade" carries the wrong connotations.

Regardless of the word used for it, eventually the messages initiated at the cell surface must be transmitted to the nucleus in order to regulate growth-and cell-cycle-dependent transcription. There is really very little evidence for the identity of the distant postreceptor signal carriers. The known oncogene products not accounted for as ras family members, growth factor receptors, and related tyrosine kinases are all located in the cell nucleus (myc, Donner et al., 1982; myb, Klempnauer et al., 1984; fos, I. Verma, personal communication). It is tempting, therefore, to tie them into the same schema and attribute the nuclear growth signal transducing role to them.

While this is pure speculation, the little we know about myc function is consistent with such a role. The addition of PDGF to resting cells

induces an increase in c-myc expression (Kelly et al., 1983); and myc, in turn, is known to regulate the expression of other cellular genes (Kingston et al., 1984). That this regulation of other genes could be a way of transducing growth signals is indicated by experiments in which myc was attached to an inducible MMTV LTR promoter and transfected into PDGF-responsive fibroblasts. At confluence these cells stopped growing (as assayed by radiolabelled thymidine incorporation). Addition of PDGF stimulated the cells to undergo a cycle of cell division. Induction of the transfected myc construct also strongly stimulated growth, although only about 25% of the response was obtained as compared to that observed with PDGF (P. Leder, unpublished results). Even if myc is not, therefore, responsible for transducing the full PDGF signal, it may play a major role in the process.

Thus all the known oncogenes may feed into a common pathway. This convergence phenomenon of "fewer and fewer oncogenes" (Weinberg, 1982) should not be surprising. After all, oncogenes are identified on the basis of their ability to do one (pleiotropic) thing: "transform" some cell type. It would be surprising indeed if cells had many totally independent systems controlling their growth. It only makes sense that there be a single (if complex) general system governing cell growth and that there be several different places where that system can be perturbed. On the other hand, all oncogenes dealing with one function is not the same thing as all of cancer dealing with one function. When we have a clearer idea exactly what oncogenes do, we will also be in a better position to figure out what other events besides oncogenes are needed for the genesis of cancer.

A.5 Multiple Hits in Carcinogenesis

We have already noted that if the probability of a particular point mutation in a single gene is calculated, one arrives at the somewhat disturbing conclusion that we all must harbor thousands of cells carrying activated oncogenes. The fact that we do not all have a multiplicity of actively proliferating neoplasms indicates that an active oncogene cannot possibly provide the full explanation of the difference between a normal and a cancerous cell. This is consistent with a large body of evidence that supports a "multiple hit" view of carcinogenesis.

Two epidemiological facts initially suggested that cancer involves more than one step. The first is that hereditary predispositions to cancer are clearly manifestations of some step toward the development of neoplasms. Yet people carrying such inherited tendencies do not develop their diseases until their cells have undergone some subsequent change after many years of life. The second indication of multihittedness comes from the fact that the likelihood of developing cancer increases with age. If a single random event were responsible for the disease, the incidence should not vary with age. In was to reconcile this age dependence with the mutational theory of cancer that it was first postulated that cancer may involve several independent mutational events (Nordling, 1954). This idea prompted several investigators to study epidemiological records to determine the kinetics of carcinogenesis. It was shown that many different forms of cancer have rates that vary with the sixth or seventh power of the age of the subject. This suggests that six or seven "hits" may be involved (Nordling, 1954; Armitage and Doll, 1954). The range of ages where this function holds is actually somewhat limited. But that could be explained by poor diagnosis of cause of death among old people, and a small

data base and hence high sampling error for young people. Thus the six-to-seven-hits model could be more general.

This mathematical model, however, required that the various steps occur with constant probabilities not dependent either on age or on the existence of other, prior hits. The consideration of either one of these possibilities changes the analysis considerably. It has been postulated that neoplastic development could involve increasing autonomy of cells containing qualitative alterations in phenotype, with each variant having a selective advantage over the previous stages (Foulds, 1954). Building mathematical models incorporating an expanding target population for secondary hits due to advantages accrued during a first hit demonstrated that the same epidemiological data betewen ages 25 and 74 could be fully explained on the basis of just two hits (Platt, 1955; Armitage and Doll, 1957).

Given the range of interpretations that can be imposed on the human epidemiological data, other investigators turned to more manipulatable animal systems. The results are again, however, less than clear-cut. For example, one study found that in vivo liver liver tumors created by continuous in vivo administration of a carcinogen showed an induction indicative of seven hits. However, a single large dose of the same carcinogen gave tumors with kinetics which could be extrapolated to suggest at most two initial concommitant hits followed by three later "background" (i.e., not carcinogen induced) hits (Emmelot and Escherer, 1977).

While the number of hits remains elusive, anywhere from two to seven, the conclusion that there are more than one is inescapable. Not all of the hits need be genetic, of course. There may be epigenetic as well as mutational events. Moreover, some of the kinetically identified "steps"

may not be phenotypic changes in the cancer cell at all. For example, a simple model of cancer could involve just two things: a mutational event with probability p and a stochastic initial escape of immune surveillance with probability q. The probability of the development of a tumor $p \cdot q$, would then give rise to second order kinetics reflective of two "hits".

Another, totally independent, line of investigation has also led to the conclusion that carcinogenesis is a multistage phenomenon. It was observed that certain benign papillomas induced by viruses or mutagenic carcinogens could be transformed into skin carcinomas by repeated application of other compounds which were not, in themselves, carcinogenic (Friedwald and Rous, 1944; Sall and Shear, 1940; Berenblum, 1941). On this basis, tumorigenesis was divided into two stages: an induction process termed "initiation" and a subsequent growth step termed "promotion". The tumor initiation can be experimentally brought about by a single application of an initiator. Promotion requires repeated application of the promoter, and it only occurs when an initiating event has preceded it. The initiation step can be provided by virally transmitted genetic information or by the mutagenic activity of such carcinogenes as 3-methylcholanthrene or urethane. The compounds active in the promotion of the tumor include certain phorbol esters, of which TPA is one of the most powerful (Hecker, 1971; Berenblum, 1975).

The mechanism by which promoters act is not yet clear. They have many physiological effects beyond tumor promotion including stimulation of anchorage independence, alterations of cell morphology, stimulation of proliferation and a variety of effects on cell differentiation (Rifkin et al., 1979; Fisher et al., 1979; Lowe et al., 1978; Diamond et al., 1977). Recalling that the protein kinase C serves as a receptor for TPA, one could

postulate that the actions may be due to repeated stimulation of the kinase C which could feed into the cells growth regulatory system and epigenetically stimulate it in conjunction with other oncogene-like perturbations already existing in the regulatory network. The existence of a previous genetic hit may interrupt some feedback loop which would otherwise limit the action of TPA on kinase C. Finally, the requirement for continued promotion may be abbrogated by another genetic hit, one which is able to statistically arise because of an expanded pool of target cells derived from the action of the promoter.

Other tumor types beside skin carcinomas exhibit similar multistep development. For example, hepatic carcinomas can be induced by initiation with a carcinogen followed by a promoting administration of phenobarbital (Peraino et al., 1973; Kitagawa et al., 1979).

A tumor also progresses through additional stages beyond those steps required to develop what is characterized as a malignancy. These stages are clinically defined on the basis of such subjective criteria as degree of differentiation, mitotic activity, amount of infiltration into surrounding tissue, amount of metastasis beyond tissue of origin, degree of vascularization, and size of the tumor (Rubin, 1973). A number of classification systems for extent of tumor progression have been established. For the clinician such formal stagings are useful in estimating prognosis, planning treatment, and exchanging information. For our purposes the established grades can not be correlated with any specific genetic or epigenetic change. Rather, they reflect two aspects of cancer that have to be kept in mind while we evaluate our work. First, unlike a clonal transfected cell line, real tumors are very heterogeneous. And second, tumors keep on evolving both in response to the host and to pressures

exerted by their own growth as long as they reside in an animal. Thus a cell may go through a short transition to a transformed state, but the development of a tumor is very complex. We have identified a mechanism by which an "oncogene" can be created and thereby transform a cell. As discussed in Chapter V, ras oncogene can be activated as an initial step in carcinogenesis, as demonstrated by their presence in benign papillomas. Once a ras hit has occurred, one would next like to know what other kinds of hits contribute to the further development of a tumor. Three possible classes of hits need to be considered: second hits within the same ras oncogene, second hits in other oncogenes, and hits other than in transfectable oncogenes.

A.6 Second ras Hits

Although all three ras oncogenes have been identified in independent isolates of the same tumor types, there are, as of yet, no examples of two different ras activations in the same tumor. This may be a simple reflection of the number of tumors assayed so far. If the probability of a transfectable oncogene participating in a given tumor is 1 in 10, then the odds of finding two in the same tumor should be 1 in 100. There have been a large number of tumor DNAs transfected until now, but not so many as to rule out this statistical explanation.

On the other hand, it is also possible that once a fully activated ras oncogene exists in a given tumor cell, there is no further proliferative advantage gained by the acquisition of a second one. While this might be true for a maximally activated ras oncogene, it is clear that not all activating lesions give an optimal growth advantage. For example, while lysine and valine substitutions at the twelfth amino acid of c-Ha-ras both

will cause transfected cells to grow in soft agar and allow them to be tumorigenenic in nude mice, the lysine substitution causes much slower growth in both assays. Moreover, the NIH3T3 cell foci caused by the valine substitution are made up of very round, densely overgrowing cells, the lysine-ras transformed cells form barely detectable foci which are easily missed in the absence of a selectable, cotransfected marker (A. Levinson, unpublished results).

Not only can different lesions lead to different kinetics of cell growth, they can also affect the pathogenesis in which the mutated oncogene can participate. A retrovirus-transduced Ha-ras gene with an amino acid 12 valine substitution or an amino acid 59 threonine substitution can cause both carcinomas and erythroleukemias in mice. An amino acid 12 arginine substitution, however, will only only cause sarcomas in a similar retroviral construct (D. Lowy, personal communication).

Thus there could be mutations in ras genes which could participate in an early stage of carcinogenesis and still leave room for a second hit in the same oncogene, which would be involved in a later stage of tumor progression, creating a "hypertransformed" phenotype. There is no direct evidence that such sequential hits have occurred in any cellular oncogenes analyzed thus far. However, very few activated oncogenes have been well characterized (as opposed to the large number which has been simply detected by a combination of transfection and Southern blot).

In Chapter V the genesis of the HaSV and KiSV viral oncogenes was considered. Both of these genomes show evidence of multiple hits in their activation. These could have been accumulated by selection for progressively stronger transforming alleles.

As discussed, there are no proven examples of progressive activation of cellular ras genes. However, weakly transforming alleles of human ras oncogenes have been observed. A method was developed for detecting cellular transforming genes based upon injection of transfected cells into a nude mouse rather than scoring foci in tissue culture (Blair et al., 1982). This assay proved more sensitive in some cases as tumors were elicited from the transfection of oncogenes which did not give rise to foci in the standard assay. Such weak activations have the potential for acquiring second ras hits.

If mild up-regulations of ras oncogenes make a selectable difference in tumor cell phenotype, then a two-step activation could have occurred in certain tumors such as the SW480 colon carcinoma where an activated Ki-ras gene is present in multiple copies (McCoy et al., 1983) and the Calu-1 carcinoma, in which a mutationally activated Ki-ras oncogene is expressed at a level five-fold above that of the normal allele coexisting in the same cell (Capon et al., 1983b). A case in which up-modulation of p21 is strongly implicated in carcinogenesis is the mouse adrenocortical tumor Y1 (Schwab et al., 1983). This tumor contains a 30-60 fold amplification of the c-Ki-ras gene both in double minute chromosomes and in a homogeneously staining chromosomal region. The amount of Ki-ras p21 is correspondingly elevated. No transfection data are presented for this tumor, but it is possible that some subset of the amplified sequences have progressively acquired a mutational activation, which could increase the ras oncogenic contribution. Such a sequence of events is known for another oncogene. Myc is amplified and its expression enhanced in the neuroendocrine tumor Colo 320. In that case, the amplified myc was subsequently mutated. The line contains copies of myc in a normal configuration and others in a

configuration containing rearrangements at the 5' end (Schwab et al., 1984).

Thus it is possible that multiple hits occur in ras oncogenes during tumor progression. An important question then becomes whether (multiple) ras activation(s) can be the full story of oncogenes or whether "hits" or other sorts are also required.

A.7 Non-ras Second Hits

The molecular evidence for several different genetic functions being required in tumor formation first came from the study of certain DNA tumor viruses. It was found that an "establishment" function (provided by the adenovirus Ela gene and the polyoma virus large-T gene) involved with immortalization of cell lines, and a second "transformation" function (provided by the adenovirus Elb gene and polyoma virus middle-t gene) involved with morphological alternations, anchorage-independent growth and ability to form tumors in animals. Both are needed to transform primary embryo cells in tissue culture (Houweling et al., 1980; Treisman et al., 1981; Rassoulzadegan et al., 1982). Unlike retroviral oncogenes, these DNA virus genes have no homology in the chromosomal DNA of normal cells. However, the involvement of more than one cellular oncogene in tumor formation was soon implicatated as well.

In ALV-induced chicken lymphomas the cellular myc gene is activated by the actions of the retroviral promoter, enhancer, and/or polyadenylation sequences. However, when the DNA prepared from such lymphomas was transfected, a different active cellular oncogene called B-lym was detected (Cooper and Neiman, 1981). In addition, activated N-ras oncogenes have been transfected out of two tumor cell DNAs which also contained altered

myc genes (Murray et al., 1983). The HL-60 promyelocytic leukemia contains amplified copies of myc (Collins and Groudine, 1982; Dalla Favera et al., 1982) and the AW Ramos American Birkitt's lymphoma contains a myc oncogene placed under the influence of an immunoglobulin regulatory region by virtue of a chromosomal translocation (Zech et al., 1976; Adams et al., 1983). (Most cancers show chromosomal abnormalities and many of the defects, such as band deletions or reciprocal translocations, are characteristic of particular malignancies (Yunis, 1983). Some of these have been tied to known oncogenes such as myc and abl, and many other shared chromosomal abnormalities may eventually be causally linked to oncogenesis.)

Combining the logic of the DNA tumor virus model and the existence of transfectable and "second" cellular oncogenes in the same tumor, several groups decided to investigate the actions of these various oncogenes in primary embryo cells (Land et al., 1983; Newbold and Overell, 1983; Ruley 1983). initially, the results seemed explainable by simple models of multiple oncogene action, but as will be discussed, as more data has been accumulated the picture has become more complex. Perhaps the most important "result" of these studies so far is that they have reinspired molecular biologists to think about carcinogenesis in terms of multiple steps after the brief period during which a one-hit acute retroviral/ cellular oncogene explanation dominated thinking.

Primary rat embryo cells when plated in vitro initially consist of a diverse population of cell types. Under standard tissue culture conditions mainly fibroblastic cells grow out. These cells are called rat embryo fibroblasts (REFs), although they may still consist of more than one cell type. REFs have a finite "lifespan" of approximately 40 population doublings. During this time the cells appear progressively "differen-

tiated" or "senescent", grow with longer and longer doubling times and exhibit poorer plating efficiencies with each passage.

When ras oncogenes are transfected into freshly plated REFs, small foci of morphologically altered cells can be seen that are not present in nontransfected dishes. These foci do not, however, overgrow the normal REF monolayer. If freed from the influence of closely surrounding cells these foci can grow out. This can be accomplished either by cotransfection with the drug resistance marker Ecogpt and killing nontransfected cells slowly with mycophenolic acid (more toxic drug markers such as neo do not work in this assay because they kill the REFs too quickly and thereby leave transfectants at densities too low to permit cross-feeding), or by plating the transfectants in semi-soft agar. This latter assay demonstrates that ras provides anchorage independence as well as morphological transformation. These ras-transfected REFs can then be expanded in culture. However, they still suffer from the finite lifespan of the untransfected REFs, and eventually they senesce and cease growing. These cells were not found to be tumorigenic when injected into animals.

Because myc oncogenes were implicated in various tumors which also contained ras activations, several laboratories attempted to determine what effect they would have on REFs. Our group found that myc transfected REFs could grow as continuous immortalized cell lines. These were not morphologically altered nor would they grow in soft agar or form tumors in animals. However, unlike ras transfectants, they could grow at low cell densities. Importantly, these immortalized lines appear at a lower efficiency than the ras foci in REFs. This could be due to a need for many transfected copies of the myc sequences that would be acquired more rarely by recipient cells, or because a different subpopulation of REF cell types

serves as the target for the two transfectants. A conflicting result was obtained in other laboratories: no myc immortalizaton was observed, but a unique morphological alteration occurred in myc-transfected REFs (B. Vennstom, unpublished results). This could mean that still another subpopulation of REFs were the target cells of myc transfection in those experiments. It may also mean that immortalization requires other, perhaps epigenetic, hits provided in our tissue culture system but not in theirs. This could also explain the lower efficiency we observed of myc immortalization relative to ras focus induction. In any event, the laboratories agree that myc transfectants fail to produce tumors when injected into animals.

Myc and ras were then cotransfected into REFs. Together they accomplished what neither could do before. Dense foci of transformed cells now overgrew the REF monolayer in the absence of selection or dispersal. These expanded into vigorous, immortal cultures of cells which were highly tumorigenic in animals. The concept of multistep cooperation between ras and myc in oncogenesis was thus vindicated.

It was subsequently found that other viral and cellular oncogenes could be grouped by their ability to "cooperate" with either ras or myc oncogenes in fully transforming REFs. It was therefore proposed that there are two complementation groups of oncogenes, one member of each being required for tumorigenicity. Two members of the same complementation class (e.g. ras and middle-t or myc and myb) do not have such an effect.

There are a number of problems with such an interpretation. While myc and ras clearly have different initial targets (being located in separate cellular compartments), both are pleiotropic in their effects and overlap in some of their consequences such as cell growth (see discussion above).

Moreover, myc and ras are not independent functions. Each has been shown to affect the expression of the other. Ras-transformed cells are known to secrete "tumor growth factors" (DeLarco and Todaro, 1976). When conditioned media from ras-transformed fibroblasts was added to nontransformed fibroblasts, myc expression was dramatically increased (M. Gilman, unpublished result). While this is clearly an indirect effect, it implies nonetheless that c-myc expression is likely to be altered in REFs transfected with ras. Conversely, myc has been shown to directly increase ras expression in cotransfections assayed 24 hours after transfection (R. Kingston, unpublished observation). This may be an artifact of transfection and not represent an in vivo interaction between nontransfected genes. However, it maybe the explanation for the cotransfection results. That is to say that much of the observed phenotype of ras/myc cotransfected cells may be attributable to the effects of ras alone, but at levels of expression not achieved in cells transfected with just ras. Unfortunately, little data presently exists on the RNA and protein levels in these various cell types.

The difficulty in interpreting the data acquired in transfection of such pleiotropic genes is exemplified by a pair of recent experiments, which are at least superficially very similar. When the selectable marker DHFR (under the SV40 early promoter) is transfected into (immortal) CHO cells, a certain number of stable colonies can be selected. Under the same set of conditions a cotransfection of DHFR with either Ela or myc results in tenfold more colonies (R. Kingston, unpublished result). Since both Ela and myc are known to effect the expression of the SV40 promoter the observation has been attributed to an increase in DHFR expression in cotransfected cells.

When another selectable marker, neo, is transfected into REFs, again a certain number of colonies are observed (although these are not immortal). When the same cells are cotransfected with neo plus p53 (a transformation-associated gene with certain myc-like properties in cotransfection with ras), once again, tenfold more colonies can be selected (L. Parada, unpublished result). In this case the result was interpreted in terms of a lowered density dependence provided by p53. Such a lowered cell density requirement has indeed been observed in myc and p53 transfected REFs and this could have allowed more neo colonies to survive. It must be noted that in the two experiments, different cell lines, selected markers, and cotransfected oncogenes were all used. The two interpretations may be valid in the two cases for which they were postulated, but the first explanation can by no means be ruled out in the second case: p53 may have acted to increase the number of G418 resistance colonies by increasing the level of neo expression.

Another problem in sorting out the myc/ras cotransfections is that under certain conditions each appears to be able to do without the other. Myc has no apparent effect when transfected into established rat cells. However, when a mammalian retrovirus is prepared transducing myc, it can create transformed, tumorigenic foci in infected rat fibroblast lines (B. Vennstrom, unpublished result). Myc can thus exhibit ras-like function. Similarly, ras-transducing virus can give rise to immortal, transformed, tumorigenic cell lines when used to infect REFs (C. Tabin, unpublished result; see below). This time ras appears to possess myc-like capabilities. Both of these experiments, seemingly at odds with the transfection data, can be explained by appealing to the existence of subpopulation of cells. Even such a resolution to the paradox is not

simple, since no (ras-like) transformed rat fibroblasts and no (myc-like) immortal REFs can be identified among the cells prior to infection.

Infection, as opposed to transfection, has the advantage that one can deliver a transduced oncogene to a much larger number of cells, including, perhaps, subpopulations of cells which are not easily transfectable. There has not been much work with retroviral infections of mammalian primary cells because of the convenience and reproducibility of using established cell lines. There are no immortal, established cell lines of chicken cells (transformed or "normal") so there have been far more extensive studies of avian transforming viruses in primary cells. By far the most work has been carried out with the src carrying retrovirus RSV.

Because chicken cells are never immortalized, other parameters such as cellular morphology, enhanced glucose metabolism and high levels of secreted plasminogen activator are used to correlate with tumorigenicity. If RSV-transformed, non-producer primary embryo cells are injected into the wingweb of a chick, the bird will develop a tumor which will eventually cause its death (Hanafusa, 1977). Once again, these tumorigenic cells are not immortal. Inversely, it can be noted that human tumors are also by and large not immortal when put in tissue culture and can only be established with some difficulty unless immortalizing agents such as Epstein-Barr virus are used. One could argue that human and chicken tumor cells are, in fact, immortal but that some factor not present in normal tissue culture media is required for the maintenance of the immortal state in those cells. However, the same appeal could be made for immortality of REFs. Immortality, like morphological transformation, must be defined under standard conditions. Thus, in a general (non-rodent-specific) sense, immortality in vitro is not a useful correlate of tumorigenicity.

Immortalization is a convenient function which can be studied in rodent cells, and which corresponds well to some activity possessed by certain oncogenes which may be required for tumorigenesis. But it should be kept in mind that immortality is the assay, not the *in vivo* required function.

While *myc* does not immortalize primary avian fibroblasts, it can transform them. The *myc* transducing virus E26, for example, transforms infected primary quail cells (Graf et al., 1979). Once again, the overlap in function between *myc* and transforming *oncs* is driven home.

A major difference between the avian and mammalian primary fibroblast systems is that, because it is so much more extensively studied, the avian system has been optimized for transforming capacity to a much greater extent. This optimization may go a long way toward explaining the differences in results achieved in the two cell types, other than immortality.

When primary chicken cells are infected with RSV, the number of foci observed is linearly proportional to the dilution of virus, indicating a single-hit induction of tumorigenicity (Hanafusa, 1977). At high virus titer, the entire plate of cells can be seen to become transformed within 48 hours. However, at low titers the transformed foci do not grow out because the nontransformed cells have a growth advantage over them and so constrain focus development. This problem can be abrogated by overlaying the cells 16 hours postinfection with crude agar which complexes with certain serum factors and gives the growth advantage to the transformed cells (Hanafusa, 1977). This might be the reason *ras*-transfected REFs form limited foci as well. It would be worth trying agar overlays in that system.

As mentioned above, the avian primary fibroblast infections have been highly optimized. To be able to observe single-hit transformed foci, the primary cells must be prepared from exactly 11-day-old embryos and plated at a particular density, and then split into secondary cultures 6-10 days later. Cells prepared from older embryos are still infectable, but do not develop transformed foci. Similarly, cells prepared as described quickly lose their ability to be transformed when carried in culture beyond 10 days (B. Mathey-Prevot, personal communication). No period of high susceptibility to one-hit-transformation exists in the mammalian REF system as it now exists. There does, however, appear to be a small subpopulation of cells which initially can be transformed in a single hit; as in the avian system, this potential is quickly lost in culture.

The analysis of REFs with retrovirally transduced ras genes has been hampered by the fact that they are 500-fold less infectable than mouse NIH 3T3 cells. This was assayed by infection with a virus transducing the neo-selectable marker (P. Jat, personal communication) and by a focus assay using low titers of HaSV or JEV (a c-Ha-ras transducing retrovirus). At low titer HaSV and JEV give rise to small foci with limited growth very similar to ras-transfected REF foci. However, at very high titer, with helper virus to allow spread, the entire plate is seen to transform morphologically. As these cells are passaged, many transformed cells senesce, but eventually a plate of immortal, transformed cells can be selected which are tumorigenic in nude mice (C. Tabin, unpublished result). This could be due to either the selection of a second "hit" during passage (even though this could not be achieved by passing an expanded focus from a single ras-transfection) or it could be due to the existence of a subpopulation of one-hit-transformable cells pre-existing in the REF

population. To test this, various numbers of REFs were infected on different-sized plates and wells to keep cell density approximately constant. The results showed that immortal, transformed lines could be produced by infection of 1-5 x 10^4 or more cells but not by infection of smaller numbers. This was taken as an indication that we were dealing with a susceptible subpopulation, which existed as 10^{-4} of the total REF culture (J. Cunningham and C. Tabin, unpublished result).

It was also observed that the ability to be transformed by infection was lost rapidly between the 5th and 9th days in culture. There was also a decrease in infectability during this period, but it was far less rapid, as shown in an XC plaque assay (C. Tabin, unpublished result).

These results could be interpreted on the basis of a primitive cell type which could have its growth regulated in such a way that ras alone would be capable of fully transforming the cell (a cell with a myc-like gene already active in the "two-hit" parlance). Some primative cells do behave as if they contain a myc-like hit. Teratocarcinoma cells are rumored to be able to support the growth of Ela mutants of adenovirus, while the differentiated progeny of those cells do not allow the mutant virus to grow. One could similarly postulate that as the REFs differentiate in culture, those cells would also modify their growth regulation (turn the myc-like gene off) and no longer be transformable by ras alone. The early transformation by ras would, according to this model, freeze the cells in the primitive state (prevent myc shut-off).

There are precedents for such a transformation-dependent freezing of differentiation. Temperature-sensitive mutants of src can be used to transform myoblasts (Fiszman and Fuchs, 1975; Holtzer et al., 1975) or retinal melanocytes (Boettiger et al., 1977). In either case, raising the

cells to the nonpermissive temperature and releasing the cells from transformation results in irreversible differentiation of the blast cells. It would be worth repeating the REF ras infections with a ts KiSV variant to test this hypothesis.

In any event, the ability of ras alone to elicit tumorigenic transformation of even a subset of primary cells calls into doubt the simple form of the obligatory two-hit, ras plus myc model of carcinogenesis.

Another complication arises when one observes the phenotypes of tumors derived from transfection of various combinations of oncogenes. Cotransfections of ras with myc, p53, or large-T each gives tumors with different growth properties. Ras and myc, for instance, give a tumor of finite dimensions. Ras and large-T, on the other hand, create a tumor which continues until it kills the host animal. This has led others to speculate about the existence of a third oncogene function, large-T being hypothesized to contribute two independent functions, perhaps acquired as a consequence of the evolutionary streamlining of the virus (Land et al., 1983b). Before the search is initiated for cellular members of this third oncogene complementation group, I would again suggest that what I consider to be more likely is that there is a large interconnected network of growth-regulating genes in the cell. By transfecting different combinations of oncogenes, one perturbs that network in slightly different ways (although all in the same general direction) with somewhat different phenotypic consequences. The difference between this and a discrete hit model is diagrammed (Figure 22) and the ramifications of the differences between the models are explored further in the legend.

However, the contention that all the "oncogenes" are likely involved in the same function does not mean that other kinds of genes are not also involved in carcinogenesis.

Figure 22

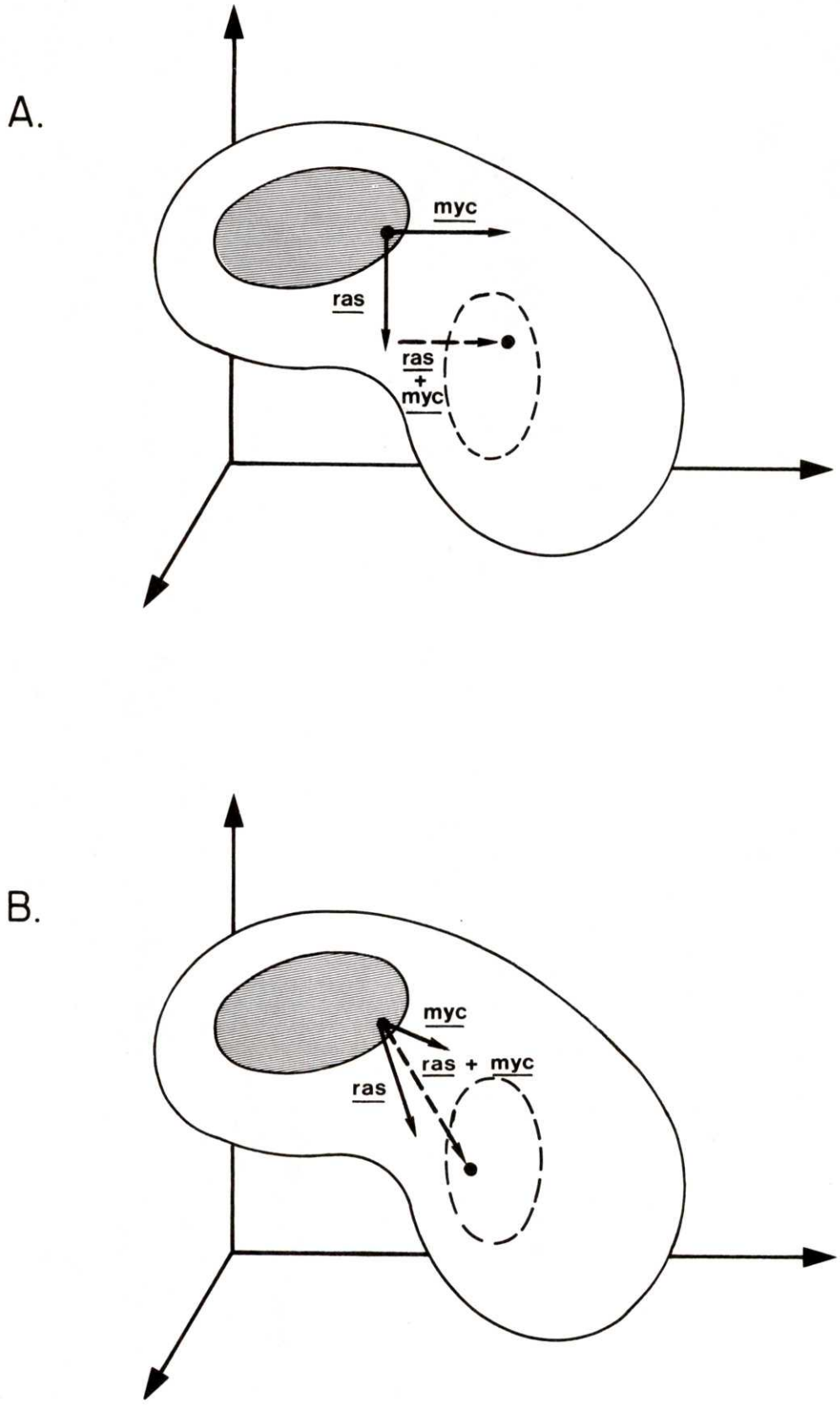


FIGURE 22

Representation of two models for oncogene cooperation in the genesis of a transformed cell. The N parameters of a cell's growth regulation are plotted in N-space; here three parameters are represented in three dimensions. Each axis represents a single parameter such as anchorage dependance or degree of unlimited growth potential ("immortality"). The solid line outlines the region of 'growth-space' in which a viable cell can exist. The hatched region is that area of growth-space occupied by a "normal" cell type (such as embryo fibroblasts in an in situ developing rat). The dotted outline is the region of space containing all cells which we define as being cancerous and which are derived from the normal lineage (in the hatched region).

In model A, each active oncogene can affect exactly one parameter. The addition of any one oncogene to a cell alters the corresponding parameter of that cell such that the coordinate of that parameter maps in the cancer region of space. For all the coordinates of a cell to place it in the cancer region multiple parameters must be altered and hence multiple oncogenes are required. The effects of multiple oncogenes are linearly additive.

In model B, each oncogene itself affects multiple parameters: The effects of different oncogenes on the same parameter may overlap extensively. The addition of any one oncogene to a cell perturbs the cells growth parameters in the general direction of the cancer region; however the vector of any single oncogene might not point directly towards the cancer region, or have a large enough effect to reach it. Multiple oncogenes are, therefore, once again required to reach the cancer region of

space. In this model we postulate that the different oncogenes affect not only the same set of parameters but also the expression of each other. Hence they are not independant and hence their effects can not be added in a linear fashion.

Thus, if model B is the more accurate view of growth control in a cell, this is an example of an instance when the reductionist program fails. The nonindependance of oncogene action means that the progression to a cancerous state can not be understood by studying the individual oncogenes. Their interaction leads to a complexity of organization that demands that they be viewed on the level of their interaction. (This does not, of course, detract from the merit of studying them in isolation as well.)

A.8 Non-Oncogene Hits

Cellular oncogenes are defined by their ability to transform cells in a transfection assay. As discussed above, only 10% of all tumors contain such a transfectable transforming activity. However, since all tumors exhibit deregulated growth, one would assume that they contain other sorts of genetic lesions which allow the same phenotypic result. Such nontransfectable oncogenic lesions could include: amplified sequences (known to exist, and discussed above), exceptionally large genes which are fragmented in transfection (a problem demonstrated by C. Shih's success in transfecting the MC5.5 cell line oncogene by chromatin transfection but not by naked DNA transfer; personal communication); genes only potent in the correct celltype (e.g., growth factor oncogenes perhaps requiring that their homologous receptor be expressed by the recipient cell for transformation to be observed); two or more unlinked genes required in concert for transformation (these could be two weakly acting alleles similar to the more powerful ones which are readily observed but whose actions are required in an additive fashion, or two disparate genes whose products function together in a cell) and recessive lesions (which clearly could not be observed in an assay which scores dominant transformation).

The existence of recessive oncogenic lesions was first indicated by genetic analysis of inherited predispositions to certain forms of cancer. As expected, DNA prepared from patients with such high cancer risk syndromes lacks transforming activity in transfections (Needleman et al., 1983; J. Cunningham, unpublished observation). It has also been shown that patients at risk for Wilms tumor or hereditary retinoblastoma are heterozygous at loci responsible for the traits. When these patients develop tumors, a high proportion of the malignancies have clearly become

homozygous at those loci (as evidenced by cytological examination), and the rest likely have less easily detectable changes which also destroy the genes at those loci on the heterologous chromosomes (Cavenee et al., 1983; Benedict et al., 1983; Koufos et al., 1984; Orkin et al., 1984; Reeve et al., 1984; Fearon et al., 1984).

Recessive lesions are likely also the reason behind observations that a normal cell phenotype can often be dominant to a malignancy in cell fusions (Harris et al., 1969; Murayama and Okada, 1970; Murayama-Okabayashi et al., 1971; Wiener et al., 1972) and that two fused malignant cells can complement each other and result in a nonmalignant hybrid (Ephrussi, 1972).

Given both the existence of this data, and the fact that 90% of all tumors fail to transfect oncogenes, recessive lesions may actually predominate in the genesis of cancer. This would be a reflection of the relative statistical ease of "hitting" a dominant gene one time in specific activating locations, as opposed to "hitting" two copies of a recessive gene anywhere that inactivates the gene. As a tumor progressed, second hits in the development of later recessive lesions would be facilitated by the karyotypic instability of tumors once they are initiated.

Ways can be devised to study all of the possible types of non-transfectable genetic lesions listed above. For example, weakly oncogenic alleles could perhaps be detected by making NIH 3T3 cells even more susceptible to transformation than they already are. Myc has no phenotypic effect on NIH 3T3 cells which acquire the oncogene by transfection, but such myc transfected fibroblasts might be an example of such a super-susceptible recipient for genomic transfection of tumor cell DNA. As mentioned earlier, very large oncogenes might be detected by chromatin transfection, and so on. One has to question whether such elaborate

experiments would actually detect any new classes of oncogenes, however, or just weaker or larger ras-type genes.

A type of lesion that might be worth the effort to isolate would be the recessive mutations, since they are likely to be in genes different from those activated in a dominant manner. A protocol which suggests itself would be the transfection of normal DNA into tumor cells and the selection of nontransformed "revertants". After the studies described above with primary embryo cells, we have reason to hope that such reverted tumor cells would retain their ability to grow in culture, since transformation and establishment are not irrevocably linked functions.

The stumbling block to such a study is the lack of a selection scheme adequate to detect revertants at the low efficiency achieved in a genomic transfection. Present selection schemes use such strategies as killing noncontact inhibited cells at confluence with metabolic toxins through several rounds of selection. These protocols, while laborious, have proved adequate for the isolation of cell lines in which oncogenes were destroyed by insertional mutagenesis (Varmus et al., 1981) and "second site revertant" cell lines, reverted in loci not linked to the transforming oncogenes (Noda et al., 1983). These latter lines will be useful in further elucidating the functions of oncogenes. Nonetheless, the schemes used in these studies are just too cumbersome for use in screening for recessive tumor lesions. That does not mean that better methods could not be developed. There has been limited success in devising selections based on differential adherence to collagen substrate (Otto and Tabin, unpublished results) and on differential surface change in adherence to glass beads (Tabin and Thilly, unpublished results). A combination of several different methods may allow efficient selection of revertants.

Once a selection scheme is developed, still better results could in theory be achieved using infection with retrovirally transduced cDNA libraries prepared from normal cell messages instead of using transfection of DNA.

The recessive mutations just considered might still turn out to be part of the cellular growth control system. Other hits, not involved with growth per se, are likely to be important in tumorigenesis. Examples of such hits would be those having to do with vascularization of a tumor, modulation of surface antigens, and cell properties important in tumor metastasis. These events are not as easily amenable to in vitro study as is "transformation". However, the demonstration that transfected oncogenes can be assayed by directly injecting transfected cells into an animal (Blair et al., 1982) indicates that an in vitro model may not be needed to study these phenomena.

On that basis, schemes can be developed to study metastasis or vascularization. Since ras transformed NIH 3T3 cells form tumors which do not metastasize and are poorly vascularized, these cells can be used as recipients for transfection of DNA prepared from metastatic, vascularized tumors. Such transfectants can then be injected directly into animals.

In vivo studies such as these get beyond the molecular biology of individual cells and address questions that involve interactions with the host animal.

A.9 The Organismal Level

Even when studying activated oncogenes, we have skirted issues at the organismal level. When we say a cell transformed by a given oncogene is or is not tumorigenic, we are referring to the result of injecting 10^5-10^6 cells into an irradiated nude mouse which lacks the immunological

competence to actively fight the tumor. But natural tumors do not originate from 10^6 cells; they develop clonally from single progenitor cells. Some "hits" in vivo may be epigenetic events that allow transformed cells to overcome architectural barriers to expand to some critical mass. In hosts other than the nude mouse, the immune surveillance must also be contended with by a developing tumor. Our present knowledge of oncogenes does not allow us to evaluate how they might relate to such events. This is very lucky because I lack the knowledge of tumor immunology that would otherwise be required to put oncogenes into such a perspective.

The other topic which needs addressing on the organismal level is the relevance of oncogene activations of cancer therapy. There was some initial hope that assays based on the presence of particular oncogenes in tumors might aid in diagnostic classifications and reflect prognosis and optimal therapy choice. The fact that ras oncogenes have been identified at varied stages of all types of tumors make this unlikely in the case of these oncogenes. As the patterns become clearer for other oncogenes, it is possible that they will have clinical applicability similar to cell surface markers have in defining tumor subtypes.

From a true therapeutic standpoint, ras oncogenes do not seem very promising targets. Since p21 is inaccessibly situated inside the cell membrane, it is not likely to be amenable to immunologically directed agents. Moreover, there are not known drugs which interact with p21. If a pharmacological discovery of such an agent were to occur, analogs could be screened for differential activity with the normal and transforming varients. In the case of ras oncogenes, however, targeted therapy remains unlikely. However, in the case of growth factor receptors altered to form oncogenes (so far only known from tumors of rats), there is the potential

for attacking an external domain of the protein which has unique determinants as a result of oncogenic activation. Such a therapeutic approach would be very attractive, since, as opposed to other tumor antigens which can be the target for immunologically directed "magic bullets", an oncogene causally related to a tumor cannot be modulated without concomitant loss of transformation. If such opportunities exist in human cancer, they unfortunately appear to be rare.

A.10 The Population Level

Surprisingly, at this highest level of biological organization reductionist study of oncogenes may have direct relevance. Since by far the majority of all human cancers are environmentally caused (when "environment" is interpreted in its broadest sense to include lifestyle and diet), knowledge of the risks of different carcinogens is important for understanding epidemiological data. While oncogenes are not "tumor-specific", we have seen that they do have a strong carcinogen-specific component. Thus in studying the incidence of a particular form of cancer it may prove invaluable to be able to plot the relative activations of various oncogenes to identify patterns affected by different carcinogenes.

If such connections do indeed prove useful it will not be because of any intentional reductionist investigation of a much higher level phenomenon, but rather because of the kind of connections that exist between levels which we can never predict in advance.

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