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GENETIC ANALYSIS OF THE TRANSFORMED PHENOTYPE IN MOUSE
CELLS

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GENETIC ANALYSIS OF THE TRANSFORMED PHENOTYPE
IN MOUSE CELLS

by
MICHAEL BARRY SMALL

A dissertation submitted to the Graduate
Faculty in Biochemistry in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy, The City
University of New York.

1984

This manuscript has been read and accepted for the Graduate
Faculty in Biochemistry in satisfaction of the dissertation
requirement for the degree of Doctor of Philosophy.

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INTRODUCTION

The causal relationship between cancer and environmental factors has long been appreciated. In 1775, Sir Percivall Pott, an English surgeon, described the high rate of scrotal cancer in chimney sweeps of London (Haagensen, 1931). Pott concluded that repeated deposits of coal soot, along with poor hygienic habits, must have caused their malignant disease. Since that time, a long list of compounds have been incriminated in a variety of cancers, most often as the unforeseen aftermath of industrial exposures (see Table 1). The rise in the occurrence of lung cancer in this century has been attributed in no small part to smoking (Selikoff and Hammond, 1975), and geographic variations in the rates of liver, bladder and gastrointestinal cancer have been correlated with cultural dietary differences (Wynder and Shigematsu, 1967). Physical agents have also been implicated in the induction of cancer (Borek, 1982). Studies of the atomic bomb survivors of Hiroshima and Nagasaki in World War II have provided compelling evidence that ionizing radiation can induce leukemia. In addition, prolonged exposure to intense ultraviolet radiation has been clearly demonstrated to increase the risk of skin cancer. Doll (1977) has estimated that 80-90% of human cancers are environmentally induced.

An impressive body of clinical evidence has been amassed invoking a genetic component in the development of human neoplasms. In particular, it has been recognized that there exist individuals who inherit, often in an autosomally dominant fashion, predisposition to a wide variety of cancer types (reviewed in Mulvihill et al., 1977). These include car-

Table 1. Chemicals recognized as carcinogenic in man^a

Chemical Mixtures	Site of Cancers
<u>Polycyclic aromatic hydrocarbons</u>	
Soots, tars, oils	Skin, lungs
Cigarette smoke	Lungs
<u>Industrial chemicals</u>	
2-Naphthylamine	Urinary bladder
Benzidine	Urinary bladder
3-Aminobiphenyl	Urinary bladder
Chlormethyl methyl ether	Lungs
Nickel compounds	Lungs, nasal sinuses
Chromium compounds	Lungs
Asbestos	Lungs
Arsenic compounds	Skin, lungs
Vinyl chloride	Liver
<u>Drugs</u>	
N,N-bis(2-chloroethyl)- 2-naphthylamine	Urinary bladder
Bis(2-chloroethyl)sulfide (mustard gas)	Lungs
Diethylstilbestrol	Vagina
Phenacetin	Renal pelvis
<u>Naturally occurring compounds</u>	
Betel nuts	Buccal mucosa
Aflatoxins	Liver
<u>Potent carcinogens in animals to which human populations are exposed</u>	
Cyclamates	Bladder
Sterigmatocystin	Liver
Cyasin	Liver
Safrrole	Liver
Pyrrolizidine alkaloids	Liver
Nitroso compounds	Esophagus, liver, kidney, stomach

^aAdapted from Heidelberger (1975).

cinoma of the breast, colorectal carcinoma, malignant melanoma, retinoblastoma, Wilm's tumor, neuroblastoma, neurofibromatosis, and chronic myelogenous leukemia. Non-random cytogenetic abnormalities (i.e. chromosomal deletion or translocation) have been associated with several of these malignancies (Harnden, 1977; Rowley, 1980).

Isozyme analysis of neoplasms in female subjects heterozygous for variant alleles of glucose 6-phosphate dehydrogenase (G6PD) has shown that, with some rare exceptions, all malignant tumors of man are of monoclonal origin (Fialkow, 1976, 1977; Nowell, 1976). Implicit in this observation is the assumption that the aberration in the initial cell, resulting in uncontrolled cell division, is stably transmitted to its progeny in the formation of a malignant tumor. This, considered together with the epidemiological and clinical evidence alluded to above, predicts that cancers, be they spontaneous in origin or induced by carcinogenic agents, arise from a heritable perturbation at the genomic level. Although chemicals known to be carcinogenic have been recognized to bind covalently to many cellular macromolecules, including DNA, RNA and proteins (Miller and Miller, 1971), the work from Ames' laboratory in particular (Ames et al., 1972, 1973; McCann and Ames, 1976; Ames, 1979), has provided strong support for the hypothesis that chemical carcinogens exert their effects by inducing somatic mutations in some relevant sequence(s) of DNA. They demonstrated that many chemical carcinogens (including all polycyclic aromatic hydrocarbons), when exposed to microsomal mixed function oxidases in rat and human liver homogenates, are activated to form potent frameshift mutagens. In their "ultimate" form, they are readily detectable by their mutagenic activity in sensitive Salmonella indicator strains. Other compounds, such as nitros-

amides and most alkylating agents, require no prior activation and cause mutations by inducing single base-pair substitutions. Various types of radiation have also been shown to be mutagenic, in the broad sense of causing some genomic change (Muller, 1954; Stewart, 1971). At least one human disorder, xeroderma pigmentosum, predisposes individuals to UV-induced skin cancer through a defect in DNA repair (Cleaver, 1968, 1977; Lehmann et al., 1975), further establishing a role for somatic mutation in cancer.

Advances in experimental biology and somatic cell genetics over the past decade have provided the tools with which we have begun to explore the mechanisms of carcinogenesis at the molecular level. Such advances form the basis for the studies to be discussed herein, and are the subject of review in the following Background section.

BACKGROUND

A. Properties of transformed cells in vitro

Neoplastic cells can be distinguished from normal cells by many criteria though they may also express features common to the normal cells of their origin. Some of these phenotypic changes found in neoplastic cells can also be recognized in cells cultured in vitro and such cells are described as "transformed". Transformation in vitro can occur spontaneously, or due to exposure of the cells to oncogenic DNA or RNA viruses, physical agents (e.g. x-rays, UV-irradiation) or chemicals.

The number of changes necessary to transform a normal cell into a tumorigenic cell cannot be strictly defined at the present. An established neoplasm in vivo is the result of two distinct processes: (i) the generation of a transformed cell, and (ii) the growth of this cell into a neoplastic mass. In the second phase, altered cells may emerge as the result of a sequence of genetic changes such as mutations, chromosomal aberrations and aneuploidy, as well as by the influence of such epigenetic factors as hormones and angiogenesis. Cells transformed in vitro can best be regarded as at least the initial precursors of neoplastic cells (Pontén, 1976).

In the case of fibroblastic cells, transformation in vitro is usually recognized by altered morphology and growth behavior of cells.

- (a) Loss of contact inhibition of movement. Transformed cells have reduced contact inhibition of movement resulting in overlapping of cells and a criss-cross pattern of cell distribution (Abercrombie, 1966).

- (b) Saturation density. Under standard experimental conditions, the number of cells per unit surface area at the stage of stationary growth is much higher for most transformed cells, although the absolute values can vary with the culture environment. The reduction in DNA synthesis and cell division characteristic of untransformed cells permits the isolation of transformed foci as a group of cells which are morphologically altered and not subject to the growth inhibition prevalent in the normal confluent culture (Stoker and Rubin, 1967).
- (c) Serum factor(s) requirement. Transformed cells may have reduced requirements for growth factor(s) provided by serum (Holley and Kiernan, 1968; Terzaghi and Little, 1976).
- (d) Anchorage dependence. Non-transformed fibroblasts are unable to grow if suspended in semi-solid medium (i.e. an attachment to a suitable surface is denied), whereas transformed cells may form grossly visible colonies (MacPherson and Montagnier, 1964).
- (e) Tumorigenicity. Many transformed cells in vitro are capable of producing tumors in vivo when injected into histocompatible or appropriately immunosuppressed hosts, whereas normal cells fail to do so (Shin et al., 1975).

Focus formation, growth in semi-solid medium (e.g. agarose or agar), or growth in medium depleted of growth factors have been the parameters most often exploited for isolation of transformed cells since they represent changes in the normal pattern of fibroblast growth. In particular, anchorage independence has been found to correlate most favorably with the tumorigenic potential of transformed cells in nude

mice (Freedman and Shin, 1974; Shin et al., 1975). It should be noted here that these criteria are not readily applicable to transformation of epithelial cells, and thus the great majority of in vitro studies have utilized fibroblastic cell lines. This points out an obvious caveat in relating in vitro findings to in vivo realities, since it is estimated that as much as 90% of all human cancers are carcinomas of epithelial origin (Harris et al., 1978).

Transformed cells can be obtained which show all, or only some, of the various parameters of transformation described above, suggesting that multiple functions are involved. Several approaches have been undertaken to assess possible interrelations of these parameters. One is the isolation of phenotypic revertant cells which have lost one or more properties of the transformed cell (Vogel and Pollock, 1974). Another has been the isolation of transformed cell lines which are temperature sensitive for a particular transformed phenotype (Renger and Basilico, 1972; Di Mayorca et al., 1973; Miyashita and Kakunaga, 1975; Yamaguchi and Weinstein, 1975). These studies have shown that loss of one phenotype is often but not always associated with loss of other transformation markers. Both approaches have provided additional concrete evidence that specific cellular genes and their products are involved in expression of the transformed phenotype, even in cells transformed by oncogenic viruses.

B. Expression of the transformed phenotype in somatic cell hybrids

Barski et al. (1961) showed for the first time that somatic cells can hybridize spontaneously in vitro. Two major developments concerning somatic cell hybridization occurred soon thereafter. Littlefield (1964)

demonstrated that hybrid cells could be selected for in vitro by co-cultivation of mouse cells deficient in thymidine kinase (TK) with those deficient in hypoxanthine:guanine phosphoribosyltransferase (HGPRT) in the presence of hypoxanthine/aminopterin/thymidine (HAT) selective medium as defined by Szybalska and Szybalski (1962). Cells resistant to the purine analogs of hypoxanthine and guanine, such as 6-thioguanine, are commonly deficient in HGPRT, which converts hypoxanthine or guanine to inosine 5'-monophosphate or guanosine 5'-monophosphate. They are, therefore, unable to utilize the hypoxanthine in HAT medium. Similarly, cells resistant to thymidine analogs, such as bromodeoxyuridine, are deficient in TK and unable to utilize the thymidine in HAT medium. Both types of drug-resistant cell lines are able to grow in non-selective medium by virtue of their de novo pathways of thymidylate and purine nucleotide synthesis. These pathways are blocked by inhibitors of folate reductase, such as aminopterin. Therefore, in HAT medium, HGPRT and TK become essential enzymes for growth and only hybrids formed by the fusion of the two different parental cells (heterokaryons) are viable. The second seminal advance came with the observation of Harris and Watkins (1965) that UV or β -propiolactone-inactivated Sendai virus was capable of markedly increasing the frequency of cell fusion; since then, other agents, such as polyethylene glycol (Pontecorvo, 1975; Davidson and Gerald, 1976), have been discovered to be equally or more effective in promoting cell fusion.

Somatic cell hybridization has been used by several investigators to determine the dominance or recessiveness of the expression of tumorigenicity. Such studies have been the subject of several reviews (Miller, 1974; Ozer and Jha, 1977; Croce, 1980). Early experiments

employing hybrids between tumorigenic and non-tumorigenic cells assessed the ability of such hybrids to form tumors in histocompatible or immunosuppressed animals. Examples of dominant, recessive and intermediate expression of tumorigenicity in such hybrids has been reported. The work of Harris and associates (Harris et al., 1969; Bregula et al., 1971), recognized as the most extensive in this field, concluded that tumorigenicity was suppressed (i.e., recessive) in hybrids between tumorigenic and non-tumorigenic mouse cell lines. Those hybrids which occasionally formed tumors were postulated to have lost chromosomes of the non-tumorigenic parent responsible for suppression, although no single chromosome was ever identified as having this capability. A problem in the study of the expression of tumorigenicity in hybrid cells is that the hybrids must be injected into immunosuppressed animals or into nude mice (Flanagan, 1966). These hosts have in the past been considered neutral. However, the discovery of natural killer (NK) cell activity in nude mice (Herberman and Ortaldo, 1981) has raised the possibility that the failure of certain cells or hybrids to form tumors is due to a cytotoxic response of the host.

More limited studies have been performed assessing the expression of the transformed phenotype in vitro in cell hybrids. Jha and Ozer (1976) demonstrated that hybrid clones derived by fusion of SV40-transformed mouse fibroblasts with untransformed mouse fibroblasts showed dominant expression of the transformed phenotype with respect to colonial morphology and ability to synthesize DNA in low serum. For use as the untransformed parent, they isolated a subline of Balb/c 3T3, designated "THO-2", which was sequentially selected by mutagenesis for resistance to 10^{-5} M thioguanine (TG^R) due to deficient activity of

HGPRT and resistance to 3 mM ouabain (OUA^R), presumably due to an altered membrane ATPase (Baker et al., 1974). This cell line retained the growth properties of the untransformed Balb/c 3T3 parent, i.e. density-dependent inhibition of growth (DDIG), low efficiency of colony formation in low (1%) calf serum, and anchorage dependence. Coupling of a recessive (TG^R) and a co-dominant (OUA^R) marker made TH0-2 a "universal hybridizer" with cells lacking any selective markers by selection in HAT medium supplemented with 1.5 mM ouabain. Over 150 hybrids between TH0-2 and SV40-transformed Balb/c 3T3 (SV/3T3), isolated under conditions that did not lead to selective advantage for the expression of the transformed phenotype, were assessed for morphology and DDIG using an autoradiographic procedure to measure DNA synthesis in situ (Fisher and Yeh, 1967). All hybrid colonies were found to express the transformed phenotype characteristic of SV/3T3. A possible objection to these findings was that TH0-2, being an established cell line with an aneuploid karyotype, might have chromosomal abnormalities making it unable to suppress the transformed phenotype. This possibility was discounted since hybrids between SV40-transformed TH0-2 and diploid secondary mouse embryo fibroblasts gave identical results. Hybrids between TH0-2 and Rous sarcoma virus (RSV)-transformed Balb/c 3T3 also displayed dominant expression of the transformed phenotype as assessed by morphology and ability to form colonies in soft agarose (Small, Jha and Ozer, unpublished results).

These studies were extended to the analysis of hybrids between TH0-2 and chemically transformed mouse fibroblasts. Jha et al. (1978) utilized as the transformed parent a cell line, designated "Clone H", derived by Quarles and Tennant (1975) by treatment of Balb/c 3T3 with

the mutagenic and carcinogenic chemical nitrosocarbaryl. Clone H exhibits its morphologic and growth characteristics associated with the transformed phenotype. Unlike its untransformed parent, it has an altered morphology, grows to high saturation density, is anchorage independent, is able to form colonies on monolayers of normal 3T3 cells, and forms tumors upon injection into athymic (nude) mice. As shown in Table 2, hybrids between TH0-2 and Clone H consistently express many of the growth properties characteristic of the transformed parent. In view of the aneuploidy of Balb/c 3T3 and the difficulty in obtaining autosomal recessive mutants in that cell line (Jha et al., 1980), these results suggested that chemical carcinogenesis in this system might well be expected to show a strong bias for a dominant (one-hit) mode of expression. Similar experiments were performed with another chemically-transformed mouse fibroblast cell line, A31/DMBA 435. This cell line was derived by Sen and Todaro (1978) by treatment of Balb/c 3T3 with the carcinogen 7,12-dimethylbenzanthracene (DMBA). The data shown in Table 3 (Small et al., 1984) lend further support to a dominant mode of expression of the chemically-transformed phenotype in the Balb/c 3T3 system. These findings led us to anticipate that upon introduction of more limited genetic information into a normal recipient cell line by gene transfer techniques to be described subsequently, one might expect to observe dominant behavior (i.e. expression) of a putative transforming gene from chemically-transformed mouse cells.

C. In vitro transformation of mammalian cells by chemical carcinogens

The first unequivocal demonstration of chemical carcinogen-induced transformation in vitro was reported by Berwald and Sachs (1963, 1965).

Table 2. Growth Properties of Cell Hybrids: TH0 x Clone H^a

Cell Line	Colonial Morphology	Saturation Density (cells x 10 ⁻⁴ /cm ²)		Colony Formation (%)		
		10% serum	1% serum	plastic	monolayer of A31	agarose
Clone H	Round/dense	30	7.0	23	18	10.0
TH02	Flat	7	3.6	8	<0.1	<0.01
Hybrid clone 1	Fibroblastoid	36	3.6	20	33	5.0
Hybrid clone 2	Round/dense	25	4.6	25	60	8.0
Hybrid clone 3	Intermediate	25	3.6	21	11	8.0
Hybrid clone 4	Round/dense	18	5.2	nd ^b	nd	18.0
Hybrid clone 5	Round/dense	18	4.6	nd	nd	0.8
Hybrid clone 6	Intermediate	21	5.3	19	33	0.8
Hybrid clone 7	Round/dense	18	3.2	nd	nd	9.3
Hybrid clone 8	Round/dense	18	4.2	nd	nd	7.0
Hybrid clone 9	Round/dense	21	3.2	nd	nd	11.0
Hybrid clone 10	Round/dense	18	3.6	nd	nd	7.0

^aData redrawn from Jha et al. (1978)

^bNot determined

Table 3. Growth Properties of Cell Hybrids: TH0 x A31/DMBA 435^a

Cell Line	Colonial Morphology	Colony Formation (%)	
		Plastic	Agarose
A31/DMBA	Dense/piled	36	30
TH02-2	Flat	10	<0.01
Hybrid clone 6	Dense/piled	22	3.1
Hybrid clone 8	Intermediate	nd ^c	2.5
Hybrid clone 10	Dense/flat	nd	0.5
Hybrid clone 12	Intermediate	30	19.0
Hybrid clone 14	Dense/flat	nd	2.0
Hybrid clone 16	Intermediate	nd	0.4
Hybrid clone 18	Intermediate	nd	1.0

^aData redrawn from Small et al. (1984).

^bAdditional colonies observed in this experiment had a dense/piled morphology characteristic of A31-DMBA and hybrid clone 6.

^cNot determined

They exposed primary Syrian hamster embryo (SHE) cells to various polycyclic aromatic hydrocarbons and obtained transformed cells which piled up in a criss-cross array. Morphological transformation was observed 1-8 days after treatment with carcinogen and was found to be dose-dependent. These transformed cells produced fibrosarcomas when injected into newborn hamsters. Since then, a great deal of work has been performed in this field and is the subject of several excellent reviews (Miller and Miller, 1974; Heidelberger, 1973, 1975; Barrett and Ts'o, 1978a).

Barrett et al. (1978), also studying transformation of SHE cells, provided the first direct evidence that a DNA-specific lesion is sufficient to induce neoplastic transformation. They exposed cells to 5-bromodeoxyuridine (BrdU) (known to incorporate exclusively into cellular DNA in place of thymidine) and near UV light, which had been shown to cause chromosome damage and somatic mutations (Chu et al., 1972). Following this procedure, morphologically transformed colonies were obtained at a high frequency, which increased with increasing doses of BrdU or UV light. In addition, they demonstrated that transformation was cell cycle-dependent; experiments with synchronized cultures showed that cells treated in the DNA synthetic period of the cell cycle (S), but not those treated in the G₁ period or in late S/G₂ phase, were induced to undergo morphological transformation.

Barrett and Ts'o (1978b, 1978c) extended these studies by analyzing both mutagenesis and carcinogenesis concomitantly in vitro. After treatment of early passage SHE cells with benzo[a]pyrene (BP) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), they compared the frequency of induced somatic mutations at the HGPRT and Na⁺/K⁺ ATPase locus with that of morphological transformation and colony formation in soft

agar (anchorage independence). They observed morphological transformation within 1 week after carcinogen treatment (fewer than 8 population doublings), in the same time frame as the appearance of mutations at the two marker loci. However, the frequency of morphological transformation was 25- to 540-fold higher than the frequency of somatic mutations. They interpreted this high frequency of transformation as being indicative of an abnormally large target size or of mutational "hot spots". Transformants capable of colony formation in agar were detected at a frequency of 10^{-5} to 10^{-6} , comparable to that of somatic mutation, but not until 6-15 weeks (32-75 population doublings) after carcinogen treatment. Barrett and Ts'o proposed that transformation of hamster embryo cells in vitro is a multistep, progressive process; aneuploid, preneoplastic cells arise from the initial carcinogenic event and acquire additional transformed phenotypes spontaneously through continued cell passage. These findings are supportive of the multistage model proposed by Foulds (1969, 1975), who based his hypothesis on pathological evidence which indicated histologically distinct stages in the neoplastic development of a variety of cancers. They are also in concert with the multistep theory of Knudson (1971, 1973), based on statistical analysis of human retinoblastoma, and with the concept of initiation and promotion as advocated by Berunblum (1954).

The theory of multistep progression, described above for the diploid, unestablished Syrian hamster cell system, has also been proposed by Smith and Sager (1982) to account for chemical transformation in the diploid, established Chinese hamster embryo fibroblast (CHEF) cell line. They reported that single step transformants, selected after treatment with MNNG for either anchorage independence or growth in low serum,

showed a low tumor-forming potential upon injection into nude mice. This potential could be greatly increased by a second round of mutagenesis. In contrast, however, are the studies of chemical carcinogenesis using aneuploid, established cell lines such as C3H/10T1/2 (Reznikoff et al., 1973) and Balb/c 3T3 (DiPaolo et al., 1972; Kakunaga, 1973) suggesting that malignant transformation is a single-step phenomenon. In these cell lines, carcinogen-transformed populations, selected on the basis of focus formation or anchorage independence, give rise to tumors as soon as they can be assayed. One can rationalize these diverse results by presuming that aneuploid, immortal cells, although "normal" by the criteria used to define in vitro transformation, in fact already resemble a "preneoplastic" stage of developing malignant cells. This, however, does not negate their usefulness in studying mechanisms of carcinogenesis as long as results are interpreted objectively.

D. Application of gene transfer to the analysis of the transformed phenotype

Major advances have been made in the last several years on the assignment of gene loci to specific chromosomes, particularly those of man, by the use of conventional (whole cell) cell hybrid techniques (McKusick and Ruddle, 1977; Ruddle, 1981), taking advantage of non-random segregation of one of the two parental donors in interspecies crosses. As discussed previously, somatic cell hybridization has proved useful in studying the expression of the transformed phenotype in virally and chemically transformed cells in vitro. However, this method has not been applicable to identification of putative mouse transforming genes, as hybrids between mouse and human cells unidirectionally segre-

gate human chromosomes, and intraspecies hybrids (rodent vs. rodent) are of little value. Another system which permits assignment to an intact chromosome is represented by the microcell hybrid approach. This technique has been described by Fournier and Ruddle (1977). It takes advantage of the fact that cells incubated for prolonged periods in the presence of a mitotic arrest agent (e.g. colcemid) form multiple micronuclei containing variable numbers of intact chromosomes without cell division. Such cells can then be enucleated with the use of cytochalasin B, and the karyoplasts separated from intact cells and cytoplasts. Such karyoplasts can be fused with intact recipient cells and hybrids isolated. Using this method, micronuclei from transformed mouse cells would be fused to whole human recipient cells, in theory, without segregation of recipient chromosomes.

Two additional techniques have been developed for introduction of limited amounts of genetic material into recipient cells, namely chromosome-mediated gene transfer (CMGT) and DNA-mediated gene transfer (DMGT). McBride and Ozer (1973a, 1973b) first demonstrated that purified metaphase chromosomes from one species (Chinese hamster) containing the normal locus for HGPRT could be taken up and genetically expressed in a recipient mouse L cell deficient in HGPRT. This technique was subsequently confirmed by a number of laboratories and extended to other marker systems (reviewed by Willecke, 1978; Klobutcher and Ruddle, 1981). However, the frequency of CMGT is generally quite low (10^{-6} to 10^{-7}) and the genetic information transferred is rarely recognizable as an intact chromosome. Miller and Ruddle (1978) reported a modification, using dimethyl sulfoxide (DMSO) during the period of cell exposure to chromosomes, which appeared to increase the frequency of transfer of cytogenetically-detectable subgenomic fragments.

DNA-mediated gene transfer (DMGT) has proven to be particularly valuable because it is amenable to the analysis of single genes. The effectiveness of this technique was initially demonstrated by several groups (Wigler et al., 1977; Maitland and McDougall, 1977; Bacchetti and Graham, 1977; Minson et al., 1978), who adopted the calcium phosphate coprecipitation transfection method of Graham and van der Eb (1973), originally developed to improve the cell infectivity of animal virus DNA genomes. This method involves mixing DNA in aqueous solution with calcium and phosphate ions which themselves form a precipitate when mixed; the DNA molecules are coprecipitated in the matrix. In this physical state they are more resistant than dissolved DNA to enzymatic degradation and to mechanical shearing and they appear to be readily taken up, by a poorly understood mechanism, by a variety of cells. The investigators referred to above reported success in using purified DNA from Herpes simplex virus (HSV), reduced to noninfectious fragments by mechanical shearing or digestion with certain restriction endonucleases, to transfer the HSV-encoded thymidine kinase (tk) gene to mouse L cells (LM tk⁻) lacking their own cell-encoded tk (Kit et al., 1963). The HSV tk gene product is biochemically and immunologically distinguishable from tk produced by mammalian cells. Transformants were detected by selection in HAT medium, which permits only cells making an active tk to grow. The presence of HSV DNA sequences in the cellular genome of these transformants was verified by nucleic acid hybridization techniques (Pellicer et al., 1978). Wigler et al. (1978) demonstrated that high molecular weight DNA isolated from HSV tk-transformed cells could transfer the viral tk activity in secondary transformation. In companion experiments, they demonstrated that the cellular tk gene from mouse,

Chinese hamster, chicken, calf thymus, or human cells would be transferred by high molecular weight DNA to LM tk⁻. Subsequently, other markers such as adenine phosphoribosyl transferase (APRT) and HGPRT were transferred into mammalian cells (Wigler et al., 1979a; Graf et al., 1979; Lester et al., 1980). The potential applications of DMGT have been the subject of several recent reviews (Pellicer et al., 1980; Scangos and Ruddle, 1981; Graf, 1982). It has become apparent that this technique is limited only by the size of the prospective gene and by the availability of an appropriate recipient cell line and selective system for the desired phenotype.

E. Oncogenic viruses

The possibility that viruses might be tumorigenic materialized early in this century, with reports that erythroleukemia and fibrosarcomas could be induced in chickens by a cell-free filtrate from chicken sarcomas (Rous, 1911). Although skepticism greeted these observations, it has dissipated over the ensuing years. The oncogenic potential of many animal viruses is well established and their study has dominated efforts to dissect the mechanisms of tumorigenesis. Somewhat less rewarding, however, has been the search for viruses that cause cancer in humans.

The oncogenic viruses are taxonomically diverse. DNA tumor viruses, both large (Herpes and adenoviruses) and small (papovaviruses), and many (but not all) members of the large family of RNA tumor viruses, can induce tumors in either experimental or natural hosts and, in some cases, transform cells in culture. Oncogenesis by both DNA and RNA tumor viruses is conceptually united by the requirement that at least a

portion of the viral genome must persist in the host cell, either as an integral part of a host chromosome or as independently replicating units. Expression of one to a few virally-encoded gene products is generally necessary for the initiation and maintenance of the malignant phenotype.

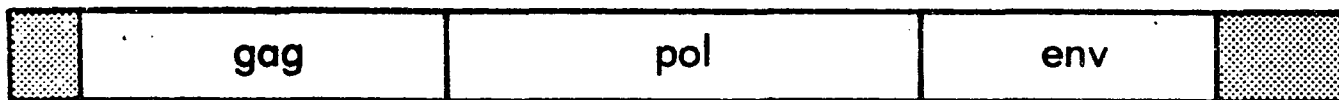
A discussion of the DNA tumor viruses is beyond the scope of this introduction; their biology has been extensively reviewed by Tooze (1981). One member of the papovavirus family, Simian virus 40 (SV40), has been employed as a model transforming vector in my studies and will be described in Chapter I. Likewise, the RNA tumor viruses have been the subject of a comprehensive treatise (Weiss et al., 1982) as well as several reviews by Bishop (1978; 1982; 1983). For the sake of later discussion, a brief introduction to their biology is warranted here.

The RNA tumor viruses, belonging to the family Retroviridae, share a similar architecture. Virion particles are spherical, 100 nm in diameter, and are covered by an envelope derived in part from the plasma membrane of the host cell. The genome, enclosed within the envelope, consists of two identical linear RNA molecules, each about 9 to 10 kilobases long, joined at or near their 5' ends by hydrogen bonding. The RNA molecules resemble eukaryotic messenger RNA's in that the 5' end has a methylated cap structure, and the 3' end is polyadenylated. The genome of all replication-competent retroviruses is comprised of three genes (as depicted in Figure 1a): gag, which encodes structural proteins of the viral core; pol, which encodes a RNA-directed DNA polymerase (reverse transcriptase); and env, which encodes the glycoprotein(s) of the viral envelope. Upon infection of appropriate host cells, the viral RNA serves as a template for the cytoplasmic synthesis of linear duplex

Figure 1. Comparison of the genomic organization of avian leukosis virus (ALV) and Rous Sarcoma virus (RSV). (From Bishop, 1982)

Figure 1

ALV



RSV



DNA directed by virally-encoded reverse transcriptase. In the process, small repeat sequences at either end of the RNA molecule are duplicated and rearranged to form long terminal repeat (LTR) sequences at the 5' and 3' ends of the linear DNA intermediate, ranging from 325 to 1300 base pairs in length depending on the species of virus. The LTR's contain potent transcriptional promoter sequences, as well as termination and polyadenylation codons (Temin, 1982). The linear duplex DNA makes its way to the nucleus, where it is converted into a closed circular DNA intermediate and integrated, apparently without regard to homology, into chromosomal DNA. Transcription of the integrated viral sequence, referred to as a "provirus", produces RNA species which serve as mRNA's for the synthesis of viral proteins, and as viral RNA for encapsulation into a budding new virion.

The retroviruses sort into two classes, chronic and acute. The non-defective chronic leukemia viruses (of which avian leukosis virus is a prototype) do not appear to contain coding sequences for proteins other than those normally implicated in virus replication (gag, pol, and env). They are capable of inducing a variety of neoplasms; however, tumors generally arise only after a long latency and the viruses have not been shown to transform cells in culture. The mechanisms by which they induce malignancy are subtle and have only recently been elucidated (see Discussion). The acute transforming retroviruses (of which Rous sarcoma virus is a prototype) all carry an additional gene implicated in neoplastic transformation (see Figure 1b); they induce tumors in host animals rapidly and are capable of transforming cells in culture. With the exception of Rous sarcoma virus (RSV), the acquisition of a transforming gene ("oncogene") involves the sacrifice of a viral gene(s) re-

quired for replication. Hence, the acute transforming retroviruses are replication-defective and can be propagated only in the company of helper leukemia viruses.

Fundamental knowledge of the origin of the retroviral oncogenes came from studies of the transforming gene of Rous sarcoma virus, designated src. Stehelin et al. (1976), using a src-specific cDNA as a nucleic acid hybridization probe, discovered that DNA from uninfected chicken cells, as well as from other birds, contained DNA sequences with extensive homology to the viral src gene; this homology was extended to DNA isolated from a variety of mammalian cells (including human) and fishes by the work of Spector et al. (1978a). Furthermore, Spector et al. (1978b) found evidence for the expression of src (i.e. RNA transcripts) in uninfected fibroblasts and tissues of several avian species. A protein identical to that encoded by viral src was also identified in uninfected fibroblasts, albeit at much lower levels than that observed in RSV-infected cells (Collett and Erikson, 1979). This 60,000 MW phosphoprotein, designated pp60^{C-src}, was found to possess tyrosine-specific protein kinase activity (identified by phosphotransfer in immunoprecipitates) similar to that of the virally-encoded gene product (pp60^{V-src}).

The evidence from studies of the RSV src model strongly suggested that the acute transforming retroviruses acquired their oncogenes as a consequence of the transduction of specific cellular DNA sequences by "non-transforming" leukemogenic viruses. It has, in fact, served as a paradigm for the evolution of the oncogenes of all acute transforming retroviruses, both of avian and mammalian origin (Duesberg, 1983). A list of the known oncogenes, first identified as viral genes and subse-

quently as their cellular homologs or "proto-oncogenes", is given in Table 4. Proof that these "proto-oncogenes" are of cellular, and not viral, origin has come from several convincing types of evidence: a) the presence of intervening sequences (introns) within most of the c-oncs - a hallmark of eukaryotic genes; b) the location of c-oncs at constant genetic loci in every member of a species, in striking contrast to the diverse distribution and positioning of endogenous proviruses; c) c-oncs are neither within nor linked to complete or defective proviruses of endogenous retroviruses; d) the c-onc sequences have been conserved across immense reaches of evolutionary time, while homologies among endogenous retroviruses can usually be found in only a limited number of closely related viral species. In particular, this impressive evolutionary preservation of c-oncs in every vertebrate species yet examined, and even in Drosophila and Caenorhabditis elegans (Shilo and Weinberg, 1981b), suggests that these cellular oncogenes are essential to the well-being of the species in which they persist.

Table 4. Viral and Cellular Oncogenes^a

Acronym ^b	Origin	Species of Isolation	Tumorigenicity	Chromosomal Location		Biochemical Activity Of Virally Encoded Protein
				Human	Mouse	
src	Rous sarcoma virus	Chicken	Sarcoma	20	2	Tyrosine kinase
yes	Y73 sarcoma virus	Chicken	Sarcoma	?	?	Tyrosine kinase
fps(fes)	Fujinami (ST feline) sarcoma virus	Chicken (cat)	Sarcoma	15	2	Tyrosine kinase
abl	Abelson murine leukemia virus	Mouse	B-cell lymphoma	9	2	Tyrosine kinase
ros	URII avian sarcoma virus	Chicken	Sarcoma	?	?	Tyrosine kinase
fgr	Gardner-Rasheed feline sarcoma virus	Cat	Sarcoma	?	?	Tyrosine kinase
erbA/erbB	Avian erythroblastosis virus	Chicken	Erythroleukemia, sarcoma	?	?	Membrane glycoprotein
fms	McDonough feline sarcoma virus	Cat	Sarcoma	5	?	?
mos	Moloney murine sarcoma virus	Mouse	Sarcoma	8	4	?
raf	3611 murine sarcoma virus	Mouse	Sarcoma	3	6	?
mil	MH2 virus	Chicken	Carcinoma	?	?	?
ras ^H	Harvey murine sarcoma virus	Rat	Erythroleukemia, sarcoma	11	7	Guanosine nucleotide binding; autophosphorylase
	Rasheed murine sarcoma virus	Rat				
ras ^K	Balb murine sarcoma virus	Mouse				
	Kirsten murine sarcoma	Rat	Erythroleukemia, sarcoma	12	6	Guanosine nucleotide binding; autophosphorylase
myc	Avian MC29 myelocytomatosis virus	Chicken	Carcinoma, sarcoma, myelocytoma	8	15	DNA binding protein
	MH2 virus					
myb	Avian myeloblastosis virus	Chicken	Myeloblastic leukemia	6	?	?
fos	FBJ osteosarcoma virus	Mouse	Sarcoma	2	?	?
rel	Reticuloendotheliosis virus	Turkey	Lymphatic leukemia	?	?	?
sis	Simian sarcoma virus	Woolly monkey	Sarcoma	22	15	Platelet-derived growth factor?
ski	Avian SKV770 virus	Chicken	?	1	?	?

^aAdapted from Land et al. (1983a). ^bNomenclature as per Coffin et al. (1981).

MATERIALS AND METHODS

Mammalian cell lines and culture conditions

Rodent cells were grown in Falcon Petri dishes and flasks in Dulbecco's modified Eagle's medium (DMEM, M.A. Bioproducts) supplemented with 10% calf or newborn calf serum (M.A. Bioproducts); they were incubated, unless otherwise noted, in a 10% CO₂ atmosphere at 37°C. The mouse fibroblast cell line, NIH 3T3, was expanded and stored at an early passage without allowing cells to reach confluence, and all gene transfer experiments were performed with freshly plated cells two passages from frozen storage. Human fibroblasts were grown in a 1:1 mixture of DMEM and Ham's F-10 (M.A. Bioproducts) supplemented with 15% fetal calf serum and incubated in a 5% CO₂ atmosphere at 37°C. These conditions are referred to in the text as 'complete medium'. The normal human fibroblast line, HS74, was utilized at the eighth passage. Cells were passaged by brief treatment with 0.05% trypsin (Trypsin-Versene, M.A. Bioproducts); cells were concentrated by centrifugation at 600 rpm for 5 minutes. Cell counts were obtained with a Royco Tissue Cell Counter after dilution in phosphate buffered saline (PBS) (0.15M NaCl, 3mM NaH₂PO₄, 7mM Na₂HPO₄, pH 7.4). Cell lines were stored in Nunc cryotubes frozen in liquid nitrogen or in a -70° freezer in complete medium containing 10% dimethyl sulfoxide (DMSO).

Anchorage-independent transformation assay (Freedman and Shin, 1974)

A solution of 1.4% (w/v) agarose (Calbiochem) in deionized water was sterilized by autoclaving and cooled to 47°C in a water bath; it was diluted with an equal volume of pre-warmed 2X DMEM supplemented with 30% fetal calf serum (for human fibroblasts) or 20% newborn calf serum (for rodent fibroblasts). Five ml of this solution was added to each 60mm Petri dish and allowed to solidify for at least 1 hour at 33°C in a 10% CO₂ atmosphere. Four ml of this 0.7% agarose solution was mixed with 4 ml of a cell suspension (the equivalent of one confluent 60mm dish) in complete medium; the resultant 0.35% agarose/cell suspension was layered on top of the solidified agarose base dishes and allowed to solidify at 33°C overnight before being incubated at 37°C for 14-21 days. Dishes were refed once a week by overlaying 2 ml of 0.7% agarose containing complete medium. Macroscopic colonies were picked from agarose with a plugged Pasteur pipette; the plug of agarose containing a single colony was dispersed into 2 ml of complete medium by vigorous pipetting and seeded into a 30mm Petri dish. Colonies were photographed under 100X power using a Polaroid microscope camera with Type 42 high contrast film.

Isolation of high molecular weight cellular DNA (Pellicer et al., 1978)

Cells were grown to confluence in 150mm Petri dishes and refed 24 hours before harvesting. Monolayers on 4-5 dishes were washed once with sterile PBS and scraped with a rubber policeman into PBS (5 ml/dish). Cells were pelleted by centrifugation for 5 min at 600 rpm in 15 ml conical polypropylene tubes. PBS was decanted and cells were resuspended in 2.5 ml of sterile lysis buffer (50mM Tris, pH 7.5, 20mM Na₂EDTA, 100mM NaCl). Sodium dodecyl sulfate (SDS) was added to a final concen-

tration of 1% and tubes were immediately incubated at 65°C for 15 min to inactivate endogenous nucleases. Tubes were cooled to room temperature and Proteinase K (Boehringer Mannheim, 5 mg/ml stock) was added to a final concentration of 50 µg/ml; tubes were inverted to mix and incubated at 37°C overnight. Cell lysate was extracted with an equal volume of buffer-saturated redistilled phenol; phases were separated by centrifugation at 2000 rpm for 15 min at room temperature. The aqueous phase was removed with a wide-bore pipette and re-extracted with phenol:chloroform:isoamyl alcohol (48:48:2) once, followed by two extractions with chloroform:isoamyl alcohol (24:1). The viscous aqueous phase was transferred to a glass beaker; 2.5 volumes of ice cold 95% ethanol was added and the mixture was poured back and forth between two beakers 15 times until the DNA precipitated into a compact white mass. The DNA was picked up with a hooked Pasteur pipette and washed once with 70% ethanol, then transferred to a sterile tube containing 2 ml of sterile TE (10mM Tris, pH 7.5, 1mM EDTA) and allowed to go into solution at 4°C overnight. The DNA concentration was determined by diluting the sample 1:50 in TE and reading the UV absorption at 260 nm (1 O.D. is equivalent to 50 µg/ml double-stranded DNA). DNA's used for Southern blot analysis were further treated with pancreatic RNase A (Worthington); RNase was boiled for 10 min to inactivate trace amounts of DNase and used at a final concentration of 50 µg/ml. Samples were incubated at 37°C for 2 hrs; the solution was then deproteinized by addition of SDS and Proteinase K to 1% and 50 µg/ml, respectively, incubated at 37°C for 2 hrs, and extracted with organic solvents as described above. DNA was precipitated with two volumes of 95% ethanol and recovered by centrifugation at 12,000 rpm in a microcentrifuge at 4°C for 15 min.

Transfection of mammalian cells by the DNA-CaPO₄ co-precipitation technique (Wigler et al., 1978)

Twenty hours prior to transfection, cells were seeded at 3×10^5 or 5×10^5 per 60mm or 100mm Petri dish, respectively, in complete medium. Four hours prior to transfection, cells were refed with DMEM supplemented with 5% newborn calf serum (4.5 ml for 60mm dishes, 9 ml for 100mm dishes). DNA precipitates were prepared as follows: a double-strength DNA-CaCl₂ solution was prepared containing DNA at 40 µg/ml in 1X TE (in the case of plasmid or viral DNA transfections, salmon testis DNA [P-L Biochemicals] was generally added as a carrier); 2.5M CaCl₂·2H₂O (Malinckrodt) was added dropwise with constant agitation to a final concentration of 250mM. This DNA-CaCl₂ solution was added dropwise, while bubbling vigorously by mouth through a 1 ml plugged plastic pipette, to a polypropylene tube (Falcon) containing an equal volume of double-strength HEPES buffered saline (2X HBS) (280mM NaCl, 50mM HEPES, 1.5mM Na₂HPO₄·12 H₂O, pH 7.05). The flocculent DNA-calcium phosphate precipitate was allowed to form without agitation for 30 min; the precipitate was then resuspended and added dropwise to recipient culture dishes (0.5 ml per 60mm dish, 1 ml per 100mm dish). Cultures were incubated for 4 hours in the case of human fibroblasts or 15 hours for mouse fibroblasts; media was aspirated and cultures were refed with complete medium.

MLV infection assay for rescue of sarcomagenic virus

Mouse cell lines were screened for rescue of sarcomagenic virus by the method described in Huebner et al. (1966). Cells were seeded at 5×10^5 cells per 100 mm Petri dish in complete medium. On the following

day, medium was aspirated and 2 ml of a filtered Moloney murine leukemia virus stock (10^7 ffu/ml; kindly provided by Dr. Dino Dina of Einstein Medical College) in DMEM with 1 μ g/ml Polybrene (Sigma) was added to each dish. After infection for 6 hours, virus was aspirated and cultures were refed with 10 ml of complete medium. Four days post-infection, the growth medium from these cultures was recovered, cleared of contaminating cells by filtration through a 0.22 μ m Millipore membrane filter, and used to infect a growing culture of NIH 3T3 fibroblasts in the same manner as described above. Four days post-infection, cultures of NIH 3T3 so treated were trypsinized and reseeded into soft agarose as previously described. Anchorage independent colonies were scored for after two weeks incubation.

Growth and preservation of bacterial strains

With one exception, the recombinant plasmids used in these studies were introduced into the E. coli rec A⁻ hosts DH-1 (Low, 1968) or HB101 (Bolivar and Backman, 1979). Both strains are routinely used as recipients in transformation and are good hosts for large-scale growth and purification of plasmids. They were propagated in LB (Luria-Bertani) medium which contains 10 g Bacto-tryptone (Difco), 5 g Bacto-yeast extract (Difco), 10 g NaCl, and 0.2 ml of 10N NaOH per liter. Medium was sterilized by autoclaving; when antibiotics were used for selection, they were sterilized separately by filtration and added to liquid medium at room temperature. The recombinant plasmids pSV or i⁻ and pSV ooi⁺ (described in Chapter I) were introduced into the E. coli strain X1776 (Curtiss et al., 1977). This strain was grown in LB medium supplemented with 5mM MgCl₂, diaminopimelic acid (100 mg/l), thymidine

(40 mg/l) and 0.5% glucose. For preparation of agar plates, 15 g of Bacto-agar (Difco) was added per liter of liquid medium just before autoclaving and poured into 85mm bacterial Petri dishes after cooling to 55°C (about 30 ml per dish). Plates were allowed to harden at room temperature and stored at 4°C until used. The antibiotics ampicillin, kanamycin, and tetracycline were used at final concentrations of 50, 50 and 15 µg/ml, respectively.

For short-term storage, bacterial strains were maintained on the surface of agar plates or in liquid culture and stored at 4°C. For long-term storage, 5 ml cultures were grown overnight; DMSO was added to a final concentration of 7% (v/v), and cultures were aliquoted into Nunc cryotubes and stored at -20°C

Introduction of plasmid DNA into Escherichia coli

Recombinant plasmids supplied to us as DNA were introduced into the *E. coli* hosts HB101 or DH-1 by the calcium chloride transformation procedure of Mandel and Higa (1970). Host strains were kindly provided by Dr. Robert Hickey (Einstein Medical College). One hundred milliliters of LB broth was inoculated with 1 ml of a dense overnight bacterial culture. Cells were grown in a 500 ml armed flask with vigorous shaking at 37°C to a Klett value of 40 corresponding to a density of approximately 5×10^7 cells/ml. The culture was chilled on ice for 10 minutes and centrifuged at 4000 g for 5 min at 4°C to pellet bacteria. The cell pellet was resuspended in 50 ml of ice-cold, sterile transformation buffer (50mM CaCl₂, 10mM Tris-HCl, pH 8.0). The cell suspension was placed in an ice bath for 15 min and then centrifuged as before. The pellet was resuspended in 6 ml of cold transformation buffer

and stored overnight at 4°C. For each transformation reaction, 50-100 ng of superhelical plasmid DNA in 100 µl TE was added to 0.2 ml of the calcium-treated cell suspension in a sterile polypropylene tube and kept on ice for 30 min. The reaction tube was transferred to a 42°C water bath for 2 min; then 1 ml of LB broth was added and the tube was incubated at 37°C for one hour to allow bacteria to recover and to begin to express antibiotic resistance conferred on the host by the introduced plasmids. The transformation mixture was spread on the surface of agar plates containing the appropriate selective antibiotic. Plates were inverted and incubated at 37°C overnight. Single colonies were picked with sterile wooden dowels and grown up as 5 ml overnight cultures for rapid analytical preparation of plasmid DNA by the method of Holmes and Quigley (1981) (see Isolation of Plasmid DNA).

Isolation of plasmid DNA

For large scale preparation of plasmid DNA, 500 ml flasks containing 100 ml of bacterial growth medium without antibiotic were inoculated with 1 ml of a dense overnight culture of the appropriate plasmid-bearing strain grown in the presence of selective antibiotic. Culture flasks were incubated at 37°C with moderate shaking in a gyratory water bath (New Brunswick Scientific) and grown to a Klett value of 100 corresponding to middle log phase (approximately 2×10^8 cells/ml). Usually 500 ml of total culture were grown for each plasmid preparation. Chloramphenicol (Sigma), prepared as a stock solution of 34 mg/ml in absolute ethanol, was added to each culture to a final concentration of 170 µg/ml; incubation at 37°C with vigorous shaking was continued over

night (12-16 hours). Treatment with chloramphenicol has been demonstrated to result in amplification of 'relaxed' plasmid copy number (Clewell, 1972). Bacteria were harvested by centrifugation in 150 ml glass Corex bottles at 6000 rpm in a Sorvall GSA rotor at 4°C for 10 min; medium was decanted and pellets were frozen at -20°C prior to DNA extraction.

For isolation of plasmid DNA, two methods were employed; the cleared lysate technique of Guerry et al. (1973) and the boiling method of Holmes and Quigley (1981); both will be described, although the latter has recently been found to be somewhat simpler and more efficient.

Cleared lysate technique

Bacterial pellets were thawed at room temperature. Cells from 500 ml of culture were resuspended on ice in 5 ml of cold buffer (50 mM Tris-HCl, pH 8.0, 20% sucrose) and divided into two 12 ml plastic Oakridge tubes to each of which was added, with gentle agitation, 0.75 ml of a freshly prepared solution of 10 mg/ml egg white lysozyme (Sigma) and 0.75 ml of 250 mM disodium EDTA, pH 8.0. After 15 min of incubation on ice, 5 ml of lysis buffer (0.2% Triton X-100, 50 mM Tris-HCl, 25 mM EDTA, pH 8.0) was added and tubes were mixed immediately by repeated inversion. Incubation on ice was continued for an additional 15 min. Tubes were centrifuged at 35,000 rpm for 20 min at 4°C in a Beckman 50 Ti rotor. The clear but slightly yellowish supernatant was recovered with a Pasteur pipette (carefully avoiding the viscous pellet) and brought to a volume of 16 ml with deionized water. Closed supercoiled plasmid DNA was purified by cesium chloride-ethidium bromide equilibrium centrifugation (Radloff et al., 1967). Eight ml of supernatant was

added to each of two Beckman polyallomer tubes; 8 grams of CsCl (Schwarz Mann Biological Grade) was dissolved in each tube and 0.8 ml of a 10 mg/ml solution of ethidium bromide was added. Tubes were capped, inverted to mix ethidium, and centrifuged at 40,000 rpm for 40 hours at 20°C in a Beckman 50 Ti rotor. DNA bands were visualized by a hand-held ultraviolet light source; the lower band, consisting of closed circular plasmid DNA, was collected with a 1 ml tuberculin syringe with a #21 needle inserted into the side of the tube at the band. Ethidium bromide was removed by repeated extraction with an equal volume of CsCl-saturated isopropanol. The DNA-containing aqueous phase was dialyzed against several changes of TE at 4°C to remove cesium. DNase-free RNase (Worthington) was added to 50 µg/ml and solution was incubated at 37°C for 4 hours. Plasmid DNA was deproteinized by two extractions with phenol:chloroform:isoamyl alcohol (48:48:4) followed by two extractions with chloroform; 0.1 volume of 2.5 M sodium acetate (pH 5.2) was added to the aqueous phase and DNA was precipitated with 2 volumes of 95% ethanol in glass Corex tubes. DNA was recovered by centrifugation at 12,500 rpm for 20 min at 4°C in a Sorvall SS 34 rotor and resuspended in TE. The yield of plasmid DNA, as determined spectrophotometrically, was generally 500 µg to 1 mg of DNA from 500 ml initial culture.

Boiling method

The bacterial pellet from a 500 ml culture was resuspended in 10 ml of buffer B (8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris-HCl, pH 8.0) and transferred to a 100 ml Erlenmeyer flask. Two ml of a freshly prepared solution of egg white lysozyme (10 mg/ml in 10 mM Tris-

HCl, pH 8.0) was added. Using a clamp, the flask was held over the open flame of a Bunsen burner, with constant shaking, until the solution started to boil. The flask was immediately immersed in a boiling water bath and held in the boiling water for 40 sec. The flask was cooled by immersion in ice water for 5 min. The viscous solution was transferred to a glass Corex tube and centrifuged at 25,000 rpm for 30 min at 4°C in a Sorvall SS 34 rotor. The supernatant was carefully removed by Pasteur pipette and the closed circular plasmid DNA was purified by equilibrium centrifugation as described previously.

For rapid, small-scale isolation of plasmid DNA, a modification of the boiling method was used. 1.5 ml of a dense overnight culture grown from a single bacterial colony was centrifuged for 1 min in an Eppendorf tube in a microcentrifuge. The medium was aspirated and the cell pellet was resuspended in 0.35 ml of buffer B. Twenty-five μ l of a 10 mg/ml solution of lysozyme was added to the tube and mixed by vortexing. The tube was placed in a boiling water bath for 40 sec and then immediately centrifuged for 10 min at room temperature. The viscous pellet was removed with a wooden dowel and discarded; 40 μ l of 2.5 M sodium acetate (pH 5.2) and 420 μ l of cold isopropanol were added to the supernatant and the DNA was precipitated in a dry ice/ethanol bath for 15 min. The DNA was recovered by centrifugation for 15 min at 4°C and resuspended in 50 μ l of TE containing 50 μ g/ml DNase-free RNase; the DNA solution was incubated at 37°C for 30 min. This technique provides plasmid DNA (approximately 2 μ g from 1.5 ml culture) suitable for restriction endonuclease digestion and analysis by agarose gel electrophoresis.

Restriction endonuclease digestion and agarose gel electrophoresis

(Southern, 1980)

Restriction endonucleases used in these studies were supplied by New England Biolabs and Bethesda Research Laboratories. The optimum buffer conditions for each enzyme, as described by Maniatis et al. (1982), are given in Table 5. Low salt buffer contained 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 mM Tris-HCl, pH 7.5, and 100 μ g/ml bovine serum albumin (BSA). Medium salt buffer contained the above plus 50 mM NaCl. High salt buffer contained 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 50 mM Tris-HCl, pH 7.5, and 100 μ g/ml BSA. Reactions were generally performed in volumes of 20–100 μ l containing DNA in TE, 0.1 volume of 10X reaction buffer (low, medium, or high), and 2 units of enzyme per μ g DNA. Reactions were performed at 37°C overnight and terminated by the addition of EDTA to a final concentration of 20 mM.

For preparation of 0.5–1.2% agarose gels, the correct amount of powdered agarose (Seakem, ME) was added to a measured amount of Tris-phosphate (TPE) electrophoresis buffer (0.08 M Tris-phosphate, 0.008 M EDTA). Agarose was melted by brief autoclaving in a pressure cooker, allowed to cool to 50°C (if desired, ethidium bromide was added to a final concentration of 0.5 μ g/ml), and poured into the agar-sealed bed of a horizontal electrophoresis apparatus. A well-forming comb was immediately set into position and the gel allowed to harden at room temperature, after which enough TPE (containing 0.5 μ g/ml ethidium bromide, if desired) was added to the apparatus to cover the gel to a depth of 1 mm. The well former was carefully removed and the gel was connected to a power supply and pre-run at 30 V (constant voltage) for at least 10 min. DNA samples were mixed by vortexing with 0.1 volume of 10X gel loading buffer (0.25% bromophenol blue, 10X TPE, 0.1% SDS, 25%

Table 5. Restriction Endonucleases

Enzyme	Buffer Conditions ^a	Recognition Sequence
BamHI	medium	G ↓ GATCC
BglI	medium	GCCNNNN ↓ NGGC
EcoRI	high	G ↓ AATTC
HindIII	medium	A ↓ AGCTT
KpnI	low	GGTAC ↓ C
PstI	medium	CTCGA ↓ G
PvuII	medium	CAG ↓ CTG
SstI	low	GAGCT ↓ C
SalI	high	G ↓ TCGAC
XbaI	high	T ↓ CTAGA
XhoI	high	C ↓ TCGAG

^alow, medium and high refer to salt concentrations as described in Materials and Methods.

Ficoll 400 [Pharmacia] in H₂O), heated at 65°C for 10 min, and applied to the slots of the submerged gel at 10 V with a micropipettor. For electrophoresis of restricted cellular DNA, 10 µg of DNA was loaded per slot. Electrophoresis was conducted, unless otherwise noted, at 20-30 V for 16-20 hours. Lambda phage DNA cleaved by HindIII was used as a molecular weight marker. Gels run in the absence of ethidium bromide were stained in deionized water containing 0.5 µg/ml ethidium bromide for 45 min at room temperature. DNA in the gel was visualized on an ultraviolet transilluminator light box and photographed with a Polaroid camera using Type 57 film.

Preparation of SV40 derivatives for transfection of HS74

SV40 viral form I (superhelical) DNA was extracted from SV40-infected monkey kidney cells (CV-1) by the technique of Hirt (1967) and purified by CsCl-ethidium bromide equilibrium centrifugation (as described for isolation of plasmid DNA). Viral form III DNA (linear) was generated by digestion of form I DNA with BamHI which cuts once in the late region of SV40. The subgenomic Eco/Bam fragment was isolated by simultaneous digestion of viral form I DNA with EcoRI and BamHI; the DNA was then electrophoresed on a 1% preparative agarose gel in TPE buffer at 25 V for 18 hours. The 4,670 base pair fragment, visualized by UV light after ethidium bromide staining, was cut out from the gel and reisolated by electroelution (McDonell et al., 1977) as follows: The gel slice was sealed in a dialysis bag filled with buffer E (100 mM sodium acetate, pH 5.2, 1 mM EDTA) and subjected to electrophoresis in a horizontal gel apparatus filled with TPE at 150 V for 3 hours. After 3 hours, the polarity of the current was reversed for 2 min to release DNA from the

wall of the dialysis bag. The buffer was recovered from the bag and extracted twice with buffer-equilibrated phenol; the DNA was precipitated overnight at -20°C after the addition of *E. coli* tRNA (as carrier) to 20 $\mu\text{g/ml}$, 0.1 volume of 2.5 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol. DNA was recovered by centrifugation at 20,000 rpm at 4°C for 20 min. The DNA was resuspended in TE and NaCl added to a final concentration of 4 M; this solution was extracted 5 times with equal volumes of NaCl-saturated isopropanol to remove ethidium bromide. The aqueous phase was diluted with H_2O , reprecipitated with ethanol, and resuspended in a minimal volume of TE.

The recombinant plasmids pSVori⁻ and pSVori⁺ were purified as form I DNA's from *E. coli* X1776 as described previously. Form III derivatives were prepared by digestion of form I DNA with XhoI (which makes a single cut within the plasmid vector, pMK 16). SVori⁻ and SVori⁺ form III DNA's (viral sequences from which plasmid sequences were removed) were generated by digestion of form I plasmid DNA with BamHI, which separates SV40 from pMK 16 sequences; viral DNA was isolated by agarose electrophoresis and electroelution as described above.

Prior to use in transfection, all DNA derivatives were deproteinized by extraction with phenol and chloroform and reprecipitated with ethanol. DNA's were resuspended in TE and analyzed by electrophoresis on a 1% agarose gel.

Isolation of low molecular weight circular DNA

The method of Hirt (1967) was employed in the isolation of SV40 viral form I DNA from SV40-infected monkey kidney fibroblasts and for

isolation of covalently closed circular DNA intermediates from MLV-infected mouse fibroblasts. The medium from each infected culture was aspirated and monolayers were washed once with 5 ml of PBS per dish. Two milliliters of Hirt lysis buffer (HLB: 0.6% SDS, 10 mM EDTA, 10 mM Tris-HCl, pH 7.6) was added to each dish, and the cells were lysed by gentle rocking. The cell lysate was poured into Oakridge plastic centrifuge tubes. Sodium chloride (as a 5 M solution) was added to a final concentration of 1 M; tubes were gently mixed by inversion and incubated at 4°C overnight. The lysate was centrifuged at 25,000 rpm for 30 min at 4°C in a Beckman Type 30 rotor, and the supernatant was collected in 50 ml polypropylene centrifuge tubes. The supernatant was twice extracted with HLB-equilibrated phenol and once with chloroform, and DNA was precipitated with two volumes of 95% ethanol overnight at -20°C. DNA was recovered by centrifugation in a Sorvall SS34 rotor at 15,000 rpm for one hour and resuspended in TE. Closed circular viral DNA was purified by equilibrium centrifugation in a CsCl-ethidium bromide density gradient (as described for isolation of closed circular plasmid DNA).

Transfer of DNA from agarose gels to nitrocellulose (Southern, 1975)

After electrophoresis, gels were soaked in 500 ml of a solution of 0.5 M NaOH and 1.5 M NaCl for 1 hour at room temperature with constant shaking to denature the DNA. Gels were rinsed twice with distilled water and neutralized by soaking in 500 ml of a solution of 0.5 M Tris-HCl (pH 8.0) and 1.5 M NaCl for 1 hour at room temperature with constant shaking.

A stack of Whatman 3MM paper (9 x 11 inches) 12 layers thick in a plastic cafeteria tray was saturated with 20X SSPE (3.6 M NaCl, 200 mM

NaH_2PO_4 , 20 mM EDTA, pH 7.4). The gel was placed on top of the stack, carefully removing any air bubbles by rolling with a glass rod. (Alternatively, a single layer of filter paper on a raised plastic support was placed in a reservoir filled with 20X SSPE, with the ends of the filter in contact with salt solution; the gel was then placed on top of the saturated filter paper.) A piece of nitrocellulose paper (Schleicher and Schuell BA 85) cut to the size of the gel was pre-wet in water and placed with forceps on top of the gel, removing air bubbles as before. Two pieces of 3MM paper, also cut to size and pre-wet in water, were placed on top of the nitrocellulose filter. A stack of paper towels (2-3 inches high) cut to the size of the gel was placed on top of the 3MM paper; a glass plate was put on top of the stack and weighed down with a 1 kg weight.

Transfer of DNA was allowed to proceed for 12-24 hours. The stack was then disassembled; the nitrocellulose filter was peeled off of the dehydrated gel and rinsed briefly in 2X SSPE. The wet filter was sandwiched between two pieces of 3MM paper and baked for 2 hours at 80°C under vacuum. Filters not used immediately for hybridization experiments were stored at room temperature in a vacuum dessicator.

Nick translation of DNA

^{32}P -labeled DNA probes with a specific activity greater than 10^8 cpm/ μg were prepared by the method of Rigby et al. (1977) as modified by Maniatis et al. (1982). The nick translation reaction was set up by the addition of the following reagents to a 1.5 ml Eppendorf tube: 1 nmole of each unlabeled dNTP (generally dGTP and dTTP), 100 pmoles of each $[\alpha\text{-}^{32}\text{P}]\text{dNTP}$ (New England Nuclear, specific activity 800 Ci/

mmole; generally dCTP and dATP), 1 μ g of DNA, 5 μ l of 10X nick translation buffer (0.5 M Tris-HCl, pH 7.2, 0.1 M MgSO₄, 1 mM DTT, 500 μ g/ml BSA), and deionized H₂O to a final volume of 47 μ l. The mixture was chilled on ice for 5 min, after which time 1.5 μ l of a 100 ng/ml solution of DNase I (Worthington) in 50% glycerol was added and mixed by vortexing. The nicking reaction was allowed to proceed on ice for 10 min, after which 1.5 μ l (10 units) of *E. coli* DNA polymerase I (Boehringer Mannheim) was added. The nick translation reaction was performed at 15°C for 60 min. The reaction was terminated by addition of 2 μ l of 0.5 M EDTA and heating at 65°C for 5 min. The nick-translated DNA was separated from unincorporated dNTP's by "spun-column" chromatography as described in Maniatis et al. (1982). The bottom of a 1 ml tuberculin syringe was plugged with a small amount of glass wool. The syringe was packed with Sephadex G-50 (Pharmacia) swelled in STE (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) by centrifugation in a 15 ml conical centrifuge tube at 1600 x g for 4 min until the packed column volume was 0.9 ml. One hundred microliters of STE was added to the column and recentrifuged under identical conditions. This step was repeated once. The nick translation reaction, brought to a volume of 100 μ l with STE, was applied to the column which was then centrifuged exactly as before, collecting the 100 μ l of effluent from the syringe in a decapped Eppendorf tube (the unincorporated [α ³²P]dNTP's remain in the syringe, which is discarded). The recovered probe DNA was diluted with TE to a final volume of 0.5 ml; a small aliquot was used to determine specific activity of the probe by scintillation counting.

Hybridization and autoradiography of Southern filters

The hybridization procedure described by Wahl et al. (1979) was followed with slight modification; it is referred to in the text as "stringent conditions".

The baked filter was immersed in 2X SSPE for 2 min, then placed into a heat-sealable plastic bag ("Seal-A-Meal", Dazey) sealed on three sides. Ten ml of prehybridization solution [consisting of 5X SSPE, 5X Denhardt's reagent (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% BSA), 0.5% SDS, and 100 µg/ml heat-denatured, sheared salmon testis DNA] was added to the bag, which was then sealed and incubated overnight submerged in a water bath at 68°C. A corner of the bag was cut off and the prehybridization solution was squeezed out. Five ml of hybridization solution [consisting of 50% formamide, 5X SSPE, 10% dextran sulfate (Pharmacia), 0.1% SDS, 100 µg/ml heat-denatured salmon testis DNA, and $0.5\text{--}1.0 \times 10^7$ cpm of heat-denatured ^{32}P -labeled probe DNA] was added to the bag. Excess air was removed from the bag, which was then heat-sealed and incubated for 16-48 hours submerged in a water bath at 42°C. The hybridization solution was decanted from the bag and the filter was placed in a glass tray containing 250 ml of a solution of 2X SSPE and 0.1% SDS. The filter was washed with gentle agitation at room temperature for one hour, with three changes of buffer. The filter was then transferred to a glass tray containing 500 ml of a solution of 0.1X SSPE and 0.1% SDS and washed with gentle agitation at 65°C for 2 hours, with one buffer change.

The filter was blotted between 2 sheets of 3 MM paper and wrapped while still damp in Saran Wrap. In a darkroom, the filter was mounted in an x-ray film holder; a single sheet of Kodak X-Omat AR-2 film was placed between the filter and an intensifying screen (Dupont Cronex

Lightning-Plus) following the method of Laskey and Mills (1977). The film holder was wrapped in aluminum foil and placed at -70°C for the duration of exposure. The x-ray film was developed manually by conventional photographic processing methods.

Cytoplasmic RNA extraction (Favaloro et al., 1980)

Cells from ten T150 mm flasks were trypsinized, pelleted by centrifugation at 600 rpm for 5 min, and washed one time in sterile cold PBS. Cells were resuspended by gentle vortexing in 50 ml of lysis buffer (0.25 M sucrose, 10 mM Mg-acetate, 250 mM KCl, 10 mM Tris, pH 7.5, 100 $\mu\text{g}/\text{ml}$ of cycloheximide, 0.5% Triton X-100) and incubated on ice for 10 min. The lysate was centrifuged at 5000 rpm for 10 min at 4°C in a Sorvall SS34 rotor to pellet nuclei, followed by centrifugation for 10 min at 10,000 rpm to pellet microsomes. The supernatant was transferred to 50 ml polypropylene tubes; EDTA and SDS were added to final concentrations of 50 mM and 1%, respectively, and mixed by gentle vortexing. An equal volume of buffer-saturated phenol:chloroform (50:50) was added; the mixture was agitated for 10 min and centrifuged at 2000 rpm for 15 min at room temperature. The aqueous phase was re-extracted twice with phenol:chloroform and once with chloroform alone, and RNA was precipitated with 2.5 volumes of 95% ethanol overnight at -20°C . RNA was recovered by centrifugation at 12,000 rpm for 30 min at 4°C and resuspended in a minimum volume of TE.

Selection of poly(A)⁺ RNA by chromatography on oligo(dT)-cellulose

(Aviv and Leder, 1972)

Oligo(dT)-cellulose (Collaborative Research, Inc.) was equilibrated in sterile loading buffer (0.5 M lithium chloride, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.2% SDS). One ml of oligo(dT)-cellulose was poured in a pasteur pipette plugged with sterile glass wool; the packed column was washed consecutively with 15 ml of sterile H₂O, 0.1 M NaOH, and sterile H₂O. (The pH of the final effluent was less than 8.) The column was then washed with 25 ml of loading buffer. The RNA sample was heat denatured by boiling for 2 min, then an equal volume of 2X loading buffer was added, the sample cooled to room temperature and applied to the column. The column was washed with 50 ml of loading buffer, followed by 25 ml of 0.1 M loading buffer (same as 0.5 M loading buffer except concentration of LiCl was 0.1 M). Poly(A)⁺ RNA was eluted with 15 ml of sterile TE plus 0.05% SDS; sodium acetate was added to a final concentration of 0.25 M and RNA was precipitated with 2.5 volumes ethanol overnight at -20°C. RNA was recovered by centrifugation and resuspended in 1X TE; RNA concentration was determined by reading the UV absorption at 260 nm (1 OD unit is equivalent to 40 µg/ml RNA). The yield from 10⁷ cells was approximately 5 µg of poly(A)⁺ RNA.

Preparation of retroviral RNA from MLV-infected cells

(Robinson et al., 1965)

Cells were seeded at 10⁷ cells per T150 mm flask. On the following day medium was aspirated and the cultures were infected with 5 ml of filtered MLV virus stock per flask as previously described. After infection for 6 hours, virus was aspirated and cultures were refed with medium (25 ml/flask). Cell culture fluid from ten flasks was collected 3 days post-infection (when cells reached subconfluence) and cooled on

ice. Medium was centrifuged at 6000 rpm for 10 min at 4°C to remove cells; the supernatant was centrifuged for 2 hours at 19,000 rpm in a Beckman Type 19 rotor at 4°C. The supernatant was discarded and the pellet containing virus was resuspended in 2 ml of TNE (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA). This crude virus preparation was further purified by centrifugation on a 6 ml 20-60% neutral sucrose gradient (in TNE) at 40,000 rpm for 2 hours at 4°C in a Beckman SW50.1 rotor. The lower virus band, visible by eye, was removed through the side of the nitrocellulose tube with a 23 gauge needle; an equal volume of TNE plus 0.2% SDS was added and the tube was incubated at room temperature for 10 min. Viral RNA was isolated by extraction twice with phenol:chloroform and once with chloroform alone, followed by ethanol precipitation as previously described.

Preparation of cDNA probes by reverse transcription

(Taylor et al., 1976)

One µg of viral RNA was dissolved in 20 µl of sterile deionized water, heated to 100°C for 5 min, and chilled rapidly in ice water. The following reagents were then added: 10 µg of oligo(dT)₁₂₋₁₈ primer (Collaborative Research), 20 µl of 5X reaction buffer (0.25 M Tris, pH 8.1, 10 mM DTT, 25 mM MgCl₂, 0.2 M KCl), 50 units RNasin (Biotec, RNase inhibitor), 100 µCi of each of the four α-³²P dNTP's (New England Nuclear, specific activity 800 Ci/mmol), water to a final volume of 98 µl, and 40 units of reverse transcriptase (Bethesda Research Labs, from avian myeloblastosis virus). The reaction was performed at 42°C for 1 hour, at which time the reaction was stopped by the addition of 2 µl of 0.5 M EDTA. Twelve µl of 3 M NaOH was added and the tube was

incubated at 37°C overnight to remove RNA templates. Twenty-five μ l of 1 N HCl was added to neutralize the solution. The entire reaction volume was chromatographed through a Sephadex (Pharmacia) G-100 column (3 ml bed volume in a 5 ml glass pipette); radioactivity was followed with a survey meter and the first peak of radioactive fractions in the excluded volume was pooled. The pooled cDNA fraction was extracted with an equal volume of buffer-saturated phenol:chloroform and DNA in the aqueous fraction was precipitated with 2.5 volumes of 95% ethanol overnight at -20°C. The cDNA probe was recovered by centrifugation and redissolved in TE. A small aliquot was used to determine specific activity of the probe by scintillation counting.

Electrophoresis of RNA through agarose gels containing methylmercuric hydroxide and transfer of RNA to nitrocellulose filters

(Bailey and Davidson, 1976)

Gels were prepared by melting 1.5 g of agarose (Sea-Kem ME grade) in 100 ml of borate gel buffer (50 mM boric acid, 5 mM sodium borate, 10 mM sodium sulfate); gel solution was cooled to 50°C and methylmercuric hydroxide (Thiokol/Ventron) was added to a final concentration of 10 mM. Gel solution was poured into a horizontal electrophoresis apparatus and allowed to harden, then immersed in gel buffer (without methylmercuric hydroxide). RNA samples (10 μ g/slot) were prepared in gel loading buffer (4X loading buffer contained 60% glycerol, 40 mM methylmercuric hydroxide, 0.4% bromophenol blue) and electrophoresed at room temperature for 8 hours at 50 volts (constant voltage). Gels were transferred to a shallow tray containing a solution of 0.5 M ammonium acetate (to complex methylmercury) and 0.5 μ g/ml ethidium bromide. Gels were photo-

graphed in UV light with a Polaroid camera using Type 57 film. RNA was transferred to nitrocellulose filters as described for transfer of DNA, except that no denaturation step was necessary. Hybridization of filters and autoradiography was performed as previously described for Southern transfers.

Size fractionation of SstI-digested JC1 DNA

The method described by Maniatis et al. (1978) was followed with minor modifications. Two hundred micrograms of high molecular weight JC1 DNA was digested to completion with SstI, deproteinized and ethanol precipitated as previously described, and resuspended in 1 ml of TE. A 38 ml, 10-40% neutral sucrose density gradient in a Beckman SW27 polyallomer tube was prepared using a Beckman gradient apparatus (the sucrose solutions were made in a buffer containing 100 mM NaCl, 20 mM EDTA, 40 mM Tris-HCl, pH 7.5). The DNA sample was heated for 10 min at 68°C, cooled to 20°C, and loaded on the gradient. The gradient was centrifuged in a Beckman SW27 rotor at 25,000 rpm for 24 hours at 20°C. One ml fractions were collected from the gradient by drip collection, mixed with an equal volume of H₂O, and ethanol precipitated overnight. The DNA in each fraction was resuspended in 0.5 ml of TE; 50 µl samples of each fraction were mixed with 10 µl of 10X gel loading buffer, heated at 65°C for 10 min, and electrophoresed in 0.7% agarose gels. DNA in the gels was transferred to nitrocellulose paper; the Southern filters were hybridized to a ³²P-labeled Kirsten ras probe (HiHi-3) as previously described, and exposed to x-ray film for 48 hours. A single restriction fragment of approximately 9 kbp in size was detected in several frac-

tions. One fraction particularly enriched for the ras^K fragment was selected for cloning.

Preparation of phage vector DNA

The method described in Blattner et al. (1977) for propagation of λ phage was followed. A single colony of E. coli 803 supF was inoculated into 5 ml of NZYCM medium [containing per liter 10 g NZ amine A (Humko-Sheffield), 5 g Bacto-yeast extract, 1 g casamino acids (Difco), 5 g NaCl, and 2 g MgSO₄·7H₂O] supplemented with 0.2% maltose and grown overnight at 37°C (maltose induces the maltose operon which contains the gene coding for the λ receptor). Three hundred microliters of λ Sst-17 phage (5×10^6 pfu) in phage dilution buffer (λ dil) (100 mM NaCl, 10 mM MgSO₄, 10 mM Tris-HCl, pH 7.9) was preadsorbed to 0.3 ml of the overnight bacterial culture at 37°C for 15 min. This mixture was diluted into 750 ml of NZYCM medium, distributed to six 500 ml flasks, and incubated for 20 hours at 37°C with vigorous shaking. Lysis, evident by the appearance of bacterial debris, was completed by the addition of 0.15 ml of chloroform to each culture flask.

Phage were purified from the lysed culture by the method of Yamamoto et al. (1970). Sodium chloride was added to a final concentration of 1 M and dissolved by swirling. The culture was allowed to stand for 1 hour on ice and then centrifuged in 150 ml glass Corex bottles in a Sorvall GSA rotor at 7500 rpm for 30 min at 4°C to pellet bacterial debris. The supernatant was decanted into a 1 liter beaker to which polyethylene glycol (PEG 6000) was added to a final concentration of 10% w/v. The PEG was dissolved on a magnetic stirrer at room temperature, after which the solution was incubated on ice water for 30 min. The

solution was centrifuged as above to pellet PEG (containing precipitated phage particles). Pellets were resuspended in λ dil in a final volume of 16 ml and extracted with an equal volume of chloroform. PEG formed a white interface between the chloroform phase and the aqueous phase (containing phage and protein). Phage particles were further purified by centrifugation of the aqueous phase on CsCl step gradients and by CsCl equilibrium centrifugation, as described by Maniatis et al. (1982).

Cesium chloride was removed from the phage preparation by dialysis against λ dil containing 1 mM EDTA overnight at 4°C. Phage DNA was isolated from phage particles by sequential extraction with equal volumes of λ dil-equilibrated phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol. The DNA was precipitated overnight after the addition of NaCl to a final concentration of 200 mM and two volumes of 95% ethanol. DNA was recovered by centrifugation in a microfuge for 15 min at 4°C and resuspended in 2 ml of TE. The yield of DNA was determined by spectrophotometry to be 1260 μ g (equivalent to approximately 2×10^{13} pfu).

Preparation of phage vector arms

The unpublished method of Brian Seed as described in Maniatis et al. (1982) was followed. One hundred μ g of λ Sst-17 DNA in a buffer containing 10 mM $MgCl_2$ and 100 mM Tris-HCl, pH 8.0, was incubated at 42°C for 1 hour to anneal λ cohesive (cos) ends. One-tenth volume of 10X ligation buffer (containing 0.66 M Tris-HCl, pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP) and 100 units of T4 DNA ligase (New England Biolabs) was added and the mixture was incubated for 2 hours at 37°C

(under these conditions, concatomeric DNA molecules are formed by ligation of the 13 bp cos ends of the vector). The DNA was deproteinized and ethanol precipitated as previously described, and was resuspended in TE. The DNA was digested to completion with SstI to separate the annealed phage arms from the 9 kbp internal "stuffer" fragment.

A 38 ml, 5-20% (w/v) potassium acetate gradient was prepared in a Beckman SW27 cellulose nitrate tube using a Beckman gradient former (the potassium acetate solutions were made in a buffer containing 10 mM Tris-HCl, pH 8.0, 5 mM EDTA). The SstI-cleaved DNA was loaded on the gradient and centrifuged in a Beckman SW27 rotor at 23,000 rpm for 20 hours at 20°C (under these conditions the annealed phage arms pellet, while the stuffer fragments remain in the body of the gradient). The pelleted arms were resuspended in 0.5 ml of TE, ethanol precipitated, recovered by centrifugation, and resuspended in 100 µl of TE. Analysis of an aliquot by agarose gel electrophoresis confirmed the purity of the recovered annealed phage arms.

Ligation and in vitro packaging of recombinant DNA molecules

Two micrograms of annealed phage arm DNA was mixed with 1 µg of the ras^K-enriched JC1 DNA fraction in TE and incubated at 68°C for 10 min to disrupt the self-annealing of SstI-generated cohesive ends, after which the mixture was cooled rapidly in ice water. One-tenth volume of 10X ligation buffer and 100 units of T4 DNA ligase was added to the DNA mixture. The reaction (in a final volume of 20 µl) was allowed to proceed at 12°C for 20 hours. Ligation was verified by electrophoretic analysis of a 2 µl sample of the reaction mixture.

A 1.5 ml Eppendorf tube containing in vitro packaging extract ("Packagene", Promega Biotec), sufficient for encapsidating 1 μg of λ DNA, was thawed on ice. Ten microliters of the ligated DNA mixture was added to the extract and mixed by gentle agitation. The packaging reaction was performed at 22°C for 3 hours, after which 0.5 ml of λ dil was added; 25 μl of chloroform was added to maintain bacteriostasis and the packaged phage were stored at 4°C until use. The titre of the in vitro packaged recombinant phage was determined by plaque assay of serial dilutions of the packaging reaction mixture. A 10 μl sample of each phage dilution was preadsorbed to 100 μl of an overnight culture of maltose-induced E. coli 803 supF as previously described. Four ml of NZYCM medium containing 0.7% agarose was added at 47°C and the solution was poured onto the surface of a 82 mm Petri dish containing 30 ml of solidified 1.5% agar. The agarose overlay was allowed to harden at room temperature for 30 min; the dishes were then inverted, incubated at 37°C overnight, and scored for plaques the following day. The phage titre was estimated to be approximately 5×10^3 pfu per 10 μl of undiluted packaging reaction mixture, corresponding to 2.5×10^5 pfu per μg of input recombinant DNA.

Screening of bacteriophage λ plaques by hybridization

The method of Benton and Davis (1977) was followed. Aliquots of the packaging reaction mixture were used to form plaques on E. coli 803 supF as described in the previous section. Dishes were chilled at 4°C for 2 hours to allow the top agarose to harden. Circular nitrocellulose filters (82 mm in diameter) were numbered with a pencil and placed onto the surface of the top agarose of each assay dish (molecules of unpack-

aged bacteriophage DNA in the plaques transfer to the filter in situ). The filters were keyed to the dishes by making 3 asymmetric holes through each filter into the basal agar layer with a 1 ml syringe containing waterproof black drawing ink. After at least one min, the filters were peeled off and immersed, DNA side up, in a shallow tray of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 60 seconds. Each filter was then transferred to neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for 5 min, rinsed in 2X SSPE, and blotted dry between 2 sheets of 3 MM paper. Filters were baked for 2 hours at 80°C in a vacuum oven to fix the DNA.

Baked filters were prepared for hybridization by wetting in a solution of 5X SSPE. They were transferred to a 1 liter beaker containing 100 ml of pre-washing solution (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1% SDS) and incubated batchwise for 2 hours at 42°C with constant agitation so as to prevent the filters from sticking to one another. The pre-wash solution was decanted, and 60 ml of prehybridization solution (50% formamide, 5X SSPE, 5X Denhardt's reagent, 0.1% SDS, 100 µg/ml heat-denatured salmon testis DNA) was added; filters were incubated at 42°C for 5 hours with agitation. The prehybridization solution was replaced with 60 ml of hybridization solution (identical to prehybridization solution with the exception that the concentration of Denhardt's reagent was 1X instead of 5X). The ³²P-labeled ras^K probe, denatured by boiling for 5 min, was added (5 x 10⁵ cpm per ml of hybridization solution) and filters were incubated for 16 hours at 42°C with constant agitation. The filters were washed under stringent conditions and exposed to x-ray film as previously described for hybridization and autoradiography of Southern filters.

Rapid isolation of bacteriophage λ DNA from single plaques

The plate lysate method of E.F. Fritsch (unpublished) as described in Maniatis et al. (1982) was employed. Single plaques were picked with sterile wooden dowels from the top agarose layer and resuspended in 1 ml of λ dil containing a drop of chloroform. Phage suspensions were placed at 4°C overnight to allow the phage particles to diffuse out of the agarose. One hundred μ l of each suspension (approximately 10^5 pfu) was preadsorbed to 100 μ l of E. coli 803 supF, suspended in 2.5 ml of 0.7% agarose, and spread on the surface of an 82 mm dish containing 30 ml of NZYCM plus 1.5% agarose. The dish was inverted and incubated at 37°C overnight until confluent lysis was observed. Five ml of λ dil was added directly to the dish and the phage particles were eluted by agitation for 2 hours at room temperature. The buffer was transferred to a 12 ml glass Corex centrifuge tube and centrifuged at 8000 x g for 10 min at 4°C to remove bacterial debris. The supernatant was transferred to another tube containing an equal volume of a solution of 20% (w/v) PEG and 2 M NaCl in λ dil and incubated for 1 hour in ice water. Precipitated phage particles were recovered by centrifugation at 10,000 x g for 20 min at 4°C. The pellet was resuspended in 0.5 ml of λ dil; 5 μ l of 10% SDS and 5 μ l of 0.5 M EDTA (pH 8.0) was added and the mixture was incubated at 68°C for 15 min. The mixture was extracted once with phenol:chloroform (1:1) and once with chloroform. DNA in the final aqueous phase was precipitated by the addition of an equal volume of isopropanol and placed at -70°C for 20 min. DNA was recovered by centrifugation in a microfuge for 15 min at 4°C, washed with 70% ethanol, and resuspended in 50 μ l TE. DNA prepared in this manner was suitable for restriction endonuclease digestion and electrophoretic analysis.

CHAPTER 1:
TRANSFORMATION OF MOUSE AND HUMAN FIBROBLASTS BY SV40:
A MODEL SYSTEM

The emphasis of this research effort has been the genetic characterization of the transformed phenotype in mouse cells transformed in vitro by chemical carcinogenesis. At the outset I chose to employ the methodology of DNA-mediated transfer; more specifically, to use naked DNA from transformed donor cells to transfer the malignant phenotype to a suitable untransformed recipient cell line by the DNA co-precipitation technique of Graham and van der Eb (1973) as modified by Wigler et al. (1978). In order to work out this method, as well as to establish a reliable selective assay for transformed cells, I elected to exploit a model system employing purified DNA from simian virus 40 (SV40), a small DNA tumor virus belonging to the papovavirus family, as the oncogenic agent. The decision to use SV40 was based on this laboratory's considerable experience with DNA tumor viruses; furthermore, SV40 has been characterized genetically in an exhaustive fashion (Tegtmeyer, 1981). Its genome, as depicted in Figure 2, can be divided into two domains or regions, early and late. The late region encodes three viral structural proteins (VP1, VP2 and VP3), which constitute the virion capsid. The early region encodes two proteins, small and large T antigen. Large T antigen is required for the regulation of viral replication during the lytic cycle of this virus. It is through the coordinate expression of both small and large T antigen that SV40 is capable of malignantly transforming a wide variety of cells in culture. Such an apparent "one-

Figure 2. SV40 genome map showing various SV40 landmarks and key restriction enzyme recognition sites. (From Fiers et al., 1978).

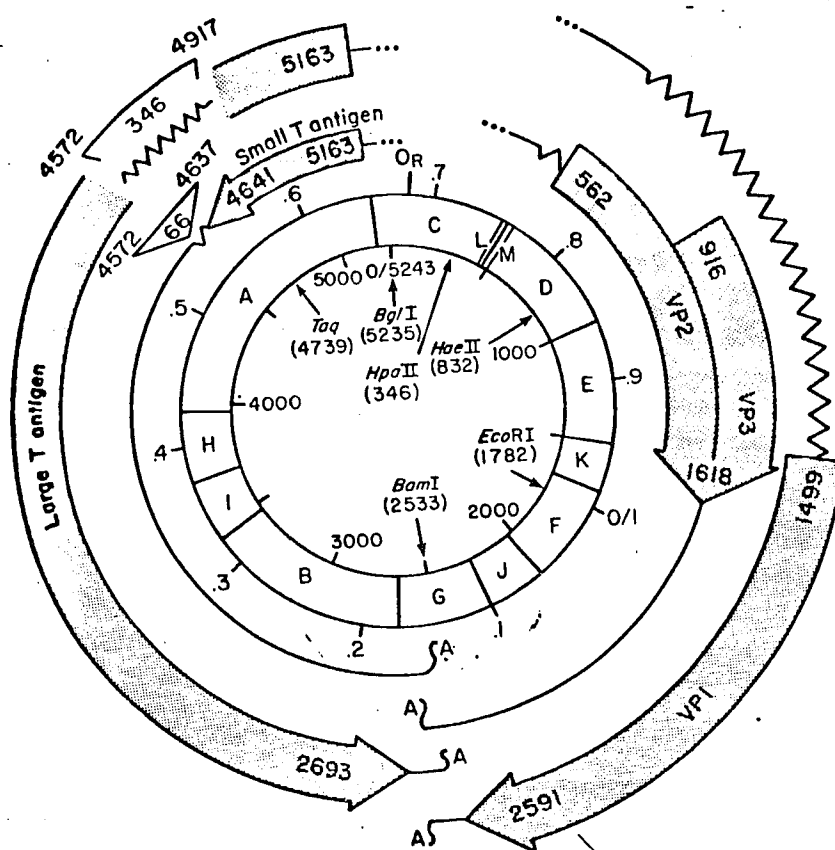


Figure 2

hit" mechanism mimics what one would expect from a dominantly acting cellular "oncogene".

Two recipient cell lines were selected for these studies. The first, NIH 3T3, is an established untransformed mouse cell line (Jainchill et al., 1969) which had been employed by two other laboratories performing related experiments (Shih et al., 1979; Cooper et al., 1980) and which had previously been shown to efficiently express retroviral transforming genes upon transfection of either proviral DNA (Andersson et al., 1979) or cellular DNA containing integrated retroviral sequences (Copeland et al., 1979). Indeed, NIH 3T3 has been the recipient cell line of choice for all cellular DNA-mediated gene transfer in both this laboratory and others in the field. At early passage, it is relatively contact-inhibited and highly anchorage-dependent; however, spontaneous transformed foci appear when even untreated monolayers are maintained at confluence for extended periods. Such foci would confound interpretation of gene transfer experiments. Consequently, I chose to rely exclusively on growth in soft agarose as a criterion for in vitro transformation (MacPherson and Montagnier, 1964). This assay is generally recognized as being more stringent than focus formation and correlates well with the tumorigenic potential of transformed cells in immune-deficient animals (Freedman and Shin, 1974). Additionally, it offers a convenient method of obtaining colonies arising from a single cell.

The other recipient chosen was a human fibroblast designated 'HS74 BM' which is derived from fetal bone marrow and displays a diploid human karyotype (Smith et al., 1976). Unlike NIH 3T3, HS74 has a finite lifetime in culture, the cells failing to survive 'crisis' after extensive

passage (about 50-70 cell generations). The reasons for using a human cell line as a DNA recipient were several: (1) Cultured human fibroblasts have proven to be relatively refractory to in vitro transformation by both physical (e.g., x-ray, UV-light) as well as chemical means, as compared to rodent cells (DiPaolo, 1983). It was our hope that transformation via transfection with oncogenic DNA sequences might bypass the cellular mechanisms which appear to impede more direct methods of carcinogenesis. (2) Some difficulty was anticipated in following the fate of introduced mouse cellular genes in a mouse recipient cell line, in the absence of a recognizable co-transferred sequence. Gene transfer of mouse genes into a heterologous genetic background such as human might enable one to identify the transgenome by virtue of interspersed mouse-specific moderately repetitive DNA sequences. (3) HS74 BM has a low saturation density without morphologically altered foci and is highly anchorage dependent, allowing one to use either focus formation in monolayer or growth in soft agarose as an assay for transformation.

One additional consideration in these studies was the host range of the recipients for SV40 replication. While mouse cells are totally non-permissive for SV40 replication (Topp et al., 1981), human fibroblasts are semipermissive (Butel et al., 1972), HS74 producing virus at an efficiency 1% that of fully permissive monkey kidney fibroblasts (Ozer et al., 1981). Transformation of human cells has been reported after virus infection (Aaronson and Todaro, 1969); however, such transformants continue to produce virus. It has been shown that purified SV40 DNA can be used to transform HS74, albeit at a low frequency relative to rodent cells, using a subgenomic fragment of SV40 DNA containing an intact early region and the origin of viral DNA replication (Zouzas et al.,

1980). Although virus proliferation was precluded by deletion of the late region encoding viral structural genes, extensive viral DNA replication was observed, likely contributing to the instability of the transformed phenotype due to excision and replication of the integrated SV40 sequences. In an effort to develop a quantitative transformation system for human fibroblasts which could be exploited as a single-gene model for cellular gene transfer, I chose to utilize a recombinant SV40 DNA molecule, pMK-SV40-BglI^R (SV_{ori}⁻) isolated by Gluzman et al. (1980a; 1980b) in which a 6 base pair deletion had been introduced within the origin of DNA replication (0.67 map units) by in vitro site-specific mutagenesis (as depicted in Figure 3). Thus it has an efficient transforming early region but would be expected to be unable to excise and replicate its DNA extrachromosomally or produce infectious virus in either permissive monkey or semipermissive human cells. The SV_{ori}⁻ mutant had been demonstrated to transform rat cells as efficiently as the SV_{ori}⁺ (wild type) derivative, but unlike the latter, is able to transform permissive monkey kidney fibroblasts (Gluzman, 1981).

Transfections were performed in the following manner. Cells were seeded at 7.5×10^5 per 60 mm Petri dish 20 hours before transfection (refer to Materials and Methods for culture conditions and experimental details). Cultures were refed 4 hours prior to transfection with 4.5 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% calf serum. DNA-calcium phosphate precipitates were prepared such that each dish received 0.5 ml containing 0.5 μ g SV40 genome equivalents and 20 μ g highly polymerized salmon testis DNA as carrier. Cells were exposed to DNA for 4 hours (HS74) or 15 hours (NIH 3T3), then refed with complete

Figure 3. Strategy for isolation of Bgl I^R (ori⁻) SV40 DNA. (From Gluzman et al., 1980a).

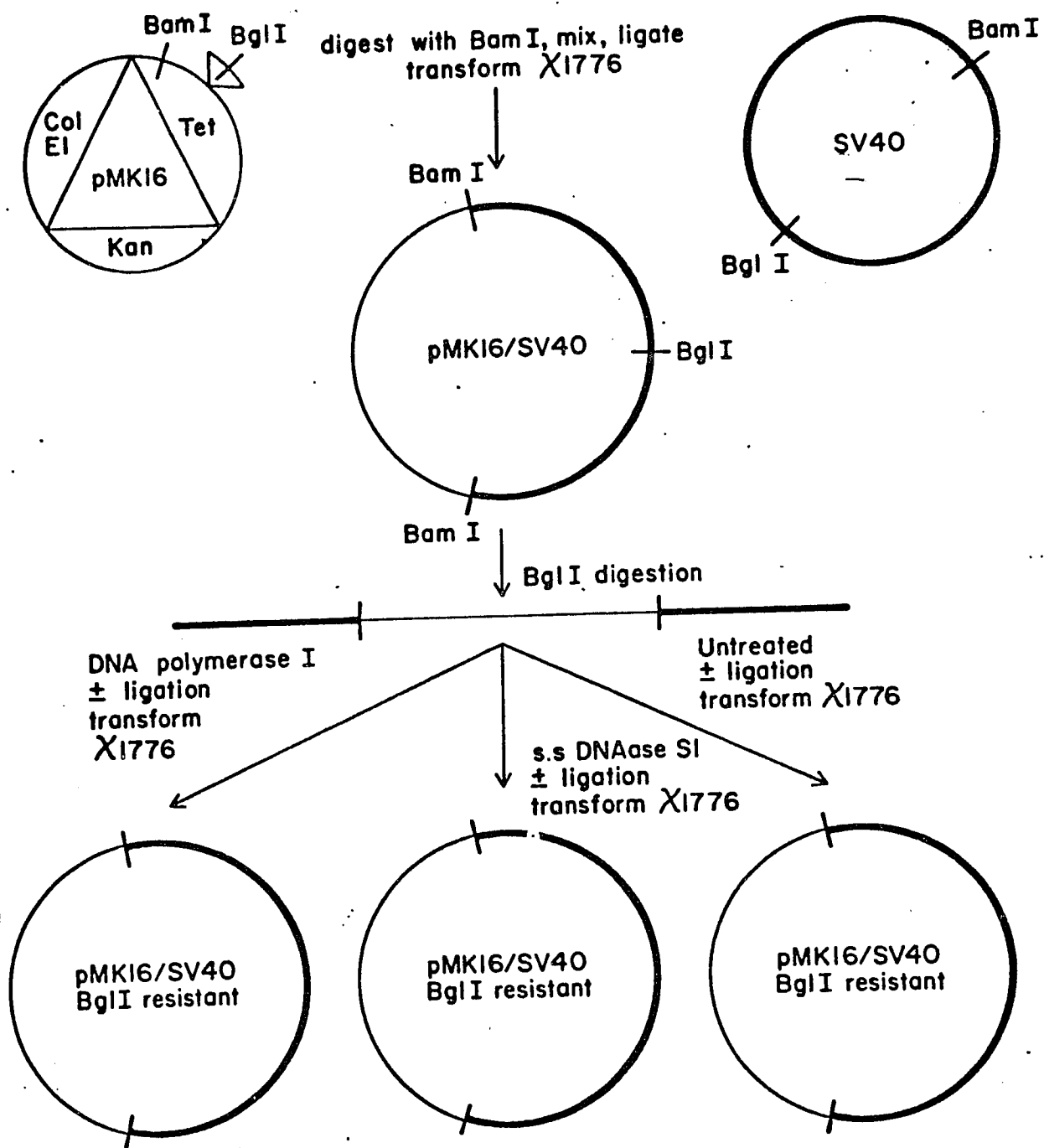


Figure 3

medium (HS74 were found to be sensitive to prolonged exposure to precipitate; 4 hours was shown to be optimal in terms of cell viability). Cultures were incubated for 3 weeks and scored for transformed foci in monolayer (in situ selection) or subcultured 3 and 7 days post-transfection; one-half of each dish was reseeded into a 100 mm petri dish and scored 3 weeks later for foci, the other half suspended in soft agarose and scored after 3 weeks for macroscopic colonies 0.1 mm or greater in diameter. When NIH 3T3 was used as a recipient, all cultures were assayed in soft agarose, with subcultivation 7 days post-transfection.

Initial experiments revealed that the SV ori⁻ recombinant molecule was capable of transforming NIH 3T3 cells at high frequency, a testimony to the extraordinary propensity of NIH 3T3 to take up and express exogenous DNA. A typical experiment as outlined above results in several hundred colonies in agarose per transfected dish. The SV ori⁻ plasmid was also able to transform HS74, although not nearly as efficiently as it did NIH 3T3. We were, however, surprised to find that selection in soft agarose yielded several colonies which, when picked from agarose and transferred to monolayer culture, attached and grew vigorously. This is contrary to our experience with transformation of HS74 by wild-type SV40 DNA, which induced transformants only rarely in soft agarose. Transformed colonies were examined for expression of SV40 large T antigen by indirect immunofluorescent staining (Pope and Rowe, 1964). Cells were grown on glass coverslips and fixed when still sparse with cold acetone: methanol (7:3). Coverslips were stained sequentially with hamster anti-tumor (anti-large T antibody) serum and fluorescein-conjugated goat antiserum directed against hamster IgG. Cells were visualized with a Leitz microscope equipped with an epillumination

system. All SV or i^- transformants were found to be positive for T antigen, consistent with typical SV40 transformants. This was confirmed independently with the cooperation of Dr. Robert Pollack of Columbia University. In addition, Dr. Pollack examined these transformants for their level of actin cable organization by fluorescent visualization with fluorescein-conjugated phalloidin, a compound which forms a complex with F-actin polymer. He found that the SV or i^- transformed human cells had fewer, finer, and shorter F-actin-containing structures than their normal counterparts, consistent with published observations (Verderame et al., 1980). A summary of his results is found in Table 6.

Several other human fibroblast lines were tested for their ability to be transformed with SV or i^- DNA. These included HS27 (normal foreskin fibroblasts), HS181 BM (fetal bone marrow fibroblasts), skin fibroblasts from a male with the Lesch-Nyhan syndrome (HGPRTase deficiency), and skin fibroblasts from a patient with hereditary adenomatosis of the colon and rectum (ACR syndrome). It has been reported that skin fibroblasts from patients with ACR, an autosomal dominant mutation, show increased susceptibility to transformation by oncogenic agents (Kopelovich, 1982). Based on a limited number of experiments, I concluded that none of these cell lines differed substantially from HS74 BM in their susceptibility to transformation with SV ori $^-$ DNA.

Transformation of semipermissive human fibroblasts by wild-type SV40 is relatively inefficient compared with SV40 transformation of non-permissive rodent cells (Topp et al., 1981). Our hypothesis is that the low efficiency of wild type SV40 is due to its ability to excise and replicate autonomously, resulting in the demise of transformants either

Table 6 . Quantitation of T Antigen and Actin Cable Organization in SV40-transformed HS74 BM.

Cell type	% T antigen positive	Actin cables by phalloidin staining: % over total number		
		I*	I + II	I + II + III
HS74 BM	0	5.1	38	55
SV-A**	96	0	14	72
SVor i- 1	76	0	2.4	69
SVor i- 2	84	0	14	69
SVor i- 3	96	0	17	68

*Roman numerals refer to the following actin cable classification:

Class I: heavy distinct cables that cross more than 90% of the central area of the cell, 50% of which span the breadth of the cell.

Class II: mostly fine cables, with at least two heavy distinct cables that enter the central half of the cell and extend more than half the breadth of the cell.

Class III: have only fine, diffuse cables.

**from Zouzas et al., 1980

by the cytotoxicity of accumulated SV40 DNA or by virtue of an unstable phenotype. We anticipated that the SV ori⁻ DNA derivative would be much more efficient than SV ori⁺ DNA in transforming HS74, and this was suggested by our initial experimental findings. In order to confirm our hypothesis, a series of experiments were designed comparing the efficiency of transformation of HS74 by SV ori⁻ DNA with that of other derivatives containing a functional viral origin of replication.

The first experiment compared intact superhelical (form I) derivatives of SV40 viral, plasmid SV ori⁺ and plasmid SV ori⁻ DNA by subculture 3 days post-transfection and by both focus and agarose assay. The results are shown in Table 7. As we had predicted, the SV ori⁻ derivative was most efficient in both transformation assays, this effect being most striking for the anchorage independence (agarose) assay. In addition, the foci generated by the SV ori⁻ mutant were considerably larger than those generated by the ori⁺ derivatives (see Figure 4), suggesting that the ori⁻-derived transformants grow more vigorously than ori⁺ transformants. The data for the focus assay, while of a preliminary nature, also suggested that the recombinant ori⁺ derivative was better than wild type viral DNA. This observation suggests that there is an additional variable effecting the efficiency of the recombinant ori⁻ and ori⁺ DNA's, namely the presence of plasmid sequences and/or the contribution of their propagation in bacteria, such as methylation (Lusky and Botchan, 1981). In order to evaluate these factors, as well as to better establish the quantitative aspects of this study, a more comprehensive experiment was performed, the results of which are shown in Table 8.

Table 7 . Transformation of HS74 by SV40 Form I DNA's

<u>DNA derivative</u>	<u>Transformed colonies per dish*: 3-day subcultivation</u>	
	<u>Focus Assay</u>	<u>Agarose Assay</u>
pMK SV40 BglI ^R (ori ⁻)	13(10-17)	6(5-7)
pMK SV40 BglI ^S (ori ⁺)	5(3-8)	0
SV40 viral DNA	2(1-3)	0.5(0-1)

*Data presented as average number of foci or colonies per dish, with range in parentheses.

Figure 4. Giemsa-stained plates of HS74 BM three weeks post-transfection with SV40 Form I DNA's: (a) no SV40 DNA; (b) SV40 viral DNA; (c) pSV ori⁺ DNA; (d) pSV ori⁻ DNA.

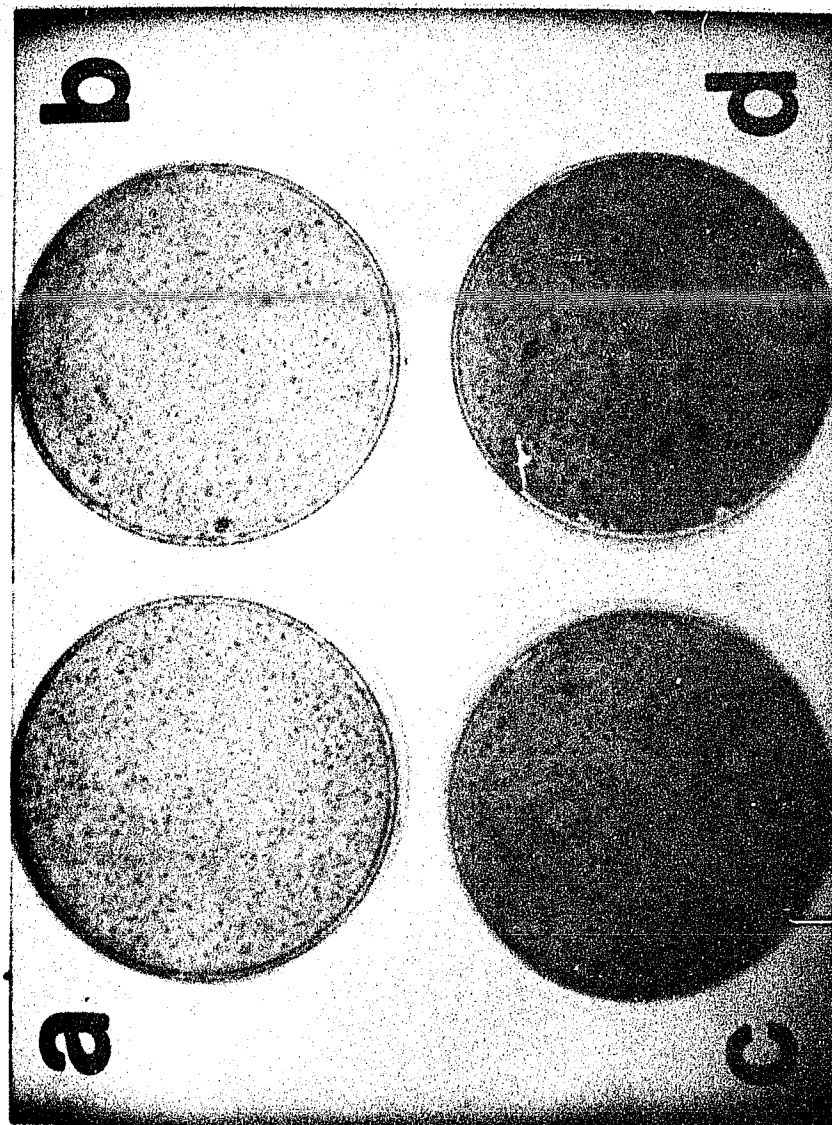


Figure 4

I compared superhelical (Form I) SV40 derivatives (lines 1, 2, 3) and once again found pSV ori⁻ to be more efficient than either viral or pSV ori⁺ DNA. Next the efficiency of transformation by linearized (Form III) SV40 viral DNA was compared with that of linear viral DNA's derived from pSV ori⁺ and pSV ori⁻ from which plasmid DNA had been removed by preparative agarose gel electrophoresis and electroelution (lines 6, 8, 9). Again SV ori⁻ was most efficient. Residual contribution from the bacterial environment appears likely as well because recombinant SV ori⁺ form III transforms with higher efficiency than form III viral DNA. However, the contribution of bacterial modification seems to be transient and almost disappears in the 7-day subcultivation protocol. One observes that linear derivatives are somewhat more efficient than their superhelical counterparts (compare lines 1, 2 and 3 with 4, 5 and 6). This effect may be due to the fact that form III derivatives have restriction enzyme-induced cohesive ends which might enhance integration relative to superhelical molecules. Another possibility is that the cohesive ends may increase the likelihood of tandem integration of SV40 sequences. Finally, I tested whether secondary infection was responsible for the reduced efficiency of viral DNA by using a viral DNA molecule which had been deleted of 750 base pairs in the late region (SV40 viral EcoRI/BamHI fragment), precluding formation of viral particles. No improvement was observed (compare 6 and 7 in Table 8).

These experiments demonstrate that the efficiency of transformation of semipermissive human fibroblasts can be markedly increased by using a viral DNA which is defective at the origin of DNA replication. Transformation with SV40 ori⁻ mutants resulted in an increased number of

Table 8. Transformation of HS74 by SV40 Form I and Form III DNA's

DNA Derivative**	In situ foci*	3-Day Subcultivation		7-Day Subcultivation	
		Foci	Colonies in agarose	Foci	Colonies in agarose
1 SV40 viral I	0.5(1,1)	0.7(0,1,0,2)	0.5(0,1,0,1)	6.0(4,8)	0.0(0,0)
2 pSV or i ⁺ I	1.5(2,1)	2.0(2,0,2,4)	2.7(6,0,2)	1.5(2,1)	0.5(0,1)
3 pSV or i ⁻ I	3.5(2,5)	8.5(5,14,10,5)	3.3(2,2,6)	10(10,10)	6.0(2,10)
4 pSV or i ⁺ III	8.0(7,9)	12(16,10,10,12)	1.0(3,0,1,0)	14(14)	0.5(0,1)
5 pSV or i ⁻ III	25(25)	31(31,28,35,32)	20(19,27,22,13)	60(60)	18(16,20)
6 SV40 viral III	2.5(1,4)	3.3(3,5,2,3)	0.3(0,1,0)	7.0(1,13)	0.0(0,0)
7 SV40 viral Eco/Bam	1.5(1,2)	2.5(2,3,2,3)	0.5(1,0,1,0)	0.0(0,0)	0.0(0,0)
8 SV or i ⁺ III	4.5(1,8)	6.7(4,7,9)	2.0(4,0,2,2)	9.0(9)	1.5(0,3)
9 SV or i ⁻ III	9.5(8,11)	8.3(10,5,8,10)	4.3 (5,6,3,3)	25(25)	20(19,22)
10 Salmon testis	0.0(0,0)	0.0(0,0,0,0)	0.0(0,0,0,0)	0.0(0,0)	0.0(0,0)

*Data presented as average number of foci or colonies per dish, with individual points in parentheses.

**DNA derivatives were prepared as described in Materials and Methods.

transformants as observed by both direct assay (in situ) and subculture assays for anchorage independence. The frequency of transformation of HS74 by pSV ori⁻ DNA increases as a function of the amount of DNA used in transfection (Table 9). However, this increase is non-linear, approximating threefold over the range 1-10 μ g per culture. Increasing the amount of SV40 viral DNA in the same manner does not substantially influence the transformation frequency.

In conclusion, I believe that the origin-defective recombinant SV40 molecule is the vector of choice for SV40 transformation of human fibroblasts. Its enhanced transformation efficiency (relative to wild-type SV40) is probably due to its inability to excise and replicate in transformed semipermissive cells. This property should increase the stability of the transformed phenotype. Furthermore, it precludes the possibility of cell death mediated by uncontrolled viral DNA synthesis or production of virus particles. It was observed that pSV ori⁻ DNA, linearized in its plasmid sequence by digestion with the restriction enzyme XhoI, routinely gave the highest transformation frequencies (1 in 10^5 or greater can be obtained upon transfection of early passage cells). Further characterization of these transformants has not been performed within this thesis because it was not my primary interest, serving merely as a model approach. However, this body of work has served as the foundation for the studies of another doctoral student, David Neufeld, who is currently examining the physical state of integrated SV40 ori⁻ sequences in HS74. In addition, he has succeeded in permanently establishing cell lines from at least one SV ori⁻-generated human fibroblast transformant, although the significance of this result as it relates to the ori⁻ defect is as yet not clear. It has

Table 9. DNA Dose Response of SV40 Derivatives

DNA dosage (μ g)	In situ foci per dish		
	SV40 viral I	pSV ori ⁻ I	pSV ori ⁻ III
0.2	0,1,0	2	----
0.5	----	2	----
1.0	1,0,1	---	14,15,10
1.5	----	2	----
2.0	----	18	----
3.0	0,0	----	18,20
5.0	----	47	----
10.0	2,1,0	----	31,26

been reported (Huebner et al., 1975; M. Botchan, personal communication) that established human cell lines transformed by SV40 harbor defective viral genomes that are incapable of initiating viral DNA replication. Given these findings, transformants of human fibroblasts generated by the ori⁻ vector may prove particularly useful in the establishment of human cell lines and in our understanding of cellular immortalization.

CHAPTER II: CELLULAR DNA-MEDIATED GENE TRANSFER

Having achieved successful results with SV ori⁻ transfection of mouse and human fibroblasts, gene transfer experiments were initiated utilizing high molecular weight cellular DNA from in vitro transformed cells as donor genetic material. Two transformed mouse cell lines were selected to focus on initially. One was a Rous sarcoma virus (RSV) transformed Balb/c 3T3 cell line designated 'B77/3T3'. This cell line has a single integrated copy of the RSV genome per haploid cell DNA complement (Varmus et al., 1973); it grows to a high cell density and forms colonies readily in soft agarose (approximately 10 colonies per 100 cells seeded). It has been made resistant to thioguanine (10^{-5}M) and ouabain (3 mM) in this laboratory by mutagenesis with ethyl methane sulfonate (Neufeld and Ozer, unpublished results). It was employed as a model donor to simulate a transformed cell line containing a highly efficient 'dominant' transforming gene. The other cell line was a nitrosocarbaryl-transformed Balb/c 3T3 line designated 'Clone H' (Quarles and Tennant, 1975). It has growth characteristics similar to the RSV-transformed B77/3T3. As discussed in the Background section of this thesis, both B77/3T3 and Clone H, upon somatic cell fusion with a normal mouse fibroblast cell line, THO-2 (Jha and Ozer, 1976), form cell hybrids which continue to exhibit the malignant phenotype characteristic of the transformed parental cell lines (Jha et al., 1978; Small, Jha and Ozer, unpublished results). This result would suggest that the responsible transforming sequences in both cell lines are likely to be ex-

pressed in a dominant fashion upon transfer to a normal, untransformed recipient cell line.

Before discussing the outcome of experiments involving the transfer of the malignant phenotype to normal mouse fibroblasts, it is appropriate to introduce the results of two related studies performed concurrently. One study was intended to provide a convenient and unambiguous method to monitor the quality of cellular DNA for transfection. High molecular weight DNA from B77/3T3 (subclone OTG) and Clone H was tested for its ability to transfer the cellular gene for thymidine kinase (tk) to mouse L cells (LM tk⁻ clone 1D) deficient in this enzyme (see Background). High molecular weight DNA was isolated from donor cells by lysis with SDS and Proteinase K, followed by deproteinization by extraction with phenol and chloroform, and concentration by ethanol precipitation (refer to Materials and Methods for additional experimental details). LM tk⁻ cells were plated in 100 mm Petri dishes at 5×10^5 cells per dish; 20 hours later 1 ml of calcium phosphate precipitate containing 20 μ g of cellular DNA was added directly to the growth medium. Following a 15-hour exposure to DNA, cultures were refed with complete medium and allowed 48 hours expression time; the medium was then replaced with HAT selective medium (DMEM/F12 in a 50:50 ratio supplemented with 10% calf serum, hypoxanthine [10^{-4} M], aminopterin [4×10^{-7} M] and thymidine [1.7×10^{-5} M]). Dishes were incubated for 14 days and then stained with Giemsa after fixation with 95% ethanol. HAT-resistant (tk⁺) colonies were found in cultures transfected with mouse DNA's as well as DNA from a hamster cell line, CHO K1. Two to ten tk⁺ colonies were routinely generated upon transfection of 10^6 cells with 20 μ g of cellular DNA. This frequency compared favor-

ably with that reported by other investigators (Wigler et al., 1978; Corsaro and Pearson, 1981a). Untreated or mock-infected cultures failed to form colonies in HAT medium; highly polymerized salmon testis DNA, when used as a donor, was unable to correct the tk^- defect. Calf thymus DNA was found to transfer the tk gene, although with an efficiency at least an order of magnitude lower than the rodent DNA's tested. This assay is particularly sensitive due to the exceedingly low spontaneous reversion rate of LM tk^- cells. Consequently, all preparations of cellular DNA used for oncogenic transfer were routinely screened for their ability to transfer the cellular tk gene. This has proven to be a reliable measure of the integrity of donor DNA, although it doesn't always correlate well with the ability of that DNA to induce the transformed phenotype; this may merely reflect the difference between LM tk^- and NIH 3T3 in their competence at taking up and expressing exogenous cellular DNA. Unfortunately, LM tk^- cells are not suitable 'oncogene' recipients because they are already morphologically transformed and grow in soft agarose.

The second study involved the assessment of the normal diploid human fibroblast cell line, HS74 BM, as a recipient for cellular DNA transfer. The advantages of using a human cell line as a gene transfer recipient have already been discussed in Chapter I. I decided to study the transfer of two different phenotypes: a) resistance to the cytotoxic drug, ouabain, and b) malignant growth as assayed by focus formation in monolayer and growth in soft agarose. Ouabain (G-Strophanthin) is a cardiac glycoside which specifically inhibits $Na^+-K^+-ATPase$. Resistance to ouabain, which can be induced by mutagenesis, is believed to be attributable to mutations which reduce the affinity of ATPase for the

drug. Resistance to ouabain is expressed in a codominant fashion (Baker et al., 1974) and the DNA-mediated transfer of the ouabain-resistance marker to sensitive mouse L cells has been reported (Corsaro and Pearson, 1981b). Human fibroblasts are exquisitely sensitive to ouabain (Baker, 1976); concentrations as low as 10^{-6} M are completely lethal. Rodent cells, being far less sensitive to the drug, are able to resist concentrations as high as 10^{-3} M. Indeed, B77/3T3 subclone OTG was selected in 3 mM ouabain. Cultures of HS74 were exposed to calcium phosphate precipitate containing 20 μ g of high molecular weight DNA from either B77/3T3 OTG or Clone H for 4 hours and then refed. After 72 hours under nonselective growth conditions, cultures were trypsinized and reseeded into 100 mm Petri dishes at a 1:5 dilution in complete medium containing 10^{-6} M ouabain for selection of drug-resistant colonies; alternatively, cultures were reseeded either into 100 mm dishes at 1:5 dilution for focus formation or into soft agarose (no dilution) for anchorage independence assay. Transfected cultures were scored after 3 weeks for either ouabain-resistant colonies or transformed foci or agar colonies. Despite numerous trials which involved the treatment, in sum, of greater than 10^8 cells, I failed to find colonies either in ouabain or in the transformation assays. The chromosome-mediated transfer of the ouabain-resistance marker into the near diploid human fibrosarcoma line, HT 1080, has been reported (Lugo et al., in press); low efficiency and high instability of the resistant phenotype was observed. Our negative findings strongly suggest that HS74 are very poor recipients for cellular DNA transfer, and this has been the experience of other investigators attempting to use human diploid fibroblast cell lines, as well. Consequently, all studies to be described henceforth utilized NIH 3T3 as the recipient cell line.

Transfer of the transformed phenotype of chemically transformed cells:Evidence for DNA mediation in the case of Clone H

Twenty hours prior to transfection, NIH 3T3 cells were seeded in 60 mm Petri dishes at 5×10^5 cells/dish in complete medium. Recipient cultures were transfected with 0.5 ml of calcium phosphate precipitate containing 20 μ g of high molecular weight DNA (a control transfection was done using pSV or ϕ DNA at 1 μ g per dish with 20 μ g of salmon testis DNA as carrier), and exposed to DNA for 15 hours. Cultures were refed 3 and 6 days post-transfection. Seven days after transfection, recipient dishes were trypsinized, pooled in sets of 10 dishes, and cells were resuspended in soft agarose (equivalent to the number of cells in the originally transfected culture per 60 mm agarose overlay). After a two-week incubation period, the agarose assay dishes were visually inspected for macroscopic colonies. The results of my initial experiments are presented in Table 10. Transformed colonies greater than 0.1 mm in diameter were found in NIH 3T3 cells transfected with DNA from clone H (see Figure 5 for photomicrograph of agarose colony). Clone H DNA was found to be as efficient in transforming NIH 3T3 as DNA from B77 (RSV) 3T3, subclone OTG. DNA from MCA 16, a 3-methylcholanthrene (3-MC)-transformed C3H/10T1/2 mouse cell line (Rapp et al., 1975), was also able to transform NIH 3T3, albeit far less efficiently. Due to the relatively long expression period before cells were assayed in agarose, as well as the fact that recipient cultures were pooled, I have made no serious effort to establish the frequency of gene transfer observed with these donors. However, I suspect that the frequency is

Table 10. Comparison of NIH 3T3 transforming efficiency of high molecular weight cellular DNA's^a

DNA	Positive dishes/total dishes (colonies per dish) ^b	
	Experiment 1	Experiment 2
Clone H	50/50 (1-10)	9/20 (1-5)
B77/3T3 OTG	17/22 (1-2)	NT ^c
MCA-16	15/50 (1-2)	NT
NIH 3T3	NT	3/20 (0-1) ^d
Salmon testis	0/15 (0) ^d	3/21 (0-1) ^d
pSV ori ⁻	10/10 (>100)	5/5 (5-10)

^aTransfections and anchorage independence assay were performed as described in the text.

^bexpressed as a range

^cNT, not tested

^dmicroscopic colonies observed in some dishes

Figure 5. Photomicrographs of primary NIH 3T3 transformants in soft agarose generated by transfection with (a) high molecular weight Clone H DNA, and (b) salmon testis DNA (100X magnification).

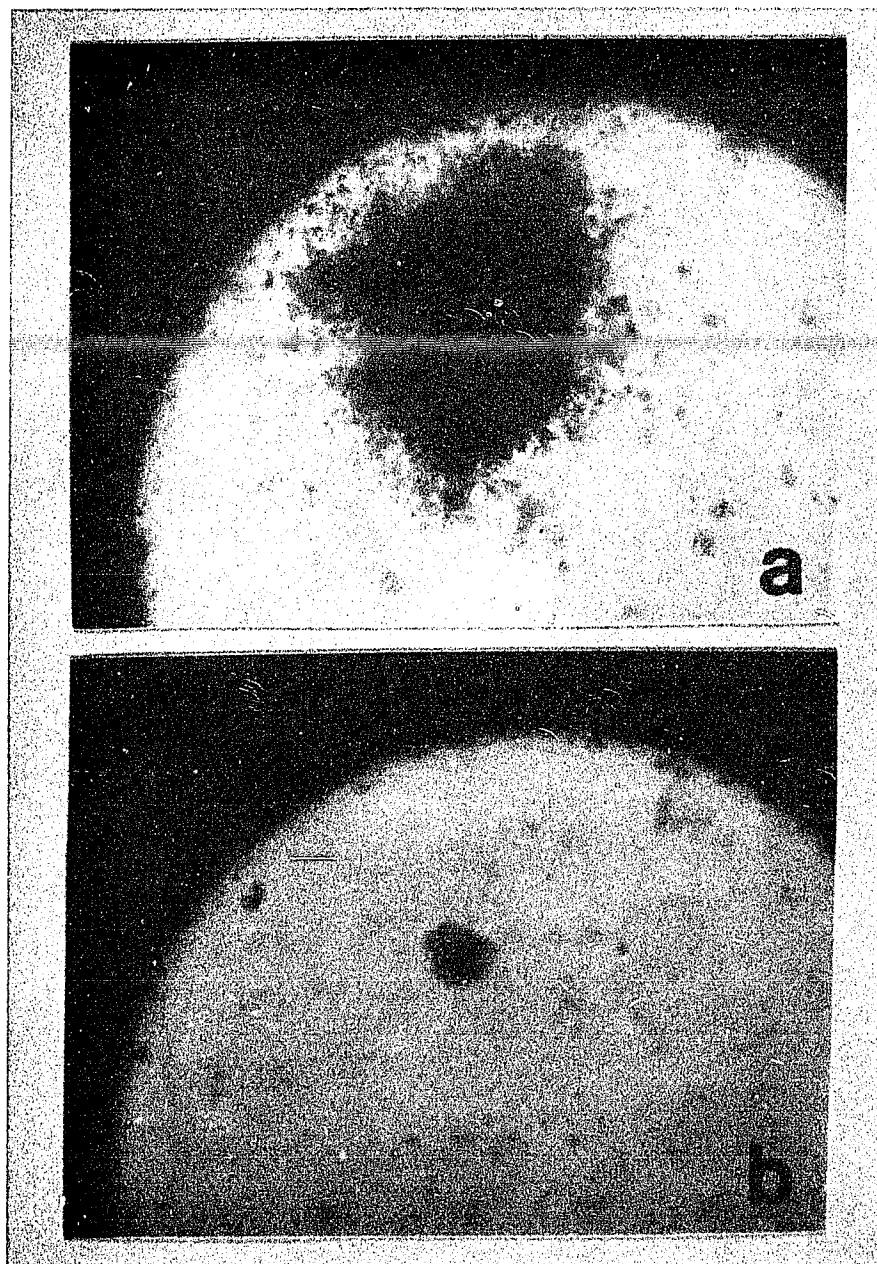


Figure 5

consistent with that obtained for transfer of cellular tk into LM tk⁻, as discussed previously. NIH 3T3 and salmon testis DNA's failed to transform NIH 3T3, although I observed a few small colonies in control cultures (see Figure 5). These colonies, when isolated and grown up in mass culture, failed to reclone in soft agarose. I suspect that these were cells which clumped together upon initial seeding in agarose. Transfection with DNA from Clone H or B77 3T3/OTG which was randomly fragmented by extensive sonication also failed to transform NIH 3T3; however, Cooper et al. (1980) have observed that even DNA from normal NIH 3T3, when sheared by sonication, is capable of inducing the transformed phenotype with a very low frequency (see Discussion).

Evidence that the passage of the transformed phenotype is a consequence of specific gene transfer and not merely an experimental artifact come from the following observations: a) mock transfection (calcium phosphate precipitate minus DNA), and transfection with normal mouse or heterologous salmon testis DNA, fail to transform NIH 3T3 as assayed by growth in agarose; b) NIH 3T3 primary transformants arising from transfection with DNA from transformed mouse cells continue to reclone in agarose upon cell passage, whereas the occasional spontaneous transformants which were observed reclone poorly, if at all, in agarose; c) the same DNA's which are able to induce the transformed phenotype in NIH 3T3 are also capable of donating their thymidine kinase gene to LM tk⁻ cells; d) Wigler et al. (1979b) demonstrated that when bacteriophage ϕ X 174 DNA (an unselected, purified DNA sequence) was physically mixed with bacterial plasmid DNA containing the cloned Herpes simplex virus (HSV) tk gene (the selectable marker) and was then applied as a co-precipitate to LM tk⁻ cells, ϕ X 174 sequences were present in the high molecular

weight DNA of the vast majority of the tk^+ transformants. Thus, cells which incorporate one selectable marker are also likely to incorporate unselected sequences, a phenomenon termed cotransformation. In one such experiment, I mixed the bacterial plasmid, pBR 322, with Clone H DNA (1 μ g of pBR 322 DNA with 20 μ g of Clone H DNA per recipient culture) prior to formation of the DNA-calcium phosphate precipitate; agarose colonies generated by transfection of NIH 3T3 with this DNA were recovered and grown in mass culture. High molecular weight DNA was isolated as previously described and was cleaved with the restriction enzyme EcoRI. DNA fragments were resolved by electrophoresis in 0.7% horizontal agarose gels and transferred in situ to nitrocellulose filters by the technique of Southern (1975) as described in detail in Materials and Methods. Filters were hybridized to a ^{32}P -labeled pBR 322 DNA probe and exposed to x-ray film. Examination of the autoradiograph from such an experiment (Figure 6) shows that all the primary transformants generated in this experiment had integrated plasmid sequences. Cotransformation is strong, if indirect, evidence that the transformants were generated by DNA-mediated gene transfer.

I proceeded to screen several independent chemically transformed cell lines as transforming gene donors by the NIH 3T3 transfection assay. The results are summarized in a qualitative fashion in Table 11. Several donor DNA's generated transformants in agarose, but all at an efficiency below that of Clone H. Four of these chemical transformants were also assayed by Weinberg and co-workers (Shih et al., 1979) and found to be positive; only two of these cell lines were positive by our criteria. However, our data are really too preliminary to conclude that any discrepancy exists. Furthermore, Shih et al. (1979) used focus for-

Figure 6. Southern blot analysis of cellular DNA's from (a) NIH 3T3 and primary NIH 3T3 transformants generated by co-transfection with pBR322, and (b) SalI-digested Clone H DNA, (c,d) XhoI-digested Clone H DNA; (e-g) BamHI-digested Clone H DNA; and (h-j) high molecular weight Clone H DNA. Ten micrograms of each DNA was restricted with EcoRI, fractionated by electrophoresis through a 0.7% agarose gel at 25 volts for 18 hours, and transferred to nitrocellulose. The filter was hybridized to 5×10^6 cpm of ^{32}P -labeled pBR322 (as described in text) and exposed for autoradiography for 4 days.

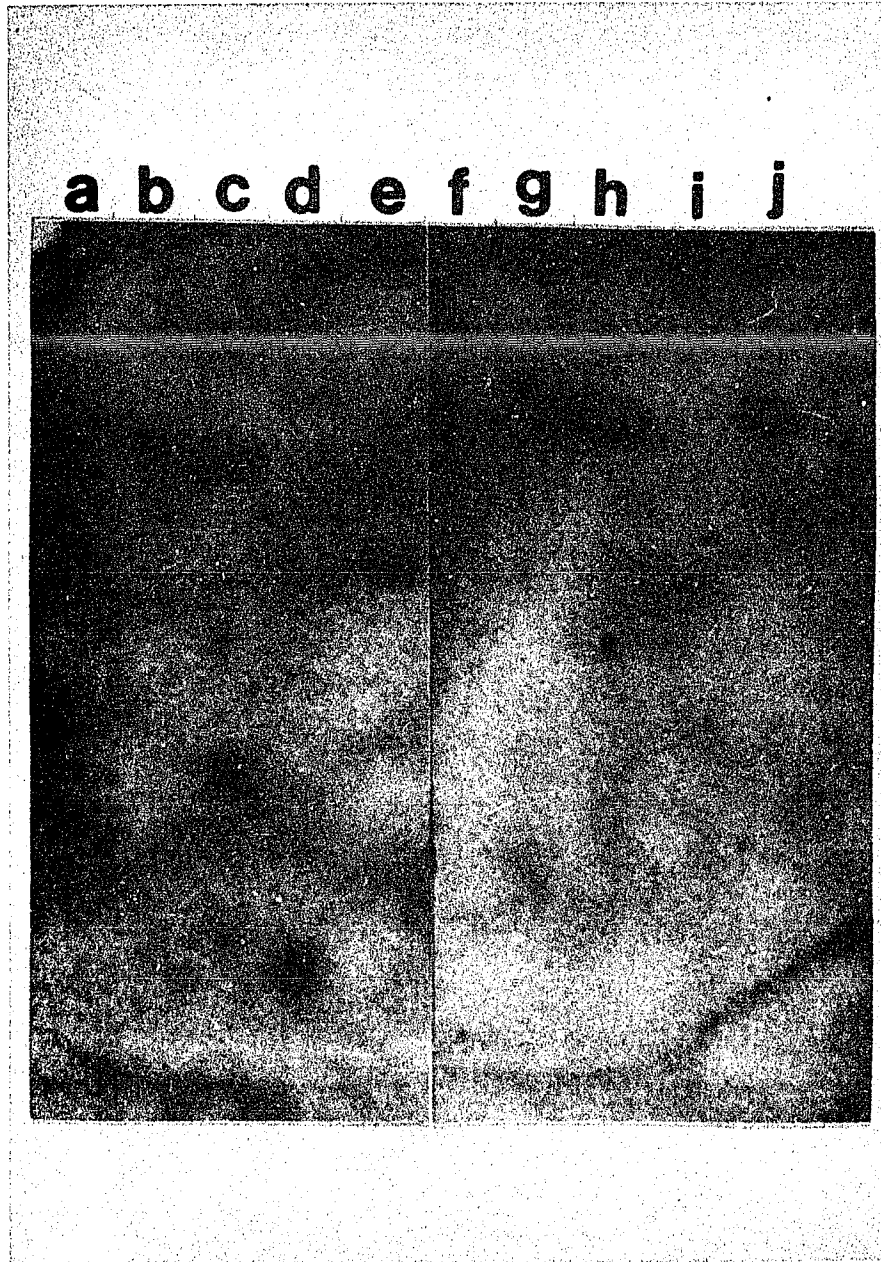


Figure 6

Table 11. Transfection By DNA From Chemically Transformed Cell Lines

Cell Line	Carcinogenic Agent	Properties			
		Agarose EOC	Cell Fusion ^a	DMGT ^c	DMGT ^d
<u>Balb/c 3T3</u>					
A31S	None	<10 ⁻⁵	NT	NT	-
Clone H	NC	>30%	D	++	NT
A31/DMBA 435	DMBA	>30%	D	+	NT
3T3/DMBA	DMBA	< 1%	D ^b	NT	+ ^e
BP/A31	BP	>30%	NT	-	NT
MC-5	3-MC	>30%	NT	-	+
<u>C3H/10T1/2</u>					
C3H/10T1/2	None	<10 ⁻⁵	NT	NT	-
MB66 MCA ACL 13	3-MC	.01%	NT	+	-
MB66 MCA C136	3-MC	.01%	NT	+	+
MCA-16	3-MC	.01%	NT	+	+
<u>NIH 3T3</u>					
NIH 3T3	None	<10 ⁻⁵	NT	-	-

^a NT, not tested; D, dominant expression of transformed phenotype.

^b Variable, with many small colonies observed in some experiments.

^c DNA-mediated gene transfer using 20 µg DNA into NIH 3T3 (-, no colonies in agarose; +, colonies in agarose; NT, not tested).

^d Results observed by Shih et al. (1979) using 75 µg DNA into NIH 3T3 (-, no foci; +, foci; NT, not tested).

^e Designated DMBA/Balb 3T3 in Shih et al. (1979); foci observed with "chromosome-transfer" but not DNA transfection.

Key to abbreviations: DMGT = DNA-mediated gene transfer
EOC = efficiency of cloning
NC = nitrosocarbaryl
DMBA = 7,12-dimethylbenzanthracene
BP = benzo[α]pyrene
3-MC = 3-methylcholanthrene

mation in NIH 3T3 as their criterion for transformation; this assay may be less stringent and subject to more subjective interpretation than the agarose assay utilized by our laboratory. I observed, as did Shih et al. (1979), that some transformants were negative in the transfection assay. Somewhat surprisingly, transferants were observed without correlation as to whether the donor cell line was highly positive for growth in agarose or not.

As a prelude to further characterization, I extended this study by examining the sensitivity of the transforming activity of Clone H DNA to digestion with site-specific (type II) restriction endonucleases (Roberts, 1982). An enzyme that cuts within a functionally essential region of the transforming gene would be expected to abolish the transforming ability of the DNA. Conversely, enzymes cutting only outside the functionally important regions of a gene should leave the biological activity of the DNA unaffected. Restriction of Clone H DNA was performed as described in Materials and Methods. Completeness of enzymatic digestion was determined by removing an aliquot of the reaction mixture, adding a small amount of bacterial plasmid DNA with a known restriction enzyme digest pattern, and monitoring the digestion products of the plasmid by horizontal agarose gel electrophoresis. The results of multiple experiments in this vein are summarized in Table 12. It was found that digestion with HindIII and EcoRI dramatically reduced the transforming activity of Clone H DNA, while digestion with XhoI and SalI had no effect. Digestion with BamHI reduced, but did not abolish the transforming activity of Clone H DNA. This differential biological sensitivity to endonuclease digestion is further strong evidence that the transformation of NIH 3T3 by transfection is mediated by integration of specific

Table 12. Sensitivity of DNA From Clone H to Restriction Enzymes^a

Restriction Enzyme	Positive dishes/total dishes (colonies per dish)		
	Expt. 1	Expt. 2	Expt. 3
None	10/10 (30.5)	4/10 (0.6)	2/5 (0.6)
XhoI	5/5 (21.8)	10/10 (2/6)	5/5 (2.4)
SalI	10/10 (11.1)	7/10 (1.6)	3/5 (1.4)
BamHI	6/10 (1.1)	4/8 (0.8)	2/4 (0.5)
EcoRI	2/10 (0.2)	1/10 (0.1)	0/5 (<0.1)
HindIII	4/10 (0.4)	0/9 (<0.1)	0/4 (<0.1)

^a Five to ten 60 mm dishes were transfected with 20 µg DNA/dish of treated or untreated DNA. Sets of 5 dishes were pooled separately for assay for *aga*⁺. No colonies were observed on control plates (untransfected, or transfected with salmon sperm or normal mouse DNA).

cellular DNA sequences. Provocatively, the pattern of sensitivity to restriction endonucleases we observed was in close agreement with the pattern established by Shilo and Weinberg (1981a) for several independently derived 3-methylcholanthrene (3-MC)-transformed mouse cell lines. This suggested that both our groups were observing transfer of the same transforming DNA sequence.

We considered the possibility that the transforming genes detected here reflected the adventitious laboratory contamination of cultures by murine sarcoma viruses. Although our laboratory did not employ retroviruses, some of our cell lines came from laboratories which did use sarcomagenic viruses in their studies. Alternatively, an endogenous type-C retrovirus genome (Todaro, 1977) might have been activated in these cells (Rapp and Todaro, 1979). In collaboration with Dr. Dino Dina of Einstein Medical College, I sought to investigate this possibility. Although acute transforming retroviral genomes are normally replication defective (Bishop, 1978), they can be "rescued" by superinfection of virus-transformed cells with a replication-competent, non-transforming murine leukemia virus whose genome allows pseudotyping and transmission of the transforming sarcomagenic genome (Huebner et al., 1966). I attempted to rescue sarcomagenic virus from our panel of transformed mouse cell lines by the following analysis, described in detail in Materials and Methods. Logarithmically-growing cultures were infected with a high-titre stock of Moloney murine leukemia virus (Mo-MLV); for each cell line so infected, a parallel culture was left untreated. Four days after infection, the cell culture medium from these cultures was recovered and cleared of contaminating cells by low-speed centrifugation and filtration; the medium from each cell line was then used to infect a

log phase culture of NIH 3T3 cells. Four days post-infection, NIH 3T3 cultures were trypsinized and reseeded into soft agarose and scored for colonies after two weeks. The results are summarized in Table 13.

Superinfection of Clone H cells with Mo-MLV resulted in the rescue of readily detectable levels of a transforming agent, this determined by the ability of cell-free medium from leukemia virus-infected Clone H to induce anchorage independence (growth in agarose) in NIH 3T3; this property defined the transforming agent as a putative murine sarcoma virus (MSV) genome. Rescuability was entirely dependent on superinfection with Mo-MLV; supernatant from uninfected Clone H cells was negative for transmission of a sarcomagenic agent within the sensitivity of this assay. Primary transformants of NIH 3T3 generated by transfection with Clone H DNA, when superinfected with MLV, also gave rise to sarcomagenic virus; this was evidence that an integrated retroviral genome of unknown origin had been transferred from Clone H to NIH 3T3 by DNA-mediated gene transfer.

All other transformed cell lines which were subjected to this analysis were found to be negative for MSV rescue, with the exception of D56, a Moloney murine sarcoma virus-transformed Balb/c 3T3 cell line (Mo-MSV/3T3) which was included as a positive control. While no attempt was made to quantitate the titre of rescued MSV from Clone H, it was clearly equivalent to, if not higher than, that observed upon MLV infection of Mo-MSV/3T3 cells. It should be noted here that Shih et al. (1979) attempted to rescue sarcomagenic virus from their 3-MC transformed mouse cell lines by this method, and found all cell lines to be negative for rescue of type-C retrovirus genomes. Thus, it would appear that the apparent similarity between the transforming activity of Clone

Table 13. MLV Rescue Assay^a

<u>Cell Line</u>	<u>Induction of Anchorage Independence in NIH 3T3^b</u>
Clone H (NCT) ^c	+
Clone H aga 13 ^d	+
NIH 3T3	-
IIHU5a ^e	+
IIJC1 ^e	+
IIHS4b ^f	+
A31/DMBA 435	-
MCA-16	-
MB66 MCAC136	-
D56 (MoMSV/3T3)	+
Clone H SCC1 ^g	+
Clone H SCC2	+
Clone H SCC3	+

^a Infection of test cell lines and assay of culture fluid for sarcomagenic virus performed as described in text.

^b -, no colonies in agarose; +, colonies in agarose.

^c Unrecloned Clone H stock culture

^d Recloned in soft agarose and used as DNA donor for all transfection experiments.

^e Primary NIH 3T3 transformants generated by transfection with HMW Clone H DNA.

^f Primary NIH 3T3 transformant generated by transfection with SallI-digested Clone H DNA.

^g SCC, single cell clones isolated as described in Chapter III.

H DNA in our lab and that of DNA from 3-MC transformed mouse cells reported by Shih et al. (1979), as deduced from the restriction sensitivity profile previously discussed, was coincidental. Comparison of the pattern of restriction sensitivity of Clone H DNA to the known restriction endonuclease maps and biological activity of various murine sarcoma viruses failed to identify a likely candidate for the responsible transforming agent in Clone H. Therefore, additional studies to characterize the transforming gene in Clone H were performed, an account of which forms the basis for Chapter III of this thesis.

Characterization of primary transformants of NIH 3T3 generated by transfection with A31/DMBA DNA

More limited gene transfer experiments have been performed with another chemically-transformed mouse cell line, A31/DMBA 435. This cell line was derived by the in vitro transformation of a Balb/c 3T3 cell line (clone A31) with the carcinogen 7,12-dimethylbenzanthracene (DMBA) (Sen and Todaro, 1978). It was selected as a DNA donor for the following reasons: a) A31/DMBA 435 was employed in our laboratory as the transformed parent in somatic cell fusion (see Table 3) with THO-2, a normal mouse fibroblast cell line previously described, and cell hybrids between these two cell lines continued to exhibit the transformed phenotype characteristic of A31/DMBA (Small et al., 1984); b) A31/DMBA, when assayed for a rescuable retroviral transforming gene by superinfection with MLV, proved to be negative (see Table 13); c) High molecular weight DNA extracted from A31/DMBA was found to be positive in the NIH 3T3 transfection assay (see Table 11). A31/DMBA 435 DNA was subjected to digestion with a limited number of restriction endonucleases and then

used for transfection of NIH 3T3 as previously described. The results of this experiment are shown in Table 14. Digestion with EcoRI or PvuII completely abolished transforming activity, while digestion with BamHI appeared to have no effect.

As previously noted, we anticipated considerable difficulty in identifying a transferant mouse transforming gene amidst a mouse genetic background. Generation of a primary transformant of NIH 3T3 by transfection with A31/DMBA DNA in which a recognizable plasmid sequence was closely linked to the transforming gene might enable one to identify the transforming gene, and perhaps isolate it, by probing for the linked plasmid sequence. Such close linkage would be greatly facilitated by exploiting the in vitro ligation by T4 DNA ligase of restriction fragments of A31/DMBA, generated by digestion with BamHI (since this enzyme does not inactivate the transforming gene), to plasmid linearized by digestion with BamHI. As a plasmid vector, we chose the recombinant DNA molecule pSV2 gpt, constructed by Mulligan and Berg (1980) and kindly provided by Paul Berg (Stanford University School of Medicine) for these studies. The recombinant contains the gene gpt, for guanine:xanthine phosphoribosyl transferase (XGPRT), derived from E. coli. It is inserted into a fragment of SV40 derived from that virus's early region containing the origin of replication and appropriate genetic signals for transcription and processing in mammalian cells, and is cloned in the bacterial plasmid pBR 322. Thus, it can be propagated as a plasmid in E. coli and is expressed in mammalian cells. The sequences encoding the SV40-transforming genes have been fully inactivated. An inhibitor of the synthesis of GMP from IMP through IMP dehydrogenase, mycophenolic

Table 14. Sensitivity of DNA from A31/DMBA 435 to Restriction Enzymes^a

<u>Restriction Enzyme</u>	<u>Total Dishes</u>	<u>Positive Dishes</u>	<u>Total Colonies</u>	<u>Colonies Per Dish</u>
None	10	6	14	1.4
EcoR1	10	1	1	0.1
PvuII	10	1	1	0.1
BamH1	10	5	28	2.8
BamH1 (partial)	60	26	115	1.9

^a Experimental procedure was same as in Table 14.

acid (MPA), makes cells totally dependent on the expression of the bacterial enzyme, which can utilize xanthine unlike its mammalian counterpart, HGPRT. The use of pSV2 gpt would permit the ready identification of primary transfectants containing it and a transforming gene. Furthermore, we envisioned its usefulness in maintaining an indirect selection for the transformed phenotype in the absence of continuous selection for transformation when colonies initially isolated in agarose are maintained in monolayer; it has been shown that DNA transfectants can show a high frequency of loss of the donor gene in the absence of selective pressure (Graf et al., 1979). pSV2 gpt has a single site for cleavage by BamHI in a region nonessential for expression in mammalian cells.

Superhelical pSV2 gpt DNA was isolated from host bacteria by detergent lysis, purified by cesium chloride equilibrium centrifugation (as described in Materials and Methods), and linearized by digestion with BamHI. The DNA was treated with calf intestinal alkaline phosphatase (CIAP) to remove terminal 5'-phosphates. Such treatment has been demonstrated to minimize the undesired recircularization of plasmid DNA, which would be the preferred reaction in the presence of T4 DNA ligase (Ullrich et al., 1977). Foreign DNA fragments with 5'-terminal phosphates can be ligated efficiently to the dephosphorylated plasmid DNA. Bam-linearized pSV2 gpt was treated with 0.5 units CIAP (Sigma) per μg DNA at 37°C for one hour in buffer containing 50mM Tris-Cl (pH 9.0), 1mM MgCl_2 , 0.1mM ZnCl_2 , 1mM spermidine. The reaction was stopped by incubation at 68°C for 15 min after addition of SDS to 0.5%. High molecular weight DNA from A31/DMBA 435 was digested to completion with 2 units of BamHI per μg DNA and mixed with the dephosphorylated plasmid

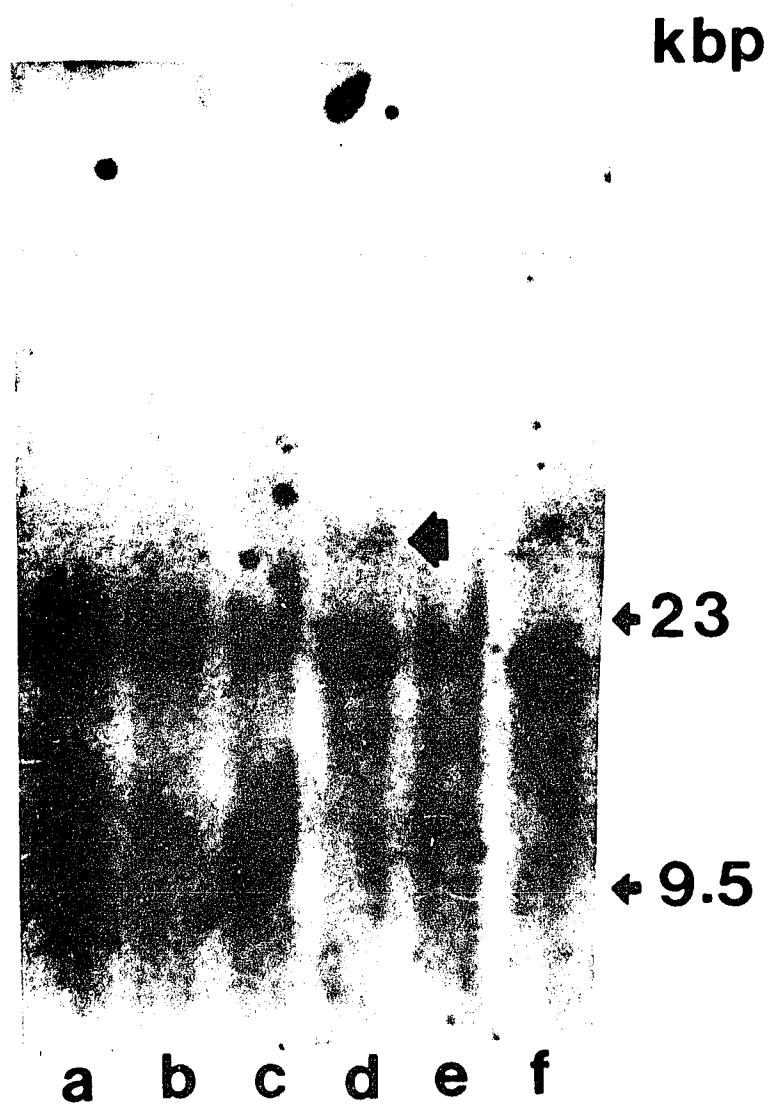
DNA such that pSV2 gpt was roughly in a two-fold molar excess. The DNA mixture was deproteinized by extraction twice with phenol and twice with chloroform, and precipitated with ethanol. DNA was recovered by centrifugation and resuspended in TE at a concentration of 500 $\mu\text{g/ml}$. The DNA was heated at 55°C for 15 min to disassociate cohesive ends, and then cooled quickly in an ice water bath. One-tenth volume of 10X ligation buffer was added containing 0.66M Tris-Cl (pH 7.5), 50mM MgCl_2 , 50mM dithiothreitol, and 10mM ATP. One hundred units of T4 DNA ligase (New England Biolabs) was added and the reaction was allowed to proceed at 8°C for approximately 20 hours. The ligation reaction was stopped by addition of EDTA to a final concentration of 20mM; DNA was deproteinized as before and precipitated with ethanol. The DNA was recovered by centrifugation and resuspended in TE at approximately 300 $\mu\text{g/ml}$. The extent of ligation was monitored by electrophoresis of a sample of the ligation reaction in a 0.7% agarose gel, followed by Southern blot transfer and hybridization of ^{32}P -labeled pSV2 gpt to the blot filter. Such analysis revealed that roughly 10-20% of the input plasmid DNA was associated post-ligation with high molecular weight DNA fragments; a control ligation of dephosphorylated pSV2 gpt in the absence of cellular DNA showed no evidence for self-ligation. The ligated DNA was coprecipitated with calcium phosphate and used to transfect sixty 60mm dishes of NIH 3T3 as previously described; 30 μg of DNA was used per dish. One week later, cultures were trypsinized and assayed by the soft agarose overlay technique for anchorage independent transformation. After 3 weeks, overlays were inspected for macroscopic colonies, and 29 colonies were observed and picked from the 60 experimental dishes. The colonies were propagated in monolayer and then tested for growth in gpt

selective medium (DMEM containing 10% newborn calf serum, 250 μ g/ml xanthine, and 25 μ g/ml mycophenolic acid). Under mass culture conditions, the majority of transformants grew to confluence in gpt selective medium. However, attempts to cultivate these cells under cloning conditions were far less successful; indeed, I was able to isolate only a single gpt-positive colony from the twenty primary transformants which were screened. This colony, furthermore, was unable to grow in agarose, making it unsuitable for further analysis. Attempts to repeat these experiments were frustrated by problems our laboratory experienced over a considerable period of time with growing cells under cloning conditions. Therefore, I decided to abandon this approach.

During the course of studies to be presented in Chapter III, several groups demonstrated that the malignant phenotype can be transferred to NIH 3T3 fibroblasts by incorporation of DNA isolated from human tumor cell lines (reviewed by Cooper, 1982). Most frequently, these transforming genes were found to bear homology to the ras transforming genes of Harvey murine sarcoma virus (Ha-ras) , Kirsten murine sarcoma virus (Ki-ras) (Parada et al., 1982; Santos et al., 1982) or a new member of the ras family (N-ras) not detectable in any known sarcoma virus (Hall et al., 1983; Shimuzu et al., 1983b). It appears from this body of work that the NIH 3T3 transfection system is remarkably sensitive, and perhaps prejudiced, to the detection of activated ras genes, and points to the possible involvement of a member of the ras family in the A31/DMBA cell line. I therefore examined, by Southern blot analysis, the DNA's from several primary transformants of NIH 3T3 generated by A31/DMBA 435 DNA which had been restricted with BamHI prior to transfection. We reasoned that analysis of these particular transformants might be most

revealing; random loss of one or both of the BamHI recognition sites flanking the transforming gene (occurring during the process of integration) would generate an altered pattern of BamHI-cleavage of the gene in primary transformants, relative to the normal cellular homolog. The newly incorporated transforming gene would be recognizable as a novel and larger restriction fragment. Such a phenomenon has been observed in our laboratory upon Southern analysis of human fibroblasts transformed with SV40 DNA linearized at its BamHI site (Neufeld, unpublished results). High molecular weight DNA's from NIH 3T3, A31/DMBA 435, and four independently derived primary transformants, were digested to completion with BamHI; 10 μ g of each DNA was subjected to electrophoresis in a 0.5% horizontal agarose gel for 60 hours at 25 volts. Such extensive electrophoresis was conducted in order to ensure that high molecular weight restriction fragments (greater than 15 kilobase pairs) would be resolvable. DNA from duplicate gels was transferred to nitrocellulose filters by the Southern method. One filter was hybridized to a ^{32} P-labeled Harvey ras-specific probe (pBS-9); a duplicate filter was hybridized to a ^{32} P-labeled Kirsten ras-specific probe (KBE-2). The filters were washed under stringent conditions (see Materials and Methods) and exposed to Kodak X-AR2 x-ray film with intensifying screens for 4 days. Figure 7 is the autoradiograph of the filter hybridized to the Ki-ras probe (KBE-2). All six cell lines share a single restriction fragment of approximately 20 kilobase pairs (Kbp) in size, corresponding to the normal cellular Ki-ras gene. However, one of the primary transformants (lane d in Figure 8) has a clearly visible, though less intense, additional restriction fragment which I estimate to be larger than 30 Kbp in size. The autoradiograph of the filter hybridized to the

Figure 7. Southern blot analysis of cellular DNA's from (a) NIH 3T3, (b) A31/DMBA 435, and (c-f) independent primary NIH 3T3 transformants generated by transfection with BamHI-digested A31/DMBA 435 DNA. A 10 μ g aliquot of each DNA was restricted with BamHI, fractionated by electrophoresis through a 0.5% agarose gel at 25 volts for 60 hours, and transferred to nitrocellulose. The filter was hybridized to 10^7 cpm of KBE-2 (ras^K) probe (as described in text) and exposed for autoradiography for 4 days. Bold arrow indicates position of novel ras^K fragment in lane d.

**Figure 7**

pBS-9 (Ha-ras) probe was completely negative; the normal cellular Ha-ras gene runs as a BamHI fragment of approximately 3.5 Kbp in size and was cut from the gel prior to blot transfer.

While subject to interpretation (see Discussion), this result tends to implicate an activated cellular Ki-ras gene as the transforming gene detected in A31/DMBA-generated NIH 3T3 transformants. This interpretation is supported by the recent report of Parada and Weinberg (1983) that the transforming gene in their in vitro 3-MC transformants (Shih et al., 1979) is an activated cellular Ki-ras gene. In addition, several laboratories have recently reported similar findings with DNA's from rodent tumors induced in vivo by chemical carcinogens (Balmain and Pragnell, 1983; Eva and Aaronson, 1983; Sukumar et al., 1983; Guerrero et al., 1984). I will reserve further comment on these results for the Discussion section of this thesis.

CHAPTER III: CHARACTERIZATION OF THE TRANSFORMING GENE OF CLONE H

Clone H is a nitrosocarbaryl in vitro transformed Balb/c 3T3 cell line derived by Quarles and Tennant (1975). Its properties were discussed in the Background section of this thesis. As described in Chapter II, high molecular weight DNA extracted from Clone H cells was used as a donor in DNA-mediated gene transfer experiments. It was found to efficiently transfer the transformed phenotype to NIH 3T3 fibroblasts, as assayed by colony formation in soft agarose. The profile of sensitivity of Clone H DNA to inactivation by restriction endonuclease digestion bore remarkable similarity to that obtained by Shilo and Weinberg (1981a) for DNA's from several independent 3-methylcholanthrene-transformed mouse cell lines. However, unlike Weinberg and co-workers, we were able to rescue sarcomagenic virus from Clone H cells, as well as from primary DNA transformants of NIH 3T3, by infection with helper murine leukemia virus. Therefore, it would appear that we have observed a transforming agent in Clone H different from that identified by Shih et al. (1979).

The possibility was considered that the Clone H cell line was heterogeneous, i.e. that it contained a subpopulation of virally transformed cells. I isolated several colonies of Clone H derived from the growth of single cells by plating out dilutions of cells on glass coverslips in microwell plates and selecting those coverslips which appeared, by microscopic inspection, to have only a single attached cell. Such coverslips were transferred to Petri dishes containing complete medium

and incubated for 2 weeks, at which time those containing a single colony were expanded into mass culture. All colonies were morphologically transformed and indistinguishable from the unrecycled cell line. Three single cell isolates were infected with MoMLV (as described in Materials and Methods); the medium from these cultures was tested for its ability to induce NIH 3T3 cells to grow in soft agarose. All three clonal isolates proved positive for sarcomagenic virus rescue; it is extremely unlikely that cell culture conditions would preferentially select for only virally transformed cells. We therefore presume Clone H to be a homogeneous cell line.

In collaboration with Dr. Stephan Hann in the laboratory of Dr. Dino Dina at Albert Einstein College of Medicine, I sought to identify the unknown MSV-like transforming agent (see Materials and Methods for experimental details). Retroviral RNA from purified viral particles was prepared from Clone H cells infected with Mo-MLV by the method of Robinson et al. (1965). Concurrently, total cytoplasmic RNA was prepared from uninfected Clone H, Mo-MLV-infected Clone H, and a MLV-infected rat fibroblast cell line (WCT-L) by the method of Favalaro et al. (1980). Polyadenylated [poly(A)⁺] RNA was selected by chromatography on oligo(dT)-cellulose as described by Aviv and Leder (1972). RNA's were electrophoresed through a 1.5% agarose gel containing methylmercuric hydroxide as a denaturant (Bailey and Davidson, 1976) and transferred to nitrocellulose paper. Duplicate blots were hybridized under stringent conditions to ³²P-labeled probes made by nick translation of recombinant plasmids bearing the cloned onc sequences of the Moloney (v-mos), Kirsten (v-Ki-ras), Harvey (v-Ha-ras) and Abelson (v-abl) murine sarcoma viruses. After washing under stringent conditions, the

filters were exposed to x-ray film overnight. A positive reaction was observed only with the v-Ki-ras probe (HiHi-3). As shown in Figure 8, the probe failed to hybridize to poly(A)⁺ RNA isolated from MLV-infected rat fibroblasts (lane a). However, the probe reacted with poly(A)⁺ RNA from both uninfected (lane c) and MLV-infected Clone H (lane d), as well as with RNA from viral particles purified from MLV- infected Clone H (lane b). Two bands were observed, the larger of which we estimate to correspond in size to 38S MLV RNA based on ribosomal RNA markers (visible in the ethidium bromide-stained gel prior to transfer to nitrocellulose). This is the size one would expect for haploid genomic retroviral RNA. The smaller band appears to be approximately 18S in size and is most likely a subgenomic species. Thus, uninfected Clone H, though a non-producer of infectious virus, contains cytoplasmic RNA transcripts which share homology with the oncogenic sequences of Kirsten MSV and are of a size consistent with their being retroviral in origin.

In tandem with the analysis described above, a ³²P-labeled cDNA probe was synthesized by in vitro reverse transcription (Taylor et al., 1976) using viral RNA isolated from MLV-infected Clone H as a template (see Materials and Methods). Plasmid DNA's isolated from recombinants bearing the cloned onc sequences of Moloney, Kirsten and Balb sarcoma viruses were digested with restriction enzymes which liberated the cloned viral sequences from the plasmid vector. p-mos8, which contains the onc sequence of Moloney MSV, was digested with PstI; pHiHi3, bearing the onc sequence of Kirsten MSV, was cleaved with EcoRI; and p-vbas, containing the onc sequences of Balb MSV (Andersen et al., 1981) and completely homologous to the onc sequences of Harvey MSV, was cleaved with BamHI. One-half µg of each restricted DNA was electrophoresed in

Figure 8. Northern blot analysis of 'rescued' virion RNA from MLV-infected Clone H and cytoplasmic RNA from various cell lines. Virus from MLV-infected Clone H was purified on neutral sucrose gradients; virion RNA was isolated by phenol extraction and ethanol precipitation. Total cytoplasmic RNA was prepared from cells and poly A⁺-containing RNA isolated by chromatography on oligo dT-cellulose. RNA's were electrophoresed through a 1.5% methyl mercury-agarose gel and transferred to nitrocellulose paper. The filters were incubated with 6×10^6 c.p.m. of ³²P-labeled v-Kirsten-ras probe and exposed for autoradiography overnight. (a) poly A⁺-RNA from WCT-L (MLV-infected rat cells); (b) virion RNA from MLV-infected Clone H; (c) poly A⁺-RNA from Clone H; (d) poly A⁺-RNA from MLV-infected Clone H.

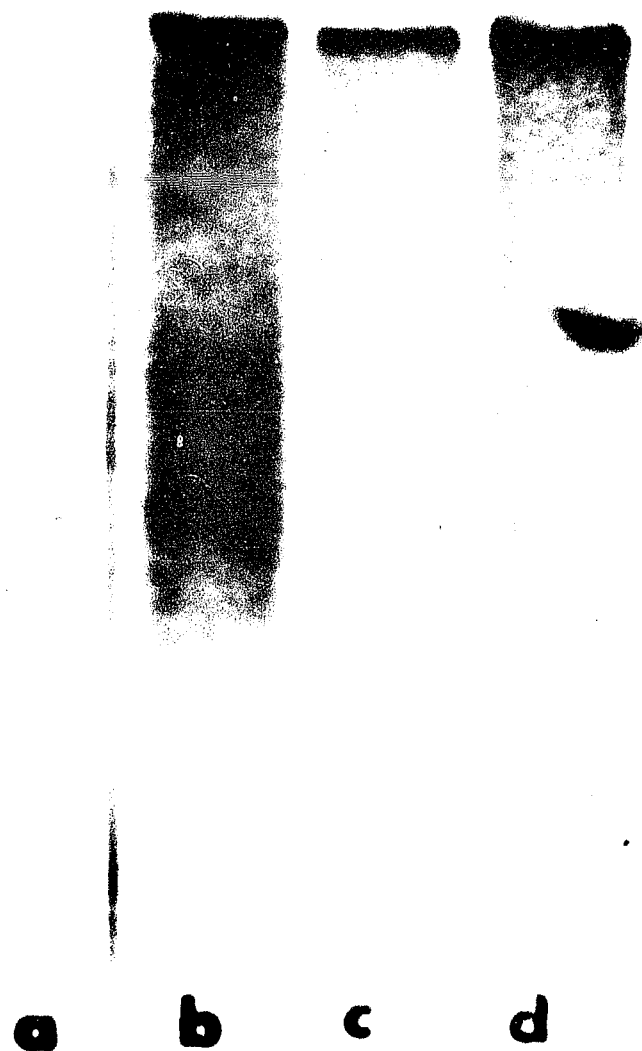


Figure 8

1% agarose gels at 25 volts for 18 hours; DNA's were transferred to nitrocellulose paper as described previously. Duplicate blot filters were hybridized to the ^{32}P -labeled cDNA probe under conditions of low (30% formamide, incubation at 37°C) and high (50% formamide, incubation at 42°C) stringency for 20 hours, washed as detailed in Materials and Methods, and exposed to x-ray film for 13 hours. Figure 9 is the autoradiograph obtained from this experiment. Comparison of lanes b and e shows that the probes reacted with the 1 Kbp onc insert of pHiHi3 (Kirsten MSV) at both stringencies; the 0.6 Kb v-bas fragment (Harvey MSV) was detected only slightly at low stringency (lane c) and not at all at high stringency (lane f). Under both hybridization conditions, a reaction was observed with fragments derived from p-mos8 (Moloney MSV) (lanes a and d). This reaction is almost certainly due to the known presence of residual murine leukemia virus sequences in the recombinant which would be detected by MLV in the heterogeneous cDNA probe.

It has been established that early in the replication cycle of retroviruses (14-20 hours post-infection), a portion of linear duplex DNA, synthesized from the viral genomic RNA template by reverse transcription, is transported from the cytoplasm to the nucleus where it is converted to a closed circular DNA intermediate (Gianni et al., 1975; Shank and Varmus, 1978). Substantial evidence points towards this DNA intermediate being the immediate precursor to the integrated proviral DNA, although this function has not been ruled out for the linear DNA molecule. Furthermore, covalently closed circular DNA prepared from infected cells has been found to be infectious (Sutkin et al., 1975; Guntaka et al., 1976; Goldfarb and Weinberg, 1981). I purified closed circular viral DNA from NIH 3T3 fibroblasts infected with the cell-free

Figure 9. Demonstration of homology between MoMLV-rescued transforming agent from Clone H and mammalian v-onc sequences. (a,d) p-mos8 digested with PstI; (b,e) pHiHi-3 (v-ras^K) digested with EcoRI; (c,f) p-bas (v-ras^H) digested with BamHI. One-half microgram of each plasmid DNA was electrophoresed through a 1.0% agarose gel at 25 volts for 18 hours and transferred to nitrocellulose. Duplicate filters were hybridized to 5×10^6 cpm of ³²P-labeled cDNA probe (prepared as described in text) under conditions of low (Panel A) and high (Panel B) stringency, and exposed for autoradiography for 13 hours.

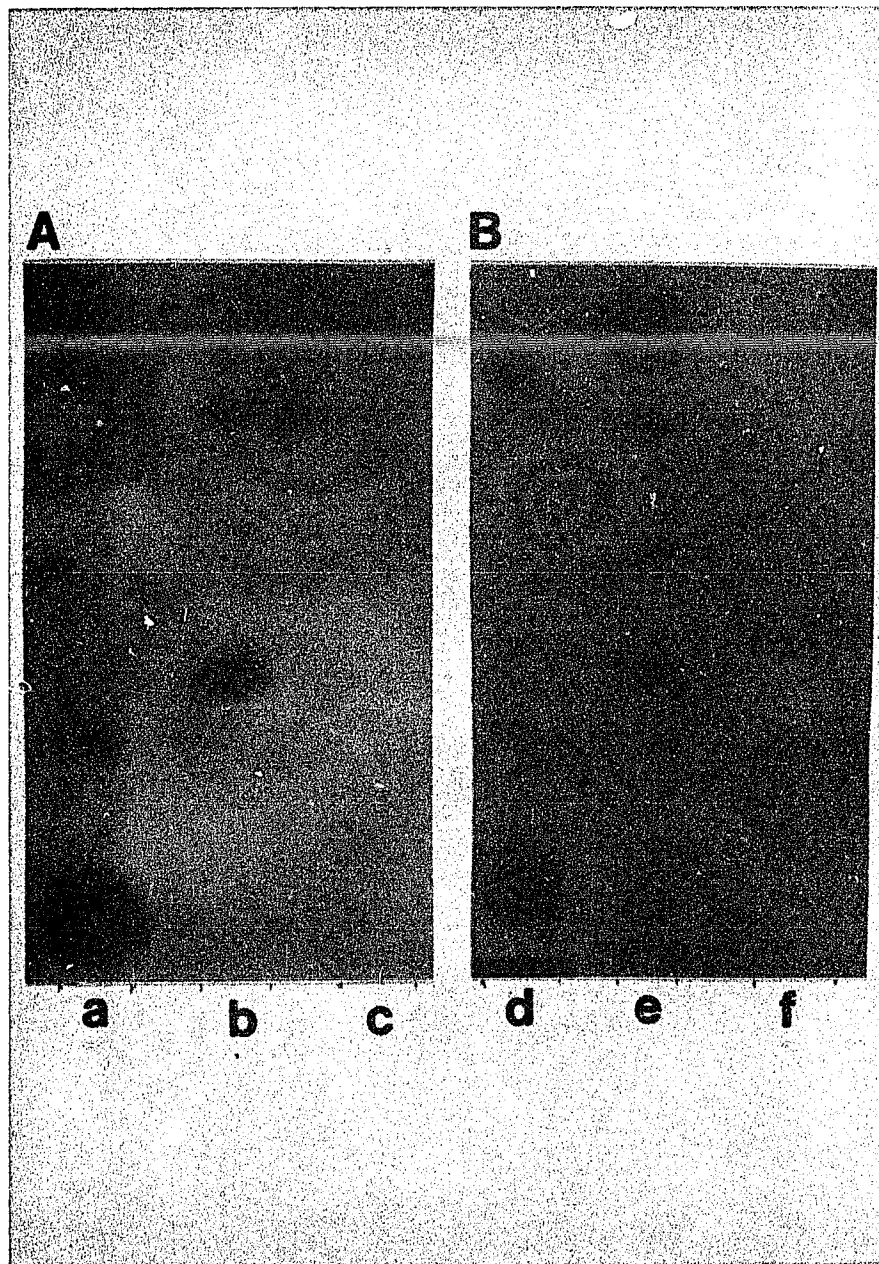


Figure 9

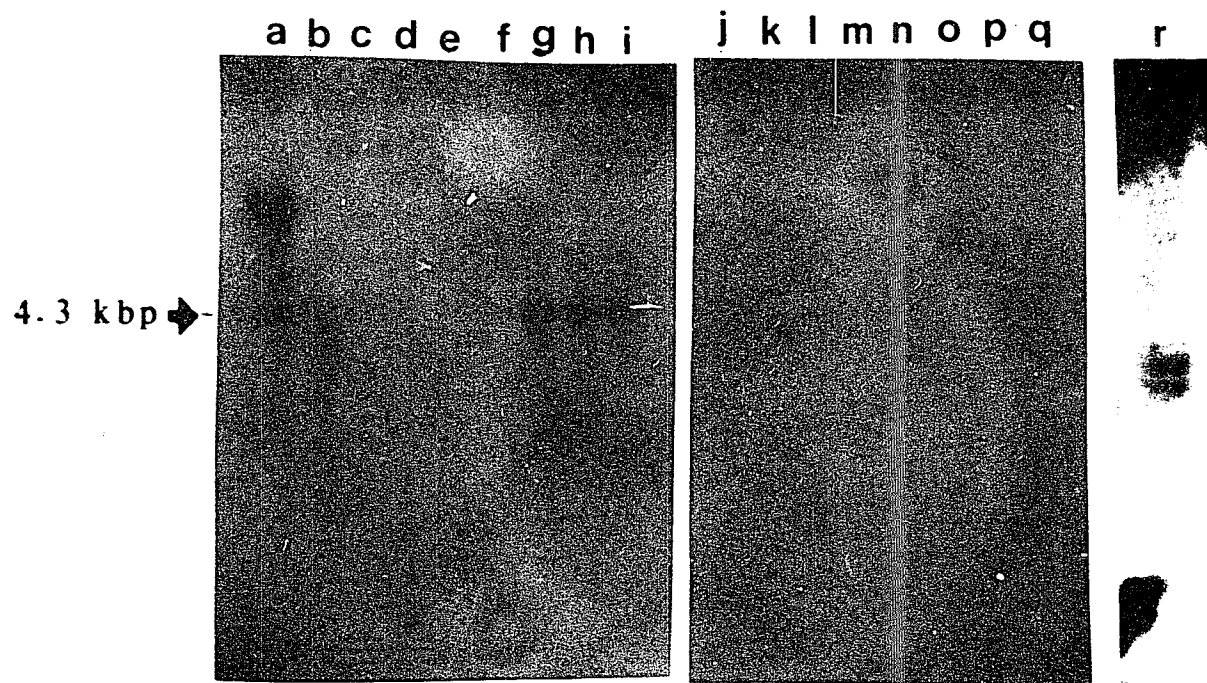
medium from Mo-MLV-infected Clone H cells following the protocol described by Tsuchida et al. (1981). Ten 75 cm² tissue culture flasks containing Clone H cells chronically infected with Mo-MLV were each refed with 10 ml of complete medium; three hours later, the medium was harvested and cleared of contaminating cells by filtration through a 0.22 µm Millipore membrane filter. In order to facilitate adsorption of viral particles, polybrene (hexadimethrine bromide) was added to a final concentration of 2 µg/ml. The medium from one hundred subconfluent cultures of NIH 3T3 fibroblasts in 100 mm Petri dishes was aspirated, and 1 ml of the cell-free polybrene-containing supernatant was added to each dish. The infection was performed at 37°C for 3 1/2 hours with intermittent rocking, followed by the addition of complete medium. Sixteen hours later, low molecular weight DNA was extracted from infected cells by the method of Hirt (1967) (see Materials and Methods). Closed circular viral DNA was purified by equilibrium centrifugation in a CsCl-ethidium bromide density gradient (Ridloff et al., 1967; Giani et al., 1975) as described previously for isolation of closed circular plasmid DNA. One-half ml fractions were collected from the gradient and extracted with isopropanol to remove the ethidium bromide. In order to identify which fractions contained closed circular DNA homologous to Ki-MSV, 50 µl samples were taken from each fraction, diluted with 200 µl of deionized water containing 10 µg of yeast tRNA (as carrier) and ethanol precipitated. DNA's in each fraction, recovered by centrifugation in a microcentrifuge, were resuspended in 25 µl of TE and electrophoresed in 1% agarose gels at 25 volts for 18 hours. The DNA in the gels was transferred to nitrocellulose paper and hybridized to a ³²P-labeled Kirsten ras-specific probe (HiHi-3) as previously de-

scribed. After washing, filters were exposed to x-ray film overnight. Figure 10 is the autoradiograph obtained from this experiment. A band with electrophoretic mobility consistent with linear markers of approximately 4.3 Kb was detected in fractions 6, 7 and 8. Such a mobility is in close agreement with the apparent size of closed circular Ki-MSV DNA (7 Kbp) as reported by Tsuchida and Uesugi (1981). These fractions were pooled and dialyzed in TE to remove CsCl. Subsequent agarose electrophoresis has resolved this single band into a doublet (Figure 10, lane r). Indeed, two forms of closed circular viral DNA, containing either one or two long terminal repeats (LTR's) are commonly observed (Shank et al., 1978; Yoshimura and Weinberg, 1979). One half of the DNA from the pooled fractions, presumed to be closed circular viral DNA by the above criteria, was tested for its ability to transform the normal rat embryo fibroblast cell line, Rat4 (Topp, 1981) by calcium phosphate-mediated transfection. Unlike NIH 3T3, these cells maintain a reasonably flat morphology upon prolonged culture, and are suitable for assay of purified transforming vectors by focus formation in monolayer. Transfections were performed as described previously, with the exception that cultures were exposed to the DNA-calcium phosphate precipitate for only four hours. Two weeks post-transfection, transformed foci were observed in cultures transfected with the viral-DNA (5 foci per 5×10^5 cells transfected); no foci were detected in control cultures. This transformation frequency is consistent with that expected for nanogram quantities of closed circular viral DNA.

The experiments discussed so far provide strong evidence that Clone H harbors a retrovirus-like sequence with homology to the oncogene of

Figure 10. Demonstration of covalently-closed circular DNA intermediates homologous to ras^K in MLV-infected Clone H. Closed circular proviral DNA was purified from total low molecular weight DNA by equilibrium centrifugation as described in text. One-tenth aliquots of each gradient fraction were electrophoresed through 1% agarose gels at 25 volts for 18 hours. The DNA in the gels was transferred to nitrocellulose, hybridized to 5×10^6 cpm of pHiHi-3 (ras^K) probe, and exposed for autoradiography for 16 hours. Lane (a) is HindIII digested λ DNA (as a size marker). Lanes b through q correspond to gradient fractions 1 to 16. Lane r shows the resolution of the single ras^K-homologous band in fractions 6, 7 and 8 into a doublet after electrophoresis of a sample of the pooled fractions in a 0.7% agarose gel at 25 volts for 24 hours (followed by Southern analysis as above).

Figure 10

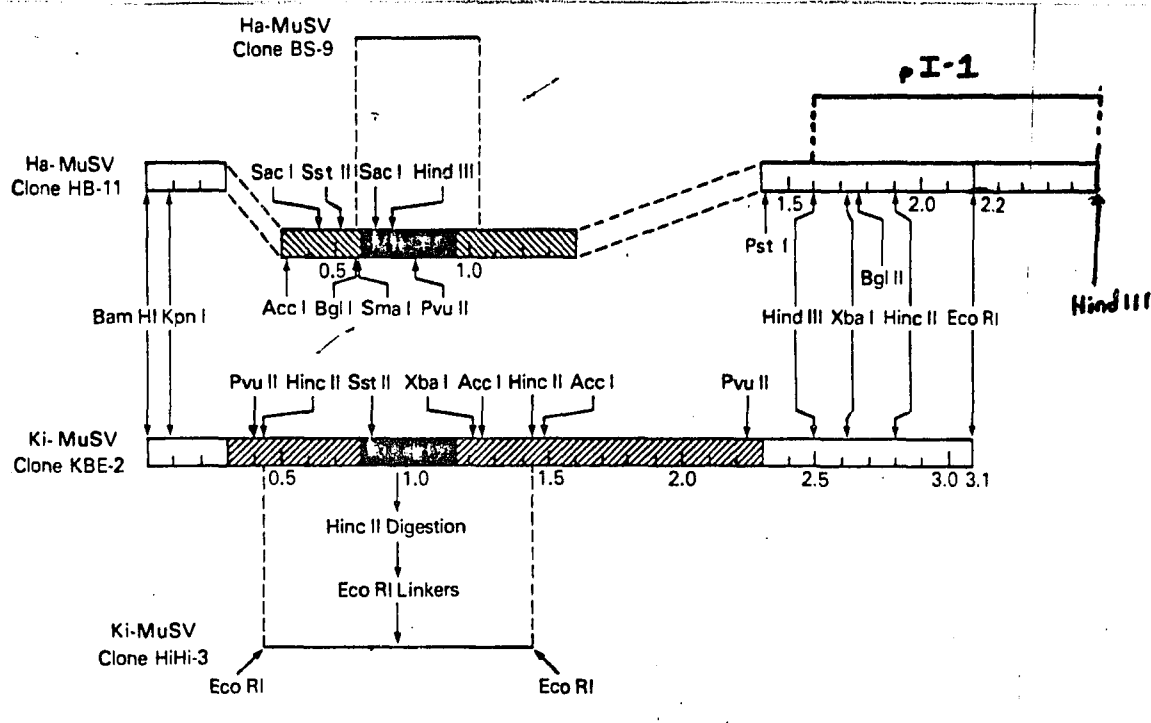


the Kirsten strain of murine sarcoma virus (Ki-MSV), and that these sequences are responsible for the transformed phenotype of NIH 3T3 fibroblasts transfected with Clone H DNA. Furthermore, this cell line produces RNA transcripts which are capable of being packaged by helper murine leukemia virus, resulting in sarcomagenic virus. However, these experiments are not in themselves definitive as to the precise nature of the retrovirus-like sequence. Several possibilities were considered. Endogenous type-C retroviruses (Temin, 1972; Pincus, 1980; Weinberg, 1980) are a part of the normal genetic make-up of many vertebrate species (Todaro, 1977); in mice, it has been estimated that as much as 0.05% of the total genome consists of endogenous virus-related information (Callahan and Todaro, 1978). Numerous studies have demonstrated the induction of endogenous proviruses by treatment with various chemical agents, including iododeoxyuridine and bromodeoxyuridine (Lowy et al., 1971; Rapp and Todaro, 1980) and 5-methylcholanthrene (Weiss et al., 1971). It is conceivable that the initial exposure of Balb/c 3T3 cells to the carcinogenic agent nitrosocarbaryl resulted in the activation of an endogenous retroviral sequence. Alternatively, the normal cellular homolog of Ki-MSV might have been so activated, although it would be unlikely that its RNA transcripts would be rescuable by infection with MLV, as we observed. A third possibility is that Clone H was derived by inadvertent transformation of Balb/c 3T3 by exogenous Ki-MSV.

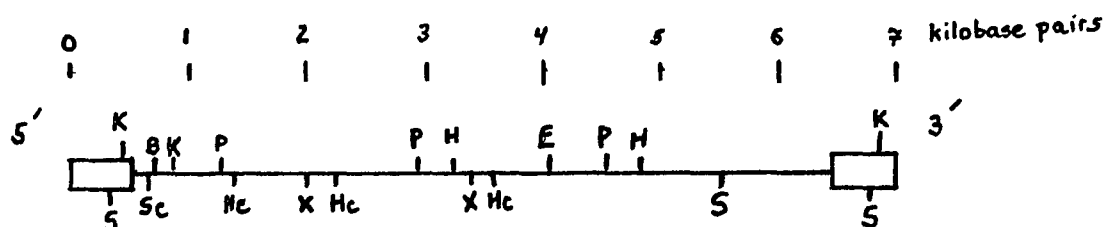
In order to distinguish among these possibilities, I took advantage of the considerable knowledge which has been gathered from studies of the evolution of the murine sarcoma viruses. The Harvey and Kirsten strains of MSV were isolated from independent rat sarcomas induced by

different strains of MLV which had been passaged through rats (Harvey, 1964; Kirsten and Mayer, 1967). The closed circular DNA intermediates of both strains have been molecularly cloned (Hager et al., 1979; Tsuchida and Uesugi, 1981) and their respective genomic restriction maps are shown in Figure 11. Approximately 1.2 Kb of DNA sequence at the 5' and 3' ends of the genomes are shared in common by both Ha-MSV and Ki-MSV and are derived from the long terminal repeats (Temin, 1982; Varmus, 1982) of mouse leukemia helper virus. 1.95 Kb (for Ki-MSV) and 1.02 Kb (for Ha-MSV) of DNA near the 5' end of the genomes arose from rat cellular DNA sequences unique to either strain; these contain the ras gene coding for the transforming protein p21 (Shih et al., 1979). These rat-derived sequences are flanked on either side by a total of 4.5 Kb (for Ki-MSV) and 3.5 Kb (for Ha-MSV) of DNA derived from replication-defective, non-transforming, retrovirus-like sequences which are highly reiterated in rat DNA and which express themselves in some rat cells as "30S" RNA (Tsuchida et al., 1974; Scolnick et al., 1976, 1979). A portion of the 30S-related sequence of Ha-MSV was isolated as a 1.6 Kb HindIII fragment from a λ clone of the entire Ha-MSV genome (Ellis et al., 1980); this fragment, subcloned in pBR322, was designated pI-1 and was kindly provided for these studies by Dr. Ronald Ellis of the National Cancer Institute. When used as a probe for hybridization to Southern blots of mouse DNA, little or no homology to mouse endogenous retroviral sequences was observed (Ellis et al., 1980). We reasoned that if the Kirsten-related sequences in Clone H arose from infection by typical exogenous Ki-MSV, they would be readily detectable by hybridization of restricted cellular DNA fragments to a radioactively-labeled rat 3 S (virus-specific) probe.

Figure 11. Detailed structure of Harvey and Kirsten ras genes indicating derivation of molecular probes (Adapted from Ellis et al., 1981).



Open box represents rat 30S RNA sequences; solid box represents *v-ras* sequences shared by Ha-MSV and Ki-MSV; hatched boxes represent *v-ras* sequences unique to either Ha-MSV (left-hatched) or Ki-MSV (right-hatched).



Restriction map of linear proviral Ki-MSV DNA. Letters refer to restriction endonuclease sites: EcoRI(E), KpnI(K), SmaI(S), SstI(Sc), BamHI(B), HincII(Hc), PvuII(P), XbaI(X), and HindIII(H).

Figure 11

High molecular weight (HMW) DNA's from Clone H and primary transformant JC1 (generated by transfection of NIH 3T3 with HMW Clone H DNA) were digested individually with several different restriction enzymes, fractionated on 0.7% agarose gels, and transferred to nitrocellulose paper. As controls, identical analyses were performed with DNA from A31/DMBA 435 and SA-1, a Ki-MSV-transformed 3T3 cell line provided by Dr. Stuart Aaronson (NIH). The Southern blots were hybridized under stringent conditions to a ^{32}P -labeled Kirsten ras-specific probe, HiHi-3 (see Figure 11). After exposure to x-ray film for 48 hours, the filters were washed at 65°C in 50% formamide, 5X SSPE for one hour to dehybridize the Ki-ras probe. After overnight exposure to x-ray film to ensure removal of the old signal, the filters were rehybridized to a ^{32}P -labeled rat 30S probe (I-1) and subjected to autoradiography for 48 hours. The autoradiographs of such an experiment are shown in Figure 12. Panel A represents DNA's digested with EcoRI; DNA's in panel B were digested with KpnI. Lanes a-d are hybridized to HiHi-3; lanes e-h are hybridized to I-1. Comparison of lanes b and c with lanes f and g clearly show that there are sequences present in Clone H and primary transformant JC1 which hybridize to both the ras and 30S probes; this is also seen with SA-1 (lanes a and e). The number and size of the fragments observed are entirely consistent with those predicted from the restriction map of exogenous Kirsten MSV (see Figure 11). DNA from A31/DMBA 435 shows positive hybridization with the ras probe (lane d) but not with the 30S probe (lane h); the 9 and 4.2 Kbp fragments detected represent portions of the normal cellular Kirsten-homologous sequence (c-Ki- ras). Amplification (perhaps 5 to 10 fold) appears to have taken place in the sequence detected by both ras and 30S probes in

Figure 12. Southern blot analysis of cellular DNA's from (a) Kirsten MSV/3T3 (S A-1), (b) Clone H, (c) primary transformant JC1 (generated by transfection of NIH 3T3 with high molecular weight Clone H DNA, (d) A31/DMBA 435. A and B are EcoRI and KpnI digests, respectively, of these DNA's fractionated by electrophoresis through a 0.7% agarose gel and transferred to nitrocellulose paper. The filters were incubated with 5×10^6 c.p.m. of nick-translated ^{32}P -labeled v-Kirsten-ras probe (pHiHi-3) and exposed for autoradiography for 48 hours (lanes a-d). The filters were dehybridized by washing at 65°C in 50% formamide and 5X SSPE, incubated with 5×10^6 c.p.m. of nick-translated ^{32}P -labeled rat 30S DNA probe (I-1), and exposed to autoradiography for 48 hours (lanes e-h).

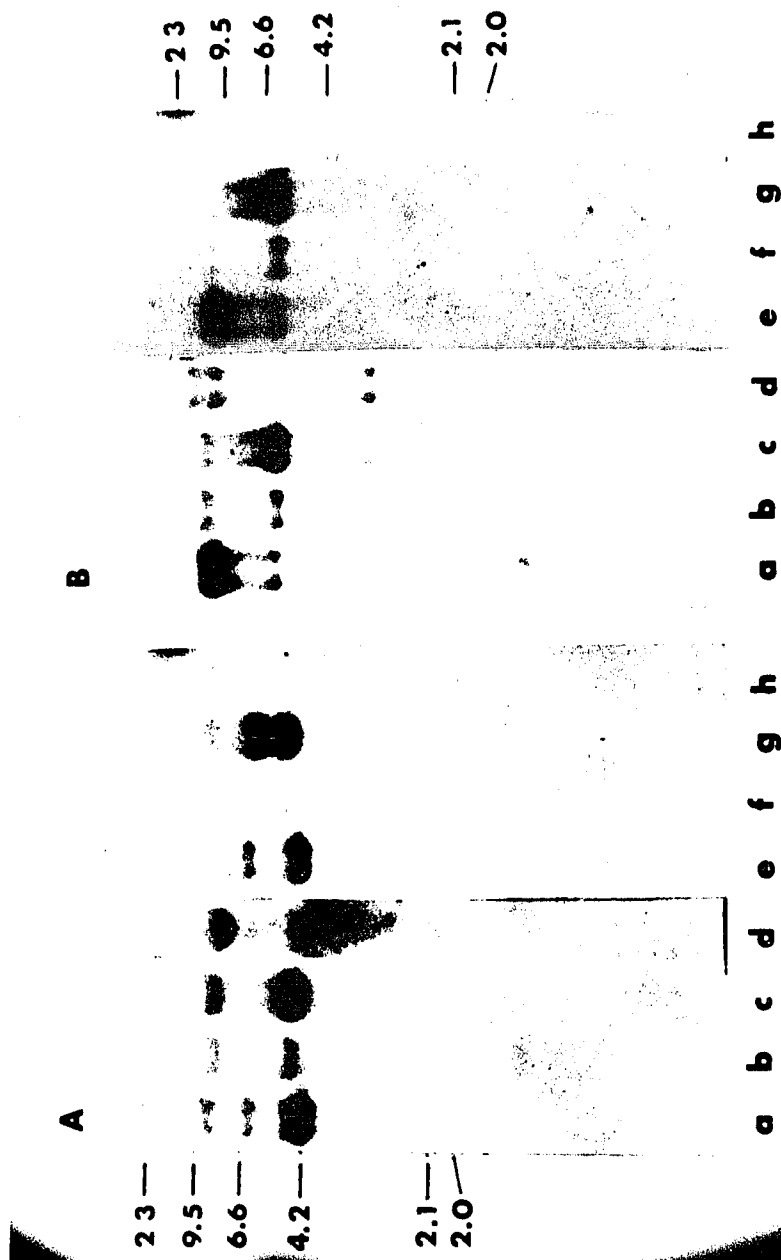


Figure 12

primary transformant JC1 (lanes c and g). Figure 13 is an autoradiograph of the Southern blots of JC1 DNA digested with HindIII and hybridized to ^{32}P -labeled HiHi-3 and I-1 (Panel A), and JC1 DNA digested with KpnI, EcoRI, and SstI and hybridized to the I-1 probe (Panel B). Examination of panel A shows that the HiHi-3 (ras) probe detected a novel 6 Kbp fragment in HindIII-digested DNA (lane a), while the I-1 (30S) probe detected another fragment approximately 1.6 Kbp in size (lane b). These are again as predicted by the restriction map of Ki-MSV (see Figure 11). Panel B indicates that digestion with SstI (lane c), which from earlier restriction mapping (Tsuchida et al., 1981) was believed to be a non-cutter of the genome, yields a single fragment estimated to be 9 Kbp in size (see below).

While the evidence I have presented strongly implicates an exogenous Ki-MSV genome as the transforming agent identified in Clone H, it fails to resolve certain discrepancies between the observed pattern of restriction enzyme sensitivity of Clone H DNA and that reported by Tsuchida and Uesugi (1981) for the cloned circular DNA intermediate of Ki-MSV (see Figure 11). The Ki-MSV genome has a single BamHI site between the 5' long terminal repeat (containing the retroviral promoter sequence) and the ras gene; digestion with BamHI would be expected to abolish activity of the transforming gene. Although we observed some reduction in transforming efficiency (Table 12), more might have been expected. EcoRI and HindIII both cut on the 3' side of the ras gene in Ki-MSV and have little or no effect on the transforming activity of cloned proviral DNA, yet our analysis has shown inactivation of Clone H DNA upon digestion with either enzyme (Table 12). It is possible to rationalize these discrepancies by proposing that Clone H har-

Figure 13. Southern blot analysis of cellular DNA from primary NIH 3T3 transformant JCl. Panel A: ten micrograms of JCl DNA was digested with HindIII and electrophoresed in a 0.7% agarose gel at 25 volts for 20 hours. DNA was transferred to nitrocellulose, hybridized to 5×10^6 cpm of ^{32}P -labeled pHiHi-3 and exposed for autoradiography for 48 hours (lane a). The filter was dehybridized (as described in Figure 13 legend) and rehybridized to ^{32}P -labeled I-1 (lane b). Panel B: ten micrograms of JCl DNA was digested with (a) KpnI, (b) EcoRI, (c) SstI, and subjected to Southern analysis as above using ^{32}P -labeled I-1 as a probe.

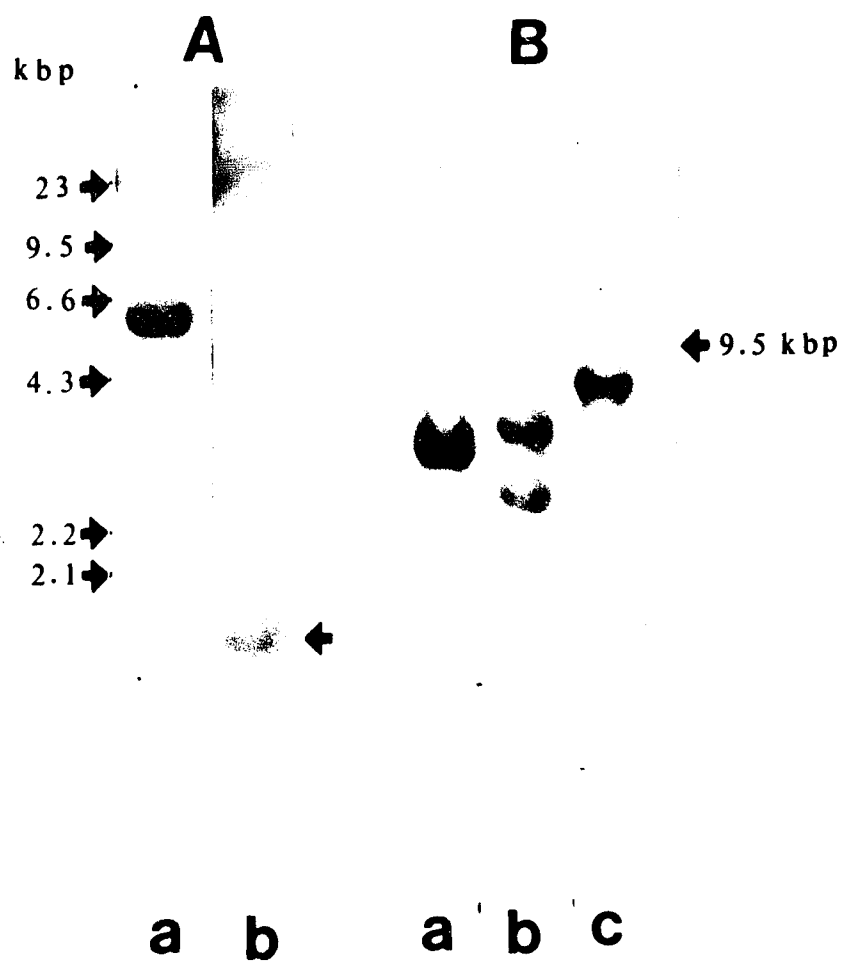


Figure 13

bors a variant of Ki-MSV. It has been reported, at least in avian systems, that evolutionary variants of retroviruses often arise through recombinational events (Weiss et al., 1973). Loss or acquisition of a restriction endonuclease recognition site by polymorphism (as has been occasionally observed by many investigators) might also account for variance in restriction enzyme sensitivity. In an effort to determine if we were dealing with a MSV variant, I attempted to isolate the Ki-MSV genome by molecular cloning techniques.

I elected to isolate the integrated Ki-MSV genome from the primary transformant JC1 which, as previously discussed, shows a modest degree of amplification of the retroviral sequence (Figure 12, lanes c and g); this amplification might enhance the likelihood of detection of the cloned fragment. The choice of a suitable restriction enzyme for cloning was limited by the fact that the most commonly used cloning enzymes (i.e. EcoRI, BamHI, HindIII) all cut at least once within the Ki-MSV genome (Figure 11). Southern analysis revealed that upon digestion of JC1 DNA with SstI, a single fragment of approximately 9 Kbp in length was detected by the I-1 (30S) probe (Figure 13). In addition, no SstI site was reported in the published restriction maps of the cloned circular DNA intermediate of Ki-MSV at the time this effort was begun [a recent report by Tsuchida et al. (1982) indicates a single SstI site near the 5' end of their isolate]. Selection of a suitable cloning vector was another consideration. No plasmid cloning vehicle with a SstI site was available. A λ phage vector, λ gt WES λ B (Leder et al., 1977), modified to accept large (5-17 Kbp) SstI-generated substitution fragments, was reported by Stringer (1981). This vector, designated " λ Sst17", was kindly provided by Dr. James Stringer (Cold Spring

Harbor). Fragments of SstI-digested JC1 DNA were enriched by size fractionation through sedimentation in a 10-40% neutral sucrose gradient by the method of Maniatis et al. (1978) for those fractions containing the Ki-MSV genome. The phage arms were isolated from SstI-digested λ Sst17 by methods described by Maniatis et al. (1982) and ligated to DNA from the Ki-MSV-enriched fraction. The resulting concatomeric DNA's were packaged in vitro into viable bacteriophage particles (Hohn, 1979; Enquist and Sternberg, 1979). An extract prepared from a complementary mixture of two induced E. coli strains lysogenic for lambda amber mutants in the D and E capsid genes, contains all of the phage proteins necessary for morphogenesis of empty phage capsids and encapsidation of linear λ DNA ranging in size from 38 to 52 Kbp (78 to 102% of the length of wild-type λ). Packaging efficiencies of 10^7 to 10^8 plaque forming units (pfu) per μ g of input wild-type λ DNA are commonly obtained by this method. Plaques arising from infection of appropriate host bacteria with recombinant phage particles were screened by hybridization of replica filters (Benton and Davis, 1977) to a ^{32} P-labeled ras probe for those carrying the Ki-MSV sequence. (For the sake of brevity, experimental procedures are detailed in Materials and Methods.)

Approximately 150,000 recombinant plaques were initially screened by replica filter hybridization. Upon autoradiography of thirty replica filters, I detected 87 ras-positive hybridization spots. However, the intensity of these spots varied greatly, suggesting that some might be due to non-specific binding of probe DNA to the filters. Furthermore, as each filter contained nearly 5000 plaques, it was impossible to realign the x-ray film to the dishes so as to pick individual plaques.

Instead, the area around each positive spot (containing perhaps 10-20 plaques) was picked and resuspended in phage dilution buffer. A second round of screening was performed using aliquots of the primary phage suspensions (some of which were pooled to reduce the number of secondary screenings) to form plaques on host bacterial lawns. Forty-eight replica filters, each containing several hundred plaques, were hybridized to the ^{32}P -labeled Ki-ras probe. Of these 48 filters, only one showed enrichment of hybridization spots on the autoradiograph (Figure 14); each spot on the x-ray film coincided with a unique plaque on the corresponding bacterial lawn. Eight independent plaques which aligned with positive hybridization spots, and two plaques which were negative, were picked and individually amplified by confluent lysis of host bacterial lawns. Phage DNA was prepared from each plate lysate, digested with SstI, and electrophoresed in a 1% agarose gel. DNA was transferred to a nitrocellulose filter, which was hybridized to the Ki-ras specific probe. The autoradiograph from this experiment is shown in Figure 15. I detected a 15 Kbp fragment common to the eight "positive" plaque isolates, as would be expected if they originated from a single primary recombinant phage. The probe failed to hybridize to either of the two "negative" plaque isolates, indicating that it was specific for the cloned insert.

The size of the cloned insert (15 Kbp) was considerably larger than the SstI fragment originally detected in JC1 DNA (9 Kbp), suggesting that I might have isolated another DNA sequence with homology to Ki-ras. Phage DNA prepared from one of the "positive" λ recombinants (λ Sst/Ki-ras) was cleaved with SstI (to separate the cloned insert from phage arms) and subjected to single and double digestion with several restric-

Figure 14. Secondary screening of partial λ SstI/Clone H phage library for ras^K-homologous recombinants. Plaque lifts, hybridization, and autoradiography were performed as described in text. (A) filter with no ras^K-positive spots; (B) filter with several ras^K-positive spots (each corresponding to a unique phage plaque).



Figure 14

Figure 15. Southern blot analysis of the cloned insert of the ras^K-positive λ SstI/Clone H recombinants. Analytical quantities of phage DNA were prepared from several phage plaque isolates as described in the text. Each DNA sample was digested with SstI, electrophoresed in a 1.0% agarose gel, and transferred to nitrocellulose. The filter was hybridized to ³²p-labeled KBE-2 (ras^K) and exposed for autoradiography for 8 hours. (a) HindIII-digested λ DNA; (b-i) DNA's from eight ras^K-positive plaques; (j,k) DNA's from two randomly selected ras^K-negative plaques.

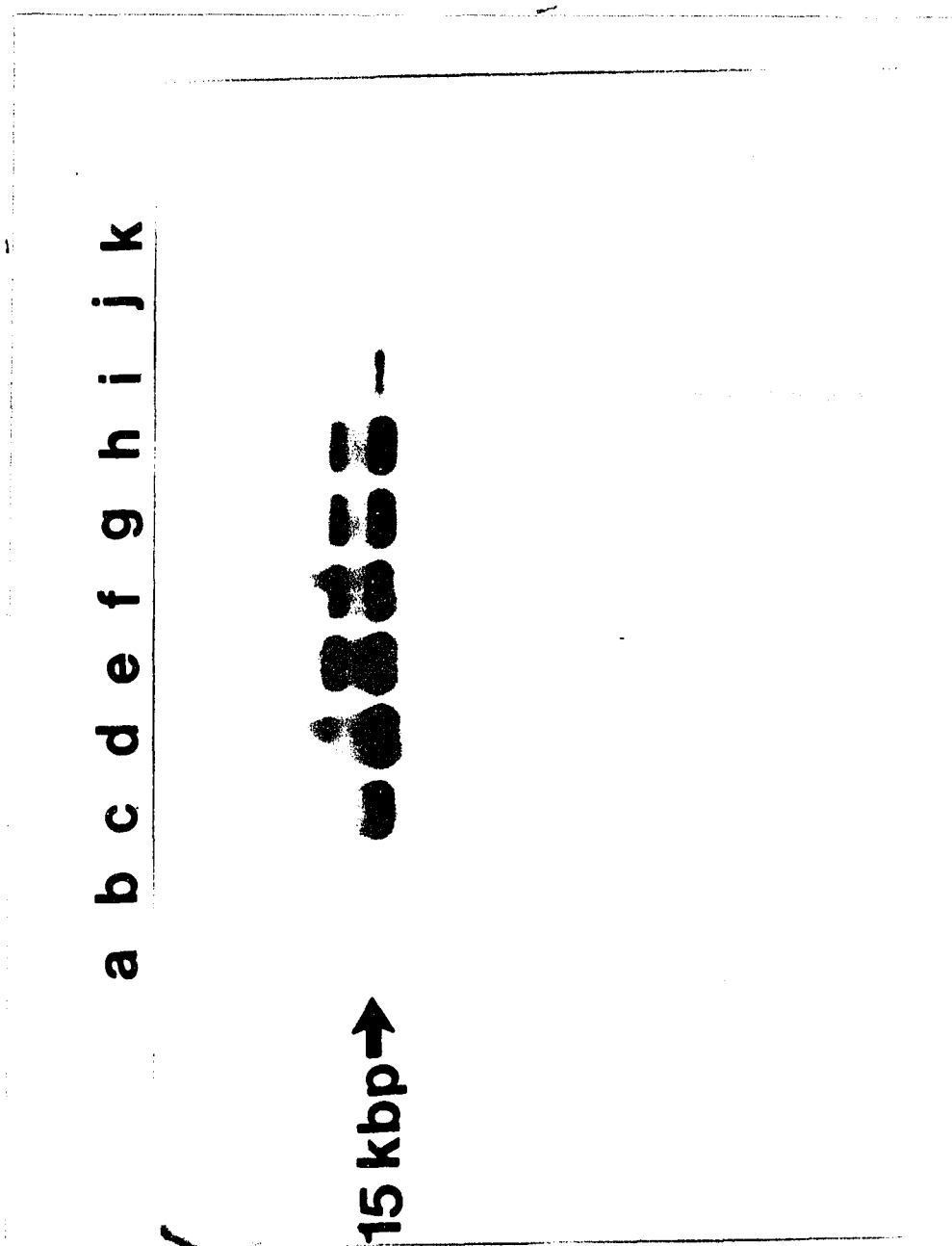


Figure 15

tion endonucleases. Restricted DNA's were electrophoresed in 0.7% agarose gels and transferred to nitrocellulose paper. These filters were hybridized to either a ^{32}P -labeled Ki-ras-specific probe (KBE-2) or a ^{32}P -labeled rat 30S-specific probe (I-1) and exposed to x-ray film. As shown in the autoradiographs in Figure 16, the Ki-ras probe hybridized to restriction fragments in each of the digests; none of these fragments were detected by the rat 30S probe. Through subsequent analysis of the partial phage library by dot blot hybridization (Kafatos et al., 1979), I was unable to detect any other recombinant phage isolates with cloned rat 30S-specific sequences. Thus it would appear that despite having taken steps to enrich for a restriction fragment containing the integrated, exogenous Ki-MSV genome, I isolated instead a fragment of the endogenous cellular Ki-ras gene (see Discussion). DNA from λ Sst/Ki-ras, either undigested or digested with SstI, was tested for its ability to transform NIH 3T3 and Rat4 fibroblasts as assayed by colony formation in soft agarose and focus formation in monolayer, respectively. I failed to detect any transformation activity of the DNA in either recipient cell line.

Figure 16. Restriction enzyme analysis of the λ Sst/ras^K recombinant.

Phage DNA was restricted with SstI to excise the cloned insert, and then subjected to single and double restriction endonuclease digestion with a variety of enzymes. DNA's were electrophoresed in a 0.7% agarose gel and transferred to nitrocellulose. Duplicate filters were hybridized to A) KBE-2 (ras^K) probe, B) I-1 (rat 30S) probe, and C) λ SstI/ras^K probe, and exposed for autoradiography for 8 hours. (a) HindIII-digested λ DNA; (b) BRL 123 base pair ladder; (c) λ SstI/ras^K DNA undigested; and λ SstI/ras^K DNA digested with (d) EcoRI; (e) EcoRI/BamHI; (f) BamHI; (g) EcoRI/HindIII; (h) HindIII; (i) EcoRI/ KpnI; (j) KpnI; (k) XhoI; (l) XbaI.

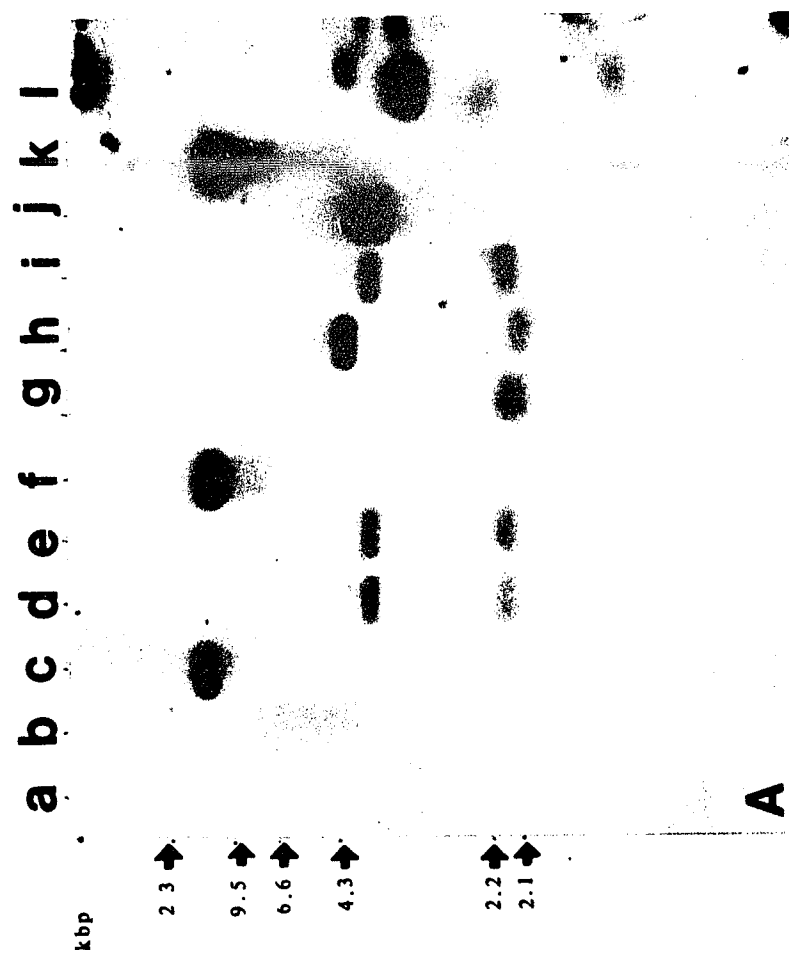


Figure 16a

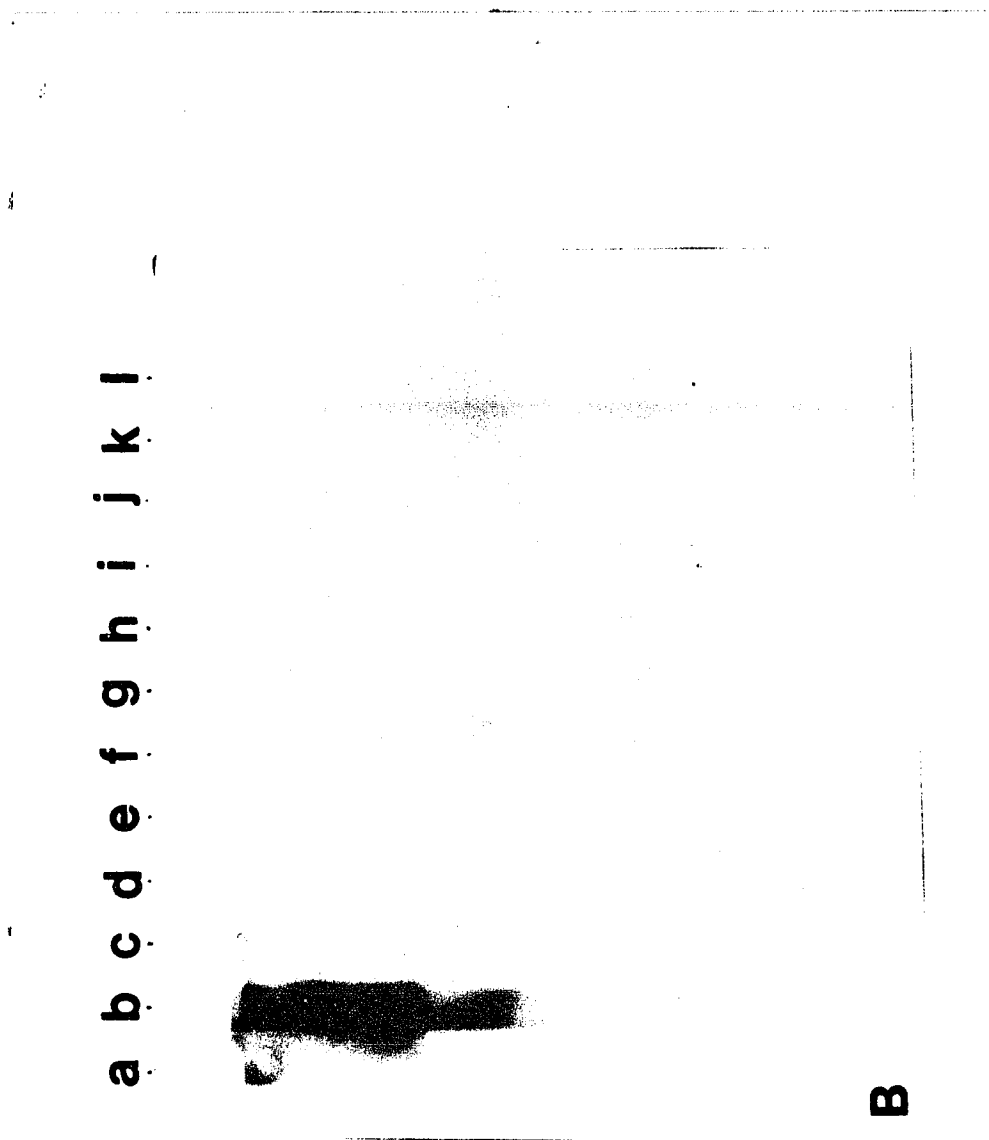


Figure 16b

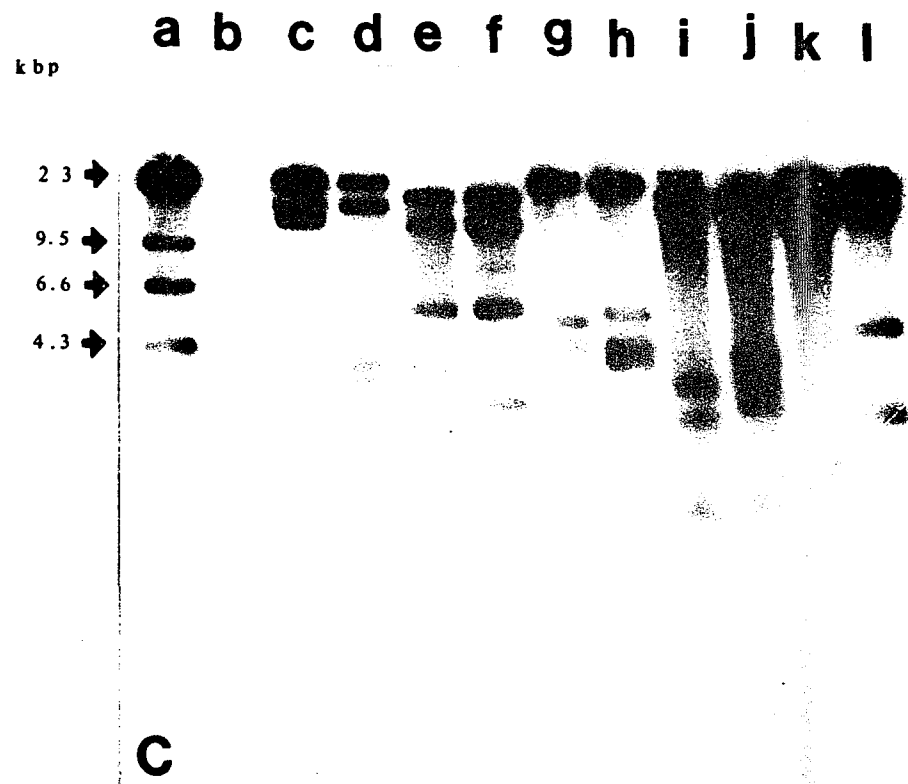


Figure 16c

DISCUSSION

DNA-mediated gene transfer has been shown to be effective in passing a variety of phenotypic markers from one cell line to another. This method was extended in these studies to the characterization of the malignant phenotype in transformed mouse cells isolated in vitro following exposure to chemical carcinogens. As described in Chapter II, high molecular weight DNA isolated from a number of independent chemically-transformed mouse cell lines was applied as a calcium phosphate co-precipitate to cultures of the untransformed mouse fibroblast cell line, NIH 3T3. DNA from some, but not all, of these donor cell lines was found to be reproducibly capable of inducing anchorage independence in NIH 3T3, a criterion of in vitro transformation well correlated with in vivo tumorigenicity. This observation confirmed our expectation, based on the analysis of somatic cell hybrids, that the genomes of at least some chemically-transformed mouse cells contain activated transforming genes which might be expressed in a dominant fashion upon re-introduction into an appropriate genetic background. My results were both supportive of and supported by the findings of Shih et al. (1979) and Cooper et al. (1980), published soon after I had made these initial observations. It should be mentioned that when these studies were first undertaken, the central role that cellular proto-oncogenes (identified through their nucleic acid sequence homology to various retroviral transforming genes) might play in carcinogenesis had yet to be appreciated. Only in the past three years, as a consequence of intensive investigation by a number of researchers, has their significance been fully realized.

Particular emphasis was placed on experiments utilizing DNA from Clone H, a transformed mouse fibroblast cell line isolated by Quarles and Tennant (1975) after treatment of Balb/c 3T3 cells with nitrosocarbaryl. Clone H was a more efficient donor in the NIH 3T3 transfection system than any other chemically -transformed mouse cell line so tested. Proof that the phenomenon I observed was mediated by transfer of specific DNA sequences, and not due to an experimental artifact, came primarily from two types of evidence. (1) Primary transformants of NIH 3T3, generated by transfection with Clone H DNA mixed with the bacterial plasmid pBR322, had integrated plasmid sequences in their DNA, as demonstrated by Southern analysis. This was a consequence of the co-transfer of a non-selected marker. (2) The transforming activity of Clone H DNA was found to be sensitive to digestion with a number of site-specific restriction endonucleases. The pattern of sensitivity and resistance to inactivation by endonuclease digestion observed for Clone H DNA was similar to that reported by Shilo and Weinberg (1981a), suggesting that a common transforming gene was activated in their 3-methylcholanthrene mouse transformants and Clone H.

As a prelude to further characterization, I sought to verify that Clone H was free of transmissible type-C retrovirus transforming genomes. Unexpectedly, I was able to rescue a murine sarcoma virus-like transforming agent from Clone H and primary NIH 3T3 transformants upon infection with nontransforming Moloney murine leukemia virus. Since Weinberg and co-workers failed to detect sarcomagenic virus in their 3-methylcholanthrene transformants, it was apparent that, despite the similarity in restriction endonuclease sensitivity, a different transforming agent might be responsible in Clone H. Because of this possible

paradox, additional characterization of Clone H was warranted. Furthermore, a preliminary analysis performed by Dr. Dino Dina (not discussed previously) indicated that Clone H had RNA transcripts with homology to the transforming gene (mos) of Moloney-murine sarcoma virus (MoMSV). Since the restriction endonuclease sensitivity profile we observed for Clone H DNA did not correlate with that expected for v-mos (based on the known restriction map of MoMSV), the possibility occurred to us that the cellular homolog of v-mos (c-mos) might have been activated in Clone H and that its RNA transcripts were capable of being packaged by helper leukemia virus particles. However, Dr. Dina was unable to reproduce his initial finding. Furthermore, by Southern analysis of DNA's from several primary NIH 3T3 transformants, I found no evidence for additional mos-related sequences acquired by transfection. Therefore, we concluded that Dr. Dina's observation was caused by an unexplained artifact.

Subsequent experiments (described in Chapter III) led to the identification of the transforming agent in Clone H as being an exogenous Kirsten murine sarcoma virus (KiMSV) genome. It is likely that prior to our obtaining Clone H from Quarles and Tennant, the cell line was accidentally contaminated either with KiMSV particles or with another cell line transformed by KiMSV. There still remain discrepancies between the restriction endonuclease sensitivity profile of Clone H DNA and that anticipated for KiMSV based on its known restriction endonuclease map. These differences can be explained by presuming that Clone H harbors a variant proviral genome, as the result of recombination or restriction recognition site polymorphism. However, Southern analysis of restriction fragments of Clone H DNA hybridized to a Kirsten onc-specific probe

failed to detect any dissimilarity between the integrated KiMSV genome in Clone H and the cloned isolate of Tsuchida et al. (1982). In an effort to resolve these discrepancies, I attempted to molecularly clone the integrated KiMSV genome (from a primary NIH 3T3 transformant which had amplified copies of the viral sequence) in a lambda phage vector. Despite manipulations which favored the cloning of the exogenous viral genome, I isolated instead an inactive fragment of the endogenous cellular Kirsten homolog. One can speculate that a cloned retroviral genome, by virtue of its inverted terminal repeat sequences, might undergo homologous recombination and excise from phage arms, leading to underrepresentation of the proviral DNA sequence in the recombinant phage library.

In retrospect, the results with Clone H underline the necessity for proper characterization of cell lines, particularly those of rodent origin, to be used as DNA donors for cellular oncogene transfer. In addition, they point out the potential hazard of relying too heavily on biological sensitivity to restriction endonuclease digestion as an indicator for gene identity. It is conceivable that inactivation of a gene might occur due to reduction in the optimal size of DNA fragments for gene transfer, rather than by cleavage within a functional region of the gene.

In parallel with the characterization of the transforming gene in Clone H, more limited studies were performed with another chemically-transformed mouse fibroblast cell line, A31/DMBA 435. This cell line was derived by Sen and Todaro (1978) by treatment of Balb/c 3T3 cells with dimethylbenzanthracene. As discussed in Chapter II, A31/DMBA 435 had qualities akin to Clone H (i.e. dominant expression in cell hybrids;

positive donor in the NIH 3T3 transfection assay). Most significantly, I was unable to rescue sarcomagenic virus from A31/DMBA by MLV infection. The restriction endonuclease inactivation pattern, though based on more limited trials than those performed with Clone H, again resembled the pattern reported by Shilo and Weinberg (1981a) for 3-methylcholanthrene mouse transformants. I isolated several primary NIH 3T3 transformants generated by transfection with BamHI-digested DNA from A31/DMBA 435. Attempts to facilitate identification of the transforming gene by ligation to a co-selectable biochemical marker (pSV2gpt), an approach considered at the time to be essential for subsequent isolation by molecular cloning, proved not to be fruitful.

During the course of these studies, several groups demonstrated that the transformed phenotype can be transferred to NIH 3T3 fibroblasts by transfection with DNA from a variety of human tumor cell lines (Krontiris and Cooper, 1981; Shih et al., 1981; Murray et al., 1981; Perucho et al., 1981; Lane et al., 1981, 1982; Marshall et al., 1982). Within the last two years, this approach has led to the molecular cloning of functional transforming genes from a human bladder carcinoma cell line (Goldfarb et al., 1982; Pulciani et al., 1982a; Shih and Weinberg, 1982), two different human lung carcinoma cell lines (Shimizu et al., 1983a; Yuasa et al., 1983), a human neuroblastoma cell line (Shimizu et al., 1983b), and a human T-cell leukemia cell line (Souyri and Fleissner, 1983). In-depth analysis has revealed that these cellular transforming genes bear homology to the oncogenes of a related pair of acute transforming retroviruses: Harvey murine sarcoma virus in the case of the T24/EJ bladder carcinoma gene (Parada et al., 1982; Santos et al., 1982; Der et al., 1982) and the Hs242 lung carcinoma gene (Yuasa

et al., 1983), and Kirsten murine sarcoma virus in the case of the Calu-1 lung carcinoma gene (Shimizu et al., 1983a) as well as the uncloned transforming gene of the SK-CO-1 colon carcinoma cell line (Shimizu et al., 1983c). In point of fact, these transforming genes are the altered cellular homologs of a family of viral oncogenes designated ras; they belong to a class of normal cellular genes whose coding sequences have been incorporated into the RNA genomes of various acute transforming retroviruses (as discussed previously in the Background section). The transforming genes isolated from the neuroblastoma and the T-cell leukemia cell line are identical and appear to be a new member of the ras family, N-ras, not detectible in any sarcoma virus (Shimizu et al., 1983c). The same gene is also activated in a human fibrosarcoma, rhabdomyosarcoma, and a promyelocytic leukemia cell line (Hall et al., 1983). All of the genes in the ras family encode a 21,000 molecular weight protein designated 'p21' whose amino acid sequence is highly conserved (Ellis et al., 1981). This protein, although its function is yet unknown, is constitutively expressed in normal cells of many vertebrate species (Langbeheim et al., 1980); it has been shown to specifically bind guanine-containing nucleotides and can catalyze the transfer of the γ -phosphate of GTP to a threonine residue on itself (Shih et al., 1980). p21 has been found in association with the inner aspect of the plasma membrane of cells transformed by HaMSV or KiMSV (Willingham et al., 1980).

These common findings indicate that the NIH 3T3 transfection assay is prone to the detection of activated ras genes, and suggested to us that the transforming gene in A31/DMBA 435 might be a member of the ras family. In order to determine if this was in fact the case, I analyzed

BamHI-digested DNA from several primary NIH 3T3 transformants (generated by DNA from A31/DMBA which had been digested with BamHI prior to transfection) by hybridization of Kirsten and Harvey ras-specific probes to Southern transfers. These particular transformants were selected based on the rationale that one or both of the BamHI recognition sites flanking the transforming gene might have been lost prior to integration, due to intracellular nuclease activity. It was anticipated that an altered pattern of BamHI-cleavage of the donor gene, relative to the resident homologous allele of NIH 3T3, would be detected. As described in Chapter II, I found a novel, high molecular weight restriction fragment with homology to Kirsten ras in one out of four primary transformants examined by this approach.

This observation is subject to other interpretations, however. For example, the extra fragment might have arisen through duplication of the endogenous Kirsten ras homolog of NIH 3T3, rather than by the integration of a donor transforming gene; it is not possible to rule out this explanation based on the present data. Alternatively, the extra fragment could represent an artifact caused by incomplete digestion of the DNA prior to electrophoresis, although I consider this less likely. I believe that my initial interpretation should be favored, however, based on evidence recently reported by several groups.

Parada and Weinberg (1983) detected amplified copies of DNA segments reactive with a Kirsten ras (ras^K) oncogene probe in NIH 3T3 transformants generated by transfection with DNA from 3-methylcholanthrene-induced in vitro transformants of the mouse fibroblast cell line, C3H/10T1/2. Taking advantage of a KpnI restriction site polymorphism in the ras^K gene of C3H/10T1/2, they were able to demonstrate that the

additional ras^K sequences were of transformed donor origin. They also observed increased levels of ras^K-homologous RNA transcripts and p21 protein in transfected cells as compared with their untransfected counterparts. Eva and Aaronson (1983) detected NIH 3T3 transforming activity in the DNA from two independent methylcholanthrene-induced mouse fibrosarcomas. The pattern of sensitivity to restriction endonuclease digestion they found for DNA from these tumors was similar to that reported by Shilo and Weinberg (1981a) for DNA from mouse cells transformed in vitro by 3-methylcholanthrene as well as to the pattern I observed for DNA from A31/DMBA 435. Eva and Aaronson, applying the same rationale as I, subjected DNA's from primary NIH 3T3 transformants to digestion with BamHI before using them in a second cycle of transfection. They detected, by Southern analysis, a novel restriction fragment in BamHI-cleaved DNA from a secondary NIH 3T3 transformant with homology to the ras^K oncogene. None of a number of other retroviral onc probes detected any additional DNA sequences in their transformants.

Balmain and Pragnell (1983) reported that transfection with high molecular weight DNA from transplanted mouse squamous cell carcinomas [induced by sequential treatment of mouse skin with an initiating agent, dimethylbenzanthracene (DMBA) and a tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA)] caused morphological transformation of NIH 3T3 fibroblasts. By Southern analysis, they were able to detect an additional copy of the cellular homolog of the Harvey ras oncogene (ras^H) in EcoRI-digested DNA's from primary NIH 3T3 transformants and secondary foci obtained after transfection with DNA from two different primary foci. Balmain and Pragnell verified that the additional copy of ras^H gene was of tumor origin by showing that a restriction site polymor-

phism, detectable in the ras^H gene of mouse carcinoma tissue but not in NIH 3T3 cells, was transferred to NIH 3T3 upon transfection with mouse carcinoma DNA. Sukumar et al. (1983) demonstrated that DNA's from mammary carcinomas induced by a single intravenous injection of N-nitroso-N-methylurea (NMU) into Buf/N female rats are able to induce morphological transformation in NIH 3T3 upon transfection. BamHI-cleaved DNA's from nine primary NIH 3T3 transformants, each generated by transfection with DNA from an independent carcinoma, were subjected to Southern analysis. A novel, amplified DNA sequence with homology to the ras^H oncogene was found in all transformants. One such sequence from a NIH 3T3 transformant was molecularly cloned into a lambda phage vector; a recombinant phage capable of malignantly transforming NIH 3T3 upon transfection was isolated. By comparison of the nucleotide sequence of the first exon of their recombinant with that corresponding to the first exon of the normal ras^H allele of Buf/N rats, Sukumar and colleagues were able to detect only a single nucleotide difference consisting of the substitution of the second deoxyguanosine residue of the twelfth codon by a deoxyadenosine. As a consequence of this point mutation, the glycine residue located at position 12 of the normal rat ras^H-encoded p21 protein is substituted by glutamic acid in the gene product of the NMU-ras^H oncogene. This finding is in accordance with the result of several groups (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982) that a point mutation resulting in the substitution of glycine by valine as the twelfth amino acid residue of p21 is responsible for the activation of a ras^H allele in the T24/EJ human bladder carcinoma cell line. Recent studies have indicated that substitution of glycine at position 12 by any other amino acid residue has the

same transforming consequences (Santos et al., 1983; Pincus et al., 1983). Furthermore, Wierenga and Hol (1983) have proposed that this point mutation alters the conformation of the nucleotide-binding site of p21, this perhaps leading to a profound effect on the activity of the protein. Since NMU is a potent alkylating agent that preferentially induces G $\rightarrow$ A transitions (Margison and O'Connor, 1979), the work of Sukumar et al. strongly suggests that the activation of the ras^H oncogene in NMU-induced rat mammary carcinomas is a direct result of the mutagenic activity of NMU. Their experimental findings are supported by the observation of Guerrero et al. (1984) that mouse thymic lymphomas (thymomas), induced by injection of mice with NMU, have activated ras^N oncogenes detectable by transfection of NIH 3T3 and established rat fibroblasts with tumor DNA. Activation of ras by specific point mutations within the coding region of the gene appears to be a common pathway in the cloned human transforming genes. Capon et al. (1983) and Shimizu et al. (1983a) have reported that activation of the ras^K oncogene in lung and colon carcinoma cell lines is due to substitution of cysteine or valine for glycine at position 12 in the encoded p21 protein. Similarly, the p21's encoded by the Harvey, Kirsten, and Balb/c murine sarcoma viruses have arginine, serine, and lysine residues, respectively, at position 12 (Capon et al., 1983). It is plausible that the retroviral transforming genes arose by a G $\rightarrow$ A transition in the twelfth codon, possibly resulting from reverse transcription errors, whereas activation of the human tumor-derived transforming genes was caused by a G $\rightarrow$ T transversion.

The demonstration by Pulciani et al. (1982b) that transfection with DNA from human tumor tissue can induce transformation in NIH 3T3 served

to reduce the likelihood that the transforming activity observed by other investigators using DNA from established cell lines was an artifact of the in vitro manipulation of cell lines. Furthermore, the findings of Sukumar et al. (1983) described above strongly suggest that ras may be an important in vivo target for chemical carcinogens, at least in the rat mammary carcinoma system. However, two lines of evidence imply that activation of ras may not be a common, or sufficient, cause of human cancers. First, Feinberg et al. (1983) have demonstrated that the point mutation at the twelfth codon of ras occurs infrequently in human malignancies. By use of restriction endonucleases sensitive to first or second base changes in this codon, they were unable to detect a point mutation in DNA's from primary tumor tissue of ten bladder, ten lung, and nine colon carcinomas. It is possible, however, that an alternate point mutation has occurred in the ras allele in these tumors, as observed by Yuasa et al. (1983) for their cloned transforming gene from a human lung carcinoma-derived cell line. Secondly, although the activated ras genes can transform established rodent fibroblast cell lines, they fail to transform efficiently, by themselves, primary embryo fibroblasts in culture and presumably, therefore, human cells in vivo. Land et al. (1983b) have reported that transformation of primary embryo fibroblasts requires cooperation between at least two oncogenes; transformation was observed only when either the cellular myc gene or the gene for polyoma large T antigen was introduced together with the activated ras^H gene. Ruley (1983) similarly found that the gene for the human adenovirus early region 1A cooperated with the T24 bladder carcinoma oncogene (ras^H) in transforming primary rat kidney cells. Both observations suggest that separate establishment and transforming functions

are required for oncogenic transformation of primary cells in culture; this interpretation fits well with the progressive, multi-stage nature of the development of human cancers.

Carcinogen-mediated point mutation(s) in a functional domain of a proto-oncogene, resulting in a qualitative change in the gene-encoded protein (e.g., altered substrate specificity), need not stand alone as the means by which c-oncs may become activated. Alternatively, overproduction of the normal gene product of a c-onc may in some cases be sufficient to trigger the profound growth alterations associated with neoplastic transformation. This has been amply demonstrated by Blair et al. (1981) and Defeo et al. (1981) who found that the cloned DNA sequences of two proto-oncogenes isolated from normal cells (c-mos from mice and c-ras from rats, respectively), non-transforming as such in the NIH 3T3 transfection assay, became activated when a retroviral promoter sequence (L_{TR}) was juxtaposed at their 5' ends, thereby vastly increasing the level of transcription of the normal gene. It should be noted, however, that other c-onc sequences from normal cells ligated to retroviral promoters have been consistently negative in the 3T3 assay (Duesberg, 1983). In addition, elevated expression of several c-onc gene products has been observed in otherwise "normal" cells and tissues (Bishop, 1983), further confusing the issue. Despite these apparent contradictions, evidence favoring a gene dosage model abounds. Several mechanisms have been proposed to account for naturally occurring quantitative changes in the expression of cellular oncogenes: a) Gene amplification. Two metaphase chromosome anomalies, long nonbanding homogeneously staining regions (HSR's) and small paired chromatin bodies known as double minutes (DM's), have been observed in a variety

of human tumor cells, but have not been observed in normal tissues (Bishop, 1983). Studies of the amplification of dihydrofolate reductase genes in methotrexate-resistant cell lines have shown that these amplified genes map to either HSR's or DM's (Kaufman et al., 1979). Based on this phenomenon, Pall (1981) concluded that the HSR's and DM's of human tumors are apt to be manifestations of amplified proto-oncogenes. He proposed that the initial step in carcinogenesis is a mutation producing a tandem duplication of a proto-oncogene. Gene amplification can then occur by successive unequal sister chromatid exchanges in several cell cycles until sufficient onc gene product is produced to transform the cell. This hypothesis has been borne out by the work of Collins and Groudine (1982) who localized amplified copies of the proto-oncogene, c-myc, to DM's in the HL-60 line of human promyelocytic leukemia and the COLO 320 human neuroendocrine tumor cell line. In addition, Schwab et al. (1983) demonstrated that amplified sequences of the ras^K gene present in a mouse adrenocortical tumor cell line reside on DM's and HSR's. However, it should be pointed out that although amplification in both cases is accompanied by increased gene expression, it is not yet clear whether there exists a causal relationship between the observed amplification and the malignant phenotype. In fact, the transforming gene activated in the HL-60 tumor cell line, as identified by the NIH 3T3 transfection assay, is not c-myc but rather N-ras (Hall et al., 1983). Furthermore, Chattopadhyay et al. (1982) found 6- to 10-fold amplification of the ras genes in certain strains of mice and hamsters, without obvious neoplastic consequences. Thus, at the present time, no consistent correlation between onc amplification and carcinogenesis has been established.

b) Insertional mutagenesis. As alluded to earlier in

the Background section, avian leukosis viruses are retroviruses without oncogenes, yet they induce B-cell lymphomas in chickens at high frequencies. An understanding of how these tumors might arise came with the discoveries of Hayward et al. (1981) and Payne et al. (1982) that viral DNA in these tumors is almost always integrated in the vicinity of a proto-oncogene, c-myc; as a consequence of the insertion, transcription from c-myc is augmented by as much as 100-fold. The insertion of viral DNA can take several forms: upstream of c-myc, in the same transcriptional orientation as the cellular gene; upstream of c-myc, but in the opposite transcriptional orientation; and downstream of c-myc, in the same transcriptional orientation. The first configuration permits the promoter of the provirus to drive transcription from c-myc directly; the other configurations must work indirectly by enhancing the activity of the c-myc promoter itself. A similar finding has recently emerged for mammary carcinomas induced in mice by the mouse mammary tumor virus (Nusse et al., 1984); in this case, insertion of the provirus within a highly conserved locus designated int-1 appears to activate expression of this putative oncogene. In the absence of further evidence, it is reasonable to speculate that transposable regulatory sequences, if present in the genomes of normal cells, might act in a similar fashion to activate other c-oncs. Alternatively, insertion of such sequences within a gene coding for a cis-acting repressor protein might disrupt the normal regulation of a proto-oncogene.

d Chromosomal translocation. The first consistent chromosomal abnormality associated with a human malignancy was reported by Nowell and Hungerford (1960). They found cytogenetic evidence for a reciprocal translocation between chromosomes 9 and 22 in 90-95% of patients with chronic myelogenous

leukemia (CML); the variant chromosome 22 has since been known as the Philadelphia (Ph¹) chromosome. Subsequently, other non-random chromosomal translocations have been identified in a variety of human neoplasms (Rowley, 1980; Rowley and Testa, 1982). Such translocations could arouse oncogenes by relocating them into transcriptionally active domains of chromatin. Cooper et al. (1980) demonstrated that DNA from normal cells, when first sheared to molecular weights of $0.3-3.0 \times 10^6$ daltons, could transform NIH 3T3 cells at a very low frequency. The transformed mouse cells in turn yielded DNA that induced transformation at much higher efficiencies, as if the original shearing of normal DNA unleashed a potentially oncogenic sequence from a cis-active regulator. More striking evidence supporting this mechanism has recently been found from studies of the B-cell tumors of Burkitt's lymphoma, which generally display translocations involving chromosome 8 and a chromosome bearing an immunoglobulin gene that is active in the tumor: 2, kappa Ig light chains; 14, Ig heavy chains; or 22, lambda light chains (Leder et al., 1983). In each case, the proto-oncogene, c-myc, is transferred from its quiescent location on chromosome 8 to come under the influence of an immunoglobulin promoter on chromosomes 14, 2, or 22. Similarly, the translocation in human CML appears to involve the juxtaposition of a proto-oncogene, c-abl, residing on chromosome 9, with the lambda light chain locus on chromosome 22; enhanced expression of c-abl in at least one CML cell line has been reported (Duesberg, 1983). The idea that such specific translocations are the cause of the malignancy, and not later events associated with tumor progression, however, is still open to vigorous debate. d) Derepression of proto-oncogenes All of the mechanisms discussed so far invoke activation of c-oncs by cis-acting

mutations, either within the functional domain of an oncogene (i.e. point mutation) or in regulatory loci (i.e. promoters) adjacent to the gene. However, an alternate pathway to oncogene activation was proposed by Comings (1973). He postulated that transforming genes are normally suppressed by a diploid pair of trans-acting regulatory genes; in order for a transforming gene to be expressed, a double mutation in the regulatory loci is required in order to overcome repression. Knudson (1971, 1973) offered a similar interpretation to explain the mechanism of tumorigenesis in human hereditary retinoblastoma, in which predisposition to malignant differentiation of retinoblasts is acquired in an autosomally dominant fashion. Knudson hypothesized that one allele with a germ line mutation in a repressor gene is inherited; a somatic mutation in the other allele results in release of a retinoblastoma tissue-specific transforming gene and onset of malignant transformation. The recent work of Cavenee et al. (1983) tends to support this hypothesis: a homozygous, constitutional deletion in the long arm of chromosome 13 was found to be common to at least 15% of all cases of inherited, bilateral retinoblastoma. It is possible that deletions too small to detect by standard cytogenetic methods might account for the other 85% of tumors.

CONCLUDING REMARKS

Based on the evidence presented in this thesis, and in large part provided by studies employing DNA-mediated gene transfer, it has been convincingly demonstrated that vertebrates and other metazoan organisms possess a family of proto-oncogenes whose altered activities play a crucial role in tumorigenesis. It is apparent that diverse mechanisms may be involved in the activation of these cellular genes; whether these can be traced to the direct action of proximal carcinogens or only to secondary events set in motion by an as yet unidentified initiating event is still subject to debate. However, there is reason to be optimistic that better understanding of the proto-oncogenes may eventually explain both the initiation and maintenance of neoplastic growth.

One should not lose sight of the broader significance of the existence of these cellular genes. It is inconceivable that they would have survived through evolution as solely noxious agents. Powerful selective forces must have been at work to assure the conservation of proto-oncogenes throughout the diversification of metazoan phyla. The proteins that these genes encode are apt to be components of a complex network that controls the growth of individual cells during the course of differentiation. In order to resolve this and other questions, we must develop strategies towards discovering the functions of these proteins as they relate to both normal and malignant cellular growth. It is most likely that important progress will come from studies of organisms more amenable to sophisticated genetic analysis and mutant selection, in particular Saccharomyces cerevisiae and Drosophila melanogaster.

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