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BIOCHEMICAL AND BIOLOGICAL ANALYSIS OF HUMAN FIBROBLASTS TRANSFORMED WITH N-ras ONCOGENES

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DANIEL MICHAEL WILSON

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Justin McCormick
Major professor

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BIOCHEMICAL AND BIOLOGICAL ANALYSIS OF HUMAN FIBROBLASTS TRANSFORMED WITH N-RAS ONCOGENES

by

Daniel Michael Wilson

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ABSTRACT

BIOCHEMICAL AND BIOLOGICAL ANALYSIS OF HUMAN FIBROBLASTS TRANSFORMED WITH N-*RAS* ONCOGENES

by

Daniel M. Wilson

N-*ras*, a member of the *ras* gene family, has been isolated as a transforming gene from many human tumors, including fibrosarcomas. To examine what role expression of an N-*ras* oncogene plays in the etiology of human tumors, I transfected plasmids containing N-*ras* oncogenes into early passage diploid human fibroblast and into an infinite lifespan human fibroblast cell line, MSU-1. Plasmid pSV N-*ras* contains the *neo* gene, coding for Geneticin resistance, and a human leukemia cell line-derived N-*ras* oncogene activated by a transversion in codon 12. The cellular promoter for N-*ras* was replaced by a viral long terminal repeat which initiates transcription of both the N-*ras* gene and the *neo* gene. Plasmid pNR-MG1 contains the *neo* gene and a human fibrosarcoma-derived N-*ras* oncogene which is activated by a mutation in codon 61. Transcription of the N-*ras* oncogene is initiated from its cellular promoter, and transcription of the *neo* gene is initiated from a simian virus-40 promoter.

Transfection of pSV N-ras into diploid human fibroblasts yielded Geneticin resistant colonies, at a frequency of 1 x 10^{-6} cells transfected per 10 ug of plasmid DNA, of which 70% were morphologically transformed. In the absence of drug selection, dense multilayered groups of cells, foci, were formed at approximately the same frequency. The transformed cells had highly anaplastic morphologies, with many multinucleated and irregularly shaped cells, but the majority either reverted to a normal morphology or senesced prematurely. However, progeny cells from two of the Geneticin resistant colonies maintained their transformed morphology for the duration of a normal lifespan in culture. Immunoprecipitation analysis indicated that high amounts of N-ras oncogene encoded protein were produced. These cell lines also exhibited anchorage independence, a characteristic of many tumor-derived cells. However, they did not form tumors when injected into athymic mice.

Transfection of early passage diploid human fibroblasts with pNR-MG1 yielded many Geneticin resistant colonies. However it was not clear that any of these were morphologically transformed. In the absence of drug selection, foci were observed, but their morphologies were not markedly abnormal. Cells from these foci yielded populations which soon reverted to a normal morphology, exhibited normal growth characteristics, and were not tumorigenic. Immunoprecipitation analysis indicated that the N-ras oncogene encoded proteins were not expressed.

Transfection of pSV N-ras into infinite lifespan MSU-1 cells yielded foci of morphologically transformed cells which maintained their morphologies, were anchorage independent, growth factor

independent, and malignant. DNA-DNA hybridization analysis indicated that the transfected oncogene was present in the tumor-derived cells, and immunoprecipitation analysis showed that high amounts of N-ras oncogene encoded protein were present in all of the focus-derived cell lines, and in all but one of the tumor-derived cell lines.

To

my parents, David and Patricia Wilson;
and
my wife, Louise Hemond-Wilson

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ABBREVIATIONS

c-H-*ras*	cellular Harvey *ras* gene
DAG	diacylglycerol
EF-Tu	elongation factor-Tu
EGF	epidermal growth factor
EJ-H-*ras*	EJ bladder carcinoma-derived Harvey ras oncogene
E1A	adenovirus early region 1A gene
G proteins	guanine nucleotide binding proteins
H-*ras*	*ras* gene homologous to transforming gene of Harvey murine sarcoma virus
IP_3	inositol-1,2,3-triphosphate
K-*ras*	*ras* gene homologous to transforming gene of Kirsten murine sarcoma virus
LTR	long terminal repeat
MSV	murine sarcoma virus
myc	gene homologous to transforming gene of avian myelocytomatosis virus
N-*ras*	cellular *ras* gene first isolated from neuroblastoma
p53	common tumor antigen of 53,000 daltons
p21	21,000 dalton *ras* protein
PIP_2	phosphatidyl inositol, 4-5-bisphosphate
PDGF	platelet-derived growth factor
PKC	protein kinase-C

SHE	Syrian hamster embryo
SV40	simian virus 40
T24-H-*ras*	T24-bladder carcinoma-derived H-*ras* oncogene
v-*myc*	*myc* oncogene of avian myelocytomatosis viru
v-H-*ras*	*ras* gene from Harvey murine sarcoma virus
v-K-*ras*	*ras* gene from Kirsten murine sarcoma virus

INTRODUCTION

Chemical and physical agents have for years been implicated in the etiology of many cancers, and in most cases these agents have induced mutations and in vitro transformation of mammalian cells with similar kinetics (Barrett and Ts'o, 1978, Chan and Little, 1978, Landolph, 1985), and have interacted with DNA (Miller, 1978). However, there were few clues as to the nature of the specific genes or regions of DNA which, when altered via mutation, were responsible for conferring transformed properties on normal cells.

The advent of many new techniques in molecular biology during the past two decades has dramatically enhanced research on the mechanisms by which cancer occurs in man. These techniques include the ability to isolate transforming genes from tumor-derived cells, to clone individual genes into various vectors, to sequence genes, to dissect DNA with restriction endonucleases, to probe DNA for the presence of specific genes by DNA-DNA hybridization, and to analyze the expression of individual genes by DNA-RNA hybridization analysis and other methods.

Several key findings have oriented much of the recent research being conducted on the origins of cancer. Included in these findings is the fact that the transforming genes of RNA tumor viruses of the ras gene family are homologous to mammalian genes and were actually derived from them (Ellis et al. 1980). Another is the recognition that

transfection of human tumor-derived DNA into an indicator cell line causes the cells which incorporate and express a transfected ras gene to form multilayered groups of morphologically altered cells, a characteristic of tumor-derived cells known as focus formation (Der et al., 1982, Parada et al., 1982, and Santos et al., 1982). This ras gene was later shown by DNA sequence analysis to be altered by only a single point mutation which caused substitution of one amino acid for another in the encoded protein (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982). Ras genes have since been identified as transforming genes in many human tumor-derived cells (Suarez, et al., 1987), and have been activated in model systems by carcinogen treatment of cloned normal ras genes (Marshall et al., 1984), by treatment of cultured mammalian cells (Sukumar et al., 1984), and by treatment of animals (Balmain and Pragnel, 1983).

In addition to the ras gene family, many other transforming retroviral genes have been shown to have homologous mammalian counterparts. One pivotal finding was that the transforming gene of simian sarcoma virus encoded a protein which was nearly identical to a mitogenic peptide known as platelet-derived growth factor, which is synthesized by some mammalian cells (Robbins et al., 1983). This discovery implied that one mechanism by which human tumor-derived cells escape the constraints which limit normal cellular division is by the synthesis of growth factors which drive replication. Genes which encode other growth factors have since been implicated as transforming genes of various human tumors (Salomon and Perroteau, 1986). In addition, genes for growth factor receptors (Ullrich et al., 1984), as well as genes which encode some of the proteins thought to couple

growth factor binding to DNA replication (Persons et al., 1988), have been identified as possible transforming genes. The portrait which has evolved from these studies is one of a tumor cell which replicates autonomously by circumventing at some point the complex network of biochemical signals which regulate cell division.

Rapid advances in cancer research have been made by manipulation of the suspected transforming oncogenes. Studies on their role have most commonly involved, first using any of various techniques to put them into a nontumor-derived cell, and then assaying the phenotype of the cell. Studies of this nature have most often employed rodent fibroblasts as the recipient cells. Such model systems have provided insight into the multistepped nature of carcinogenesis (Land et al., 1983), and have provided critical information on the biochemical action of the oncogene products.

The ultimate aim of most cancer research, and of the bioassays which are used to assess the carcinogenicity of a particular agent, is to protect human life. However, the majority of basic cancer research and most short-term assays, have used either animals or nonhuman cells. Although carcinogenicity tests which use animals are irreplaceable for some purposes, and much valid information has probably come from cell culture assays using nonhuman cells, the use of only these systems to analyze the risk to human life would seem unacceptable if more test systems using human cells, such as model transformation systems using cultured fibroblasts, could be devised.

This thesis was undertaken (1) to investigate the multistepped process of carcinogenesis, by transfecting cloned oncogenes into diploid human fibroblasts in culture in attempts to obtain cells

capable of forming malignant tumors in athymic mice; (2) to determine the biological and biochemical characteristics associated with transformation of diploid human fibroblasts with an N-ras oncogene; (3) to determine the biological and biochemical characteristics of transformed cells obtained by transfection of an N-ras oncogene into indefinite lifespan human fibroblast cell line MSU-1.

Chapter I of the thesis reviews the literature that covers the relationship between environmental agents and carcinogenesis, including discussions of epidemiology, animal models for cancer, studies on transformation of cells in culture, and the relationship between mutations and cancer. Also discussed in Chapter I is literature dealing with the involvement of ras genes in carcinogenesis and with the biochemical properties of ras gene products. Chapter II consists of a manuscript to be submitted to the journal Carcinogenesis. This manuscript details transfections of N-ras oncogenes into finite lifespan diploid human fibroblasts and characterization of the two stable transformants obtained. I carried out all the studies described in the manuscript and am the principal author on the paper. Dennis G. Fry, Ph.D., a senior research associate in the Carcinogenesis Laboratory, assisted with the research by conducting related pilot studies and by providing training in the molecular biology techniques. Chapter III consists of a manuscript to be submitted to the journal of Molecular and Cellular Biology. This manuscript reports the results of transfections of an N-ras oncogene into infinite lifespan human fibroblast cell line MSU-1, which was derived in this laboratory. I carried out the major studies included in the manuscript. Dajun Yang,

M.D., carried out the cytogenetic analysis and John Dillberger, D.V.M., conducted the pathology analysis of the tumors.

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CHAPTER I

LITERATURE REVIEW

A. Studies implicating environmental agents in carcinogenesis.

Research into the causes and cures for cancer has evolved into an in-depth effort at understanding the myriad of metabolic processes, and genetic and epigenetic events that interact during carcinogenesis, and also trying to sort out those phenomena that are critical from those that are not. The desired outcome of this intense effort is a pronounced reduction in the number of cancer deaths through combined improvements in prevention, early detection, and intervention in the course of the disease. Such improvements may well affect the quality of the lifestyle and environment of human beings as well as other aspects of human life.

1. Cancer frequencies and epidemiology.

Cancer currently accounts for approximately 22% of all deaths in the United States (Silverberg and Lubera, 1987). Epidemiological reports were originally the only data available to determine if certain agents had the potential to cause cancer in humans. The first of two hallmark studies in the 1700's related nasal cancer to the use of tobacco snuff (referred to in Redmond, 1970). Shortly thereafter Pott showed a correlation between scrotal cancer in chimney sweeps and exposure to products in coal tar (Pott, reprinted 1963).

Some epidemiological data provide overwhelming evidence that certain forms of cancer are caused by a particular environmental agent. That skin cancer is often caused by excessive exposure to ultraviolet light from the sun has been well documented (Emmett, 1973). A very

strong correlation has also been shown between tobacco smoking and lung cancer, both for males (US Public Health Service, 1964), and more recently for females (US Public Health Service, 1978).

Epidemiological data implicating certain agents as being carcinogenic has often been sufficient to prompt interventive measures by regulatory agencies. Pott's early observations yielded guidelines on hygiene for chimney sweeps (Butlin, 1892). The correlation between tobacco smoking and lung cancer was convincing enough to lead the US surgeon general to place restrictions on advertising of tobacco products and to require written warnings on their packages, to impel legislatures to regulate smoking in some communities, and to promote initiation of anti-smoking laws in some businesses. Quite often the results of epidemiological studies suggest that a correlation exists between a given type of cancer and a particular factor, but do not convincingly demonstrate a cause and effect relationship. Although epidemiological research is still widely carried out, there are inherent difficulties with such studies. These problems include improper diagnosis of the type of cancer, false estimates of cancer incidence, unreliability of data that must be drawn from the memory of the persons interviewed concerning lifestyle and prior exposures to carcinogenic agents, and trying to relate recent cancer occurrence to carcinogen exposures that may have occurred many years previously. Although epidemiological studies provide invaluable information concerning the cause of some cancers, the lack of mechanistic information and the inherent problems with such studies, combined with many attractive features of laboratory experiments, led to the creation

of model systems utilizing whole animals, cell culture, and other in vitro assays, that might yield new insight into the causes of cancer.

2. Treatment of laboratory animals with suspected carcinogenic agents.

Reports that exposures to some agents were associated with unusually high incidences of certain cancers prompted some early workers to administer suspected carcinogens to laboratory animals. Fisher (1906) showed that application of the dye scarlet red to rabbits caused tumor-like growths on the exposed areas. The pivotal findings that dermal application of coal tar to rabbit ears (Yamagiwa and Ichikawa, 1915 - referred to in Haddow, 1947) and that extracts of residues from the pyrolysis of hydrocarbons (Kennaway, 1925) caused skin cancer in experimental animals, fostered an intense effort to identify the carcinogenic agents present in such material. Polycyclic aromatic compounds, such as benzo(a)pyrene (Cook et al., 1933) and derivatives of benz(a)anthracene (Kenneway and Hieger, 1930) that were formed during combustion of hydrocarbons, were implicated as the causative agents.

Many classes of carcinogens, both organic and inorganic, naturally occurring and man-made, were shown to cause cancer in treated animals. Early investigations, such as those of Berenblum (1940), indicated that carcinogenesis was a multistepped process. He showed that repeated application of croton oil to skin that had previously been initiated with as low as 0.05% benzo(a)pyrene served to "promote" the formation of a cancerous lesion at the site of application. Initiation is regarded as a permanent change that can occur after the single application of a subthreshold dose of a carcinogenic agent that is

insufficient by itself to cause cancer (Boutwell, 1974). Promotion is broadly defined as a reversible process whereby a tumor is elicited by application of a promoting agent subsequent to administration of an initiating agent (Boutwell, 1974). This might occur by growth enhancement of previously initiated cells into larger populations with a proportionally larger probability of undergoing a subsequent initiation-like event. Later studies went on to show that promotion could be subcategorized into at least two discrete steps (Slaga et al., 1980). According to Scribner and Suss (1978), evidence that initiating and promoting events actually take place in humans is shown by the strong probability of developing bronchiogenic carcinoma in asbestos workers who also smoke tobacco, and by the decline with time of the risk of developing lung cancer in people who have quit smoking.

Experiments utilizing live animals are commonly carried out to test for toxicity, mutagenicity, and teratogenic and carcinogenic effects of compounds, and are often required by law to screen new products before market approval. Although there are some obvious advantages in using live animals, including weighing the effects of uptake, metabolism, and excretion, and generation of dose response data, there are also disadvantages. These include host specificity, expense, latency periods that are often long, sacrifice of many animals, and a poor ability to resolve questions of the mechanisms involved. Similar to the need for epidemiological studies, tests utilizing live animals are also essential to determine some aspects of the toxicity and carcinogenicity of various agents. However, various in vitro strategies for the study of carcinogenesis, including a battery of short term tests to determine the mutagenic potential of agents,

treatment of cultured cells to determine the capacity of different agents to cause changes characteristic of tumor-derived cells (transformation), and elaborate molecular biology techniques (see below) have been developed.

3. Treatment of mammalian cells in culture with suspected carcinogens.

There have proved to be many advantages of using cultured mammalian cells in mutation and transformation assays. Some of the advantages include a relatively short duration between treatment time and endpoint, ease of growing many cell types, cost effectiveness of the experiments, and the capability of ascertaining "mechanisms", etc. The cell type most widely utilized for in vitro tissue culture experiments is the rodent fibroblast because early attempts to culture these cells were very successful. In addition, well characterized assay systems were developed based on the use of such cells, which enabled the identification of mutagenic or carcinogenic agents by virtue of their causing discernable effects.

Berwald and Sachs (1963) made the initial discovery that cells could be transformed in culture by chemical carcinogens when they showed that polycylic aromatic hydrocarbons could cause alterations in the colony growth pattern of primary Syrian hamster embryo fibroblasts in culture. These cells are diploid and have a finite lifespan in culture (Meyer, 1983). Much work has been done to further elaborate the best assay conditions for in vitro transformation assays using these cells. DiPaolo et al. (1969, 1971) refined the assay, showed it was sensitive to many carcinogens, and showed that some of the transformants were tumorigenic. Pienta et al. (1977) developed the

assay further by utilizing cryopreserved primary SHE fibroblasts for target cells and feeder layers. Since SHE cells have a finite lifespan and do not spontaneously transform in culture, Barrett's group has extensively employed them to study the process of neoplastic progression after carcinogen treatment (Koi and Barrett, 1986, Oshimura et al., 1985).

The first mouse fibroblast cell line that was developed for transformation studies was derived from the ventral prostate of a C3H mouse (Chen and Heidelberger, 1969a). This cell line was selected because it exhibited density dependent contact inhibition of growth and was therefore suitable for focus assays of the transforming potential of a number of suspected carcinogens (Chen and Heidelberger, 1969b, 1969c). Subsequently, a highly contact inhibited cell line designated C3H10T1/2Cl8, was established from a C3H mouse embryo (Reznikoff et al., 1973a), and has since been extensively employed to assay the capacity of various agents to cause focus formation (Reznikoff et al., 1973b; Benedict et al., 1977; Landolf and Heidelberger, 1979). The ability to form a multilayered group of cells called a focus is one characteristic of transformed fibroblasts which distinguishes them from normal fibroblasts that grow to a confluent single layer of cells in tissue culture dishes. A focus assay involves treating normal cells at low density with an agent suspected of causing cancer, and then allowing the cells to grow to confluence. They are stained after an appropriate expression interval and any foci present are evaluated. The types of foci observed in the C3H10T1/2 system have been categorized into three classes, Types I - III. Type I foci stain lightly and are not considered significant, Type II foci stain darkly

because of multiple dense cell layers, but have smooth edges, and Type III foci also stain darkly because of multiple dense cell layers, but have irregular edges resulting from the criss-cross orientation of the cells (Landolf, 1985b). This assay is deemed a relatively valid predictor of carcinogenic potential because, when the focal derived populations of cells are harvested, expanded, and injected into immunosuppressed C3H mice, 50% of those derived from Type II foci and 80% of those derived from Type III foci form tumors (Landolf, 1985b).

4. In Vitro Transformation of Human Cells.

Since evidence strongly suggests that most human cancers are caused by exposure to environmental carcinogens, cultured human cells would, in principle, be the most logical choice for use in developing in vitro screening assays for carcinogens, and for studying the mechanisms by which transformation occurs. Although the majority of human tumors are of epithelial origin, human epithelial derived cells have proved to be difficult to maintain in culture and so reproducible methods to culture such cells have only recently been established. Therefore the majority of transformation experiments with human cells, as with all mammalian cells, have utilized cultured fibroblasts.

Normal diploid human fibroblasts do not, as a rule, spontaneously transform in culture to either indefinite lifespan cell lines or malignant cells. This stability makes them ideal candidates for use in studying the process of carcinogen-induced neoplastic transformation. There are numerous reports of partial transformation of human fibroblasts in culture following carcinogen treatment. The end-points most often assayed are growth in soft agar or the acquisition of

infinite lifespan in culture. Namba et al. (1978, 1981) have successfully transformed human fibroblasts to infinite lifespan following repeated treatment with either 4-nitroquinaline-1-oxide or X-rays. Many workers have used growth in soft agar to assay for transformation of human cells after treatment with suspected carcinogens. For example, Milo and DiPaolo (1978) used a variety of carcinogens to induce anchorage independent growth in human fibroblasts. Similar studies have been done by Landolf's group using carcinogenic metals (Biedermann and Landolph, 1987), and by McCormick and Maher and their coworkers using radiation and chemical carcinogens (Silinskas et al., 1981, Wang et al., 1986).

There are several reports that human fibroblasts can be malignantly transformed after exposure to carcinogens. However, results of a recent study have indicated that most of these reports are invalid. For example, the most widely known study on the malignant transformation of human fibroblasts is that of Kakunaga (1978) who reported the in vitro transformation of a cell line designated KD by exposure to 4-nitroquinoline-1-oxide or N-methyl-N'-nitro-N-nitrosoguanidine. Careful analysis of the reported transformants has revealed that the transformed cells were not derived from KD cells, but were most likely derived from a contaminating human fibrosarcoma cell line. Other reports of transformation of human fibroblasts did not convincingly demonstrate that the tumors were, in fact, malignant (Borek, 1980; Min et al., 1980; Ming et al., 1986).

5. Relationship Between Mutation and Cancer.

As noted above, most known human cancers are thought to be caused by environmental carcinogens. The combined results of many experiments using both human and nonhuman cells suggest that these agents exert their carcinogenic effects by interacting with and altering the genome. Many chemical carcinogens are either electrophilic or can be metabolically activated to electrophilic species that bind covalently to DNA to form "adducts" (Miller, 1981). Such adducts can subsequently be converted to mutations, i.e., permanent changes in DNA. Quite often the mutation induction curve for a carcinogen parallels its dose-response curve for transformation of cells treated in culture (Barrett and Ts'o, 1978; Landolph, 1985a). In recent years many workers have concentrated on identifying particular genetic sequences that, when modified by carcinogen treatment, result in genes being able to cause changes in cells that are characteristic of transformed cells. One of the major accomplishments of modern cancer research has been finding that the genetic material in certain tumor viruses that is capable of causing cancer in animals is homologous to normal endogenous human genes, i.e., protooncogenes (see below).

B. Involvement of the *ras* genes in carcinogenesis.

1. Evolution of RNA tumor virus *ras* genes and identification of homologous cellular genes.

Four murine sarcoma viruses (MSV) of the "*ras*" (*ra*t *s*arcoma) family of RNA tumor viruses have been identified. Harvey-MSV was isolated from a BALB/c mouse after it had been inoculated with a virus that was obtained by infecting rats with Maloney murine leukemia virus (Harvey,

1964). Kirsten-MSV was obtained from rats that had been inoculated with an extract from a C3H mouse lymphoma (Kirsten and Mayer, 1967). BALB-MSV was isolated from a BALB/c mouse hemangiosarcoma that was obtained after injection of a filtrate from a BALB/c mouse chloroleukemia (Anderson et al., 1981). Rasheed-MSV was isolated after cocultivation of a chemically transformed rat tumor cell line with spontaneously transformed Sprague-Dawley rat embryo cells that were releasing a slow-transforming endogenous ecotropic type C virus, a virus which normally occurs as a stably integrated provirus, but which when it is induced to replicate, does so best in rat cells (Rasheed et al., 1978).

Scolnick and Parks (1974) showed that Harvey-MSV contained genetic sequences of Maloney murine leukemia virus origin and also sequences that had been transduced from a rat genome during evolution. Scolnick et al. (1973) showed that Kirsten-MSV was also a recombinant virus which had evolved by transduction of rat DNA into Kirsten murine leukemia virus. Harvey-MSV and Kirsten-MSV are oncogenic to rats and were shown to transform in vitro a commonly utilized mouse fibroblast cell line developed at the National Institutes of Health (NIH/3T3). Transformation of NIH/3T3 cells following transfection of subgenomic clones of these viruses, followed by hybridization of the transfected transforming gene with rat-specific probes, indicated that the transforming 21 kd protein was the product of a rat gene (Chang et al., 1980). De Feo et al. (1981) used sequences derived from the transforming gene of Harvey-MSV to probe rat genomic DNA. Two related genes homologous to the viral probe were identified, isolated, ligated to a Harvey-MSV long terminal repeat (LTR), and found to induce

transformation when transfected into NIH/3T3 fibroblasts. When Chang et al. (1982) probed human genomic DNA with the transforming sequences from Harvey-MSV and Kirsten-MSV, a total of four homologous genes were identified. These cellular genes which are often referred to as protooncogenes, were named c-Ha-ras1 and 2 and c-Ki-ras1 and 2. Later, c-Ha-ras1 and c-Ki-ras2 were shown to be functional genes, and c-Ha-ras2 and c-Ki-ras1 were shown to be processed pseudogenes, genetic sequences which does not actually code for a functional protein (DeFeo et al., 1981; McGrath et al., 1983).

2. Isolation and molecular characterization of cellular ras oncogenes.

A test that has been widely used to detect activated oncogenes in malignant tissue is the NIH/3T3 transfection assay. NIH/3T3 cells are efficient recipients of transfected DNA, and form readily detectable foci with activated ras and other oncogenes (Lemoine, 1987). The focus-derived cells form malignant tumors when injected into athymic mice, whereas nonfocus-derived NIH/3T3 cells usually do not form tumors in athymic mice. To isolate activated oncogenes, tumor cell-derived genomic DNA from a species other than mouse is transfected into NIH/3T3 cells which are allowed to grow to confluence and assayed for focus formation. DNA from focal derived populations can then be used in subsequent rounds of transfection of NIH/3T3 cells, to enrich for the transforming gene of interest. Libraries can then made of genomic DNA from the transformed NIH/3T3 cells and probed for highly repetitive sequences characteristic of the donor species.

Using the above protocol, Shih and Weinberg (1982) cloned a transforming gene from the human EJ bladder carcinoma line, and Goldfarb et al. (1982) cloned a transforming gene from the human T24

bladder carcinoma cell line. Each group found that when transfected into NIH/3T3 cells, their cloned gene caused transformation. It was subsequently realized that these two cell lines were derived from the same original tumor (Shimizu et al., 1983). Der et al. (1982), Parada et al. (1982), and Santos et al. (1982), independently found that the transforming gene isolated in the above manner from the human EJ bladder carcinoma cell line was homologous to the transforming gene of Harvey-MSV. Shortly thereafter, the genetic alteration that activated the H-ras oncogene in the EJ cell line was identified by first constructing hybrid vectors between cloned transforming and nontransforming alleles, and then determining the nucleotide sequence of a short restriction fragment that was sufficient to confer transforming ability when ligated in place of its homologous counterpart into the normal c-H-ras gene (Tabin et al., 1982, Reddy et al., 1982, and Taparowsky et al., 1982). These groups found that a point mutation which caused a base substitution in codon twelve of the c-H-ras protooncogene, was responsible for the transforming activity of the ras sequence. Point mutations in several critical codons of ras genes which cause the transfected DNA to cause focus formation in NIH/3T3 cells are now often referred to as "activation" of a ras gene. In addition to the above findings, there are many other examples of H-ras activations that have been detected in human tumor-cell DNA that were capable of causing focus formation in NIH/3T3 cells. For example Yuasa et al. (1983) found that c-H-ras was activated by a single base substitution in codon 61 in cells from lung carcinoma cell line Hs242.

Since the only protooncogene activating mutations in ras genes known at the time were codon 12 mutations in the c-H-ras gene,

Feinberg et al. (1983) analyzed 29 human tumor DNA samples by restriction enzyme analysis to detect a given point mutation at codon 12. None of the 29 samples analyzed had the specific point mutation assayed for, so the authors concluded that previous results obtained with the human EJ bladder carcinoma cell line provided a good model for human carcinogenesis, but were not representative of the majority of human cancers. Subsequent to the findings of Feinberg's group, many other point mutations have been found that are capable of activating cellular ras genes.

Most often, when a human oncogene has been identified by virtue of its causing focus formation in NIH/3T3 cells, it has been found to be a cellular Kirsten ras gene (c-K-ras). For example, Der (1982) first reported that a cellular gene homologous to the transforming gene of Kirsten-MSV was identified in DNA from the human lung carcinoma cell line LX-1 by virtue of its ability to cause focus formation in NIH/3T3 cells. Capon et al. (1983) then reported that the human lung carcinoma cell line Calu-1 has an activated c-K-ras2 gene which resulted from substitution of cysteine for glycine at the amino acid specified for by codon 12, and that the human colon carcinoma cell line SW480 is also activated at codon 12 of c-K-ras2, but the product of this gene has a valine in place of glycine.

Shimizu et al. (1983) found that DNA from the human neuroblastoma cell line SK-N-SH also induced foci on NIH/3T3 cells. When the human transforming gene was cloned by this group, it was found to be a third cellular member of the ras gene family distantly related to Harvey and Kirsten murine sarcoma viruses, but not detected by viral derived probes to their transforming genes. Shimuzu et al. (1983a) named this

newly identified gene N-ras, and established its orientation of transcription and approximate exon coding regions. Marshall et al. (1982) had reported that DNA from the human fibrosarcoma cell line HT1080 and the human rhabdomyosarcoma cell line RD was capable of causing focus formation in transfected NIH/3T3 cells. Soon after Shimuzu's report, Hall et al. (1983) reported that N-ras had also been identified as the transforming gene of the HT1080 and RD cell lines, and also of the human promyelocytic leukemia cell line HL60. The activating mutation of the HT1080 N-ras oncogene was localized by testing the ability of chimeric molecules between the cloned N-ras gene from normal human fibroblasts and the HT1080 fibrosarcoma cell line to transform transfected NIH/3T3 cells, and then was sequenced (Brown et al., 1983). The only difference between the normal and activated gene was shown to be a C to A transversion altering the 61st amino acid from glutamine in the normal allele to lysine in the HT1080 gene. The identical activating base substitution was also identified in the cell line SK-N-SH (Taparowski et al., 1983). In a similar fashion the N-ras oncogene of the RD cell line was shown to be activated by an A to T transversion which caused substitution of histidine for glutamine at codon 61 (Chardin et al., 1985). In humans, N-ras has been shown to be activated predominantly in hematopoietic malignancies (Bos et al., 1987, Needleman et al., 1986). Souyri and Fleissner (1983) found that N-ras was an activated oncogene in the three human T-cell leukemia cell lines RPMI 8402, CCRF-HSB2, and p-12.

To investigate whether the presence of an activated N-ras oncogene correlated with the clinical course of acute myeloblastic leukemia, Gambke et al. (1984) evaluated its occurrence in bone marrow cells

from a patient at the outbreak of the disease. An active N-ras oncogene was detected in the bone marrow cells during the acute phase of the disease. However, an N-ras oncogene was not present in non-affected fibroblasts of the same individual. This N-ras oncogene was later found to be activated by a point mutation in codon 12 that caused substitution of aspartic acid for glycine (Marshall, 1985).

Bos et al. (1985) found activating mutations in codon 13 of peripheral blood cells in four out of five patients with acute myeloid leukemia. Peripheral blood cells from one patient tested in remission no longer had a detectable N-ras mutation. Senn et al. (1988) analyzed DNA isolated from the cells of blood or bone-marrow samples from 18 patients with acute non-lymphocytic leukemia and 14 patients with acute lymphocytic leukemia. The only N-ras mutations that were found were in cellular DNA derived blood and bone marrow cells from five acute non-lymphocytic leukemia patients. In a follow-up study on three affected patients, the two individuals in remission no longer had detectable levels of a mutant N-ras gene in DNA isolated from peripheral blood cells, whereas the other affected patient exhibited detectable levels of a mutant N-ras gene (codon 13) in DNA from peripheral blood cells.

Hirai et al. (1987) analyzed bone marrow cells from eight patients with myelodysplastic syndrome, a disease which occasionally progresses to frank leukemia, and found three of those patients had marrow cells containing an active N-ras oncogene with a point mutation in codon 13. These investigators monitored the progression of the disease in all eight individuals and found that the three patients who had an activated N-ras gene later developed acute myeloid leukemia, whereas

the other five patients exhibited no progression of the disease within one year following diagnosis.

3. Mechanisms of activation of cellular ras genes.

Ras oncogenes were detected in many early analyses of human tumor cells by virtue of their ability to cause focus formation in transfected NIH/3T3 cells. The transforming potential of the isolated ras genes was attributed soley to various point mutations that caused single amino acid substitutions in the gene product. But there was certainly precedence for investigating other possible mechanisms of ras gene activation, since DeFeo et al. (1982) had found that induction of high expression of the normal c-H-ras gene was capable of causing transformation in transfected NIH/3T3 cells. Tahara et al. (1986) found that expression of c-H-ras was higher in metastatic nodules of patients with gastric carcinomas than in the primary tumors, and that those patients that had the highest expression of c-H-ras had the poorest prognosis. One possible mechanism by which higher than normal expression of a gene might occur is by amplification of that gene. There are numerous examples of amplification of ras genes in DNA obtained from tumor cells. The cellular K-ras gene was amplified 5-fold in the colon carcinoma cell line SW480 (Capon et al., 1983; McCoy et al., 1983), 40-fold in DNA from a primary bladder tumor (Fujita et al., 1985), and 10-fold in the giant cell carcinoma cell line Lu65 (Alitalo et al., 1984; Taya et al., 1985). The cellular H-ras gene was amplified up to 20-fold in cell line SK-2 derived from a malignant melanoma patient (Sekiya et al., 1985), and 10 fold in cells from a primary bladder carcinoma (Hayashi et al., 1983).

Another proposed mechanism by which cellular ras genes might be activated is through alterations in the methylation status of genomic cytosine residues. The restriction endonucleases HpaII and HhaI do not cleave the sequence 5′-C-G-3′ if the cytosine is methylated, whereas MspI will cleave that sequence. By digesting DNA purified from human cancers and adjacent normal tissue with these enzymes, followed by hybridization with labeled K-ras and H-ras probes, it was ascertained that there were marked decreases in the methylation status of some tumor DNAs compared to DNA obtained from nontumor-derived cells of the same patients (Feinberg and Vogelstein, 1983). Another possible mechanism of ras oncogene activation was suggested by Cichutek and Duesberg (1986) who showed that in many human tumor DNAs a noncoding exon of c-H-ras is altered.

4. Studies of multistepped carcinogenesis by transfection or infection of ras oncogenes into mammalian cells.

Many studies on the multistepped nature of carcinogenesis have involved ras oncogenes. Some workers have transfected cloned ras oncogenes into cultured normal cells in attempts to create cancer cells in vitro. Most of these attempts have required that more than just an activated ras gene be transfected, or that the recipient cells already possess some attributes of a tumor-derived cell, such as an extension of the normally limited lifespan usually observed once the cells are cultured. Land et al. (1983) showed that transfection of finite lifespan, rat embryo fibroblasts with an activated H-ras oncogene resulted in morphological transformation, but with limited potential to grow in soft agar. The ability to grow in semisolid

medium such as soft agar is an attribute of tumor-derived cells and is often used as an assay to predict the malignant potential of a cell. Cotransfection of the T24-H-ras oncogene and either the v-myc or polyoma virus large-T antigen oncogenes into finite lifespan rat embryo fibroblasts resulted in the formation of transformed foci. When the cells from the foci were isolated, expanded in culture, and injected into athymic mice, they gave rise to tumors. The tumors induced by cells containing the cotransfected ras and myc oncogenes were benign and stopped growing before ever becoming sufficiently large to kill the mouse, whereas the tumors induced by the cotransfected ras and polyoma virus large-T antigen oncogenes formed a malignant tumor that killed the mouse. When infinite lifespan Rat-1 cells were cotransfected with T24-H-ras and v-myc oncogenes, foci composed of morphologically transformed cells were formed. When isolated, expanded in culture, and injected into nude mice they formed invasive malignant tumors.

Newbold and Overell (1983) transfected the c-H-ras oncogene into either normal finite lifespan Syrian hamster fibroblasts, as well as four infinite lifespan hamster fibroblast cell lines. Following transfection, transformed foci were observed against a confluent monolayer for each of the infinite lifespan cell lines, but no foci were evident in the normal diploid cell line.

Ruley (1983) found that the adenovirus early region-1A gene could cooperate with either the T24-H-ras oncogene or the polyoma virus middle-T gene to cause focus formation in finite lifespan baby rat kidney cells transfected in culture, whereas neither the T24-H-ras or polyoma virus middle-T genes caused any noticeable effects on their

own. Ruley et al. (1986) later showed that the E1A gene cooperated with T24-H-ras by a mechanism other than merely facilitating the acquisition of an infinite lifespan. This is because the T24-H-ras was not sufficient by itself to transform an infinite lifespan rat embryo fibroblast cell line, but did so when co-transfected with the E1A gene.

Parada et al. (1984) found that the gene coding for the p53-tumor antigen was also capable of cooperating with the EJ-H-ras oncogene in the tumorigenic transformation of transfected rat embryo fibroblasts. p53 is a protein that is expressed in high levels in many different tumor-derived cells.

Barrett's group found that transfection of Syrian hamster embryo cells with the v-H-ras oncogene caused morphological transformation, but that the cells were not immortal and senesced shortly after being isolated (Thomassen et al., 1985). However cotransfection of v-H-ras and the v-myc oncogene yielded transformed cell populations that formed progressively growing tumors after being injected into nude mice. On subsequent karyotypic analysis all tumors that were formed showed a consistent loss of chromosome 15, suggesting that loss of a possible suppressor gene was also necessary for malignant transformation (Oshimura et al., 1985). Support for this concept was given by Geiser et al. (1986) who showed that fusion of normal human fibroblasts with EJ-bladder carcinoma-derived cells caused suppression of tumorigenicity, even though expression of the c-Ha-ras oncogene was maintained.

Contrary to the reports that the activation of multiple protooncogenes are necessary for complete transformation of cells in culture, Spandidos and Wilkie (1984) found that transfection of early

passage Chinese hamster fibroblasts or rat embryo fibroblasts with ras genes cloned into high expression vectors was capable of causing either immortality or malignant transformation, depending on the ras gene used. When cells were transfected with the normal c-H-ras gene which had been cloned into the Homer plasmid, a plasmid designed to give high expression, clones with a normal morphology were obtained after drug selection. When these clones were isolated and propagated in culture some of the resulting populations acquired immortality. When rodent fibroblasts were transfected with the T24-H-ras oncogene which had been cloned into the Homer plasmid, morphologically transformed clones were obtained that gave rise to tumorigenic populations of cells. When these two H-ras genes were cloned into low expression vectors and then transfected into rodent fibroblasts, no transformation was observed under any circumstances. Pozzatti et al., (1986), also reported that malignant cells could be generated by transfection of early passage rat embryo fibroblasts with the cloned T24-H-ras oncogene.

5. Transformation of human cells in culture by ras oncogenes.

Various workers have utilized human cells as recipients for exogenous ras oncogenes, with limited success. Newbold et al. (1983) reported that finite lifespan human fibroblasts could not be transformed by transfection of the cloned T24-H-ras oncogene. Similarly, Sager et al. (1983) reported that diploid human fibroblasts were resistant to transformation after transfection of the EJ-H-ras oncogene, even though the transfected DNA was shown by Southern hybridization analysis to be incorporated intact into the genome. Sager (1986) later showed that even though the mutant form of the H-ras

oncogene product was being expressed in the transfected human cells, they still were not transformed.

Sutherland et al. (1985) found that the ability to grow in soft agar was conferred on normal human fibroblasts that were transfected with the cloned T24-H-ras oncogene. However these investigators did not analyze the recipient cells to ensure that the transfected plasmid had been integrated within the genome and was actually being expressed.

Feramisco et al. (1984) microinjected high levels of mutant H-ras protein into both rodent and human fibroblasts. Although the rodent fibroblasts exhibited a transformed morphology until the transient effects of the microinjected protein subsided, the human fibroblasts remained normal in morphology. Similarly, Marshall et al. (1983) observed no morphological alterations in normal human fibroblasts that had been transfected with the activated N-ras oncogene which was isolated from the HT1080 human fibrosarcoma cell line and cloned into the plasmid pSV2neo.

Recently Hurlin et al. (1987) working in this laboratory, observed morphological transformation, focus formation, and anchorage independent growth of diploid human fibroblasts by the transfected T24-H-ras oncogene which was cloned into the Homer high expression plasmid. Their results contradict those of Spandidos (1986) who reported that, in his hands, transfection of human fibroblasts with this construct had failed to cause detectable alterations. The Homer vector was designed to be a high expression vector, and Hurlin et al. showed that the levels of mutant H-ras protein expressed in the transformed cells were several fold higher than the levels of the endogenous ras proteins.

Sager et al. (1986) has also recently reported the transformation of SV40 virus immortalized human fibroblasts by the v-K-ras oncogene. However, this group found that non-established human fibroblasts infected with v-K-ras soon senesced.

Two groups have reported transformation of human-derived cultured epithelial cells following transfection. Yoakum et al. (1985) reported that v-H-ras was capable of causing malignant transformation of normal human bronchial epithelial cells, and Rhim et al. (1985) reported the v-K-ras induced malignant transformation of epidermal keratinocytes that had previously been immortalized by an adenovirus 12 - SV40 hybrid.

6. Activation of ras genes by treatment with known carcinogens.

To determine how protooncogenes become activated, several groups have studied the ability of known carcinogens to activate ras. Balmain and Pragnell (1983) used the well-developed mouse skin carcinoma model to study the role activation of oncogenes had in the several discrete stages that lead to tumor development. They found that as expected, sequential treatment of mouse skin by the initiator dimethylbenzanthracene and a promoting phorbol ester caused epidermal tumors (papillomas) at the site of application. Some of these became carcinomas. Transfection of NIH/3T3 cells with high molecular weight DNA isolated from the malignant tumors yielded transformed foci as a result of transfer of an activated c-H-ras oncogene. Later, Balmain et al. (1984) determined that the c-H-ras gene had become activated within the time interval between treatment and the early stage of the benign papilloma.

Eva and Aaronson (1983) found that the fibrosarcomas which arose by treating mice with the chemical carcinogen 3-methylcholanthrene contained a c-K-ras oncogene. That these were K-ras oncogenes was established by isolation and characterization of the transforming gene present in focus-derived populations of cells obtained after transfection of NIH/3T3 cells with genomic DNA from the fibrosarcomas.

Gullino et al. (1975) found that a single injection of methylnitrosourea into Buf/N rats was capable of causing mammary carcinomas in 90% of the animals within 60 days. Sukumar et al. (1983) used this model system to study oncogene activation and found that the c-H-ras gene was activated in each of nine mammary carcinomas induced by carcinogen treatment. In one tumor analyzed in detail they showed that a G to A transition caused glycine to be replaced by glutamine at amino acid 12 of the p21 protein. Later this same group analyzed the rest of the tumors and found that they all had this same mutation (Zarbl et al., 1985). These authors concluded that since methylnitrosourea is highly labile in vivo, and is known to cause G to A transitions greater than 99% of the time, it must have directly initiated the mutation within hours after being injected. They also showed that in tumors induced by dimethylbenzanthracene, a carcinogen that does not specifically induce G to A transitions, the c-H-ras oncogene was not mutated at the guanine in codon 12.

Guerrero et al. reported in 1984 that carcinogen induced mouse lymphomas contained genes capable of transforming transfected NIH/3T3 cells. Lymphomas induced by gamma-radiation contained an activated c-K-ras oncogene and those induced by the chemical carcinogen methylnitrosourea contained an activated N-ras oncogene. Guerrero et

al. (1984a, 1985) later showed that the ras genes were activated by somatic mutations, with c-K-ras at codon 12 resulting in substitution of glycine for aspartic acid, and N-ras at codon 61 resulting in substitution of lysine for glutamine.

After treatment of fetal guinea pig cells with 3-methylcholanthrene, benzo(a)pyrene, N-methyl-N'-nitroso-N-nitrosoguanidine, or diethylnitrosamine, transformed cells were selected by their ability to grow in soft agar, and genomic DNA extracted from each group was found to induce foci when transfected into NIH/3T3 cells (Sukumar et al., 1984). The transforming gene in cells derived from each of those foci was shown to belong to the ras gene family since each was detected by a probe derived from BALB-MSV.

Marshall et al. (1984) showed that reaction in vitro of the ultimate carcinogen benzo(a)pyrene diol-epoxide with the cloned c-H-ras1 proto-oncogene activated the ras gene to a form capable of causing transformation of transfected NIH/3T3 cells. Subsequently, this group used oligonucleotide probes which would detect specific point mutations in the 12th and 61st codons of c-H-ras1 to show that they were activated by point mutations after in vitro exposure to either benzo(a)pyrene diol-epoxide or N-acetoxy-2-acetylaminofluorene (Vousden et al., 1986). Benzo(a)pyrene predominantly caused G-C to T-A and A-T to T-A base substitutions, whereas N-acetoxy-2-acetylaminofluorene caused G-C to T-A substitutions.

C. Biochemical properties of *ras* proteins.

1. Structure and biochemical properties of the *ras* genes and their products.

The viral and cellular *ras* genes encode structurally related proteins (p21s) that have a molecular weight of approximately 21 kd (Shih et al., 1979) and consist of 188-189 amino acids (de Vos et al., 1988). The cellular *ras* genes are homologous to each other in sequence, and each has four coding exons (Chang et al., 1982). The highest degree of homology between the *ras* genes is found in the first coding exon with the amount of homology decreasing through the fourth exon (Brown et al., 1984) which codes for the carboxyl-terminal region of the protein (Barbacid, 1987). In addition, genes quite homologous to mammalian and viral *ras* genes have been found in species as diverse as *Drosophila melanogaster* (Shilo and Weinberg, 1981) and *Saccharomyces cerevisiae* (DeFeo-Jones et al., 1983). Such widespread conservation among quite divergent species suggests that a critical role for *ras* proteins exists, which is essential for viability of the organism.

Monoclonal antibodies were developed against the *ras* p21s (Furth et al., 1982) and these antibodies have been used to help elucidate the biochemical properties of the *ras* proteins. By conjugating these antibodies to fluorescent dyes, researchers determined by immunocytochemistry that *ras* p21s were predominantly localized to the inner surface of the cytoplasmic membrane (Furth et al., 1982). This work confirmed the observations of earlier workers who, using antisera obtained from tumor-bearing rats that had been infected with Harvey MSV, also used immunofluorescence microscopy to determine the location

of the p21s (Willingham et al., 1980).

Scolnick et al. (1979) found by adding radioactive guanine to extracts of cells that were transformed by Kirsten or Harvey sarcoma virus, that viral p21s bind guanine nucleotides. Shih et al. (1980) then showed that the ras oncogene of Harvey MSV had an autophosphorylating activity at threonine 59, and used guanine nucleotides as the phosphoryl donors. Papageorge et al. (1982) next compared cellular and viral ras p21s and found that both were synthesized as precursor proteins which became localized to the inner plasma membrane, that cellular ras p21s also bound guanine nucleotides, but that cellular ras p21s did not exhibit an autophosphorylation on threonine as did the viral p21s.

Sefton et al. (1982) reported that the ras p21 of Harvey MSV was posttranslationally acylated and contained tightly bound lipid. These authors proposed that localization of ras p21s to the inner cellular membrane was due to such acylation. Buss and Sefton (1986) later determined that the lipid attached to p21 was palmitic acid. Finkel et al. (1984) investigated the biochemical properties of the products of the ras genes from normal or tumor-derived human cells. These authors found that the subcellular localization, posttranslational acylation, and affinity for binding guanine nucleotides were the same for the normal and oncogenic cellular ras proteins. Shortly thereafter, McGrath et al. (1984) reported that the ras p21 encoded by the T24-H-ras gene, a p21 which is activated by a single amino acid substitution at position 12, displays an impaired ability to hydrolyze bound GTP compared to the normal ras p21. These authors speculated that this impaired ability to hydrolyze GTP played a crucial role in the process

of transformation. They further speculated that ras p21s behaved analogously to the G proteins which regulate adenylate cyclase, and which were involved in the transduction of information across the plasma membrane. The G proteins of adenylate cyclase are in an active state when they are bound to GTP, and are subsequently inactivated upon hydrolysis of the GTP (Gilman, 1984). If normal ras p21s are activated by a similar mechanism, then an amino acid substitution in a ras protein which inhibited GTP hydrolysis might constitutively activate the p21. Gibbs et al. (1984) confirmed the above findings of McGrath et al. (1984) by using highly purified p21s isolated after expression of normal and transforming c-H-ras genes in Escherichia coli.

To further assess the biochemical properties of the ras p21s, Ulsh and Shih (1984) measured the metabolic turnover of the viral p21s, and both normal and transforming cellular p21s. Both of the cellular p21s as well as the nonphosphorylated form of the viral p21 were found to have a half-life of 20 hours. However, the half-life of the phosphorylated viral p21 increased to 42 hours, and the authors proposed that this increased stability may effect the increased oncogenicity of viral p21s compared to the cellular ras p21s.

To study the relevance of certain biochemical features of ras p21s, Willumsen et al. (1984) constructed a series of deletion mutants of v-H-ras that coded for p21s which were altered near their C-terminus. This group found that the amino acids located at or near the C-terminus of ras p21s were required for membrane association, lipid binding, and transformation.

Seeburg et al. (1984) determined that an alpha-helical structure was essential in the region near amino acid 12 of the cellular H-ras

p21 for normal function of the protein. Using in vitro mutagenesis to construct 20 different mutant c-H-*ras* genes which included every possible amino acid at position 12, they found that all amino acids except glycine (which is the encoded by the normal c-H-*ras* gene) and proline activated the p21 to a transforming protein. Since proline interrupts the alpha helical structure of peptides its inability to activate *ras* is perhaps not surprising. When H-*ras* p21s of tumor-derived cells have been analyzed and found to contain substituted amino acids at position 12, these substitutions have only required one nucleotide change in the codon. This is most likely because the other amino acids which would activate the p21 would require two nucleotide changes within one codon, and this would be a very rare event.

Evidence that *ras* p21s were involved in normal growth and development was obtained from work using *S. cerevisiae*. De Feo-Jones et al. (1983) found and isolated from this yeast, genes named *ras*1 and *ras*2 that were very homologous to cellular *ras* genes of eucaryotes. Papageorge et al. (1984) subsequently isolated proteins from *S. cerevisiae* that were 30K in size which were quite homologous to mammalian *ras* p21s, and were immunoprecipitated with a monoclonal antibody against a mammalian *ras* p21. Toda et al. (1985) found that some *ras* function was essential for the continued growth and viability of the yeast. These authors also found, by using *ras* genes with missense mutations, that the *ras* proteins controlled adenylate cyclase in *S. cerevisiae*, functioning analogously to the G proteins described above. DeFeo-Jones et al. (1985) showed that mammalian-derived *ras* genes could function to replace *ras* genes in *S. cerevisiae*. In addition, a deletion mutant yeast *ras*1 gene, when transfected into and

expressed in NIH/3T3 cells, was shown to transform them.

McCormick et al. (1985) developed a model for the tertiary structure of p21s to help visualize how the proteins functioned and how they might be activated by oncogenic mutations. EF-Tu, a bacterial elongation factor, was found to be 42 percent homologous to mammalian ras p21s (Halliday, 1984). McCormick's group based their model on the tertiary structure that had been determined for EF-Tu by x-ray crystallography (la Cour et al., 1985), together with supporting biochemical, immunological, and genetic data. The model predicted that p21 was made of a central core with a beta-pleated sheet structure, which was connected by loops and alpha-helices. The authors determined that the guanine nucleotide binding site was comprised from four of the loops, that the phosphoryl binding region was made up of amino acid 10 to 16 and 57 to 63, and that specificity for guanine binding was determined by amino acids Asn-116 and Asp-119.

More recently, the crystal structure of the normal human c-H-ras p21 was determined by x-ray diffraction analysis (De Vos et al., 1988). The structure agrees for the most part with that predicted by McCormick et al. (1985), and provides many details of the structure that were not previously known. The ras protein consists of a six-stranded beta sheet, four alpha helices, and nine connecting loops. Four of the loops were found to be involved in interactions with bound guanine nucleotides, and most of the transforming p21s were found to have single amino acid substitutions at one of a few key positions in three of the four loops.

2. The role of ras proteins in transmembrane signaling.

Much work has been carried out to determine what role, if any, that ras proteins have in the process of cellular division. In culture, mammalian cells initiate DNA synthesis in response to a variety of peptide growth factors, including epidermal growth factor (Carpenter and Cohen, 1976), platelet-derived growth factor (Robbins et al., 1983), and others, depending on the cell type and the species from which it was derived. These growth factors bind to specific receptors located on the external surface of the plasma membrane. Binding of the growth factor to its receptor triggers a cascade of biochemical events ultimately resulting in cell division. Some of the details of this cascade have been elucidated, however just how the ras p21s interact in this pathway is still a matter of speculation. One of the earliest events known to occur upon binding of some growth factors to the membrane is phospholipase-C catalyzed hydrolysis of phosphatidylinositol 4,5-bis-phosphate (PIP_2), a minor component of some lipids in the plasma membrane (Berridge, 1984). Of the two major hydrolysis products of PIP_2, 1,2-diacylglycerol (DAG), synergizes with calcium ions to activate protein kinase-C (PKC) (Kikkawa et al., 1983), and inositol 1,4,5-triphosphate (IP_3) causes release of calcium from intracellular stores (Batty et al., 1985). PKC is considered to be a key regulatory enzyme, the activation of which causes a myriad of biological phenomena, including phosphorylation of many cellular macromolecules (Nishizuka, 1980; Connolly et al., 1986; Gould et al, 1986), and the secretion of numerous diverse peptides (Nishizuka, 1986). The events necessary for the initiation of DNA synthesis which occur subsequent to PKC activation are not clear. In many cell types

the cellular myc and fos genes are transiently expressed within several hours of growth factor binding (McCaffrey et al., 1987). In addition, a membrane bound Na/H^+ antiporter is soon activated which causes cytoplasmic alkalinization, an event considered critical for DNA replication (Hesketh et al., 1985).

One of the first reports of interactions between ras p21s and a growth factor was by Kamata and Feramisco (1984) who showed that EGF stimulated the ability of ras p21s to bind guanine nucleotides, and also stimulated the autophosphorylation of the p21 encoded by v-H-ras. Feramisco et al. (1984) found that microinjection of the ras p21 encoded by T24-H-ras into quiescent cells caused morphological transformation and rapid cellular proliferation. These authors claimed that this effect was observed in immortal cells, but was not observed in normal human fibroblasts nor when the protooncogenic ras p21s were used. Mulcahy et al. (1985) found by microinjection of anti-ras antibodies into NIH/3T3 cells, that the protooncogenic ras p21s were required for serum stimulated growth of these cells.

Fleischman et al. (1986) reported that compared to nontransformed cells, ras-transformed rodent fibroblasts showed over a 2.5-fold higher ratio of DAG to PIP_2. These authors suggested that the ras p21 in ras-transformed cells affected the levels of PIP_2 through regulation of the enzyme phospholipase-C. Support for the concept that ras p21s were regulating phospholipase-C was given by the results of Wakelam et al. (1986) who showed that nontransforming N-ras p21 was capable of coupling several growth factors to hydrolysis of PIP_2. By ligating the N-ras gene to a dexamethasone-inducible promoter, these authors showed that induction of N-ras expression caused enhanced PIP_2 hydrolysis in

response to added growth factors. Further support for the concept that ras p21s regulated phospholipase-C was given by Lacal et al. (1987) who found that microinjection of H-ras p21 into Xenopus oocytes stimulated DAG production.

There is also evidence that transforming ras p21s can cause production of some growth factors by transformed cells. Spandidos (1985) measured the anchorage independent growth of normal rat kidney cells as an assay to determine the presence of transforming growth factors in conditioned medium collected from Chinese hamster lung fibroblasts transformed by H-ras and N-ras oncogenes. He found that cells transformed by ras secreted high levels of transforming growth factors if the ras genes were expressed at high levels in the transfected cells. Later, Dickson et al. (1987) reported that transfection of the v-H-ras oncogene into the MCF-7 human breast cancer cell line induced production of transforming growth factor-alpha, transforming growth factor-beta, and insulin-like growth factor. Similarly, Durkin and Whitfield (1987) concluded that v-K-ras caused production and secretion of PDGF. These authors found that proliferation of normal rat kidney fibroblasts that were transformed by a temperature-sensitive mutant of v-K-ras was inhibited at the permissive temperature by protamine sulfate, a compound which is reputed to specifically block PDGF action.

In summary, the aberrant control of cellular division, a key feature of transformation, often appears to be attributable to inappropriate expression of ras proteins. Although the mechanism by which ras proteins transform cells is unknown, they are thought to act on an effector molecule, with hydrolysis of bound-GTP attenuating

their action (Gibbs et al., 1984). The nature of this second molecule is speculated upon by Trahey and McCormick (1987), who discovered a cytoplasmic protein in Xenopus oocytes which appeared to cause an increased GTPase activity for normal ras p21s, but had no effect on the ability of ras proteins which bore activating amino acid substitutions to hydrolyze GTP.

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CHAPTER II

STABLE TRANSFORMATION OF NORMAL DIPLOID HUMAN FIBROBLASTS BY A TRANSFECTED N-RAS ONCOGENE

Daniel M. Wilson, Dennis G. Fry, Veronica M. Maher,
and J. Justin McCormick

Carcinogenesis Laboratory - Fee Hall
Department of Microbiology and Department of Biochemistry
Michigan State University, East Lansing, MI 48824-1316

Abstract

Early passage diploid human fibroblasts were transfected with a N-ras oncogene isolated from human leukemia cell line 8402 cloned into a high expression plasmid, pSV N-ras, or with the N-ras oncogene from human fibrosarcoma cell line HT1080 cloned into pNR-MG1 with transcription driven from the endogenous promoter. When the transfected cells were selected for Geneticin resistance, 70% of the colonies formed after transfection with pSV N-ras consisted of very morphologically altered cells. A much lower frequency of the drug resistant colonies formed after transfection with pNR-MG1 had altered morphology and the alterations were much less distinct. If the population of transfected cells was not selected for Geneticin resistance, but was simply allowed to grow to confluence, very distinct foci could be seen against a contact-inhibited monolayer with the pSV N-ras-transfected population, but foci formed by the pNR-MG1 population were subtle and indistinct. The majority of the morphologically transformed colonies from the pSV N-ras plasmid either reverted to a normal phenotype or senesced prematurely; those from the pNR-MG1 plasmid exhibited a normal lifespan, but reverted to a normal morphology soon after being isolated. However, progeny cells from two of the pSV N-ras colonies did not senesce prematurely and maintained their transformed morphology stably. These cell strains exhibited anchorage independence and formed distinct foci. DNA-DNA hybridization analysis confirmed the presence of new N-ras specific DNA sequences and immunoprecipitation analysis showed that the cells produced much larger amounts of N-ras protein than did the age-matched control

cells. However, these transformed cells did not exhibit an infinite lifespan in culture nor the ability to form tumors in athymic mice.

Introduction

Epidemiological evidence indicates that most human cancer is the result of carcinogen exposure (1). However, by their very nature, such studies cannot provide direct insight into the mechanisms by which human cells become malignantly transformed. The strength of cell culture techniques is precisely their usefulness in studying mechanisms. It is for this reason that we and our associates (2-5), as well as other workers (6-9), have been studying the changes involved in the transformation of human fibroblasts in culture (see ref. 10 for review). These cells are ideal for such studies since thay grow well in culture and do not spontaneously transform. In humans, fibroblasts are found in all organs but are not the major cell type of any organ. Most imporantly, fibroblastic tumors (fibrosarcomas) arise in humans, demonstrating that fibroblasts do become malignantly transformed. A further advantage of using fibroblasts for such studies is that there is an extensive body of literature on the transformation of rodent fibroblasts in culture which can serve as a reference.

Recent evidence indicates that there is no reproducible system for transforming diploid human fibroblasts into malignant cells by carcinogen treatment (10). One explanation for the lack of success in attempting to transform such cells could be that investigators have not been able to recognize the phenotypes of cells that are intermediates in the process of malignant cell transformation. This explanation is supported by the fact that, unlike foci formed by carcinogen-treated rodent fibroblast cell lines, the foci formed by carcinogen-treated human fibroblasts are subtle and indistinct (11). Our approach to this problem has been to transfect diploid human fibroblasts with oncogenes

known to be altered and/or expressed in human fibrosarcomas (3-5), examine the phenotypic alterations and characteristics induced and determine the number and kinds of changes involved in the malignant transformation of such cells. In the present study, we transfected diploid human fibroblasts with plasmids containing N-ras oncogenes isolated from human tumors, i.e., pNR-MG1 which contains the N-ras from fibrosarcoma cell line HT1080,which is mutated in codon 61 (12), and pSV N-neo which contains the N-ras oncogene from leukemia cell line 8402 mutated in codon 12 (13). The results showed that mutant N-ras expressed at high levels can cause the transformation of diploid human fibroblasts to morphologically-altered, focus-forming cells which exhibit several of the characteristics of tumor-derived cells, but which still possess a normal lifespan in culture and do not form tumors in mice.

Materials and Methods

Cells and culture medium

Normal diploid human fibroblast cultures were derived from neonatal foreskins as described (14). The cells were routinely grown in Eagle's minimal essential medium supplemented with 0.2 mM L-serine, 0.2 mM L-aspartate, 1.0 mM sodium pyruvate, 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY), penicillin (100 units/ml), and streptomycin (100 ug/ml) (culture medium). The cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Plasmids

The pSV N-*ras* plasmid containing an N-*ras* oncogene and the *neo* gene coding for Geneticin resistance, was provided by Dr. E. Fleissner of Sloan-Kettering Memorial Cancer Center. The pNR-MG1, plasmid which contains an N-*ras* oncogene and the *neo* gene, was obtained from Dr. D. Spandidos. The pSV2*neo* plasmid used as a control contains the *neo* gene coding for Geneticin resistance, but no oncogene. The p6A1 plasmid, which contains exons 2,3, and 4 of the human c-N-*ras* gene was used for preparing probe and was obtained from the American Type Tissue Collection (Rockville, MD).

DNA transfection and Geneticin selection

Fibroblasts were transfected using the dimethylsulfoxide/Polybrene method adapted for use with human fibroblasts (15). Cells were plated at 1-2 x 10^5 cells per 100 mm-diameter dish and transfected 24 h later with 1-10 ug of plasmid DNA. If cells were to be selected for resistance to Geneticin (Grand Island Biological Co.), 200 ug active Geneticin per ml medium was added the next day. The cells were refed with selective medium after 1 wk and the macroscopic drug resistant

colonies that developed two wk post-transfection were examined for morphological alterations. The majority were isolated and propagated in selective medium for further characterization.

Focus assay

Transfected cells were allowed to grow to confluence. The medium was replaced every 7 days and the dishes were examined for foci formation.

Anchorage independence

Growth in soft agar was assayed essentially as described (11). 2500 cells in 1.5 ml of top agar composed of Ham's F10 medium with 2.2 g/l $NaHCO_3$, supplemented with 12% fetal bovine serum and antibiotics, and containing 0.33% Noble agar (Difco, Detroit) was plated into 60 mm diameter dishes containing 3 ml of solidified bottom agar. The bottom agar was prepared with this same medium but with 2% agar. The frequency of colonies greater than 40 um in diameter, i.e., composed of 50 or more cells, was determined by light microscopy after 4 weeks. At the time cells were plated in agar, an aliquot of the cell suspension that was to be used as the top agar layer was also appropriately diluted with agar-free culture medium and plated at cloning density to determine the cloning efficiency of the cells that were being assayed in soft agar. This value was used to compare the viability of the cells used in the assay.

Lifespan analysis and assay for tumorigenicity

These two parameters related to cellular transformation were assayed using the procedures described previously (3).

Immunopreciptation of p21 ras

The amount of p21 ras protein was assayed using either of two methods modeled after the method described by Furth et al. (16). With the first method 1 x 10^6 cells were grown for 24 hr in the presence of [^{35}S]-methionine (800 uCi in 3 ml of medium). The cells were lysed in Staph A buffer (0.2 M phosphate buffer, pH 7.4, 1% TRITON-X 100, 0.1% SDS, 0.1% NaN_3, 0.1 M NaCl 12 mM sodium deoxycholate) containing 2 mM phenylmethyl-sulfonyl fluoride and 100 Kallekrein inactivator units of apoprotein, and were centrifuged for 30 min at 35,000 rpm at 4^{o}C. Cellular extracts containing 1 x 10^8 trichloracetic-acid-precipitable cpm were incubated with 10 ul of antibody v-H-ras (Ab-1) or v-H-ras (Ab-2) (Oncogene Science Inc., Manhasset, NY) for 2 h at 4^{o}C on a shaker. Protein-A sepharose (Pharmacia, Piscataway, NJ) was coated with anti-rat IgG (Cooper Biomedical, Malvern, PA) following the manufacturer's instructions and 200 ul of this suspension was added to the incubation mix and allowed to react for 2 h at 4^{o}C. The samples were microfuged for one min, and the pellet was washed twice with 1 ml of Staph A buffer. The pellet was incubated for 10 min with 1 ml 50 mM Tris, pH 7.5, 1 M $MgCl_2$, microfuged one min, and washed with 1 ml of Staph A buffer. The pellet was resuspended in 50 ul of 90 mM Tris buffer, pH 6.8, containing 21.8% glycerol, 0.14% SDS, 0.014% Bromphenol blue, 8.6% beta-mercaptoethanol and heated for 5 min at 95^{o}C. Gel electrophoresis was performed on 1.0 mm thick 12% gels (16 cm x 16 cm) using a Tris-glycine buffer system (17). After electrophoresis the gels were fixed in methanol:acetic acid:water (5:2:5), rinsed in Enlightning solution (NEN Research Products, Boston, MA), dried and analyzed by autoradiography.

The second method differed from the first in the following steps. Cells (9×10^5) were metabolically labeled for 20-24 h in 4 ml of McM medium (20) containing hydrocortisone (10 ug/ml) and 750 uCi Tran[^{35}S]-label (ICN Biomedicals, Costa Mesa, CA). Gel electrophoresis was performed on 0.75 mm thick (16 cm x 16 cm) 14% gels. After electrophoresis the gels were soaked in RESOLUTION solution (EM Corp., Chestnut Hill, MA) for 0.5 h, soaked in distilled ice cold water for 1 h, then dried and analyzed by autoradiography.

Results

Morphological transformation by N-ras

To determine if the N-ras oncogene can cause transformation of normal diploid fibroblasts, we transfected several early-passage foreskin-derived cell lines using pSV N-ras and pNR-MG1. pSV N-ras is a high expression vector containing two Maloney leukemia virus long terminal repeats (LTR's). The cellular promoter of the N-ras oncogene has been eliminated and the gene inserted between an LTR and the neo gene coding for Geneticin resistance (13). Since transcription initiated at the first LTR must transcribe the N-ras gene before transcribing the neo gene, the likelihood that cells selected for Geneticin resistance will also express the N-ras oncogene is very high. pNR-MG1 contains the N-ras oncogene from HT1080 cells with its endogenous promoter and also the neo gene which is transcribed separately from an SV-40 promoter (12). As a control, the cells were transfected with pSV2neo which lacks an oncogene. The target population was selected for resistance to Geneticin to determine the frequency of transfection and the drug resistant colonies were then examined for evidence of morphological transformation and those that appeared to be morphologically altered were isolated and propagated for further study. Figure 1 shows a typical example of the morphologically altered cells which developed following transfection with pSV N-ras. The majority of the cells were multinucleated, vacuolated, and of highly irregular shape, and the cells did not exhibit the oriented growth pattern characteristic of normal human fibroblasts.

When cells were transfected with the pNR-MG1 plasmid, the frequency of Geneticin resistant transfectants was 10-fold higher than with the

Figure 1. Morphology of normal fibroblasts (A) and pSV N-*ras* transformed fibroblasts (B). Cells stained with crystal violet. Magnification 40X.

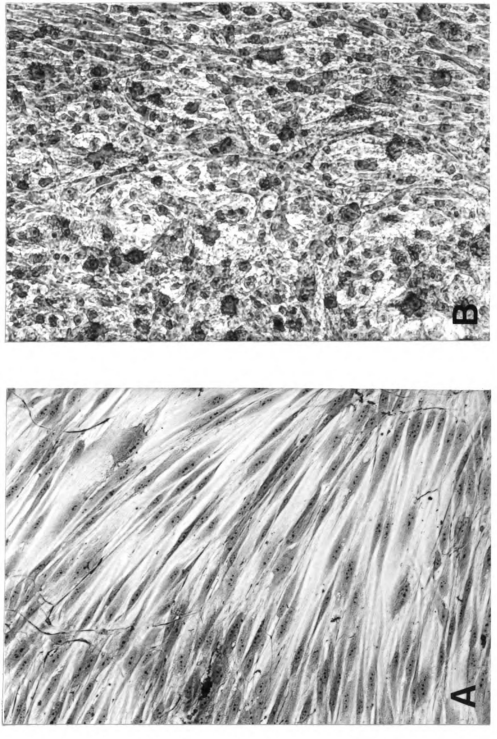

Figure 1

pSV N-ras, i.e., 225 per 10^6 cells treated, but the majority of the colonies had a normal morphology and the changes in those that appeared to be morphologically altered were very much less distinct than those transformed with pSV N-ras (data not shown). In contrast to the results with pNR-MG1, at least 70% of the drug-resistant colonies formed following transfection with pSV N-ras exhibited morphological transformation. This is consistent with the fact that for transcription of the neo gene in that plasmid to occur, it must be initiated from the LTR which preceeds the N-ras oncogene.

Transformation of human fibroblasts to focus formation

The ability of the N-ras oncogene to transform human fibroblasts into focus-forming cells was also assayed. Cells were plated into a series of 100 mm-diameter dishes and 24 h later were transfected with pSV N-ras, pNR-MG1, or pSV2neo. In these experiments the target population was not selected for Geneticin resistance, but assayed directly for focus formation by being allowed to grow to confluence with weekly refeeding. Within two weeks post-transfection, very distinct, dense foci could be observed on a monolayer of contact-inhibited fibroblasts that had been transfected with pSV N-ras (Fig. 2) at a frequency of 15 per 10^6 treated cells. By day 10 post-transfection, foci could also be seen on the lawn of cells transfected with pNR-MG1 at a frequency of 30 per 10^6 cells, but these were very much less distinct and by 2 wk they were often indistinguishable from the confluent background. No foci were ever observed in dishes containing cells transfected with the control plasmid.

Figure 2. Foci of human fibroblasts following transfection with pSV N-*ras*. (a) Cells transfected with pSV N-*ras* (b) cells transfected with pSV2*neo*. Cells (2×10^5) were plated into 100 mm-diameter dishes and after 24 h transfected with pSV N-*ras* or pSV2*neo*. They were fed with culture medium following transfection and allowed to grow to confluence with one refeeding. The dishes were stained with crystal violet 2 wk after plating cells.

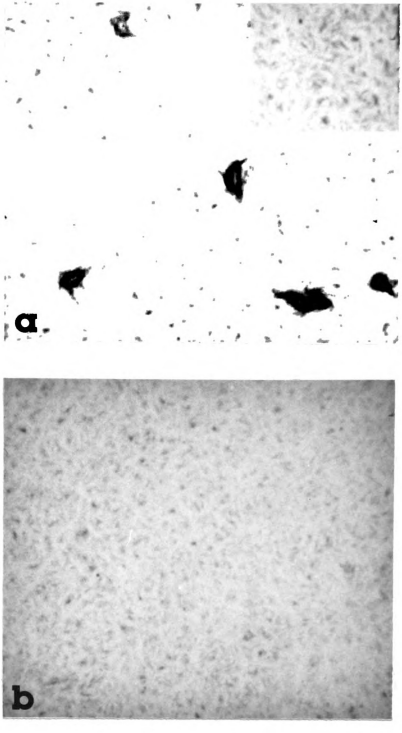

Figure 2

Biological characterization of the cells from morphologically transformed colonies

A large number of the morphologically transformed, Geneticin-resistant colonies derived by transfection with the N-ras plasmid, as well as age-matched, Geneticin-resistant colonies of control cells with normal morphology derived with pSV2neo, were isolated and propagated in selective medium. Similarly, cells were isolated from a number of foci which developed on top of a confluent lawn of cells transfected with pNR-MR1. These were propagated in non-selective medium.

Within 5 to 10 generations after being isolated, the majority of the morphologically transformed cells derived from populations transfected with pSV N-ras either reverted to a normal phenotype or senesced. Age-matched control colonies which had been obtained by transfection with the pSV2neo plasmid and morphologically transformed cells derived from transfection with the pNR-MG1 plasmid did not senesce prematurely, but the latter cells reverted to a normal morphology within 5 to 10 generations. However, progeny cells from two of the colonies formed after transfection with pSV N-ras did not senescence prematurely and maintained their transformed morphology stably throughout a normal lifetime in culture. Therefore, their progeny, designated cell strain 1 and 2, were used for further biological and biochemical characterization. Two independent normal age-matched cell lines derived from drug-resistant colonies formed after transfection of the same diploid fibroblast cell line (designated LG1) with the pSV2neo plasmid were selected as controls for these characterizations.

Using a reconstruction assay, we investigated whether the Geneticin-resistant, morphologically transformed cells from cell strain 1 could form foci on a background of normal cells. A total of 200 or 1,000 transformed cells were seeded into 60 mm-diameter dishes along with 1,000 control cells and the populations were allowed to replicate. They were fed with fresh culture medium after three days and four days later the medium was exchanged for medium in which the serum level had been reduced from 10% to 2.5%. The cells were refed weekly with this latter medium and were stained after three weeks and examined for foci. Dense foci could be seen in the dishes seeded with the morphologically transformed cells (Fig. 3). No foci were observed in the dishes seeded only with control cells.

The two stable cell strains and their age-matched control cell strains were also assayed for anchorage independence. The morphologically transformed cell strains formed colonies in agar at a 16-fold higher frequency than the control cells, even though their ability to form colonies on plastic was 4 to 5 times lower than the control.

Evidence of expression of the N-ras oncogene

To assay for the presence of N-ras oncogene-encoded protein, we used v-H-ras (Ab-1) which in human cells will precipitate H-ras, K-ras, and N-ras p21s, and v-H-ras (Ab-2), which will precipitate human H-ras and K-ras p21s, but not human N-ras p21 (18). Since neither antibody specifically recognizes the N-ras p21 in human cells, a method was devised to obtain an immunoprecipitate containing N-ras p21, but little or no K-ras or H-ras proteins and was used to analyze N-ras transformed cell strain 1 and its age-matched control. To remove the

Figure 3. Reconstruction assay for focus formation. Dishes a-d were seeded with 10^3 pSV2*neo* transfected age-matched control cells. A total of 200 N-*ras* transformed cells (strain 1) were added to dish b and 1000 were added to dish d. The cells were allowed to grow and were stained after 3 wk.

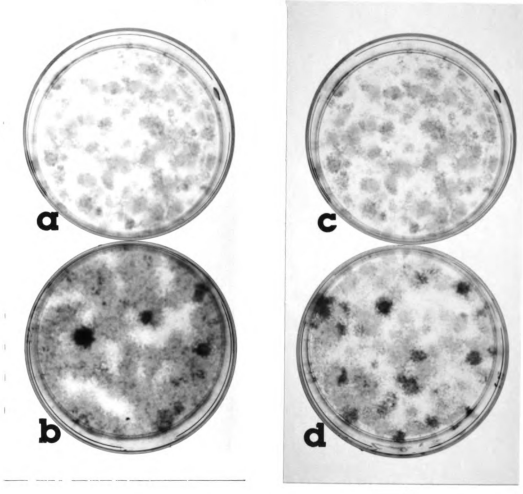

Figure 3

H-ras or K-ras p21s, labeled cell extracts were reacted twice with v-H-ras (Ab-2). The extracts were then reacted with v-H-ras (Ab-1) which should precipitate only N-ras p21 since the H-ras and K-ras p21s had been eliminated. As shown in Fig. 4 (lane b) pSV N-ras-transformed cell strain 1 contained a high amount of this material, but its control cell strain (lane a) had very little. A further indication that the band shown in lane b represents N-ras p21 is the fact that when an equivalent sample of cellular extract was reacted twice with v-H-ras (Ab-2) and was then analyzed, it did not yield any ras p21 bands (data not shown).

To detect expression of the transfected N-ras oncogene in stable cell strain 2, a second approach was used. Extracts from cell strain 2 and its age-matched control were precipitated with v-H-ras (Ab-1) which reacts with all 3 ras p21s. As shown in Figure 4, the transformed cells (lane c) contained much more total p21 protein than the control (lane e). A second set of cellular extracts was precipitated using v-H-ras (Ab-2), which in human cells only recognizes H-ras and K-ras. This time there was no difference in the level of p21 between the transformed cells (lane d) and the control (lane f).

Other parameters related to transformation

The lifespan in culture of the two cell lines stably transformed by pSV N-ras was assayed and found not to be significantly different from that of the parallel, age-matched pSV2-neo-derived control cells. Injection into X-irradiated athymic mice of 1-2 x 10^6 N-ras transformed cells along with 9 x 10^6 control cells did not yield any tumors. The injected animals were examined for tumors for more than nine months.

Figure 4. Immunoprecipitation evidence of N-ras oncogene expression. Cell extracts from [^{35}S]-methionine-labeled N-ras transformed cell strains 1 and 2 and their corresponding control strains were immunoprecipitated and analyzed by SDS/PAGE. Method one described in Materials and Methods was used for cell strain 1 and its control; method two for cell strain 2 and its control. Extracts from cell strain 1 (lane b) and its control (lane a) were precipitated twice with v-H-ras (Ab-2) followed by v-H-ras (Ab-1) in order to obtain N-ras p21 specifically (see text). Extracts from cell strain 2 (lane c) and its control (lane e) were precipitated with v-H-ras (Ab-1) in order to obtain all 3 ras p21s. Extracts of transformed cell strain 2 (lane d) and its control (lane f) were precipitated with v-H-ras (Ab-2) to obtain only H-ras and K-ras (see text).

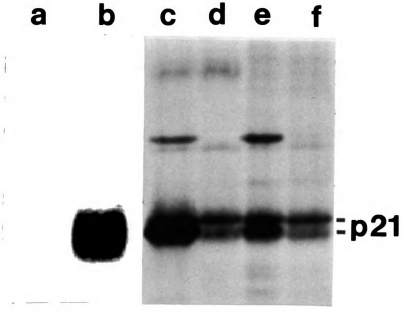

Figure 4

Discussion

Our results suggest that the level of expression of the N-*ras* oncogene is critical for transformation of diploid human fibroblasts in culture. Although we only measured the level of expression of N-*ras* in the transformed cells that remained stably transformed, not in those that reverted to a normal morphology soon after being isolated, cells transfected with an N-*ras* oncogene cloned into pSV N-*ras* gave distinct foci and marked morphological alterations, whereas those transfected with an N-*ras* oncogene cloned into pNR-MG1 gave foci that were much less distinct and composed of cells with subtle morphological changes. The pSV N-*ras* plasmid was designed to give high expression of N-*ras* by replacing the endogenous promoter of the cellular oncogene with a Maloney murine leukemia virus LTR (13); pNR-MG1, on the other hand, contains an N-*ras* oncogene transcribed from its own promoter. Insufficient expression may also explain why Marshall (21) failed to observe transformation following transfection of human fibroblasts with the N-*ras* oncogene from HT1080 cells inserted into the plasmid pSV2neo, in which transcription of the N-*ras* oncogene is initiated from an SV40 promoter. Sager *et al*. (6), using the EJ H-*ras* oncogene cloned into the same plasmid, pSV2neo, also failed to observe any evidence of transformation of finite lifespan human fibroblasts. Dennis Fry, of this laboratory, found that focus formation in human fibroblasts transfected with a v-*sis* oncogene was 50- to 100- fold less frequent if *sis* was transcribed from an SV40 promoter than if it was transcribed from the simian virus-LTR (D. G. Fry, unpublished observations). Hurlin *et al* (4), on the other hand, showed that the EJ H-*ras* oncogene, if cloned into a high expression vector, could transform such cells

into morphologically-altered, focus-forming, anchorage independent cells which still exhibited a finite lifspan in culture and were not tumorigenic.

Our data also support the hypothesis that high expression of transfected ras oncogenes can cause premature senescence of cells in culture. This is because the transformed colonies we obtained after transfection with pSV N-ras almost always underwent crisis within 10 generations of being isolated, whereas, the transformed colonies we isolated after transfection with pNR-MG1 did not senesce prematurely. Similar results were observed by Land et al. (22) who reported that transfection of primary rat embryo fibroblasts with cloned ras oncogenes yielded transformed colonies which as a rule senesced prematurely. In addition, Ruley et al (23) reported that transfection of baby rat kidney cells with highly expressed ras oncogenes almost invariably resulted in rapid senescence in the transformed colonies.

Many of the transformed cells obtained after transfection by either pSV N-ras or pNR-MG1 reverted to a normal morphology. Reversion of transfected cells to their prior phenotypes shortly after being subcultured is a common phenomenon, but the explanation for this reversion is not known. Hurlin et al. (4) who observed this with diploid human fibroblasts transfromed by the T-24 H-ras oncogene, showed that the transfected oncogene was still present in the genome of the revertants, but that they no longer expressed the T-24 p21. One possible explanation for loss of expression of the transfected oncogene is expression is down regulated by methylation of the gene within the cell. Support for this hypothesis is given by the studies of Rinehart et al. (22) who reported that human endometrial cells transfected with

ras and oncogenes were tumorigenic only after treatment of the transfected cells with 5-azacytidine, an agent which interupts the ability of a cell to methylate cytosines (23).

A possible explanation for how N-ras oncogene p21s cause focus formation is that an enhanced mitogenic signal is being generated either in response to serum supplied growth factors or by the production of growth factors. Fry et al. (3) found that human fibroblasts also form foci when transfected with pSSVneo, which contains the Simian sarcoma provirus carrying the v-sis oncogene coding for a protein homologous to the B chain of human PDGF (24). Unlike normal human fibroblasts, pSSVneo-transfected focus-derived cells express v-sis RNA and grow in the absence of exogenous protein growth factors (3). Other groups have reported that ras transformed rodent fibroblasts produce growth factors such as PDGF (25), TGF-alpha (12), and others (26).

Another possible explanation for focus formation by the N-ras oncogene is that its p21's enable the transformed cells to respond better to growth factors already present in serum. Numerous reports indicate that ras proteins interact in the signaling which occurs in response to mitogens, and suggest that oncogenic ras proteins constitutively activate phospholipase-C, a key enzyme in one signal pathway (27-29). If a major effect of the oncogenic N-ras protein is enhanced generation of a mitogenic signal, than this could also explain why the two stably transformed cell strains we obtained exhibited anchorage independent growth. Palmer et al. (30) has reported that PDGF induces anchorage independent growth of human fibroblasts when the medium is supplemented with growth factor-inactivated serum. In

addition, Stevens et al. (31) found that transfection of diploid human fibroblasts with a highly expressed human c-sis gene was capable of causing them to exhibit anchorage independent growth.

The fact that production of high levels of N-ras oncogene protein was insufficient to transform human fibroblasts in culture into tumorigenic cells supports the hypothesis that carcinogenesis is a multi-stepped process. Our findings agree with those of Weinberg's group (32) and others (33-35) who showed that transfection of a second oncogene, such as myc, was needed if finite lifespan rodent cells were to be transformed to immortality and tumorigenicity by H-ras. As discussed above, Hurlin et al. (4) also showed that transfection of finite lifespan, diploid human fibroblasts with the T24 H-ras oncogene cloned into a high expression vector conferred on the cells some transformed characteristics, but not tumorigenicity.

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CHAPTER III

MALIGNANT TRANSFORMATION OF INFINITE LIFESPAN, HUMAN FIBROBLAST CELL LINE MSU-1 BY A TRANSFECTED N-*RAS* ONCOGENE

Daniel M. Wilson, Dajun Yang, John E. Dillberger,
Veronica M. Maher, and J. Justin McCormick

Carcinogenesis Laboratory - Fee Hall
Department of Microbiology and Department of Biochemistry
Michigan State University, East Lansing, MI 48824-1316

Running Title: Malignant Transformation of Human Cells by N-*ras*

ABSTRACT

We showed previously that transfection of finite lifespan, diploid human fibroblasts with a N-ras oncogene cloned into a high expression vector, pSV N-ras, caused them to exhibit morphological transformation, focus formation, and anchorage independence, but not to acquire an infinite lifespan in culture or become tumorigenic. Recently, a near diploid, non-tumorigenic cell line, MSU-1, with an infinite lifespan in culture was developed in this laboratory following transfection of diploid human fibroblasts with a plasmid containing a v-myc oncogene. We transfected these infinite lifespan cells with pSV N-ras and obtained foci of morphologically transformed cells which formed colonies in soft agar at a frequency 500 times higher than the parental cells and which replicated vigorously in serum-free medium containing a reduced level of calcium, and lacking exogenously-added protein growth factors. This medium will not support growth of diploid human fibroblasts and allows only minimal growth of the parental MSU-1 cells. The focus-derived transformed cells, injected subcutaneously into athymic mice, formed malignant tumors (fibrosarcomas, undifferentiated sarcomas and giant cell sarcomas) after a latency period of 1 to 4 weeks. Intravenous inject of focus-derived or tumor-derived cells resulted in metastatic tumors. The tumor-derived cells were Geneticin resistant and had the two marker chromosomes present in the parental MSU-1 cell line.

INTRODUCTION

A large body of data, including epidemiological studies on human cancer (5), supports the concept that carcinogenesis is a multistepped process. Experimental studies with animals have shown that induction of tumors by carcinogens usually requires multiple exposures, or a single application followed by multiple treatments with a tumor promoter (1,28). To examine the question of the mechanisms involved in the development of cancer in humans, we and our associates have been utilizing diploid human fibroblasts (9,12,36). We chose these cells because they grow well in culture, and do not transform spontaneously in vitro (18), and yet in vivo, they are the progenitor cell for malignant fibrosarcomas. An additional reason for using human fibroblasts for studies on the changes required in a cell to cause it to become malignantly transformed is that there is an extensive body of literature on the transformation of rodent fibroblasts in culture which can serve as a reference.

Experience in numerous laboratories over the past decade indicates that it has not been possible to cause the malignant transformation of human cells in culture by exposing them to carcinogens (see ref. 18 for review). Therefore, several groups of investigators (9, 12, 22, 25, 31, 36), have sought to determine if these cells can be transformed to the malignant state by being transfected with one or more oncogenes. It is known that DNA from many human tumors or tumor-derived cell lines can cause transformation of NIH3T3 cells into focus-forming malignant cells and this assay has led to the isolation and identification of human oncogenes. In addition, there is evidence that the protooncogenic cellular counterparts of these genes can be targets for

carcinogenic agents (2,17,33,35,38). Most studies utilizing finite lifespan rodent fibroblasts in culture indicate that transfection of a single oncogene, such as ras, is not sufficient to transform these cells to the malignant state (15,16,23,34). However, transfection of a ras oncogene into an infinite lifespan rodent cell line (16), or co-transfection of finite lifespan rodent cells with a ras oncogene along with an immortalizing oncogene, such as myc (3,15,16), has resulted in malignant transformation.

Wilson et al. (36) recently reported that transfection of finite lifespan diploid human fibroblasts with an N-ras oncogene cloned into a high expression vector pSV N-ras caused them to exhibit many tumor-cell related characteristics, but did not confer infinite lifespan in culture nor malignancy. A similar result was obtained by Hurlin et al. (12) using an H-ras oncogene in another high expression vector. Recently, however, a near-diploid, non-tumorigenic cell line which has a normal morphology, does not form foci, or exhibit anchorage independence but which has acquired an infinite lifespan in culture, was developed in this laboratory following transfection of normal diploid fibroblasts with a plasmid containing a v-myc oncogene (T. L. Morgan, D. G. Fry, D. Yang, V. M. Maher, and J. J. McCormick, unpublished studies). Transfection of this cell line, designated MSU-1, with the N-ras oncogene in this high expression vector caused the cells to form foci. The cells from the foci were morphologically transformed, exhibited anchorage independence and growth factor independence, and formed progressively-growing malignant tumors in athymic mice.

MATERIALS AND METHODS

Cells. The derivation of the MSU-1 cells used in these experiments will be described in detail elsewhere. In brief, they were derived from a diploid, foreskin-derived cell line, designated LG1, initiated in this laboratory from a normal newborn male, which had been transfected with a plasmid carrying the neo gene, as well as a v-myc gene. An infinite lifespan, near-diploid cell line arose from the Geneticin resistant population. It expresses the transfected myc gene and carries two marker chromosomes.

Growth factors. Epidermal growth factor (EGF) was obtained from Bethesda Research Laboratories (Gaithersburg, MD). Platelet-derived growth factor (PDGF) was obtained from PDGF Inc. (Boston, MA). An aqueous solution of purified human serum albumin (5 mg/ml) (Sigma Chemical Co., St. Louis, MO) was prepared and used as the solvent to make stock solutions of EGF (3 ug/ml) and PDGF (0.75-1.5 ug/ml). Plastic bottles were used to prevent adsorption of the growth factors to glass and aliquots of the stock solutions were stored at -20^{o}C until use. PDGF was used at a final concentration of 1.5 ng/ml of medium. Basic fibroblast growth factor (bFGF) was obtained from Collaborative Research Inc. (Lexington, MA), and was used at a concentration of 3 ng/ml of medium.

Culture medium. The cells were routinely grown in Eagle's minimal essential medium supplemented with 0.2 mM L-serine, 0.2 mM L-aspartate, 1.0 mM sodium pyruvate, 10% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, NY), penicillin (100 units/ml), and streptomycin (100 ug/ml) (culture medium) and maintained at 37^{o}C in a humidified incubator with 5% CO_2. Studies of growth factor and calcium

requirements were done using McM medium (24), a modified version of the MCDB110 base medium of Bettger et al. (4), formulated in this laboratory for use with serum supplements in studies requiring the absence of serum. For the majority of the studies, the McM medium was prepared with a calcium concentration of 0.1 mM instead of the usual 1.0 mM, and is referred to as low-Ca^{++} McM. The serum replacement supplements used were those of Ryan et al. (24), but lacking EGF, and are referred to here as SR_2. Where noted, medium was supplemented with 0.1% FCS which aided in cell attachment, but did not affect the cells response to exogenous growth factors. All growth factor experiments were repeated 3 or 4 times.

Plasmids. pSV N-ras, which contains the N-ras oncogene from human leukemia cell line 8402 (29), was provided by E. Fleissner of Memorial Sloan-Kettering Cancer Center. Plasmid pNR-MG1, which contains the N-ras oncogene from the HT1080 human fibrosarcoma cell line, was provided by D. Spandidos (32). Plasmid pSV2neo, used as a control, contains the neo gene coding for Geneticin resistance, but no oncogene (20). Plasmid p6a1, which contains a partial cDNA clone of the human N-ras gene, was obtained from the American Type Culture Collection (Rockville, MD).

DNA transfection and selection for focus formation. Fibroblasts were transfected using the dimethylsulfoxide/Polybrene method adapted for use with human fibroblasts (19). Cells were plated at 2 $x10^5$ per 100 mm-diameter dish, transfected 24 h later with 1-10 ug of plasmid DNA, and fed weekly thereafter with culture medium. Dishes were observed twice weekly for focus formation. Foci were isolated approximately 3 wk post-transfection by transferring them to separate 25 cm^2 flasks by using sterile filter paper wetted with trypsin.

Anchorage independence. Cells in 1.5 ml of top agar, composed of Ham's F10 medium with 2.2 g/l $NaHCO_3$, supplemented with 6% FCS, 100 U/ml penicillin and 100 ug/ml streptomycin and containing 0.33% Noble agar (Difco, Detroit, MI) were plated into 60 mm-diameter dishes on top of 3 ml of solidified bottom agar prepared with the same medium, but containing 2% Noble agar. The next day the cultures were overlaid with 2 ml of this medium, lacking agar and containing hydrocortisone (HC) at 32.5 ug/ml to yield a final HC concentration of 10 ug/ml. The cells were grown at 37°C in a humidified atmosphere of 3% CO_2 in air. The cultures were refed weekly by replacing the liquid medium layer with 2 ml of fresh medium containing HC. An aliquot of the cell suspension that was to be used as the top layer was also appropriately diluted with agar-free culture medium and plated into a series of 100 mm-diameter culture dishes at cloning density to determine viability. Growth in agar was evaluated 3 wk after plating using a CUE-2 Image Analyzer (Olympus) to count and record the number of colonies greater than 80 um in diameter.

Assay for tumorigenicity. Balb-c athymic mice 6 to 8 weeks old that 24 h previously had been given 350 rads of gamma irradiation from a ^{60}Co source were used and tumor growth was monitored on a weekly basis. Focus-derived populations of cells or tumor-derived cells were injected subcutaneously ($5 - 10 \times 10^6$), or intracardially (1×10^5), or into the tail vein (2×10^6.

Cytogenetic analysis. Karyotypes were analyzed using the G-banding technique of Yunis and Chandler (37). Briefly, cells were subcultured at 1.5×10^6 cells per 75 cm^2 flask. Colcemid (25 ng/ml) was added 24-48 h later. Cells were harvested after 2-4 h, centrifuged

8 min at 1000 rpm, resuspended 0.5 ml of medium containing colcemid, and diluted into 15 ml of 0.075 M KCl at 35°C. After 30 min the cells were centrifuged as before, and the pellet was resuspended in freshly prepared methanol:glacial acetic acid (3:1 v/v) (Carnoy's fixative) that was added dropwise to a volume of 1 ml. The volume was adjusted to 20 ml with Carnoy's fixative at 25°C. After 20 min, the fixing procedure was repeated twice, using Carnoy's fixative for each rinse. The pellet was resuspended in a small volume of Carnoy's fixative, and the cells were dropped onto an ice-cold wet microscope slide using a siliconized pasteur pipette. After air drying the slides were heated at 67°C for 2 h or maintained for several days at 35°C. The slides were dipped in a solution containing 0.05% trypsin and 0.09% NaCl for 10-20 sec, then stained with 0.25% Wright's stain in Gurr buffer (1:3) at pH 6.8 for 3-5 min. At least 25 G-banded karyoptypes were examined per cell line and 100 conventionally stained metaphases were counted for determining the modal chromosome number.

DNA hybridization analysis. The presence of transfected DNA sequences was determined by Southern hybridization analysis (30). DNA was extracted (11), digested with EcoRI according to the directions of the supplier (Bethesda Research Laboratories, Bethesda, MD), and electrophoresed through a 0.7% agarose gel. The fragments were transferred to a Zetaprobe membrane (Biorad Laboratories, Rockville Ctr, NY) with 0.4 M NaOH. The filter was rinsed in 2x-SSC, (SSC consists of 15 mM sodium citrate/0.15 M NaCl), dried at 80°C under vacuum for 0.5 hr, and prehybridized for 1 h at 42°C with a solution containing 50% formamide, 1.0 M NaCl, 0.01 M EDTA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin (BSA), 3% sodium

dodecylsulfate (SDS), and 100 ug denatured salmon sperm DNA per ml. Hybridization was carried out for 24 h at 42°C using this solution containing denatured probe DNA (labeled with ^{32}P-dNTPs by extension of random hexamer primers) (6). The probe for N-ras sequences was a 650 bp BamHI-EcoRI fragment of plasmid p6a1 homologous to exons 2-4 of the human N-ras gene. After hybridization, the membrane was washed in 2x-SSC, 3% SDS for 10 min at 25°C, for 60 min at 65°C with 0.5x-SSC, 3% SDS, 0.5% BSA, and for an additional 60 min at 25°C with 0.1x-SSC. The location of hybridizing bands was determined by autoradiography at -70°C using Kodak XAR-film with intensifying screens (Cronex Lightning Plus, Dupont Inc., Wilmington, DE).

Immunoprecipitation of p21 ras. The amount of p21 ras protein was assayed by a variation of the method described by Furth et al. (10). Exponentially growing cells were maintained for at least 3 days in McM medium containing 10% FCS, hydrocortisone (10 ug/ml), penicillin (100 units/ml), and streptomycin (100 ug/ml), and then replated in this medium at 9 x 10^5 cells per 100 mm-diameter dish. The medium was replaced 20-24 h later with methionine-cysteine-free McM medium, containing 1% FCS, penicillin and streptomycin. Two h later the medium was replaced with 4 ml of this medium containing 750 uCi of Tran ^{35}S-label (ICN Biomedicals, Costa Mesa, Ca). After 20-24 h, the cells were rinsed twice in methionine-cysteine-free McM medium, lysed in 1 ml of Staph A buffer (0.2 M phosphate, pH 7.4 with 1% Triton X 100, 0.1% SDS, 0.1% NaN_3, 0.1 M NaCl, 12 mM sodium deoxycholate) containing 2 mM phenylmethanyl-sulfonyl fluoride and 100 Kallekrein inactivator units of apoprotein, and centrifuged for 30 min at 35,000 rpm at 4°C. A 10 ul aliquot of supernatant was added to 10 ml aqueous scintillation

fluid, and counted in a Beckman LS-9000 scintillation counter. For immunoprecipitation, the volume of supernatant containing 0.75 x 10^7 cpm of incorporated Tran ^{35}S-label was incubated with 10 ul aliquots of antibodies v-H-ras (Ab-1) or v-H-ras (Ab-2) (Oncogene Science, Inc., Manhasset, NY) for 2 h at 4°C on a shaker. Protein-A sepharose (Pharmacia, Piscataway, NJ) was coated with goat anti-rat IgG (Cooper Biomedical, Malvern, PA) following the manufacturer's instructions and 200 ul of this suspension was added to the incubation mix and allowed to react for 30 min at 4°C. The samples were microfuged 1 min, and the pellet was washed twice with 1 ml of Staph A buffer. The pellet was incubated for 10 min with 1 ml 50 mM Tris, pH 7.5, 1 M MgCl, and then microfuged 1 min, and washed with 1 ml of Staph A buffer. The pellet was resuspended in 50 ul of 90 mM Tris buffer, pH 6.8, containing 28.8% glycerol, 0.14% SDS, 0.014% Bromphenol blue, and 8.6% beta-mercaptoethanol, and heated for 5 min at 95°C. Gel electrophoresis was performed using 14% polyacrylamide gels that were 0.75 mm thick (16 cm x 16 cm) and a Tris-glycine buffer system (14). 25 ul of sample was loaded per lane. After electrophoresis the gels were soaked in RESOLUTION solution (EM Corp., Chestnut Hill, MA) for 0.5 h, in distilled water at 0°C for 1 h and then dried and analyzed by autoradiography as described above.

RESULTS

Transformation of MSU-1 cells to focus formation by transfection of an N-ras oncogene. We transfected the infinite lifespan cell line, MSU-1, with the N-ras oncogene from human lymphoma cell line 8402 in the high expression plasmid pSV N-ras (29). This plasmid contains two Maloney leukemia virus long terminal repeats (LTRs). The endogenous promoter of the N-ras oncogene has been eliminated and the gene inserted between an LTR and the neo gene coding for Geneticin resistance (13). Transcription of both genes is initiated from the first LTR. The transfected population (2×10^5 cells per dish) was allowed to grow to confluence and examined for the formation of foci. As a control, MSU-1 cells were similarly transfected with pSV2neo which lacks an oncogene. After seven days, several foci could be seen on top of the confluent monolayers of cells that had been transfected with pSV N-ras. An example is shown in Figure 1. No foci were found in the control. The morphology of the cells in the foci was highly anaplastic, with many being epitheloid, refractile and irregularly shaped. Eight independent foci were isolated and propagated in culture medium. These morphologically-altered, focus-derived populations were designated MSU-1-N-ras cell strains 1 through 8. For propagating these cell strains, hydrocortisone (10 ug/ml) was added to the culture medium because it was found to facilitate attachment.

Anchorage independence of MSU-1-N-ras cell strains. Anchorage independence, the ability to form colonies in semi-solid media, is a characteristic of many tumor-derived cell lines and has been shown to correlate with the potential of rodent cells transformed in vitro to form tumors (8). Therefore, it was of interest to determine if these

Figure 1. Photomicrograph of crystal violet stained human fibroblasts. (A) Focus induced by transfection of immortal MSU-1 cells with pSV N-ras. (B) Non-transfected MSU-1 cells. Magnification 80X.

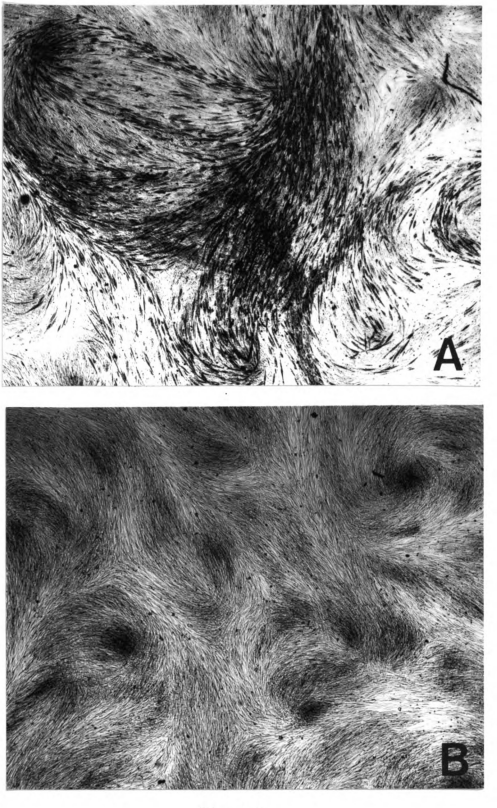

Figure 1

Table 1. Anchorage independence of focus-derived MSU-1-N-*ras* cell strains

Cell strain	Colonies per dish[a]
2	218 (11.4)
3	515 (17.6)
4	140 (8.9)
5	292 (13.2)
MSU-1	1 (1)

[a] 5×10^4 cells were plated per dish.

Colonies greater than 50 um diameter were counted. Numbers in parenthesis indicate standard error of the mean.

MSU-1-N-ras cell strains could form colonies in soft agar. The results are shown in Table 1. The N-ras transformed cells exhibited anchorage independence, their parental cell strain did not.

Growth factor requirements of MSU-1-N-ras cells. Schilz et al. (26) showed that normal diploid early-passage human fibroblasts in culture replicate approximately every 24 h in low Ca^{++} McM medium which is supplemented with 10% FCS. They found a similar 24 h population doubling time when these normal cells were grown in McM medium with its standard concentration of Ca^{++} (1 mM) in the presence of the serum replacement supplements specified by Ryan et al. (24), but lacking EGF (designated SR_2) (26). These serum replacement supplements include insulin, but normal human fibroblasts replicate just as rapidly in such medium lacking insulin (26). They cannot replicate in low-Ca^{++}-McM medium supplemented with SR_2 but addition of EGF (3 ng/ml) or PDGF (5 ng/ml) or bFGF (1.5 ng/ml) will drive replication (26).

Schilz et al. (26) also showed that, unlike normal human fibroblasts, 7 out of 7 human fibrosarcoma-derived cell lines can replicate in low-Ca^{++} McM+SR_2 with no need for exogenous protein growth factors. In fact, for many of these tumor-derived cell lines, addition of the growth factors does not increase their rate of growth, which is equal to their rate of growth in 10% FCS. Therefore, we examined several focus-derived MSU-1-N-ras cell strains for their growth factor independence. Representative results of their response to low-Ca^{++} McM supplemented with SR_2 and 0.1% FCS is shown in Fig. 2. The parental MSU-1 cell line replicated only minimally under these conditions (open circle), whereas MSU-1-N-ras doubled every 24 h (open squares), i.e., at the same rate as the parental MSU-1 cell line did in the presence

Figure 2. Growth factor requirements of N-*ras*-transformed cells (closed symbols) and their parental cell line MSU-1 (open symbols). Cells were plated at 2 x 10^4 cells per 60-mm diameter culture dish in McM medium supplemented with 1% serum. 4 h later the number of cells which had attached to the culture dish was determined for each cell line, and the medium was replaced with low-Ca^{++} McM medium supplemented with SR_2 and 0.1% FCS (open circles and open squares), or with with 10% FCS (closed circles and closed squares). Cells were refed with designated media on days 3 and 7.

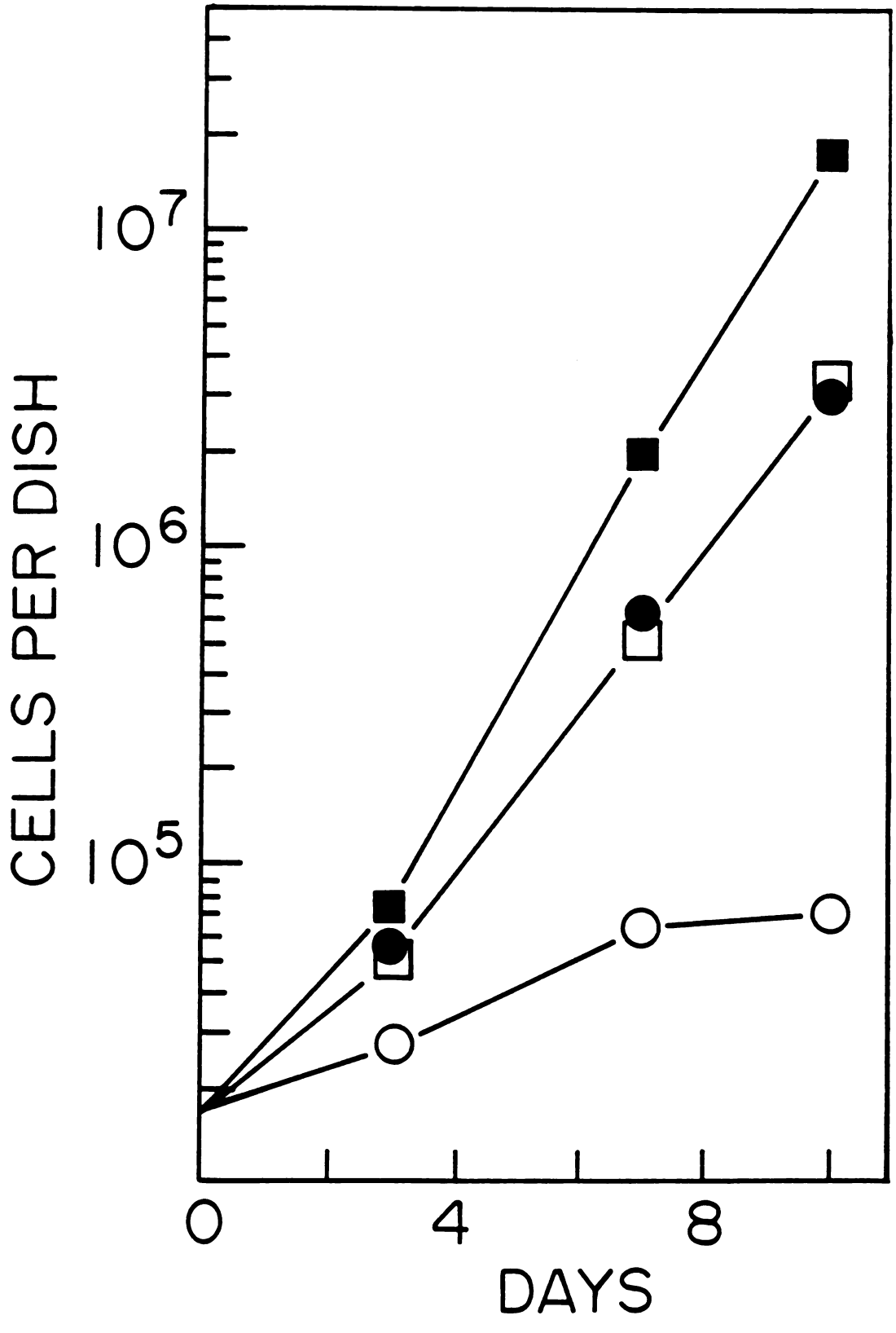

Figure 2

Figure 3. Cell number of MSU-1 cells and N-*ras*-transformed cell strains as a function of Ca^{++} concentration and response to growth factors. Cells were plated at 2 x 10^4 per 100-mm diameter culture dish in McM medium supplemented with 1% serum. 4 h later the number of cells which had attached to the culture dish was determined for each cell line, and the medium was replaced with low-Ca^{++} McM medium supplemented with SR_2, or with SR_2 plus either bFGF, PDGF, or EGF, or with SR_2 plus 10% FCS. Cells were refed with designated media 4 days later. The number of cells per dish (duplicate dishes) was determined 7 days after seeding cells.

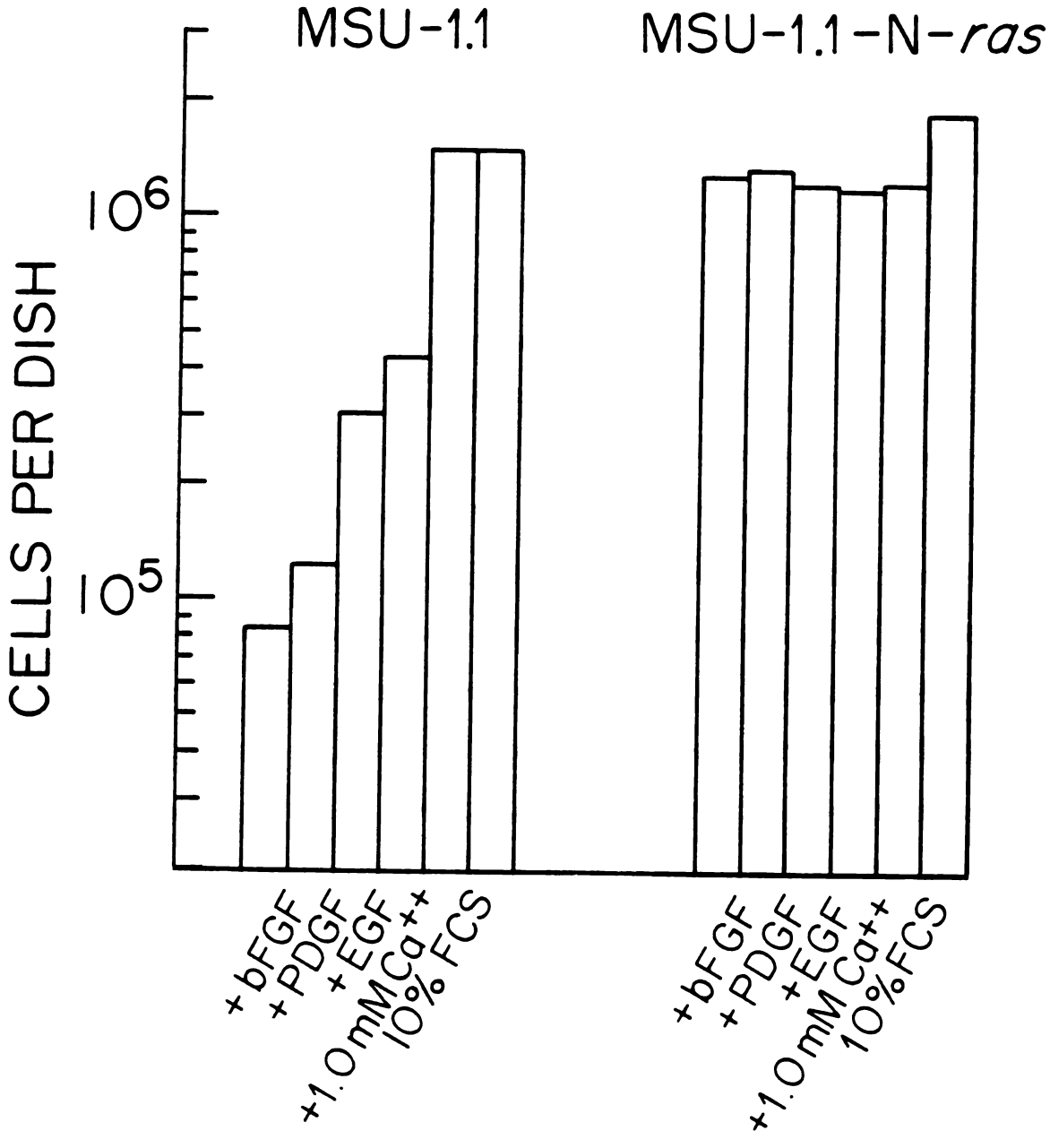
MSU-1.1
MSU-1.1-N-ras
CELLS PER DISH
10^6
10^5
+bFGF
+PDGF
+EGF
+1.0mMCa++
10%FCS
+bFGF
+PDGF
+EGF
+1.0mMCa++
10%FCS

Figure 3

of 10% FCS (closed circles). The MSU-1-N-ras cells responded to 10% FCS by replicating twice in 36 h (closed squares). Fig. 3 shows that the MSU-1-N-ras cells were independent of the need for exogenous EGF, PDGF, or bFGF, whereas their parental cell line responded to such growth factors by an increased rate of replication.

Tumorigenicity of the MSU-1-N-ras transformed cell strains. Five MSU-1-N-ras cell strains were injected subcutaneously into athymic mice to examine their tumorigenic potential. Extensive unpublished studies from this laboratory had shown that the parental MSU-1 cell line does not give rise to tumors. As shown in Table 2, all five cell strains tested gave rise to malignant tumors after a relatively short latency period. Figure 4 gives examples of their histology. Mice were necropsied at various times after injection, and tumor specimens were collected for pathological evaluation. In all cases, the tumors were invasive and grew progressively indicating they were truly malignant. At that time portions of some tumors were also minced and replated in culture medium containing Geneticin. All tumor samples which were reintroduced into culture in this manner gave rise to Geneticin-resistant populations or morphologically transformed fibroblasts.

To further characterize these N-ras-transformed cells, five mice were injected via the tail-vein with cells derived from a subcutaneous tumor that had formed after s.c. injection of MSU-1-N-ras cell strain 3. Four mice developed sarcomas in their rear legs and abdominal wall, and the fifth mouse had a tumor in its dorsal thoractic cavity that did not involve the lungs. Either the injected cells traversed the pulmonary capillary bed, entered the systemic circulation, and escaped to form tumors in the muscles of the body wall and rear legs, or they

Table 2. Tumorigenicity of focus-derived MSU-1-N-*ras* cell strains.

Cell strain	Mice with tumors[a]	Latency period[b] (days)	Type of tumor
2	4/4	34	Undifferentiated sarcoma
3	4/4	9	Giant cell sarcoma, Fibrosarcoma
4	4/4	13	Giant cell sarcoma
5	4/4	c	Undifferentiated sarcoma
8	3/3	29	Undifferentiated sarcoma

[a]Mice injected subcutaneously with 10-12 x 10^6 focus derived cells.

[b]Length of time it took the tumor mass to reach an average diameter of 6 mm.

[c]N-*ras*-strain 5 formed tumors which regressed and then reappeared as invasive malignant tumors.

Figure 4. Photomicrograph of formalin fixed tissue sections of subcutaneous tumors formed by N-ras-transformed cell strains. (A) Undifferentiated sarcoma, (B) giant cell sarcoma, (C) fibrosarcoma. Sections stained with hematoxylin and eosin. Magnification 80X.

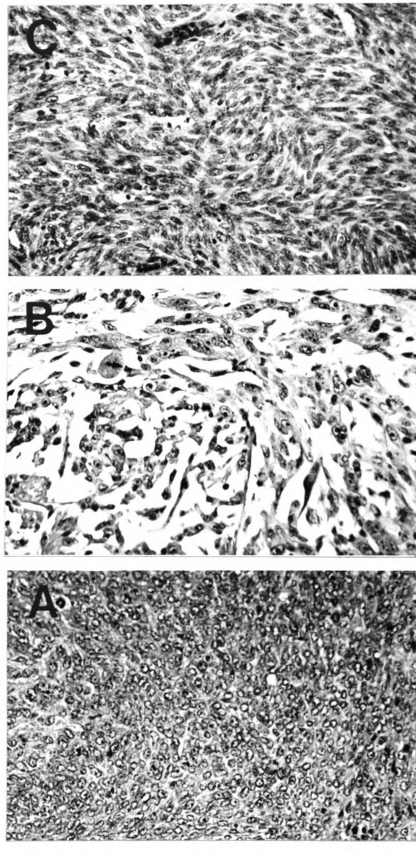

Figure 4

escaped from the tail vein at the injection site into the lymphatic system and entered peripheral lymphatics to form tumors in the muscle and were carried via the thoracid duct to form a tumor in the thorax.

To distinguish between these possibilities, two mice each were injected intravenously (tail vein) or intracardially, with focus-derived MSU-1-N-ras cell strains 3 or 4, with cells derived from subcutaneous tumors formed by injection of these two cell strains (designated $3T_{sc}$ and $4T_{sc}$), or with cells derived from one of the tumors that arose following the original tail vein injection experiment (designated $3T_{iv}$). Only cell strain 4 produced tumors when given intravenously, ie. one of the two mice developed multiple fibrosarcomas in its lungs. Cell strain 3, $3T_{sc}$ and $3T_{iv}$ cells all produced sarcomas following intracardial injection, although none of the tumors produced were giant cell sarcomas. Tumors occurred at various sites, including the endocardium, thoracic cavity, abdominal cavity, kidneys and adrenal gland. The $4T_{sc}$ cells did not yield tumors in this experiment.

Cytogenetic analysis. Karyotypes were determined for fibroblast cell line LG1, the parental MSU-1 cell strain which was derived from LG1, MSU-1-N-ras cell strains 3 and 4, and the tumor-derived cell strains $3T_{sc}$ and $4T_{sc}$. Fibroblast cell line LG1 has a modal chromosome number of 46 as expected. The parental MSU-1 cells have a modal chromosome number of 45 and 2 distinctive marker chromosomes designated M1 and M2. Each of the focus-derived MSU-1-N-ras cell strains and the tumor-derived cell lines also contain the marker chromosomes M1 and M2, clearly indicating that MSU-1 cells were the cell strain of origin.

There was essentially no difference between the karyotypes of MSU-1

cells and any of the focus-derived cell strains analyzed. In 12 of 29 MSU-1-N-ras$3T_{SC}$ cells evaluated, the karyotype was identical to that of MSU-1 cells. Eleven cells had randomly lost either chromosomes 4, 6, 17, 20, or 21. The remaining six cells were either hypoploid or hyperploid. Among the cells which contained less than 45 chromosomes, 4 of 11 had a marker chromosome (M3) which resulted from rearrangement involving chromosomes 9 and 17. Of the six hyperploid and hypoploid cells found, three contained new giant dicentric chromosomes.

Compared to their focus-derived progenitors, many karyotypic changes have occurred in MSU-1-N-ras-$4T_{SC}$ cells. The chromosome number of these cells varies from hypodiploid (2n-, 39%), diploid (2n, 16%), near triploid (3n, 27%) hyperdiploid (4n +/- or greater, 28%). In most of the 4T cells analyzed, two or additional dicentric chromosomes were present. These rearranged chromosomes varied from cell to cell, with several cells exhibiting chromosomes most of them involving C, E, or F group chromosomes which formed a giant dicentrid chromosome that had homogeneous staining regions. Most of the rearranged chromosomes were hard to identify.

From a total of 29 cells observed for each cell strain, 27% of the $3T_{SC}$ cells, and 74% of $4T_{SC}$ cells displayed aberrant chromosomes, consisting of dicentric chromosome fragments, double minutes, and breaks or gaps.

Expression of the N-ras oncogene p21. Our earlier findings (36), and those of Hurlin et al. (12), showed that transfection of finite lifespan, diploid human fibroblasts with ras oncogenes yielded foci of morphologically transformed cells only when the ras oncogenes were cloned into high expression vectors, and their encoded proteins were

Figure 5. Immunoprecipitation evidence of N-*ras* oncogene expression. Cell extracts from Tran-[^{35}S]-methionine-labeled MSU-1 cells, N-*ras*-transformed cell strains, or their subcutaneous-tumor-derived strains were immunoprecipitated and analyzed by SDS/PAGE. Antibody v-H-*ras* (Ab-1) recognizes human H-*ras*, K-*ras*, and N-*ras* p21s; v-H-*ras* (Ab-2) recognizes the human H-*ras* and K-*ras* p21's, but not the human N-*ras* p21.

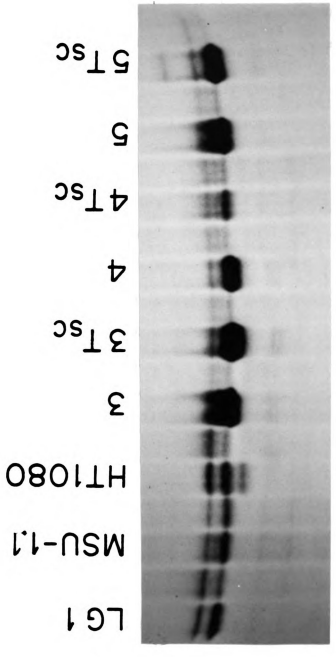
Ab 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
LG 1
MSU-1.1
HT1080
3
3 T_{sc}
4
4 T_{sc}
5
5 T_{sc}

Figure 5

produced in high ammounts. We, therefore, expected that the MSU-1-N-ras cell strains would exhibit levels of N-ras oncogene products several times higher than the levels of endogenous ras gene products. The level of expression was determined using immunoprecipitation. As shown in Figure 5, the focus-derived MSU-1-N-ras cell strains had much higher levels of a protein of 21,000 daltons than did the parental MSU-1 cells. The overexpressed protein can be identified as N-ras since it is precipitated by Ab-1, an antibody which recognizes human H-ras, K-ras, and N-ras gene products, but not by Ab-2, which for human cells recognizes only H-ras, and K-ras gene products (10). Compared to LG1 cells from which the infinite lifespan MSU-1 cell strain arose, MSU-1 cells did not have any detectable levels of endogenous N-ras encoded protein (Fig. 5). We also failed to detect N-ras gene expression in one of the tumor-derived cell lines ($T4_{SC}$), although MSU-N-ras cell strain 4, which gave rise to the tumor, expressed high levels of N-ras. The former cells ($T4_{SC}$) were derived from a giant cell sarcoma which contained multiple new chromosomal rearrangements.

Evidence of the presence of N-ras oncogene DNA sequences. Two focus-derived MSU-1-N-ras cell strains and five tumor-derived cell lines were analyzed for the presence of N-ras oncogene sequences using Southern blot hybridization analysis and the patterns were compared with those of the parental MSU-1 cell strain and LG1 cells, as shown in Fig. 6. When probed for N-ras specific sequences, hybridizing fragments of 8.8 kb and 7.0 kb, corresponding to fragments of the endogenous N-ras gene, were present in the DNA from all of the cell lines analyzed. Digestion of DNA obtained from normal human fibroblasts, with the restriction enzyme Eco RI has been reported to

Figure 6. Southern hybridization analysis for N-*ras* specific gene fragments in N-*ras*-transformed human fibroblasts. *Eco*RI digested DNA samples were analyzed as described with a probe specific for exons 2-4 of the N-*ras* gene.

cleave the N-ras gene into fragments of 8.8 kb containing exons 1 and 2, and 7.0 kb containing exons 3 and 4 (27). The LG1 cell line most likely had a polymorphic Eco RI site on one allele of chromosome 1 which yielded a new N-ras specific fragment, as seen in all EcoRI digests (Fig. 6). Digestion of the plasmid pSV-N-ras with Eco RI should yield three fragments that are 9.6 kb, 6.0 kb, and 22.44 kb in length. The two focus-derived cell lines analyzed, and two of the tumor-derived cell lines contained a 6.0 kb Eco RI fragment that was diagnostic of the presence of the transfected N-ras oncogene (Fig. 6). Of the remaining tumor cell lines analyzed, one contained the 9.6 kb Eco RI fragment noted above, and the rest contained other new N-ras specific fragments which are likely to have resulted from random breakage of the plasmid during integration into genomic DNA, and subsequent production of hybrid fragments between plasmid and genomic DNA after cleavage by EcoRI.

DISCUSSION

Data from several groups of investigatgors including Ruley (23) and Land et al. (16), indicate that acquisition of an infinite lifespan is a necessary prerequisite for malignant transformation of cells in culture by a ras oncogene. We found previously (36) that transfection of finite lifespan human fibroblasts with pSV N-ras gave rise to morphologically transformed focus-forming cells, but that when clones of such cells were isolated and propagated, the majority exhibited premature senescence, entering crisis within several weeks of the initial transfection, or else reverted to a normal morphology. Premature senescence of primary rodent fibroblasts transfected with ras oncogenes has been observed (8,16) suggesting that it may be a general characteristic of primary cells. However, the several stably transformed clones that we obtained proved to have a normal lifespan in culture and were non-tumorigenic (36). A likely explanation for the lack of tumorigenicity in the normal diploid human fibroblasts that had been stably transformed by N-ras is that by the time the cloned population was exapanded to a size large enough to be tested for tumorigenicity by being injected into athymic mice, the cells were nearing senescence and were incapable of multiplying to a population large enough to give an observable tumor.

In the present investigation, transfection of the MSU-1 cells with pSV N-ras yielded foci of morphologically transformed cells which gave rise to malignant tumors when injected into athymic mice. None of these cells reverted to normal morphology or senesced prematurely.

The mechanism responsible for the stability of the transformed phenotype when pSV N-ras was transfected into MSU-1 cells, in contrast

to what was observed in the finite lifespan parental cells is not known. The stability of the MSU-1-N-ras transfectants could be related to any of the unique properties of this cell line. These properties include the related infinite lifespan phenotype, the expression of the v-myc protein, or the failure of these cells to express measurable levels of the normal N-ras gene.

Since the parental LG1 cell was not malignantly transformed by the transfected ras plasmid, but the derivative MSU-1 cell was, our study suggests that immortality is required for malignant transformation by a transfected N-ras gene. Studies by Namba et al. (21) show that a human carcinogen-induced infinite lifespan human fibroblast cell line can be transformed to a malignant cell by infection or transfection of an H-ras oncogene. Similar results have been obtained by O'Brien et al. using an SV40 immortalized human fibroblast (22). Cells lacking the infinite lifespan characteristic could not be malignantly transformed. Similar results have been obtained in this laboratory by Hurlin et al. (13) who transfected an H-ras oncogene into three infinite lifespan human fibroblasts, i.e., MSU-1 cells, KMST-6 cells transformed to an infinite lifespan by ^{60}Co (21) and an SV40-transformed cell line GM637. The MSU-1 cell line has also been malignantly transformed by the v-K-ras gene (D. G. Fry, V. M. Maher, and J. J. McCormick, unpublished studies). Although the mechanism by which the infinite lifepsan characteristic arose in these three cell lines is not known, the fact that each one was generated by a distinct protocol suggests that it is the resulting infinite lifespan phenotype that is important for subsequent transformation to malignancy, not the mechanism used.

In addition to the necessity of utilizing an infinite lifespan cell

line, transformation of human cells by a human N-ras oncogene required more than just normal expression of an N-ras gene activated by a point mutation in codon 12. The levels of protein product produced from the N-ras oncogene cloned into the ZIP vector are at least 4-9 times higher than the levels of N-ras protein found in normal human fibroblasts (Fig. 5) (quantitative data not shown). This enhanced expression most probably results from construction of the pSV N-ras plasmid, with its Malony leukemia virus LTRs (29). Our data suggest that this high level of expression was necessary for the transformation related characteristics observed in these cells, including focus formation, tumorigenicity, and perhaps metastatic potential. This is because when MSU-1 cells were transfected with a plasmid containing the mutant N-ras oncogene from the fibrosarcoma cell line HT1080, in which transcription was initiated from the gene's own promoter instead of from a viral long terminal repeat as in pSV N-ras, only one transformed focus could be found in a total of 5×10^6 transfected cells, and the cells from the focus were not tumorigenic. Preliminary data suggest that the level of N-ras protein product is not greatly elevated in these latter cells, whereas it was substantially increased in all MSU-1-N-ras-transformed cell strains examined in the present study. The N-ras oncogene conferred growth factor independence on MSU-1 cells, enabling them to replicate as rapidly in medium containing no serum or protein growth factors, and only 0.1 mM calcium, as non-transformed MSU-1 cells did in medium supplemented with high concentrations of serum or calcium. Although the growth factor independence studies reported in Fig. 2 and 3 contained insulin, we have also determined that MSU-1-N-ras-transformed cells do not require insulin for growth,

nor do they respond to it (data not shown). The mechanism by which N-ras confers growth factor independence on MSU-1 cells is under study.

The giant cell sarcomas observed after inoculation of athymic mice with some of the focus-derived pSV N-ras transformed cells are of special interest. The focus-derived population of cells that gave rise to these tumors expressed high levels of the mutant N-ras oncogene (Fig. 5). However, the giant cell sarcoma-derived strain cell $4T_{SC}$ no longer exhibited enhanced expression of N-ras (Fig. 5) even though they were able to produce tumors when reassasyed. High expression of N-ras may have initially led to chromosomal instability in the cells and this instability could have eventually yielded giant cell sarcomas. If this is true, we would have to postulate that new genetic lesions which occurred as a result of those chromosomal rearrangements were responsible for maintenance of the malignant phenotype.

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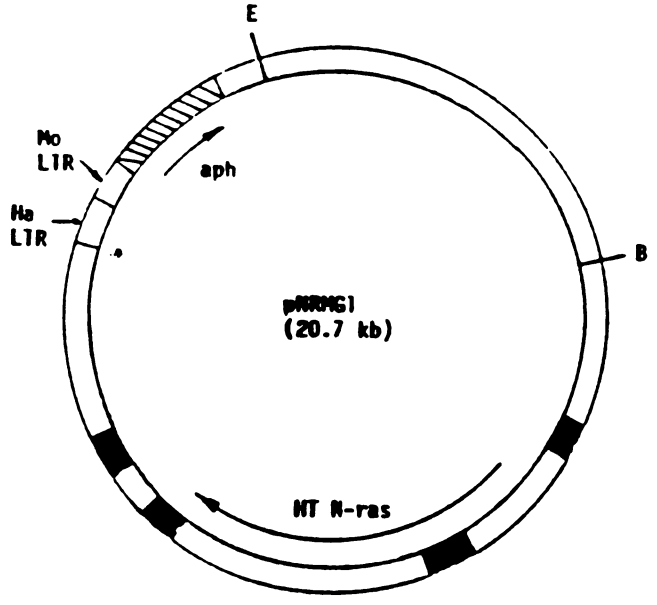
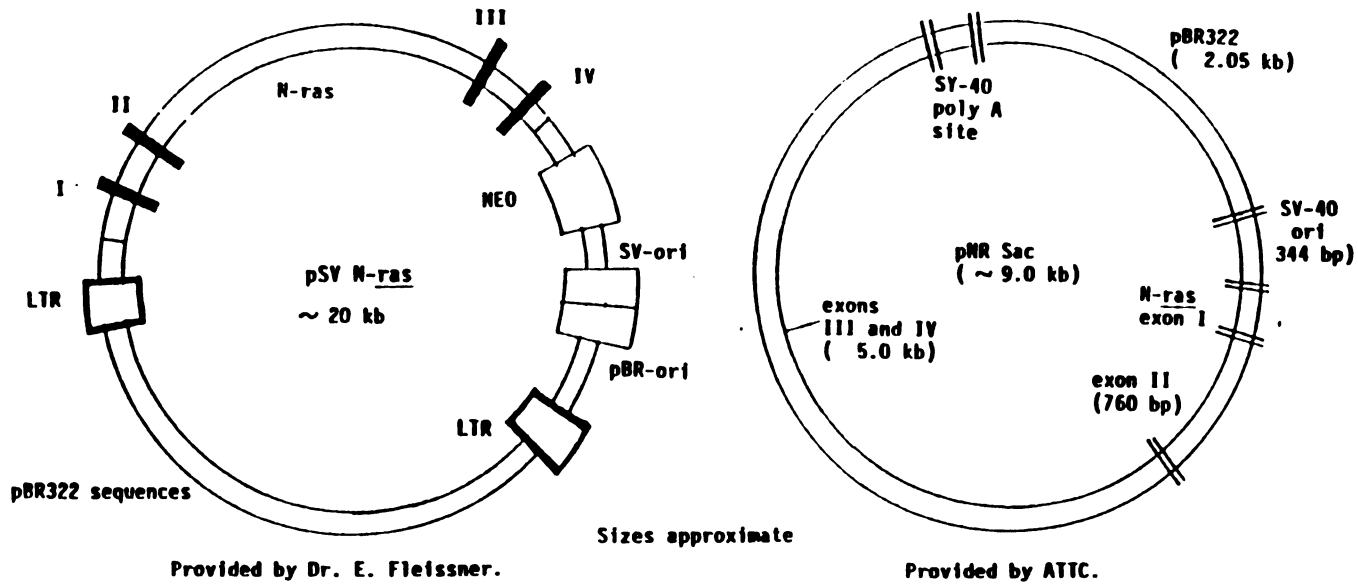
APPENDICES

APPENDIX A

Plasmids used in transfection experiments.



Provided by Dr. D. Spandidos



Plasmids used in transfection experiments

APPENDIX B

Discussion of controls used in transfection experiments.

The phenotypes of the human fibroblasts which are transformed after transfection with pSV N-ras are most likely the result of a highly expressed N-ras oncogene, and are not just the effects of the Maloney leukemia virus long terminal repeats. The best evidence for this is that transfection of human fibroblasts with the H-ras oncogene cloned into the Homer plasmid, a high expression vector also containing LTRs, is capable of causing transformation, whereas the Homer plasmid lacking the inserted oncogene does not cause transformation (P. Hurlin, personal communication). In addition, Hurlin found that the non-mutated cellular H-ras gene, when cloned into the Homer plasmid, does not cause transformation of transfected human fibroblasts. It is unlikely therefore, that transfection of human fibroblasts with the cellular N-ras gene cloned into the ZIP plasmid would cause transformation of human fibroblasts.

Transfection of human fibroblasts with N-ras oncogenes having other activation mutations, or with other ras oncogenes such as H-ras and K-ras would most likely cause transformation if the genes were cloned into vectors capable of giving high expression in human fibroblasts. Evidence for this is that I found the HT1080 N-ras oncogene which is mutated at codon 61 causes morphological transformation of human fibroblasts when cloned into a vector with two LTRs, and Hurlin has found that the H-ras gene cloned into the Homer plasmid can cause transformation of human fibroblasts. The presence of a different LTR could have caused quite different results since the activity of LTRs

varies depending on the origin of the LTR and the species of donar of the cells that the LTR has been introduced into.

There is strong evidence for the cause and effect relationship of transformation of human fibroblasts by a transfected N-ras oncogene. When pSV N-ras is transfected into diploid human fibroblasts and these cells are then selected for resistance to Geneticin, most of the drug resistant colonies are morphologically transformed. If the transfected cells are not selected for drug resistance, but allowed to grow to confluence, the frequency of focus formation is approximately the same as the frequency of morphologically transformed Geneticin resistant colonies obtained in dishes containing cells that were selected for Geneticin resistance. When expression of the transfected N-ras oncogene is evaluated, it is high in those cells which remain transformed, but undetectable in cells which reverted to a normal phenotype after showing initial morphological transformation.

The control cells, either diploid human fibroblasts transfected with pSV2neo which does not contain an oncogene, or immortal MSU-1 cells, grow to only a limited extent in soft agar, and do not form foci or tumors. Since the amount of growth of normal human fibroblats in soft agar can be regulated by the amount of serum which is supplied to the agar-medium, the fact that the control cells grow poorly is the result of titering the serum concentration to a point where normal fibroblasts have only a low background growth. This allows us to measure the increased capacity for growth in agar exhibited by the human fibroblasts that were transformed by N-ras. Normal human fibroblats do not form foci and have not been induced to do so by the addition of high concentrations of serum to the culture medium. Yet

human fibroblasts transformed by the Simian sarcoma virus oncogene, coding for a mitogenic peptide related to platelet-derived growth factor, form distinct foci. Therefore the phenotype of focus formation appears to be attributable to enhanced mitosis in the focus forming cells in relation to the background cells. Simple addition of more serum to the culture medium would not cause focus formation in the control cells because all of the cells should respond equally well, and the background of confluent cells should only become more dense.

The phenotype of tumorigenicity is very complex and not well understood. The control cells may or may not remain viable at the site of injection without giving rise to a tumor. However, when the cells are first injected a small nodule is present at the site of injection which is not apparent by 10 days post-injection. Therefore, the majority of the control cells have probably been removed by the immune system of the mouse by this time, or else they did not have the capacity to remain viable in the mouse.