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MALIGNANT TRANSFORMATION INDUCED IN HUMAN FIBROBLASTS BY EXPRESSION OF A TRANSFECTED T24 H-ras ONCOGENE: REQUIREMENT FOR AN INFINITE LIFESPAN

by

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ABSTRACT

MALIGNANT TRANSFORMATION INDUCED IN HUMAN FIBROBLASTS BY EXPRESSION OF A TRANSFECTED T24 H-ras ONCOGENE: REQUIREMENT OF AN INFINITE LIFESPAN

by Peter J. Hurlin

This study was undertaken to identify the phenotypes induced in human fibroblasts by expression of the T24 H-ras oncogene, one of several members of the ras family of genes freqently detected in human tumors. When the T24 H-ras oncogene, in a plasmid containing two sets of enhancer sequences, was transfected into early passage, finite lifespan human fibroblasts, colonies of morphologically transformed cells were generated. Progeny of these cells expressed T24 H-ras p21, formed foci, and developed colonies in soft agar. No such transformation was observed following transfection with the normal H-ras gene. The majority of transformed populations reverted to the normal phenotype, which correlated with loss of expression of T24 H-ras p21, even though the DNA persisted. The stably transformed cells exhibited a finite lifespan and were not tumorigenic. To determine whether the finite lifespan of these cells was a factor in preventing tumor formation, the T24 H-ras oncogene was transfected into an infinite lifespan human fibroblast strain, MSU-1.1, generated in this

laboratory following transfection with a v-myc oncogene. Foci of morphologically transformed cells developed, and progeny of these cells expressed the v-myc p110 and T24 H-ras p21, formed colonies in soft agar, proliferated rapidly in serum-free medium containing 0.1 mM calcium, which does not support growth of the parental cell line, and formed progressively growing malignant fibrosarcomas in athymic mice. No such transformation was observed with the control plasmid. Transfection of additional infinite lifespan human fibroblast cell lines with the T24 H-ras oncogene also resulted in malignant transformation. The results demonstrate the potent ability of the T24 H-ras oncogene to induce in human fibroblasts characteristics of tumor cells and the fundamental and complementary role that the infinite lifespan phenotype plays in T24 H-ras oncogene-induced malignant transformation.

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I also would like to express my graditude to my parents, Shirley and Kenneth Hurlin, whose friendship, guidance and love have been constant throughout this endeavor. Finally, the love, support and smile of Patricia Pillsbury, my companion through the last two years, is gratefully acknowledged.

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Introduction

Malignant transformation of cultured mammalian cells is generally recognized as a multistep process (Barrett and Ts'o, 1978; Thommasson and DeMars, 1982; Perez-Rodrigez et al. 1982: Newbold et al., 1982; Land et al., 1983; Ruley, 1983), as is in vivo carcinogenesis (Peto, 1977; Doll, 1978). Overwhelming evidence exists indicating that agents which damage DNA are capable of mediating steps in the pathway leading to malignancies in vivo and transformation in vitro. The first clues to the identification of potential genetic targets for such agents came from studies on RNA tumor viruses. Such studies showed that cellular genes, transduced by RNA tumor viruses, were responsible for greatly enhancing the ability of these viruses to cause tumors in host animals (Varmus, 1982). Studies on RNA tumor viruses converged with DNA transfection studies early in this decade resulting in the discovery that human tumors contained mutated versions of cellular genes, which were homologous to some of the cellular transforming genes of RNA tumor viruses (Bishop, 1983; Varmus, 1984). Cellular oncogenes were first detected in the DNA of tumors by their ability to induce characteristics of tumor cells, for example focus formation, when transfected into mouse NIH3T3 cells (Shih et al., 1979). The oncogenes most frequently detected in human tumors using the NIH3T3 transfection assay are homologous to the transforming ras genes of Harvey and Kirsten murine sarcoma viruses, and members of a family of cellular genes which have been designated H-ras, K-ras and N-ras (Varmus, 1984). Sequence analysis of these oncogenes indicated that

point mutations occurring at or near codons 12 (Tabin et al., 1982; Reddy et al., 1982; Taparowski et al., 1982) or 61 (Taparowski et al., 1983; Yuasa et al., 1983) were responsible for their ability to transform NIH 3T3 cells. The detection of ras oncogenes in approximately 10-15% of human tumors (Barbacid, 1987), together with the known association between carcinogen exposure and the generation of point mutations (Ames, 1979), provides strong circumstantial evidence of a role for ras oncogenes in the development of human malignancies.

Our understanding of the biological properties associated with expression of ras oncogenes has come primarily from transfection studies using rodent fibroblasts. Early transfection experiments using finite lifespan diploid rodent fibroblasts indicated that ras oncogenes could induce several phenotypes characteristic of tumor cells, including morphological transformation, focus formation and anchorage independence, but not an infinite lifespan in culture or tumorigenicity (Land et al., 1983; Ruley, 1983; Newbold and Overell, 1983). However, co-transfection of such cells with an H-ras oncogene and any one of a number of genes thought to cause induction of an infinite replicative lifespan in culture resulted not only in morphological transformation, focus formation and anchorage independence, but also in an infinite lifespan in culture and tumorigenicity (Land et al., 1983; Ruley, 1983). These results, along with results showing that transfection of ras oncogenes into rodent fibroblasts already exhibiting an infinite lifespan caused tumorigenic transformation (Land et al., 1983; Overell and Newbold, 1983), led to the hypothesis that oncogenes that induce the infinite lifespan phenotype act in a complementary or cooperative fashion with ras oncogenes in a two step model of in vitro carcinogenesis.

Although the use of rodent fibroblast transformation systems has been instrumental in the identification and preliminary characterization of oncogenes, it is not clear whether the results obtained using rodent fibroblasts are directly applicable to cancer development in humans. This is because exposure to various carcinogens (Barrett and Ts'o, 1978), transfection of cloned oncogenes (Land et al., 1983; Ruley, 1983; Newbold and Overell, 1983), or direct microinjection of oncogene-encoded products (Feramisco et al., 1984; Stacy et al., 1984) can cause transformation of rodent fibroblasts in culture, but attempts to reproduce such results using human fibroblasts for the most part have failed (DiPaolo, 1983; Sager et al., 1983; Spandidos et al., 1984; Feramisco et al., 1984; Stacy et al., 1984). The failure of human fibroblasts to transform as readily as rodent fibroblasts in culture may result from a fundamental biological difference between the species, or may be related to the fact that, unlike human fibroblast cell lines, many rodent fibroblast cell lines can spontaneously transform into cells that exhibit characteristics of tumor cells (Heidelberger, 1973). Because of this latter property of rodent fibroblasts, one cannot dismiss the possibility that some transformed characteristics attributed to the mutagenic effects of a carcinogen, or expression of a transfected oncogene may actually be the result of, or related to some spontaneous transformation event(s).

The goal of this thesis was to define the biological properties induced in human fibroblasts following transfection of these cells with the T24 H-*ras* oncogene, an H-*ras* oncogene cloned from a human bladder carcinoma. Results from earlier studies by others who tried to accomplish this same goal suggested that human fibroblasts in culture are resistant to

transformation by ras oncogenes (Sager et al., 1983; Spandidos, 1985). However, the considerable circumstantial evidence involving this family of oncogenes in the development of human malignancies, as discussed above, encouraged me to carry out a more comprehensive examination of the problem. Transfection studies with the T24 H-ras oncogene cloned into various vectors were carried out using both finite lifespan, and infinite lifespan human fibroblasts in order to permit comparison of the results to those of ras transfection studies that used comparable rodent fibroblast cell lines as recipients.

Chapter I of the thesis reviews the literature that provides the conceptual framework of our understanding of how oncogenes can be activated and how they may be involved in the induction of cancer and reviews the literature directly concerning ras oncogenes. Chapter II is primarily a manuscript by P.J. Hurlin, D.G. Fry, V.M. Maher and J.J. McCormick that was published in the November, 1987 issue of **Cancer Research** 47, 5752-5757, which describes my work showing that transfection of the T24 H-ras oncogene into diploid human fibroblasts induces several characteristics of tumor cells. The published manuscript has been modified in Chapter II to include data showing that cells stably transformed following transfection of the T24-H-ras oncogene are not tumorigenic. These latter data were published in a review article by D.G. Fry, P.J. Hurlin, V.M. Maher and J.J. McCormick which appeared in a special issue of **Mutation Research** (vol. 199, 341-351, 1988) on the transformation of human fibroblasts. Chapter III is a manuscript by P.J Hurlin, V.M. Maher and J.J. McCormick submitted in May, 1988 for consideration for publication in the **Proceedings of the National Academy**

of Sciences USA, which describes my work showing that the T24 H-ras oncogene induces malignant transformation when it is transfected into infinite lifespan human fibroblasts. Data relevant to this study that were not shown in the submitted manuscript are shown in Appendices B and C. Appendix A shows maps of plasmids important to these studies.

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CHAPTER I

LITERATURE REVIEW

A. Role of Oncogenes in Tumorigenesis

1. The Genetic Basis of Cancer

Cancer incidence data indicate that human populations living in different geographical regions of the world tend to develop different kinds of cancer (Waterhouse, 1976). For example, the inhabitants of third world countries often have a high incidence of liver cancer, those of western nations have a high incidence of cancer of the breast and colon, and those living in Japan have a high incidence of stomach cancer. Migrant populations tend to take on the pattern of cancer that is characteristic of their new homes (Waterhouse, 1976), suggesting that such variation in cancer incidence is due to diet, customs, or what can be generally considered environmental factors.

It is now well established that the two most common cancers in the western world, skin cancer and lung cancer, are caused primarily by known environmental factors, i.e., sunlight exposure and cigarette smoking respectively. Components of cigarette smoke and ultraviolet irradiations, like most carcinogenes interact with DNA and are mutagenic (Haseltine, 1983). Because skin, lung and most other types of cancer are strongly age dependent, it has been hypothesized that cancer induction must be a multistep process, and that the accumulation of critical mutations, resulting from carcinogen exposure or otherwise, over the life of an individual is part of this process (Doll, 1978; Cairns, 1981; Moogavkar and Knudson, 1981). Support for this hypothesis has come from a wide

range of studies including those on the induction of cancer in animals by chemical carcinogens (Berenblum, 1940; Berenblum and Shubik, 1947), studies on the relationship between carcinogen exposure and the induction of mutation and cell transformation of cells in culture (Barrertt and Ts'o, 1978; Spandidos and Siminovitch, 1978), and studies on the increased mutagenicity of carcinogens in cells derived from individuals who have a hereditary predisposition to cancer (Maher et al. 1976). There is now experimental evidence to show that point mutations and deletions, gene amplification and chromosomal translocation- -events that are known or suspected to be induced by carcinogens- -can cause the "activation" of specific cellular genes, referred to as "proto-oncogenes", into oncogenes whose protein products contribute to the malignant phenotype.

2. Identification of Oncogenes in the Genomes of Acutely Transforming Retroviruses

It was hypothesized by Temin in 1964 that as part of the lifecycle of RNA tumor viruses, RNA acted as a template for the synthesis of a DNA provirus which integrated into the host cells genome. The discovery of the reverse transcriptase enzyme by Temin and Baltimore in 1970 explained how the DNA-provirus mechanism could work. Experiments by Neiman (1972), showing the presence of proviral DNA in the genome of infected cells, provided more formal proof of the hypothesis. These discoveries led to explanations for the profound transforming properties of some RNA tumor viruses such as Rous sarcoma virus (Rous, 1911). It was proposed by Temin (1976) that the genome of acutely transforming retroviruses evolved, in part, from normal cellular sequences that had been previously mutated in the host cell by exposure to carcinogens.

A similar hypothesis, the "viral oncogene hypothesis", proposed by Huebner and Todaro in 1969, stated that the cells of all or most vertebrate species contain genomes of potentially oncogenic RNA tumor viruses that are transmitted vertically from parent to offspring. In a modified version of this hypothesis (Todaro and Huebner, 1972) it was proposed that the occurrence of cancer may be determined by the activation of normally repressed viral genes by, perhaps, exposure to carcinogenic agents or the normal processes of aging. Experiments by Neiman (1972) and later Spector (1978) showing that sequences within the acutely transforming Rous chicken sarcoma virus hybridized with the DNA of uninfected cells appeared to support this hypothesis. However, it is now known that the life cycle of RNA tumor viruses predisposes them to rare genetic events that lead to the incorporation in their genome a group of cellular genes that provide a selective advantage for proliferation to cells that are infected by such transforming viruses. Ultimately, this is manifested as malignant neoplasia.

Although it is not true, as originally hypothesised by Huebner and Todaro (1969), that retroviral genomes are present in all of the cells of any given host species, it is true that specific genes (proto-oncogenes) which have the potential to contribute to the malignant phenotype do exist in all cells. This concept, and the concept, put forth by both Huebner and Todaro (1969) and Temin (1976), that carcinogen induced mutations can be responsible for activating the transforming potential in such cellular genes are now well established.

Since the first v-oncogene, v-<u>src</u>, was identified in 1976 (Stehelin et al., 1976) there have been more than 20 other v-oncogenes identified in the genomes of RNA tumor viruses isolated from tumors, mostly sarcomas,

formed in a number of different vertebrate species. Each of these oncogenes has been found to be homologous to a cellular counterpart in the genome of the viruses host species. Such cellular counterparts or proto-oncogenes exist in evolutionarily distant eukaryotes including yeast and drosophila and are sometimes regulated in a cell cycle or lineage-specific manner, suggesting that they might play a central role in processes that control the normal cellular growth and proliferation, processes that are altered in cancer cells.

3. Identification of Cellular Oncogenes in Human and Animal Tumors

There are many possible mechanisms by which cellular proto-oncogenes can be activated into oncogenes. These include overexpression, inappropriate or ectopic expression (e.g., expression in cell type that it is normally not expressed in), unscheduled expression, altered biochemical properties caused by structural alterations within coding sequences, or some combination of these mechanisms. In most cases, putative cellular oncogenes have been detected in human and animal tumors by the identification of genes that possess one or more of these properties, as well as by exibiting homology to recognized retroviral oncogenes.

a. Oncogenes Identified in Tumors by the NIH3T3 Transformation Assay

Experiments by Anderson et al. (1979) demonstrated that DNA fragments of acutely transforming retroviruses, that carried putative v-oncogenes, were capable of causing cellular transformation when transfected into normal cells. These experiments suggested to Weinberg and his colleagues that if cellular counterparts (c-oncogenes) existed in

chemically transformed cells, they too should be capable of exerting such dominant transforming effects on normal cells. Experiments by Shih et al. (1979), demonstrated that when DNA, isolated from chemically transformed mouse cells, was transfected into non-transformed mouse NIH 3T3 cells using the the calcium phosphate transfection technique developed by Graham and Van der Eb (1973), foci of morphologically transformed cells were generated. When DNA, isolated from normal mouse cells, was transfected into the same recipient cells, it did not cause such transformation. Although there was no formal proof, it was concluded that the induced transformation was the result of the transfer of cellular alleles (oncogenes) that had their transforming potential activated by carcinogen induced mutations (Shih., 1979). The existence of such transforming sequences has since been demonstrated in the DNA of a large number of different human tumor cell lines and human tumor biopsy material.

The identification of tumor-derived sequences that caused focus formation and malignant transformation when transfected into mouse NIH 3T3 cells was first achieved by four separate groups (Parada et al., 1982; Santos et al., 1982; Der et al., 1982; Goldfarb et al., 1982), using DNA derived from human bladder carcinoma cell lines. By using molecular probes developed from v-oncogenes, each of these groups discovered that the sequences responsible for transformation in each case were homologous to the v-oncogene, v-H-<u>ras</u>, of the Harvey strain of murine sarcoma virus. Literature concerning <u>ras</u> genes and their products is reviewed in detail in later sections.

Several other putative oncogenes have been detected in human tumor cell DNA by the NIH3T3 transformation assay (Padhy et al., 1982; Cooper

et al., 1984; Padua et al., 1984; Shimizu et al., 1985; Eva and Aaronson, 1985; Martin-Zanca et al., 1986; Fusco et al., 1987). For at least two of these oncogenes the putative mechanism of activation has been identified. The neu oncogene, isolated from a rat neuroblastoma, codes for a transmembrane, receptor-like protein and shows a 50% sequence identity with the human epidermal growth factor receptor gene (Schechter et al., 1984) appears to be activated by point mutations (Bargman et al., 1986). The trk oncogene, isolated from a human colon carcinoma, is a fusion gene consisting of sequences derived from the tropomyosin gene and a gene that codes for a protein tyrosine kinase (Martin-Zanca et al., 1986). This fusion gene appears to have been generated by a mechanism involving chromosomal translocation (Martin-Zanca et al., 1986).

b. Identification of Oncogenes by Genetic Rearrangements

i. Activation by Proviral Insertion

Retroviruses that do not have oncogenes may nevertheless be involved in tumor induction. This occurs by means of insertional mutagenesis as was first shown in the study of chicken lymphomas (Hayward et al., 1981). In these tumors, the cellular myc gene has been activated by insertions of retroviral DNA upstream, or within the gene (Hayward,et al., 1981; Payne et al., 1981; Neel et al., 1981). Altered transcriptional regulation of c-myc is generally thought to be one of several steps in tumorigenesis (Thompson and Neiman, 1987). Such altered regulation and/or overexpression of a previously regulated or silent gene results because the integrated viral DNA provides a strong promoter from which transcription of the cellular gene is initiated and/or a transcriptional enhancer that stimulates increased transcription from the cellular gene's

own promoter (Bishop, 1987). Because proviral integration is thought to occur randomly throughout the host cells genome (Varmus, 1983), commonly interrupted cellular domains identified in tumor cells are likely to represent sites that are functionally significant to tumor induction. In addition to c-myc, c-mos (Gattoni-Celli et al. 1983; Cohen et al., 1983;) and c-erb B (Fung et al., 1983), which has been identified as the epidermal growth factor receptor gene (Downward et al. 1984), are frequently activated in animal tumors by proviral insertional mutagenesis. Further support for a role in tumor induction by these genes is based on the fact that each is homologous to v-oncogenes (Varmus, 1984). Other genetic loci that do not show homology to v-oncogenes have also been identified as putative oncogenes based on the observance of repeated adjacent retroviral insertion (Varmus, 1984).

ii. Activation by Chromosomal Translocations

Although most human tumors fail to show specific chromosomal translocations, there are highly regular translocations associated with some tumor types, most notably chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), and several B-cell malignancies (Sandberg et al. 1980). In approximately 95% of CML patients and 20% of ALL patients (Sandberg, 1980), there exists a cytogenetic marker, the Philadelphia chromosome, that is generated by a reciprocal translocation between chromosomes 22 and 9 (Nowell and Hungerford, 1960). The presence of the Philadelphia chromosome in such patients has important prognostic and diagnostic value (Sandberg, 1980). The c-abl gene, a homologue of the v-abl oncogene that codes for a protein tyrosine kinase (Davis et al. 1985), has been localized to the portion of chromosome 9 that contributes

to the Philadelphia chromosome (de Klein et al., 1982). The c-abl gene is translocated into a region of chromosme 22, designated the breakpoint cluster region (bcr), and the biochemical consequence of this molecular rearrangement is the production of an abnormal fusion protein, bcr-abl p210, with enhanced protein kinase activity (Konopka and Witte, 1985; Chan et al. 1987; Kurzrock et al. 1987).

Whereas the translocations that characterize the formation of the Philadelphia chromosome in CML and ALL apparently activate the abl gene by altering the biochemical properties of its product, translocations characteristic of a number of B-cell malignancies appear to activate the c-myc gene by altering its normal transcriptioal regulation (Leder et al., 1983, Marcu et al., 1983).

The specific translocations existing in a large number of B-cell tumor lines join the c-myc gene on chromosome 8 with the an immunoglobulin locus on one of three different chromosomes (2, 14 and 22) (Croce et al., 1979; Erickson et al., 1982; Malcolm et al., 1982). Altered transcriptional regulation of the c-myc gene (overexpression and/or unscheduled expression), caused by the influence of adjacent transcriptional regulatory elements at the immunoglobulin loci, appears to be the mechanism of activation of the c-myc gene in such B-cell malignancies (Leder, 1983).

iii. Activation by Amplification

Several cellular genes, that are homologous to v-oncogenes, have been identified in some tumors and tumor-derived cell lines in multiple copies. For example, the myc proto-oncogene is amplified 30 to 50-fold in the human promyelocytic leukemia cell line HL-60 (Dalla-Favera 1982),

and in small cell carcinoma-derived cell lines (Little et al., 1983). The k-ras proto-oncogene is amplified 3- to 5-fold in a human colon carcinoma cell line (McCoy et al., 1983), and a member of the myc family of genes designated N-myc is amplified in many human neuroblastomas (Schwab et al., 1983). Such amplification has been found to correlate with advanced disease stage (Brodeur et al., 1984). In these cases, the increased gene copy number correlates with an increased level of transcription.

4. Function of Oncogene Encoded Products in Tumorigenesis

For the most part, the physiological function of the products of oncogenes is unknown. However, the preliminary characterization of oncogene encoded products has revealed that they all appear to share properties with various gene products that are known to play a role in cellular mechanisms that control growth. The following descriptions of oncogene products are not meant to be complete, but to relate the above sections on mechanisms of oncogene activation to their role, or speculated role, in the transformation process.

a. Growth Factors and Growth Factor Receptors

The only retroviral oncogenes identified as genes that code for a peptide growth factor is the v-sis oncogene of simian sacoma virus (SSV)(Devare et al., 1983) and the transforming gene of the Parodi-Irgenes feline sarcoma virus (Besmer et al., 1983), which both code for the B-chain of platelet-derived growth factor (PDGF). In vitro studies of SSV-transformed cells indicate that the mature v-sis gene product enters the cellular secretion pathway and interacts with the PDGF receptor to cause unrestricted growth in an autocrine fashion (Heldin et al., 1987).

Expression of the c-sis gene is not detectable in most normal cell lines or in most tissues (Eva et al., 1982), but has been found expressed in a number of human tumor cell lines (Heldin et al., 1987). In such cells expressing PDGF receptors, activation of c-sis gene expression may affect cells in a manner similar to SSV transformation, i.e. induce autocrine growth stimulation.

A connection between oncogene products and growth factor receptors was established with the discovery that the v-erb oncogene of avian erythroblastosis virus, and the v-fms oncogene of the McDonough strain of feline sarcoma virus code for products homologous to the epidermal growth factor receptor (Ullrich et al., 1984; Xu et al., 1984: Lin et al., 1984) and colony stimulating factor-1 receptor (Rettenmeier et al., 1985) respectively. In addition, the product of the neu oncogene, mentioned above, is thought to be the rat homolog of the human EGF receptor (Shector et al., 1984). The features common to the EGF and CSF-1 growth factor receptors, as well as to the growth factor receptors for PDGF and insulin, is a glycosylated extracellular domain that binds ligand, a transmembrane domain consisting primarily of hydrophobic amino acid residues, and an intracelluar domain that exhibits intrinsic protein kinase activity. The tyrosine kinase activity of these receptors is dependent on the binding of the appropriate growth factor (Heldin et al., 1987), and appears to be an integral part of their function in stimulating cellular proliferation. However, there are many substrates and it has been difficult to find a molecular scheme to explain which tyrosine phosphorylations are important for such stimulation. In the case of the v-erb B oncogene, the region coding for the extracellular, ligand binding domain is deleted (Downward et al., 1984) and, as a consequence, its tyrosine kinase activity is

activated (Gilmore et al., 1985; Kris et al., 1985). Thus, alterations in the proportions of phoshorylated derivatives of EGF receptor-targeted intracellular proteins may act to cause cellular transformation by v-erb B.

b. Intracellular Modulators of Cell Growth

In addition to the growth factor receptor products of oncogenes, several other oncogene products that reside at the plasma membrane or in the cytoplasm code for protein tyrosine kinases, as well as serine/threonine kinases. Although the functions of these kinases are not known, it is thought that they control cell proliferation by modulating the activity or function of their various cytoplasmic and plasma membrane substrates. The oncogenic versions induce transformation through inappropriate or excessive phosphorylation of such substrates (Hunter, 1987). Therefore, it is not suprising that the gene that codes for protein kinase C, whose protein product is known to induce profound effects on cell growth and proliferation by substrate phosphorylation, can induce transformation of NIH3T3 cells if it is linked to strong viral transcriptional elements (Housey et al., 1988).

The protein products of ras oncogenes, which are localized to the plasma membrane, appear to exert their profound effects on cell proliferation through a pathway involving protein kinase C and possibly other protein kinases. This will be discussed in a latter section.

c. Nuclear Proteins Involved in Controlling Gene Expression

The viral and cellular myc genes code for proteins that are located in the nucleus and have DNA-binding properties (Abrams et al. 1982). The

rapid increase of c-myc gene expression following mitogenic stimulation of resting cells (Leder et al. 1983) suggests that the c-myc protein plays a role in the cell cycle, possibly regulating entry into S-phase. As discussed above, the transforming properties of myc oncogenes are activated by unscheduled expression and/or overexpression. Thus, it is thought that such activation leads to the continuous supply of signals for entry into S-phase, and an inability of cells to cease dividing.

Another nuclear oncogene product, that of the jun oncogene, functions as a trans-acting transcriptional regulator (Angel et al., 1988). It was recently discovered that the cellular jun product forms a complex with the cellular product of another known nuclear oncogene, fos, and that this complex can interact with the regulatory sequences of many different genes (Rauscher et al, 1988). Like the cellular myc product, the cellular fos gene product is rapidly induced in various cell types in responce to mitogens (Mitchel et al., 1985; Muller et al., 1985; Treisman, 1985). Thus, interactions between the jun and fos gene products appear to be a part of a network of mechanisms that regulate the expression of genes involved in normal, and potentially abnormal, cellular proliferation in mammalian cells.

B. Biochemical properties of ras

Using the NIH 3T3 transformation assay, ras oncogenes have been detected in approximately 10-15% of human tumors (Barbacid, 1987). The recent development and employment of more sensitive analytical methodologies for detection of ras genes indicate that a higher percentage of human tumors may contain ras oncogenes (Winter et al., 1985; Bos et al., 1986; Bos et al., 1987; Forrester et al., 1987). However, it is not possible to demonstrate a causative role for ras oncogenes in the development of human tumors simply by being able to detect their presence there. The use of carcinogen-induced animal tumor model systems provides one means of determining if there is a causal relationship between tumor induction and somatic mutation of ras genes. Several studies have shown that tumors which arise in animals following administration of chemical or other carcinogens contain activated ras genes, and that such activation is reproducible and carcinogen-specific (Balmain and Pragnell, 1983; Sukumar et al., 1983; Balmain et al., 1984; Zarble et al., 1985; Quintanilla et al., 1986; Roop et al., 1986). These in vivo studies provide strong evidence that ras genes play a role in mammalian tumorigenesis.

1. The ras Genes

a. Structural Features of ras Genes

Three functional ras genes have been identified in the human genome. These are designated H-ras, K-ras and N-ras (Shimizu et al., 1983). The H-ras gene, and the K-ras gene were found to be homologous to the

transforming genes of Harvey murine sarcoma virus (Der et al., 1982, Parada et al., 1982; Santos et al., 1982) and Kirsten sarcoma virus (McCoy et al., 1983) respectively, hence their names. The N-ras gene was originally identified as a transforming gene in a human neuroblastoma cell line by the NIH 3T3 transformation assay (Hall et al., 1983). The three mammalian ras genes code for proteins that have a molecular weight of approximately 21 kilodaltons with 187 or 188 amino acid residues (Capon et al., 1983; Fasano et al., 1983; Reddy, 1983; Hall and Brown, 1984). The p21 coding sequences of each of the human ras genes is contained in 4 exons (K-ras also has an alternative 4th exon) with the first three exons being nearly identical, and each of the exon splice junctions for all three ras genes being identical (Capon et al., 1983; Fasano et al., 1983; Reddy, 1983; McGrath et al., 1983; Shimizu et al., 1983). Such sequence conservation suggests a common origin of ras genes from one ancestral gene. Because variation in the size of the intervening sequences exists between H-, K- and N-ras genes, the genetic locus of these genes ranges in size from 4.5 kilobase pairs for H-ras to 50 kilobase pairs for K-ras (Fasano et al., 1983; McGrath et al., 1983; Shimizu et al.,1983; Capon et al., 1983). The promotor regions of each of the three functional mammalian ras genes lack a characteristic TATA or CAAT box (Breathnach and Chambon, 1981), but contain multiple transcriptioal start sites and copies of the hexanucleotide sequence CCGCCC or GGGCGG (GC box) (Hall and Brown, 1985; Ishii et al. 1985; McGrath et al., 1983). The GC box consensus sequence was originally found in the simian virus 40 early promoter (Byrne et al., 1983) and is characteristic of a number of cellular housekeeping genes (Ishii et al., 1986),

c. Mechanism of Activation of ras Genes

Mammalian ras genes acquire the ability to transform cultured mammalian cells by single missense point mutations within their coding sequences (Tabin et al., 1982; Reddy et al., 1982; Taparowski et al., 1982). Sequence analysis of ras oncogenes derived from carcinogen induced tumors in animals, carcinogen transformed rodent cells, and from naturally occurring human tumors indicate that the mutations responsible for conferring transforming properties are located at codons 12 (Tabin et al., 1982; Reddy, et al., 1982; Taparowski et al., 1982), 13, (Bos et al., 1985), 59, (Dhar et al., 1982), and 61 (Yuasa et al., 1983). Missense mutations at several other positions, introduced by in vitro mutagenesis, also can confer transfoming properties to ras genes (Fasano et al., 1984; Walter et al., 1986; Sigal et al., 1986).

Activation of ras genes by alterations affecting position 12 have been studied most thoroughly. Analysis of the transforming potential (focus formation in transfected Rat 1 cells) of 20 different H-ras genes, each encoding a different amino acid at this position, revealed that substitution of the normal amino acid (glycine) with any one of the other 20 amino acids, except proline, resulted in an activated p21 (Seeburg et al., 1984).

There is some evidence that overexpression of normal ras genes, can play a role in cell transformation in vitro and tumor development in vivo. For example, it has been shown that normal ras genes can cause malignant transformation of NIH 3T3 cells when linked to a strong promotor (Chang et al., 1982), or when multiple copies are incorporated into the genome

of these cells (Pulciani et al, 1985). Transformation in each of these cases correlated with a 30-100-fold increase in the level of ras expression over that observed in non-transfected NIH 3T3 cells (Chang et al., 1982; Pulciani et al., 1985). Amplification and overexpression of normal ras genes, usually K-ras, has been observed in a number of human tumors (Kahn et al., 1987), but the levels of expression were much below that which was capable of causing transformation of NIH 3T3 cells. Furthermore, there has been no consistent results from studies examining ras expression in tumor tissues that would implicate a role for overexpression of normal ras alleles in tumorigenesis (Barbacid, 1987).

2. The ras Proteins

a. Structure of ras Proteins

Several different functional domains of ras p21 proteins have been identified (Figure 1). These domains were identified using primarily three approaches: comparing the deduced amino acid sequence of p21s that exhibit transforming properties and those that do not (Taparowski et al. 1982; Dhar et al., 1982; Seeburg, et al., 1984; Fasano et al., 84; Walter et al., 1984; Sigal et al., 1986); comparing the biochemical properties of mutant proteins expressed in E. coli with their biological properties in cells microinjected with the mutant protein or in cells transfected with the mutated gene (Willumsen et al., 1985; Willumsen et al., 1986; Sigal et al., 1986; Der et al., 1986); and determining the biochemical and biological properties of ras p21 following reaction with antibodies with known p21 epitopes (Clark et al., 1985). The binding domain of the Y13-259 monclonal antibody (Furth et al., 1981), which has been utilized extensively for characterization of ras proteins, is located in a highly

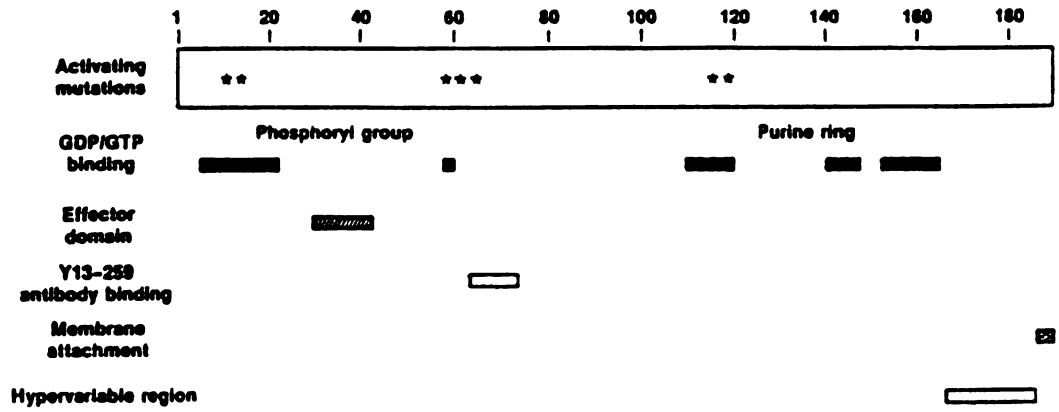

Figure 1. Schematic representation of the structural and functional domains within mammalian p21 ras proteins. Printed with permission from Ann. Rev. Biochem. Inc.

conserved, but functionally expendible region (Willumsen et al., 1986; Sigal et al., 1986; Lacal et al., 1986). The only region of ras proteins that shows sequence diversion (hypervariable region) is near the carboxy terminus, suggesting that some as yet unknown feature of this region will provide an explanation as to why three so functionally similar ras genes are expressed in mammalian cells.

b. GTP Binding and Hydrolysing Activities

The known biochemical activities associated with ras p21 proteins include GDP and GTP binding (Scolnick et al, 1979; Shih et al. 1980) and GTPase activities (McGrath et al., 1984; Gibbs et al., 1984; Sweet et al., 1984). In addition, ras proteins possesing threonine at amino acid position 59 (v-H-ras p21) exhibit autophosphorylation at that residue (Shih et al., 1982). In all ras p21's, palmitic acid is post-translationally attached to a cysteine residue near the carboxyl terminus which facilitates their association with the inner surface of the plasma membrane (Willingham et al., 1980; Shih et al., 1982; Sefton et al., 1982; Willumsen et al., 1984). A biochemical marker that seems to correlate well with mutations that activate the transforming properties of ras oncogenes is decreased GTPase activity (McGrath et al., 1984; Gibbs et al., 1984: Sweet et al., 1984).

The biochemical properties of ras p21 proteins closely resemble those of the G proteins involved in transmembrane signaling systems (Gilman, 1984). Such G proteins cycle between active GTP-bound forms, and inactive GDP-bound forms, and are involved in the stimulation or

inhibition of adenyl cyclase (Gilman, 1984). The observation that many oncogenic mutants of p21 are reduced in GTPase activity led to the proposal that transformation by p21 is the result of abnormally high levels of p21 in the active GTP-bound state (Gibbs et al., 1984; McGrath et al.,1984; Sweet et al., 1984). However, analysis of GTPase activities associated with a large number of p21 mutants failed to reveal a clear quantitative relationship between GTPase activity and transforming potential (Trahey et al. 1987; Colby et al., 1986; Der et al., 1986). Furthermore, no such association between mammalian ras p21 proteins and adenylate cyclase has been observed (Beckner et al., 1985).

c. Interaction with a Putative Effector Protein

The putative ras p21 effector domain, the region or regions that interact with molecules either upstream or downstream in the ras pathway, was ascribed to a region of the NH_2-terminal end of ras proteins (Figure 1) by analyzing deletion mutants (Sigal et al., 1986). Mutations in the region that includes amino acids 35-40 reduce the biological effects of ras proteins in NIH 3T3 cells, but do not disrupt the in vitro biochemical properties of E. coli expressed ras proteins (Sigal et al., 1986).

Recently, it was discovered that a protein, designated GAP (GTPase activating protein) interacts with the ras p21 (Trahey et al., 1987), and that such interaction occurs at the putative effector domain (Cales et al., 1988). The GAP protein is a cytosolic protein that stimulates the GTPase of normal ras p21 by 100-fold with no apparent effect on the GTPase of oncogenic ras p21 (Trahey et al., 1987). The studies by Cales et al. (1988) suggest that association of GAP with the normal ras p21-GTP bound

complex leads to a conformational change in the protein that increases its intrinsic GTPase activity, and that due to the mutations in the oncogenic forms of ras p21, such conformational changes are prevented from occurring, resulting in a reduced intrinsic GTPase activity. Formal proof of a role for GAP as the target or a regulator of ras p21 has yet to be obtained.

d. Involvement in the Control of Cell Proliferation

The normal physiological role of the ras proteins remains unknown. Studies on the expression of ras genes in mammalian cells indicate that they are expressed at low levels, in most, if not all cell lineages (Chesa et al., 1987; Furth et al., 1987). Such data imply that the physiological role of ras proteins is shared by most cell types. Because the biochemical properties of ras genes are similar to those of proteins involved in signal transduction, and because expression of ras oncogenes in various rodent cells lead to the abnormal growth characteristics exhibited by transformed cells, ras genes are thought to participate in biochemical pathways essential to normal cellular proliferation.

It has been known for a long time that various growth factors cause changes in inositol lipid metabolism (Durell et al., 1969). More recently, it has been shown that biochemical activities correlated with the action of GTP binding/hydrolyzing proteins are involved in pathways linked to phosphotidylinositol turnover (Brown et al., 1984). Intermediates in this pathway, particularly diacylglycerol (DAG) and inositol 1,4,5-triphospate (IP_3), can act as second messengers to mediate cellular responses initiated by the binding of growth factors, as well as

neurotransmitters and hormones to their cell surface receptors (Berridge, 1984). In ras transformed NIH3T3 cells the steady state levels of phoshotidylinositol 4,5-diphosphate (PIP_2) and DAG are elevated compared to their untransformed counterparts (Fleischman et al., 1986), and it has been suggested that ras p21 may act to affect such levels by activation of a specific enzyme, phospholipase C (Fleischman et al., 1986), which catalyses the breakdown of PIP_2 to IP_3 and DAG. IP_3 itself stimulates release of calcium into the cytosol from intracellular stores (Berridge, 1984) and DAG activates a calcium- and phospholipid-dependent kinase (protein kinase C) (Nishizuka, 1986) and acts as a substrate for arachidonic acid release during prostaglandin synthesis. Both the DAG-activated protein kinase C and the IP_3-stimulated Ca_{++} release cause a plasma membrane Na^+-H^+ exchanger, which elevates cytoplasmic Na_+ and raises the pH (Streb et al., 1983). Recent evidence suggests that generation of these signals through stimulation of phosphinositol turnover is a critical event in growth factor stimulation of cell proliferation (Berridge et al., 1984). Although there is little direct data supporting a role for ras genes in this pathway, it has been speculated that the loss of GTPase activity in oncogenic ras proteins causes these proteins to activate phospholipase C and the concommitant production of IP_3 and DAG in an uncontrolled way, independent of signals supplied by growth factor binding (Berridge, 1984; Fleischman et al., 1986).

3. Biological Properties Associated with Expression or ras Oncogenes in Mammalian Cells

a. Biological Properties Induced in Rodent Fibroblasts

Studies by several groups (Land et al., 1983; Ruley et al., 1983; Newbold and Overell, 1983; Thomassen et al., 1985) have indicated that transfection of ras oncogenes into early passage rodent fibroblasts can cause such cells to exhibit some properties of tumor cells, such as a transformed morphology and anchorage independence, but not the infinite lifespan phenotype or tumorigenicity. Malignant transformation of finite lifespan rodent fibroblasts can be attained if a ras oncogene is transfected along with any one of a number of different genes, such as v-myc, c-myc or polyoma large T (Land et al., 1983), adenovirus E1A (Ruley, 1983), N-myc (Schwab et al., 1985), or p53 (Parada et al., 1985), that are thought to be capable of causing the infinite lifespan phenotype in rodent cells in culture. Malignant transformation can also result following transfection of ras oncogenes into rodent fibroblasts that already exhibit an infinite lifespan in culture (Land et al., 1983; Newbold and Overell, 1983). Together, these results suggest that the infinite lifespan phenotype is a prerequisite for malignant transformation of rodent fibroblasts induced by expression of a transfected ras oncogene. However, it has been reported that

transfection of a ras oncogene alone is sufficient for the malignant transformation of finite lifespan mouse, rat, or Chinese hamster fibroblasts (Pozatti et al., 1986; Spandidos and Wilkie, 1984; Land et al., 1986; Spandidos, 1985), and it appears that a high level of ras oncogene expression was necessary for such transformation. Such malignant transformation has not been achieved following transfection of ras oncogenes into Syrian hamster fibroblasts (Thomassen et al., 1985, Newbold and Overell, 1983), which have a more stable karyotype and a lower incidence of spontaneous transformation into cells having an infinite

lifespan than do mouse, rat or Chinese hamster fibroblasts (Newbold and Overell, 1983; Thomassen et al., 1985). This latter result has led to speculation that the malignant transformation reported to occur following transfection of a ras oncogene into finite lifespan mouse, rat and Chinese hampster fibroblasts was caused by the increased propensity of such fibroblasts to aquire an infinite lifespan (Thomassen et al., 1985). Therefore, the hypothesis that the acquisition of an infinite lifespan in culture is a prerequisite for the malignant transformation of rodent fibroblasts is still widely accepted.

b. Biological Consequences of Expression of ras Oncogenes in Transgenic Mice

An important limitation of experiments involving the use of cultured cells for evaluating the action of oncogenes, is that they do not accurately reflect transformation as it occurs in vivo. Such experiments cannot assess the influence of various factors, such as blood supply, humoral and cell-mediated growth controls, and physical barriers to growth, that are thought to be crucial to the development of tumors in vivo (Stewart et al., 1984). In contrast, the use of transgenic mice, i.e., mice containing exogenous sequences introduced into their germ lines, permits the evaluation of the action of oncogenes in the context of the living organism.

Three studies utilized transgenic mice to investigate the effects of ras oncogenes (Quaife et al., 1987; Andres et al., 1987; Sinn et al., 1987). In each of these studies an H-ras oncogene, linked to transcriptional regulatory elements that direct expression in a tissue-

specific manner, was introduced into the germ line of mice (Quaife et al., 1987; Andres et al., 1987; Sinn et al., 1987). Expression of an H-ras oncogene under the control of the rat elastase regulatory elements which direct transcription only in the pancreas (MacDonald et al., 1987), resulted in the neoplastic proliferation of all, or most of the pancreatic acinar cells (Quaife et al., 1987). However, such neoplastic proliferation does not necessarily represent a true malignant tumor, but more likely represents a benign, but lethal neoplasm. In contrast to these results, expression of an H-ras oncogene under the control of either a mouse mammary virus tumor (MMTV) promoter (Sinn et al., 1987), or under the control of the mouse whey acidic protein promotor (Andres et al., 1987), each of which directs transcription primarily in mammary epithelium, resulted primarily in the development of mammary tumors (adenocarcinomas) that were apparently of clonal origin and occurred only after a relatively long latent period (usually 6-12 months) (Sinn et al., 1987; Andres et al., 1987). Such results suggest that the cells of certain tissues are capable of responding directy to the transforming effects of ras oncogenes by exhibiting benign neoplastic growth and that other cells require additional changes at the cellular level before neoplastic proliferation or malignant tumor development occurs.

In experiments by Sinn et al. (1987), mice matings were performed between transgenic mice that harbored an H-ras oncogene linked to the MMTV promoter, and mice that harbored a cellular myc gene linked to the same promoter (Stewart et al., 1984), to examine the combined effects of these genes in target cells of progeny animals. Coexpression of these genes resulted in a change in the spectrum of cell types that developed tumors

and a decrease in the time of tumor onset compared to that observed in animals expressing the ras or myc transgenes separately (Sinn et al., 1987). Nevertheless, the tumors formed appeared to be of clonal origin, and tumor incidence in these animals increased in an age dependent manner (Sinn et al., 1987), suggesting that events additional to co-expression of the ras and myc transgenes were required for tumor development by the targeted cells.

c. Use of Human Cells to Study the Biological Effects of ras Oncogene expression

For obvious reasons humans cannot be experimentally manipulated in the same manner as mice or other animals. Therefore, in the case of the human species, cell culture transformation systems take on special relevance for the study of cancer. However, no reproducible method has been developed to bring about the malignant transformation of human cells in culture using carcinogenic agents or transfection oncogenes (DiPaolo, 1984; McCormick and Maher, 1988). The failure of human fibroblasts to transform as readily as the rodent cell lines to date prevents a direct comparison of the tranformation process in human and rodent cells, and raises the question of whether the results achieved using rodent cells can be applied to cancer development in humans. Such dissimilarities between rodent and human cell transformation systems may reflect a fundamental biological difference between the two species, or may merely relect a significant difference in the frequency of acquiring some required phenotype, such as infinite lifespan (McCormick and Maher, 1988).

There have been several studies that have attempted to identify the

biological consequences of expression of transfected ras oncogenes in human fibroblasts. These studies are discussed in Chapters II and III of the thesis.

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CHAPTER II

Morphological Transformation, Focus Formation and Anchorage Independence Induced in Diploid Human Fibroblasts by Expression of a Transfected H-ras Oncogene[1]

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[2]The abbreviations used are:
DMSO, dimethylsulfoxide; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; Staph A buffer, 0.2 M phosphate buffer, pH 7.4, 1% triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.1% NaN_3, 0.1 M NaCl.

[3]Peter J. Hurlin, Dennis G. Fry, Veronica M. Maher and J. Justin McCormick, unpublished data.

SUMMARY

In an attempt to determine how normal human fibroblasts respond to high expression of the T24 H-ras oncogene, we transfected such cells with the plasmid vector pHO6T1 (Spandidos and Wilkie, Nature 310:469-475, 1984), containing the T24 H-ras oncogene with 5' and 3' enhancer sequences, and the aminoglycoside phosphotransferase gene which confers resistance to the drug G418. Approximately 1.5% of the G418 resistant colonies obtained after transfection and selection consisted of cells exhibiting obvious morphological transformation, i.e., they were highly refractile, and more rounded than normal fibroblasts. DNA hybridization analysis showed that the morphologically transformed cells contained the transfected T24 H-ras oncogene, and radioimmunoprecipitation analysis showed that they were expressing the T24 H-ras protein product, p21. Morphologically transformed cells formed colonies in soft agar at a frequency at least 60 times higher than that of cells that had been transfected with the control plasmid containing the normal cellular H-ras gene. Cells transfected with plasmid pHO6T1 could also be identified by their ability to form distinct foci when grown to confluence in non-selective medium following transfection. This study demonstrates that normal diploid human fibroblasts in culture can be transformed by transfection with a H-ras oncogene, and that such transformation correlates with expression of the mutant p21.

INTRODUCTION

A significant proportion of human tumors from various sites in the body have been shown to contain activated oncogenes from the ras family (1-3). This finding, which suggests that the ras oncogenes are involved in causing such tumors, has prompted investigations into the role ras oncogenes play in bringing about transformation of cells in culture. Transfection studies using primary or early passage rodent fibroblasts as recipients of ras oncogenes indicate that these genes act in a dominant manner to cause transformation (for review see ref. 4). The transformed phenotypes observed in ras oncogene-transfected rodent fibroblasts include morphological alteration (5,6), focus formation (6), anchorage independence (5,8,9), and, in some instances, tumorigenicity (6-9). Several of these studies indicate that the level of expression of the ras oncogene can be an important factor governing the degree of transformation by ras oncogenes (7-9) and that high expression of the transfected ras oncogene is required for the induction of the tumorigenic phenotype in such fibroblasts (6-9).

In contrast to the results achieved using rodent fibroblasts, the results of most DNA transfection studies in which early passage diploid human fibroblasts were used as the recipients suggested that these cells are more resistant to transformation by ras oncogenes. For example, several groups have reported that following ras oncogene transfection, human fibroblasts failed to exhibit morphological transformation (9,10), focus formation (10), anchorage independence (9), or tumorigenicity (9-11). Sutherland et al. (11) reported that

transfection of human fibroblasts with the T24 H-ras oncogene in a plasmid devoid of transcriptional enhancer sequences caused the target population to exhibit a very high frequency of cells able to form colonies in soft agar. Sager et al. (10,12) reported that following transfection of normal human fibroblasts with the same oncogene, but in a plasmid which contains SV40 transcriptional enhancer sequences, expression of the H-ras p21 could be detected. However, this did not result in morphological transformation or focus formation, or tumorigenicity. These investigators did not assay the transfectants for anchorage independence.

An explanation for the negative results with human fibroblasts, suggested by the transfection studies with animal fibroblasts, is that the transfected ras oncogene was not expressed or that the level of expression of the ras oncogene was not high enough to cause measurable effects. In an attempt to achieve high expression of the T24 H-ras oncogene in early passage, diploid human fibroblasts we used a plasmid, pHO6T1, obtained from Spandidos and Wilkie (8), in which the oncogene is inserted between two sets of transcriptional enhancer sequences. We here report that the transfectants exhibited transformation to morphological alteration and focus formation, that progeny of morphologically altered colonies exhibited anchorage independence, and that such transformation was correlated with expression of the T24 H-ras oncogene protein product p21 in the human fibroblasts.

MATERIALS AND METHODS

Cells and Culture Conditions. Diploid human fibroblasts were initiated from foreskins of clinically normal newborn infants as described previously (13). The human bladder carcinoma cell line T24 was purchased from the American Type Culture Collection (Rockville, MD). EMEM[2], supplemented with 0.2 mM serine, 0.2 mM aspartate, 1.0 mM pyruvate, 10% FBS (Grand Island Biological Co., Grand Island, NY), penicillin (100 units/ml), and streptomycin (100µg/ml) (complete EMEM) was used for routine culturing of cells. Cells were grown at 37°C, in 5% CO_2, water saturated incubators.

Plasmids. Homer 6 plasmids (8) containing the T24 H-ras oncogene (pHO6T1), the cellular H-ras gene (pHO6N1), or no additional sequences (pHO6) were kindly provided by Dr. Neil Wilkie. These plasmids contain the selectable marker gene, aminoglycoside phosphotransferase, which confers resistance to the drug G418.

DNA Transfection. The Polybrene/DMSO method, adapted for transfection of diploid human cells by Morgan et al. (14) was used. Briefly, 1 x 10^5 cells (passage 7-10) were plated into 100-mm diameter dishes in complete EMEM and incubated at 37°C. After 16-24 h, the medium was removed and replaced with transfection medium, i.e., 2.5 mls of complete EMEM containing 7.5 µg Polybrene per ml (Sigma Chemical Co., St. Louis, MO) and 1.2-2.0 µg plasmid per ml, and the dishes were returned to 37°C. The transfection medium was distributed over the cells by shaking once every hour. After 6 h the transfection medium was removed and the cells were treated for 4 min at room temperature with 5 mls of complete EMEM containing 30%

freshly-distilled DMSO. Following DMSO "shock" the cells were rinsed 2 times with EMEM, refed with complete EMEM, and incubated at 37°C.

Selection of G418 Resistance. If the transfected population were to be selected for drug resistance, 24 h after DMSO shock the medium was exchanged for complete EMEM containing 200 µg of active G418 (GIBCO) per ml. Colonies were allowed to develop for 14 days, with one refeeding with this selective medium.

Assay for Focus Formation. If the transfected cells were to be assayed for the ability to form macroscopic foci piled up on top of a monolayer of contact-inhibited cells, they were not selected for G418 resistance, but instead were allowed to grow to confluence in complete EMEM following DMSO "shock". The cells were fed once per week for three weeks and then stained with methylene blue and the foci counted.

Assay for Anchorage Independence. Cells were trypsinzed and resuspended in a modified version of Ham's MCDB110 (15) formulated in this laboratory for use with serum replacements in studies requiring absence of serum (16). For the present assay, 1% FBS was supplied. The cells were counted electronically (Coulter Corp., Hialeah, FL) and plated into top agar at a density of 4,000 cells per ml. The top agar consisted of the modified MCDB110 medium supplemented with 1% FBS and 0.33% SeaPlaque agar (FMC Corp., Rockland, ME). The bottom agar was prepared with the same medium, but supplemented with 2% agar. One ml of top agar was plated per 35-mm diameter dish on top of 2 ml solidified bottom agar. The following day and weekly thereafter 1 ml of modified MCDB110 medium with 1% FBS was added to the top agar to compensate for evaporation. After 3 weeks the

number of colonies with a diameter larger than 60 μm was determined microscopically.

Southern Blot Analysis. Genomic DNA was isolated as previously described (17). DNA was digested with restriction enzymes under conditions recommended by the supplier (New England Biolabs, Beverly, MA). Ten μg of digested genomic DNA or 5.6 μg plasmid DNA was electrophoresed in 0.8% agarose gels and transferred to nylon filters (Gene Screen Plus, New England Nuclear, Boston, MA) using standard techniques (18). Nylon filter prehybridization, hybridization and high strigency washing conditions were carried out as recommended by the supplier. The 2.9 kb Sac 1 fragment of the T24 H-ras oncogene, nick translated with ^{32}P-labeled dNTPs (New England Nuclear, Boston, MA, 600Ci/mmol) to a specific activity of 1-2 x 10^8 cpm/μg, was used for hybridization. Filters were exposed to Kodak XAR X-ray film (Kodak, Rochester, NY) with the aid of intensifier screens (Cronex Lightning Plus, Dupont, Inc., Wilmington, DE) for 72 h at -70°C.

RNA Analysis. Cytoplasmic RNA dot blots were performed as described by White and Bancroft (19).

Detection of p21. Protein labeling and immunoprecipitation were carried out essentially as described previously (20,21) with the following modifications. Cells growing exponentially in the modified version of MCDB110 medium supplemented with 10% FBS were plated into 100-mm diameter dishes at 10^6 cells/dish. After 24 h the medium was exchanged for 3 ml of this medium lacking methionine and FBS, but supplemented with serum replacements (16). After 45 min, 1.0 mCi of ^{35}S-methionine (1148 Ci/mmol, New England Nuclear, Boston, MA) was added and the cells were incubated for 24 h. Cells

were lysed with 1.0 ml Staph A buffer[2] containing 2.0 mM phenylmethanosulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) and 135 kallikrein inactivator units/ml aprotinin (Sigma Chemical Co., St. Louis, MO). Lysed cells were centrifuged at 35,000 rpm in a Beckman 50Ti rotor for 35 min. The supernatant, containing 10^8 cpm of trichloroacetic acid precipitable counts, was immunoprecipitated for 2 h at 4°C with the monoclonal antibody Y13-259 (22), kindly supplied by Dr. M. Anderson. Protein A-sepharose (Pharmacia Chemical Co., Piscataway, NJ) coated with goat-anti-rat IgG (Cooper Biomedical, Malvern, PA) was added and the mixture was incubated for 30 min at 4°C. The mixture was centrifuged, the supernatant was removed and the sepharose fraction was washed twice with Staph A buffer, once with a solution of 50 mM Tris-Cl (pH 7.5), 1 M $MgCl_2$ and one more time with Staph A buffer. Electrophoresis sample buffer was added and the samples were heated at 90°C for 5 min. The samples were centrifuged and aliquots of the supernatant, along with pre-stained protein molecular weight markers (Bethesda Research Laboratories, Bethesda, MD), were electrophoresed (23) through 12.5% SDS-polyacrylamide gels for 4 h at 200 V. The gels were fixed, treated with autoradiographic enhancer (Enlightning, New England Nuclear, Boston, MA), dried, and exposed to X-ray film at -70°C for 24-72 h.

RESULTS

Frequency of Transfection to G418 Resistance. Plasmid pHO6T1, which contains the T24 H-ras oncogene, was chosen for transfection of diploid human fibroblasts because of its demonstrated ability to transform early passage rodent fibroblasts into malignant cells (8) and because in our preliminary experiments, other plasmids containing the T24 H-ras oncogene proved to be ineffective in transforming human fibroblasts[3]. Plasmid pHO6N1, which contains the normal, endogenous H-ras gene, and plasmid pHO6 which has no ras gene were used as controls in the transfection experiments. Recombinant plasmids were introduced into early passage (passage 7-10) diploid human fibroblasts using a modified Polybrene/DMSO transfection technique. This transfection technique was chosen because the frequency of G418 resistant clones obtained was shown to be 10 to 40 times that acheived using calcium phosphate (1). To increase the probability that colonies selected for resistance to G418 represented unique transfection events, transfection and selection of cells were performed in the same culture dish. The frequency of G418 resistant colonies obtained after transfection of human fibroblasts with the various Homer 6 plasmids is shown in Table 1. Each plasmid construction yielded approximately the same number of G418 resistant colonies per μg of transfected plasmid, i.e., 200 per 10^6 target cells.

Transformation to Altered Morphology. G418 resistant colonies were microscopically examined for evidence of altered cellular morphology two wks following transfection and selection in G418 medium.

Table 1. Frequency of transformation to altered cellular morphology and focus formation

Plasmid	Number of dishes used in transfection[a]	Total G418 resistant colonies detected	G418 resistant colonies per 10^6 cells transfected[a]	Morphologically altered colonies per 10^6 transfected[b]	Foci[c] per 10^6 cells transfected
pHO6	22	573	260	0	0
pHO6N1	10	175	175	0	0
pHO6T1	119	2,461	201	3	39

[a] 10^5 cells per 100-mm diameter dish were transfected with 3μg plasmid DNA.

[b] Determined after two weeks of G418 selection following transfection.

[c] Determined after two weeks growth in complete EMEM following transfection.

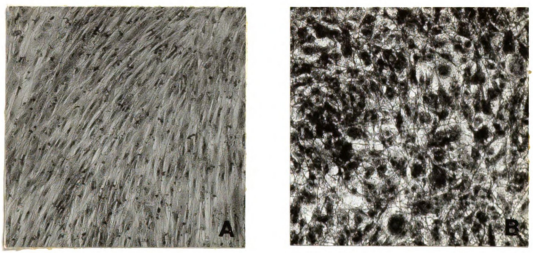


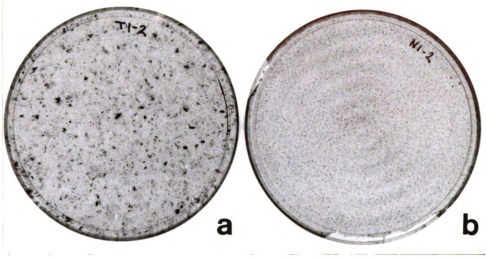
Figure 1. Comparison of normal and transformed morphologies. (a) normal human fibroblasts; (b) fibroblasts transfected with pHO6Tl. Confluent cultures were fixed with methanol and stained with methylene blue. (Magnification, 100X.)

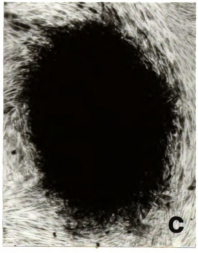
Only pHO6T1 induced morphological transformation, as indicated in Table 1. A representative colony of morphologically transformed cells is shown in Fig. 1. The morphologically transformed cells divided rapidly, were highly refractile and grew in irregular patterns, rather than the highly-oriented pattern of normal human fibroblasts.

Transformation to Focus Formation. Cells transformed following transfection with pHO6T1 could be also identified by their ability to grow into a 3-dimensional array of cells, a focus, on top of a monolayer of contact-inhibited cells. This type of aberrant growth behavior is a common characteristic of tumor cells, including those derived from human fibrosarcomas[3]. Macroscopic foci, and an individual focus of pHO6T1 transfected fibroblasts surrounded by adjacent non-transformed fibroblasts are shown in Fig. 2. The number of foci observed per 100-mm diameter dish following three weeks of growth under non-selective conditions was approximately 10-fold greater than the number of morphologically transformed colonies observed after two weeks of G418 selection (see Table 1). No foci were generated following transfection of the control plasmids pHO6N1 and pHO6 which lack the oncogene. Microscopic inspection of the morphology of the cells making up the focus revealed that not all cells capable of forming foci exhibited the transformed morphology. Thus, the number of foci is a more sensitive determination of the frequency of transformed cells following transfection with pHO6T1 than is the number of morphologically transformed colonies.

Induction of Anchorage Independence. Morphologically transformed, G418 resistant colonies obtained following transfection with pHO6T1 and G418 resistant colonies obtained following transfection with

Figure 2. The focus forming ability of pHO6T1 transfected fibroblasts. Following transfection with pHO6T1 which contains the T24 H-ras oncogene, or pHO6N1 which contains the normal cellular H-ras gene, cells were allowed to grow for 3 weeks under non-selective conditions. Confluent cells were trypsinized and replated at a 1 to 3 dilution and then allowed to grow for an additional 3 weeks before being stained with methylene blue. (a) Foci generated in a dish containing pHO6T1 transfectants; (b) confluent monolayer in a dish containing pHO6N1 transfectants; (c) 40x magnification of a focus from the dish shown in (a).


T1-2
a
M1-2
b

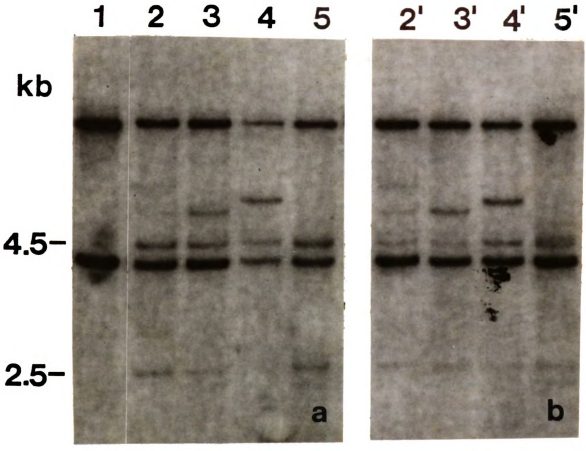

c

the control plasmid, pHO6N1, were compared for their ability to form colonies in soft agar. The cell populations assayed were derived from pooled colonies that had been isolated 24 h earlier. No colonies were observed in the control dishes, representing a total of 8,000 cells assayed (frequency, $<1.25 \times 10^{-4}$). In contrast, 122 colonies with a diameter equal to or larger than 60μm were observed with the oncogene transformed cells, out of a total of 16,000 cells assayed (frequency, 76×10^{-4}).

A concomitant comparison of the ability of the two populations to form colonies on plastic showed that the cloning efficiency of the control cells was twice that of the transformed cells (data not shown). Therefore, when measured under the stringent conditions (1% FBS) employed in this assay, cells exhibiting the transformed morphology formed colonies in soft agar at a frequency at least 60 times that of the control cells.

Analysis for the Presence of Transfected Sequences. G418-resistant colonies that exhibited the transformed morphologically were isolated, pooled in groups of several clones, and expanded for several population doublings. High molecular weight DNA was extracted and digested with restriction endonucleases Xba I and Hind III (double digestion) to liberate specific size fragments (2.5kb and 4.5kb) that are diagnostic for an intact region of plasmid pHO6T1 containing the T24 H-ras oncogene, its endogenous promoter and exogenous 5' and 3' enhancer sequences (8). The DNA was analyzed by the method of Southern (18). As shown in Fig. 3, cells exhibiting the transformed morphology contain the 4.5 kb and 2.5 kb fragments. The intensity of these two fragments was approximately half that of the endogenous

Figure 3. Southern blot analysis of cellular DNA from morphologically transformed cells obtained following transfection with pHO6T1. Following transfection of pHO6T1, and G418 selection, colonies of morphologically transformed cells were isolated and pooled so that each pool represented 3-4 separate clonal populations. The clonal populations were expanded and high molecular weight DNA was extracted from them and from the parental strain of normal diploid human fibroblasts used for transfection. 10μg of DNA from each sample was digested with Xba I and Hind III (double digest), electrophoresed through a 0.8% agarose gel, and transferred to nylon filters. The filters were hybridized with the 2.9 kb Sac I fragment of the T24 H-ras oncogene radioactively labeled by nick translation. (a) Lane 1, non-transfected normal diploid human fibroblasts; lane 2-5, pooled populations of morphologically transformed cells. Hybridizing fragments of 4.5 and 2.5 kb are diagnostic for the presence of transfected T24 H-ras oncogene sequences. (b) Southern blot analysis of high molecular weight DNA extracted from progeny cells of those analyzed in Fig. 4a, but which had reverted to the normal fibroblast morphology. DNAs were analyzed as above. Lanes 2'-5' are the progeny cells from the respective lanes in Fig. 4a, kb, kilobases.


1
2
3
4
5
2'
3'
4'
5'
kb
4.5–
2.5–
a
b

ras sequences, indicating that the cells contain only one copy of the transfected ras gene.

We found that if the morphologically transformed G418-resistant cells were passaged extensively, even in the presence of selective medium, the majority of them eventually reverted to a normal morphology. Of the 38 morphologically transformed colonies analyzed, 36 reverted to a normal morphology within 25 population doublings following transfection. These reverted cells were assayed for the ability to form foci, to form colonies in soft agar, and for the presence of the T24 H-ras oncogene and its protein product. They did not form foci or colonies in agar. However, Southern blot analysis showed that the revertants still possessed the transfected oncogene (Fig. 4b).

Expression of the T24 H-ras Oncogene in Transformed Cells. Substitution of valine for glycine at the 12th amino acid position of the T24 H-ras-encoded p21 alters its electrophoretic mobility, making it distinguishable from normal, endogenous p21 (24). Seven independent G418 resistant, morphologically transformed clonal populations obtained after pHO6T1 transfection and 5 clonal populations that reverted to the normal fibroblast morphology were analyzed for the presence of the mutated, form of p21 by radioimmunoprecipitation analysis using monoclonal antibody Y13-259 (22). Fig. 4 shows a comparison of p21 expression in pHO6T1 transfectants exhibiting a transformed morphology, and in cells which reverted to a normal morphology. In cells transfected with the control vector (lanes 1 and 7) and in pHO6T1 transfectants that reverted (lanes 3-5), the characteristic p21 doublet (20) is expressed (see dashes). The pHO6T1

Figure 4. Analysis of ras gene expression. [^{35}S]-methionine labeled cellular extracts were immunoprecipitated with monoclonal antibody Y13-259, the immunoprecipitates electrophoresed on 12.5% polyacrylamide gels, and the gels analyzed by fluorography. Lanes: 1 and 7, cells transfected with the control vector, pHO6; 2 and 8, the human bladder carcinomoa cell line T24; 3-5 independent clonal populations that reverted from a transformed morphology to a normal morphology following transfection with pHO6T1; 6 and 9, pHO6T1 transfected, morphologically transformed cells. Lanes 7-9 represent a second analysis of samples 1, 2, and 6, respectively. Dashes: endogenous p21 doublet. Arrows: mutant (T24) H-ras p21.

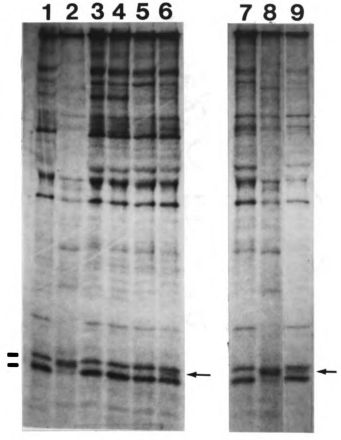
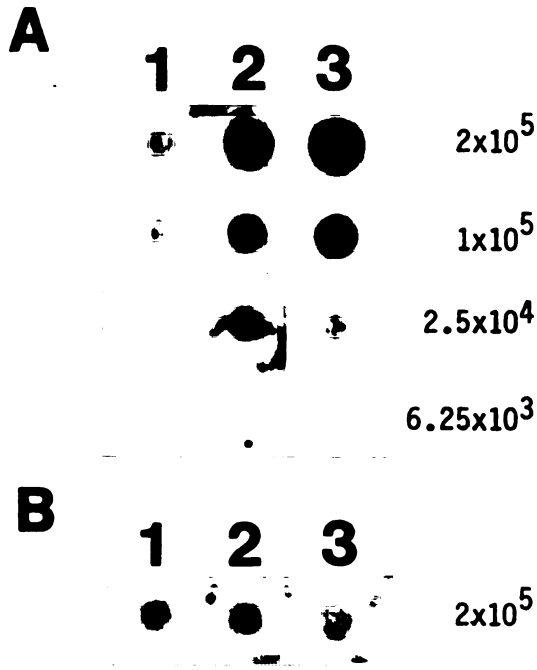

1 2 3 4 5 6
7 8 9

Figure 5. H-ras RNA expression. Cytoplasmic samples were extracted from 8 x 10^5 cells, denatured, diluted to the equivalent cell number indicated, and spotted onto nitrocellulose filters. (a) Hybridization with the Sac I 3kb T24 H-ras fragment, nick translated with [^{32}P]-NTPs. Lanes: 1, cells transfected with the control vector, pHO6; 2 and 3, cells transfected with pHO6T1 and exhibiting a transformed morphology. (b) Hybridization of the same samples as in (a) with a β-2 microglobulin probe to control for variability in the amount of cytoplasmic extract loaded.


A
1 2 3
2x10^5
1x10^5
2.5x10^4
6.25x10^3
B
1 2 3
2x10^5

transfectants exhibiting a transformed morphology expressed an additional p21 species that co-migrated with a p21 species expressed in the human bladder carcinoma cell line T24. The mutant species migrated immediately below the slower migrating, normally present p21 species (see arrow). A similar pattern of expression has been observed in all seven of the morphologically transformed populations analyzed. The additional p21 species was not observed in any of the reverted populations, or in control, vector (pHO6) transfected populations tested.

RNA dot blot analysis of cytoplasmic RNA samples indicated that transfectants maintaining the transformed morphology expressed higher than normal levels of H-ras specific RNA. Fig. 5 shows that samples from such cells (lanes 2 and 3) expressed approximatley four times the amount of H-ras RNA expressed in control cells (vector transfected; lane 1). This higher level of expression of H-ras RNA correlates with expression of the T24-H-ras p21 species in pHO6T1 transfected, morphologically transformed cells.

Tumorigenicity. Two G418-resistant, morphologically transformed clonal populations obtained following transfection with pHO6T1 that maintained their altered morphology when expanded into large populations were assayed for tumorigenicity in athymic mice as described previously (25). Three mice were used per test and 10^7 cells were injected subcutaneously in each animal. No tumors or indication of growth at the site of injection were observed over a period of greater than one year. When the cells were continuously passaged in culture as part of a lifespan assay, they senesced at a population doubling level comparable to that of normal fibroblast populations.

DISCUSSION

Results of the present study show that normal diploid human fibroblasts can be transformed into morphologically altered, focus forming, and anchorage independent cells following transfection of the T24 H-ras oncogene. Our results, showing that the transformed cells exhibited anchorage independence, support the results obtained by Sutherland et al. (11) and extend that study by showing that the transformed cells expressed the T24 H-ras protein. However, unlike Sutherland et al. (11) we did not use anchorage independence to identify transformed populations of cells directly following transfection. Therefore, it is not possible to directly compare our frequency of anchorage independent cells with theirs.

If the anchorage independence exhibited by cells transfected with the T24 H-ras oncogene in the enhancer-free plasmid used by Sutherland et al. (11) is an indication that the transfectants were expressing the mutant p21 protein, then it is difficult to understand why Sager et al. (10,12), did not observe any evidence of transformation. They used the same oncogene, but in a plasmid which contains a set of transcriptional enhancer sequences, and they found expression of mutant p21. An obvious explanation for the difference between the results of Sager et al. (10,13) and those we present here is a difference in the level of expression of p21 resulting from the use of different plasmid constructions containing the T24 H-ras oncogene. When we began transfection studies using normal diploid human fibroblasts as recipients, like Sager et al. (10,12) we constructed and used a pSV2-derived plasmid containing the T24

H-ras oncogene and found, as they did, no evidence of morphological transformation or focus formation[3]. What is more, there was no induction of anchorage independence[3]. The positive results reported in the present study were generated using a plasmid (pHO6T1) that was specifically designed by Spandidos and Wilkie (8) to elicit high expression of the T24 H-ras oncogene in mammalian cells by inserting the oncogene between two sets of transcriptional enhancer sequences. Therefore, we interpret our results to indicate that a sufficiently high level of expression of the T24 H-ras oncogene was achieved to cause the transformation of normal human fibroblasts, and that the pSV2-T24 plasmid of Sager et al. (12) was not capable of generating such levels. This interpretation does not explain the negative results of Spandidos (9), who recently reported that transfection of normal human fibroblasts with pHO6T1 did not cause anchorage independence or any obvious morphological transformation. However, Spandidos used a different method of transfection (calcium phosphate) then we did (DMSO/Polybrene), and examined a relatively small number of independent G418 resistant colonies ($\sim$200) compared to the total number we examined (>2461).

The results of numerous ras oncogene transfection studies carried out with rodent fibroblasts suggest that high expression of the T24 H-ras oncogene can be attained, and that with such expression the tumorigenic phenotype is induced (6-9). Our studies with human fibroblasts indicate that the transfected oncogene is expressed at low levels, compared to what has been observed in rodent fibroblasts, and that such expression is lost in most cells as they are passaged. Only two populations of pHO6T1 transfectants out of 38 analyzed could

be isolated that maintained a transformed morphology when extensively passaged, and both of these proved to have a finite lifespan and were not tumorigenic. However, there is evidence that ras oncogenes are involved in neoplastic transformation of human fibroblasts. For example, transfection of diploid fibroblasts derived from Bloom's syndrome patients with DNA from NIH 3T3 cells that had been transformed by Harvey murine sarcoma virus (Ha-MSV) DNA resulted in cells able to form nodules in athymic mice (26). These nodules subsequently regressed (26). Also, Harvey (27) or Kirsten murine sarcoma virus (28) infection of human fibroblasts that had acquired an indefinite lifespan in culture following SV40 virus transformation or repeated exposure to gamma radiation resulted in cells able to form tumors. One group (27) indicated that the tumors regressed, the other (28) found they did not. Three groups (29-31) have succeeded in causing neoplastic transformation of human epithelial cells by ras oncogene transfection. Further studies to determine if diploid human fibroblasts that continue to express the T24 H-ras oncogene are tumorigenic and whether this gene cooperates with other oncogenes (e.g., myc) in causing malignant transformation of human fibroblasts are in progress.

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CHAPTER III

Malignant Transformation of a Human Fibroblast Cell Strain, MSU-1.1, By Expression of a Transfected T24 H-*ras* Oncogene

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SUMMARY

We showed previously that normal diploid human fibroblasts that express a transfected T24 H-ras oncogene exhibit several characteristics of transformed cells, but are not tumorigenic. To extend these studies of the transforming ability of the T24 H-ras oncogene in human cells, we have utilized an infinite lifespan, but otherwise phenotypically normal human fibroblast cell strain, MSU-1.1, which was developed in this laboratory following transfection of diploid fibroblasts with a plasmid carrying a v-myc oncogene. Transfection of MSU-1.1 cells with the T24 H-ras oncogene flanked by 2 transcriptional enhancer elements (pHO6T1) caused foci of morphologically transformed cells to develop. No such transformation occurred if the plasmid containing the T24 H-ras oncogene had only one enhancer or none at all, or if the normal H-ras gene was transfected in the pHO6 vector. Cells derived from such foci expressed high levels of T24 H-ras p21, formed colonies in soft agar at high frequency, proliferated rapidly in serum-free medium containing 0.1 mM calcium, which does not support growth of the parental cell line, and formed progressively growing, invasive fibrosarcomas in athymic mice. No karyotypic changes accompanied the transformation of MSU-1.1 fibroblasts, suggesting that chromosome instability did not play a role in the conversion of the cells to malignancy. Transfection of two other infinite lifespan fibroblast cell lines with the T24 H-ras oncogene in pHO6T1 also resulted in malignant transformation, suggesting that the infinite lifespan phenotype of MSU-1.1 cells, and not necessarily expression of the v-myc oncogene, was the factor that complemented T24 H-ras expression to cause malignant transformation.

INTRODUCTION

Oncogenes of the ras family have been detected in numerous and diverse human malignancies. A growing body of evidence from both in vivo and in vitro studies suggests that the p21 products of ras oncogenes are involved in the genesis of such malignancies. For example, transgenic mice harboring the T24 H-ras (1), or v-H-ras oncogenes (2) linked to a murine mammary protein gene promoter developed mammary tumors following a long latency. These tumors were clonal in origin, suggesting that additional changes at the cellular level were required to cause tumor formation. Clues to the nature of the steps or events that may complement mutational activation of ras genes in tumorigenesis have come primarily from transfection experiments utilizing rodent fibroblasts in culture (for review see ref. 3). When primary or early passage rodent fibroblasts are transfected with a combination of a ras oncogene and any one of a number of genes thought to be involved in causing cellular immortalization, malignant transformation results (4-6). Transfection of ras oncogenes into infinite lifespan rodent fibroblast cell lines usually results in malignant transformation (4,7,8), but not in all cases (9). However, several studies have reported that high levels of ras oncogene expression can convert finite lifespan, early passage rodent fibroblasts into infinite lifespan malignant cells without the need for transfection of another oncogene (10-12).

To test models proposed for ras transformation generated using rodent fibroblasts for their relevance to human cell transformation, we have been using human fibroblasts as recipients of transfected ras oncogenes (13,14). Several groups reported that early passage diploid human

fibroblasts are resistant to transformation by transfected ras oncogenes (15,16), or microinjection of ras oncogene encoded p21 (17,18). But, Hurlin et. al. (13) showed that if such cells are transfected with the pHO6T1 plasmid which contains the T24 H-ras oncogene flanked by Moloney murine sarcoma virus LTR and SV40 virus transcriptional enhancer sequences, they become morphologically transformed, develop distinct foci, and form colonies in soft agar. In a related study, Tubo and Rheinwald (19) showed that diploid human fibroblasts transfected with the T24 H-ras oncogene in a plasmid which contains an SV40 virus enhancer become morphologically transformed and exhibit reduced growth factor requirements. However, the transformed cells in these two studies did not acquire an infinite lifespan and were not tumorigenic (14,19).

For the most part, oncogenes have been detected and defined by their transforming effects on rodent fibroblasts which have an infinite lifespan in culture, but are otherwise normal. No comparable human cell line has been available for use in testing the applicability of the results to human fibroblasts. This is because human fibroblasts in culture are extremely difficult to transform into cells with an infinite lifespan (20,21), even by SV40-virus infection (22) and the few infinite lifespan, non-tumorigenic fibroblast cell lines that have been generated (22-24) consist of cells that are morphologically transformed and exhibit other transformation characteristics. Recently, a human fibroblast cell strain with an infinite lifespan in culture, designated MSU-1.1, was generated in this laboratory in experiments examining the properties induced in finite lifespan, diploid human fibroblasts by transfection of a plasmid carrying a v-myc oncogene. MSU-1.1 cells have a normal morphology and a stable, near diploid karyotype. They do not form foci, or produce colonies

in soft agar, and are non-tumorigenic. Details on the generation of this cell strain, and its properties will be described eleswhere. In the present study we show that transfection of MSU-1.1 cells with the T24 H-ras oncogene in pHO6T1 caused morphological transformation and focus formation and that cells derived from such foci exhibited reduced growth factor requirements, formed colonies in soft agar at high frequency and gave rise to rapidly growing, malignant fibrosarcomas in athymic mice.

METHODS AND MATERIALS

Cells and Culture Conditions. Cell line LG1, from which the MSU-1.1 cell strain arose was initiated from foreskin material of a clinically normal newborn male as described (25). The infinite lifespan cell strain MSU-1.1, described above, is resistant to G418 (Grand Island Biological Co., Grand Island, NY) because the plasmid used to transfect the original LG1 population carried the aminoglycoside phosphotransferase gene. The infinite lifespan human fibroblast cell lines KMST-6 and SUSM-1, which were generated following repeated carcinogen treatment (23,24), were obtained from M. Namba. The SV40 virus transformed, infinite lifespan human fibroblast cell line GM0637B was purchased from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ. The human bladder carcinoma cell line T24 was purchased from the American Type Culture Collection (Rockville, MD), and the chicken hepatoma, MC29 virus-producer cell line LSCC-DU72 was provided by K. Nazerian. The cells were routinely cultured in Eagle's minimum essential medium, supplemented with 0.2 mM serine, 0.2 mM aspartate, 1.0 mM pyruvate, 10% fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin, and 100 ug/ml streptomycin at 37°C, in 5% CO_2, in water saturated incubators. For studies of growth factor independence, a serum-free medium, McM, a version of MCDB110 (26) formulated for use with serum-replacements (27), was employed.

Plasmids and DNA Transfection. Homer 6 plasmids (10) containing the T24 H-<u>ras</u> oncogene (pHO6T1), the normal human H-<u>ras</u> gene (pHO6N1) or no additional sequences (pHO6), were kindly provided by N. Wilkie. Plasmid pT24 was purchased from the American Type Culture Collection, and plasmid

pSV2-T24 was constructed in this laboratory by inserting the T24 H-ras oncogene into the BamHl site of the plasmid pSV2neo (28). Target cells (2 x 10^5) were transfected with plasmid DNA at 1.3 ug per ml in transfection medium, using the Polybrene/dimethylsulfoxide method (29) as previously described (13).

Assay for Growth Factor Independence. Cells (5 x 10^4) were plated in McM medium supplemented with 1% FBS. After 24 h, the number of cells was determined by electronic counting and the medium was exchanged for McM medium containing 0.1 mM calcium and supplemented with the serum replacements of Ryan et al. (27), but lacking epidermal growth factor. The medium was renewed 3 days later, and the number of cells was determined after another 3 days.

Assay for Anchorage Independence. Cells were assayed for the ability to form colonies in soft agar as described (13) with the following modifications. Cells (5000 per dish) were plated in top agar consisting of McM medium supplemented with 2% FBS and 0.33% SeaPlaque agar (FMC Corp., Rockland ME.) McM medium containing 2% FBS was provided to cultures weekly. Colonies having a diameter larger than 80 um were counted and sized electronically (Olympus Cue-2 Image Analyser, Olympus Corp. Lk. Success, NY) after 3 wk.

Tumorigenicity Assays. The ability of cells to form tumors was assayed by injecting 1 x 10^7 exponentially growing cells subcutaneously into 4 wk to 8 wk old BALB/c athymic mice which had been irradiated with 300 rads from a ^{60}Co source 24 h previously. To test for the ability to metastasize,

5 x 10^4 cells were injected into the tail vein of athymic mice, and after 8 mo the mice were sacrificed and complete necropsies were performed.

DNA Analysis. DNA was isolated as previously described (30) and analysed by the method of Southern (31) for transfected sequences.

Analysis of ras p21 and v-myc p110. Cells to be analysed for expression of ras p21 were labeled with [^{35}S]-Met (New England Nuclear, Boston, MA) at 250uCi per ml for 18 h in McM medium lacking methionine, but supplemented with 1% FBS. Cell lysis, immunoprecipitation with the ras p21 specific antibody Y13-259 (32), NaDodSO$_4$-PAGE and fluorography were performed as previously described (13). For analysis of v-myc p110, cells were labeled as above, but for only 1.5 h. Cells were washed once in ice-cold phosphate-buffered saline, lysed in lysis buffer (10 mM Tris hydrochloride (pH 8.2), 150 mM NaCl, 1% sodium deoxycholate, 1% TRITON X-100, 0.1% NaDodSO$_4$, 5 mM EDTA, and 2 mM phenylmethanosulfonyl fluoride), and clarified by centrifugation at 10,000 x g for 20 min. Supernatants containing 7.5 x 10^7 cpm were immunoprecipitated with a sheep antibody (Cambridge Research Biochemicals, Valley Stream, NY, antibody OA-11-801) that reacts broadly with the proteins of the myc family. Protein A-Sepharose (Pharmacia Chemical Co., Piscataway, NJ) coated with goat anti-sheep IgG (Cooper Biomedical, Malvern PA) was added, and the mixture was shaken for 30 min at 4°C. The Sepharose fraction was washed twice with lysis buffer and once with 10 mM Tris hydrochloride (pH 7.5), 1 M $MgCl_2$, and one more time with the lysis buffer. NaDodSO$_4$-PAGE sample buffer containing 8 M urea was added, and the samples were heated for 5 min at 95°C. The samples were centrifuged, and aliquots of the supernatant, along

with prestained molecular weight markers (Bethesda Research Laboratories, Bethesda, MD) were electrophoresed (33) in 10% NaDodSO$_4$-polyacrylamide gels. The gels were fixed, treated with autoradiographic enhancer (ENHANCE, NEN, Boston, MA), dried, and exposed to X-ray film at -80°C.

Karyotype Analysis. G-banded chromosomes were prepared as described (34). For each cell line, at least 25 G-banded karyotypes were prepared to examine for chromosomal rearrangements, and 100 conventially stained metaphases were counted to determine the modal chromosome number.

RESULTS

Transformation to Focus-Formation by the T24 H-ras Oncogene. Following transfection of MSU-1.1 fibroblasts with the various plasmids, the cells were cultured for 3 wk in Eagle's medium containing 10% FBS, and screened for colonies of cells that overgrew the monolayer (focus formation). With cells that had been transfected with pHO6T1, distinct foci of morphologically altered cells were found at a frequency of approximately 10 per 10^6 cells transfected. No foci, or indications of transformation were observed following transfection with control plasmids containing either the normal human H-ras gene (pHO6N1), or lacking a ras gene (pHO6). Transfection of 5 x 10^6 MSU-1.1 cells with plasmids containing the T24 H-ras oncogene, but no enhancer sequences (pT24), or with only one SV40 transcriptional enhancer (pSV2-T24) did not induce focus-formation. Six populations of morphologically transformed fibroblasts (designated MSU-1.1-T24 strains 1-6) were isolated from foci in separate dishes, and the populations were expanded for subsequent characterization. An example of the morphology of MSU-1.1-T24 cells, which are smaller, rounder and more refractile than their parental cells, is shown in Fig. 1. These cells have maintained their transformed morphology for more than 40 population doublings thus far. This stability contrasts with what Hurlin et al. (13) found with finite lifespan cells transformed following transfection of the T24 H-ras oncogene in pHO6T1.

Growth of MSU-1.1-T24 Fibroblasts in Serum-Free Medium. Schilz et. al. (35) showed that human fibrosarcoma-derived cell lines, but not normal diploid human fibroblasts, were capable of proliferating in McM medium

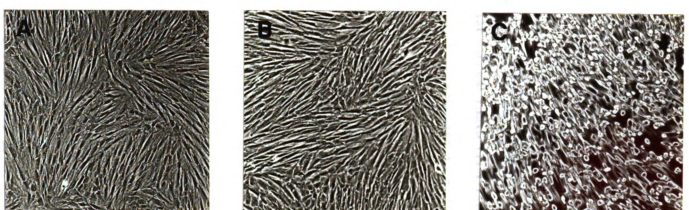


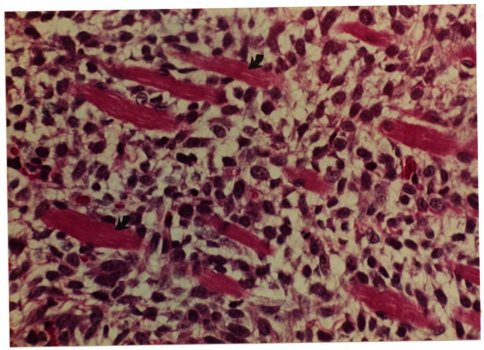
Figure 1. Morphology of LG1 human fibroblasts (A); MSU-1.1 fibroblasts (B); and MSU-1.1-T24 strain 3 fibroblasts (C). Magnification 150x.

containing 0.1 mM calcium, instead of the usual 1 mM, and supplemented with the serum replacements of Ryan et. al. (26), but lacking epidermal growth factor. The six MSU-1.1-T24 cell strains, the parental MSU-1.1 cells and the LG1 cell line from which the latter arose were compared for their ability to proliferate in such medium. MSU-1.1 cell strains proliferated to numbers 6 to 7 fold higher than the parental MSU-1.1 cells did, and as expected, LG1 cells could not proliferate under these conditions (Table 1). The number of cells attained by the MSU-1.1-T24 cell strains indicated that, under these conditions, they double at a rate approximately equal to that achieved in medium containing 10% FBS, i.e., once every 24 h.

Anchorage Independence of MSU-1.1 T24 Cell Strains. The six MSU-1.1-T24 cell strains were tested for their ability to form colonies in 0.33% agar medium. LG1 cells, and the parental MSU-1.1 cell strain formed colonies with an efficiency of less than 0.007%, i.e., no colonies were detected out of a total of 15000 cells plated. The six MSU-1.1-T24 cell strains formed colonies in agar at efficiencies ranging from 3.5% to 23% (Table 1). There was variation in the size range of colonies detected. For example, although MSU-1.1-T24 strain 6 exhibited a cloning efficiency of only 3.5%, it gave rise to the highest proportion of very large colonies (i.e., having diameters of >300um).

Tumorigenicity of MSU-1.1-T24 Cell Strains. Four of the six MSU-1.1-T24 cell strains tested for the ability to form tumors in athymic mice formed progressively growing tumors (Table 1). Four to 5 wk were required for the tumors to reach 1 cm in diameter. They were diagnosed as

Figure 2. Histology showing a poorly-differentiated human fibrosarcoma formed in an athymic mouse by MSU-1.1-T24 strain 3 cells. A 2.5 cm diameter tumor was removed 8 wk following subcutaneous injection of 10^7 cells, and was sectioned, and stained with hematoxylin-eosin. Mouse skeletal muscle is indicated by the arrows. Magnification; (A) 40x, (B) 200x.



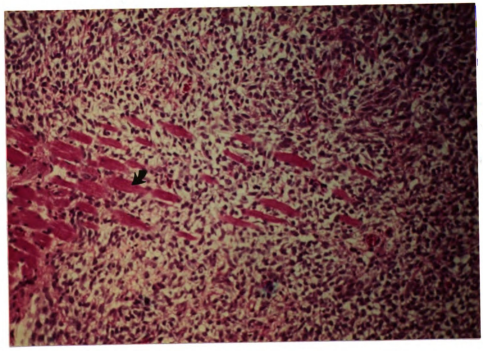


Table 1. Growth properties and tumorigenicity of MSU-1.1-T24 cell strains

Cell strain	Cloning efficiency in agar (%)	Cell no. attained in serum-free 0.1 mM Ca^{++} medium ($x10^{-5}$)[a]	Tumor incidence[b]	Days for tumor to reach 1 cm diam
1	15	16	2/2	36
2	23	NT[c]	6/6	30
3	10	NT	6/6	30
4	18	17	0/5	--
5	8.5	19	0/3	--
6	3.1	17	4/4	34
MSU-1.1	<0.01	2.7	0/9	--
LG1 cells	<0.01	0.4[d]	0/9	--

[a]Duplicate cultures were counted 7 d after plating 5 x 10^4 cells/dish.

[b]Animals with tumors per animals injected subcutaneous. Animals were examined for tumor formation for at least 8 mo following injection.

[c]NT, not tested.

[d]This is the same no. of cells as was determined 1 d after plating.

poorly-differentiated, invasive human fibrosarcomas with a high mitotic index and a moderate degree of anaplasia. A low proportion of giant multinucleated cells were observed in several of the tumors. A histological section of a representative tumor is shown in Fig. 2. Two criteria indicated that the tumors were formed by the injected MSU-1.1-T24 fibroblasts. First, the tumor-derived cells that were returned to culture were capable of proliferating in medium containing G418, and second, they contained the marker chromosomes characteristic of MSU-1.1 cells (see below). One of the tumorigenic MSU-1.1-T24 strains was tested for metastatic properties by being injected into the tail vein of mice. No tumors were found from necropsies performed on 5 such mice 8 months later.

Presence of T24 H-ras and v-myc Oncogenes. Following digestion of genomic DNA with Xba I and Hind III, fragments of 4.5 and 2.5 kb that hybridize with the Sac I, 2.9 kb fragment of the T24 H-ras oncogene are diagnostic for plasmid pHO6T1 sequences which include the T24 H-ras oncogene and flanking 5′ and 3′ transcriptional enhancer sequences (10). As shown in Fig. 3, genomic DNA from each of the MSU-1.1-T24 cell strains contained H-ras hybridizing fragments close to these predicted sizes. Although the data are not shown, v-myc hybidizing DNA fragments were detected in the six MSU-1.1-T24 cell strains and they were of identical size and intensity as those detected in the parental MSU-1.1 cell strain, indicating that no rearrangements or amplification of this oncogene had occurred subsequent to introduction of plasmid pHO6T1.

Expression of T24-H-ras and v-myc Oncogenes. Radioimmunoprecipitation analysis of the MSU-1.1-T24 cell strains (Fig. 4) using the ras p21

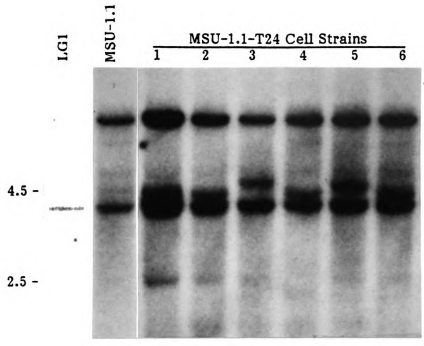
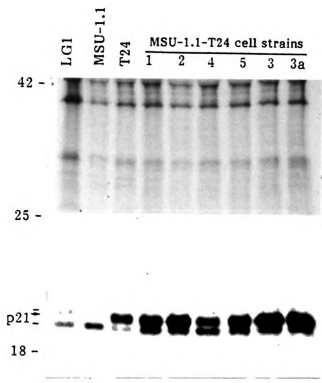


Figure 3. Southern blot analysis of plasmid pHO6T1-derived T24 H-ras oncogene sequences in MSU-1.1-T24 cell strains. LG1 is the diploid human fibroblast cell line from which the MSU-1.1 cell strain was derived. DNA was digested with Hind III and Xba I and hybridized with the 2.9 kb Sac I fragment of the T24 H-ras oncogene. The locations of the expected 4.5 kb and 2.5 kb plasmid pHO6T1 specific fragments are indicated.

Figure 4. Expression of T24 H-ras p21 in MSU-1.1-T24 cell strains. The ras encoded p21 proteins were immunoprecipitated with antibody Y13-259 (25) from [^{35}S]methionine-labeled cell lysates and analysed by electrophoresis in 12.5% SDS-NaDodSO$_4$ polyacrylamide gels and flourography. The location of the endogenous ras encoded p21 protein doublet is indicated by the bars, and the T24 H-ras encoded p21 location is indicated by the arrow. T24 is the human bladder carcinoma cell line. 3a is a clonal derivitive of MSU-1.1-T24 strain 3. The location of molecular weight (kilodaltons) markers on the gel is indicated.


LG1
MSU-1.1
T24
MSU-1.1-T24 cell strains
1
2
4
5
3
3a
42 -
25 -
p21
18 -

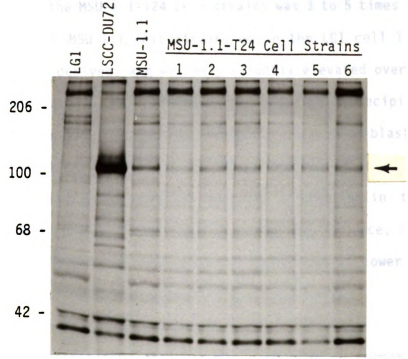


Figure 5. Expression of v-myc encoded p110 in MSU-1.1-T24 cell strains. v-myc p110 was immunoprecipitated from [^{35}S]methionine-labeled cell lysates with an antibody that reacts with proteins of the myc family and analysed by electrophoresis in 10% polyacrylamide gels and fluorography. The location of the v-myc encoded p110 is indicated by the arrow. LSCC-DU72 is a chicken hepatoma, MC29 virus-producer cell line. The location of molecular weight (kilodalton) markers on the gel is indicated.

specific monoclonal antibody Y13-259 (28), indicated that each of them overexpressed a 21 kd protein (see arrow) that migrated to the same position as the overexpressed T24 H-ras oncogene encoded p21 of the T24 bladder carcinoma cell line. To determine the relative levels of ras p21 expression, the radiolabeled p21 bands were cut out of duplicate gels and quantitated by scintillation counting. The level of total ras p21 expression in the MSU-1.1-T24 cell strains was 3 to 5 times that expressed in the parental MSU-1.1 cell strain, or in the LG1 cell line from which the latter was derived, and was only slightly elevated over that observed in T24 bladder carcinoma-derived cells. Radioimmunoprecipitaion analysis of ras p21 was also carried out on MSU-1.1-T24 fibroblast cell strains derived from tumors generated in athymic mice. T24 H-ras p21 levels in these cells were comparable to levels determined in their parental focus-derived cells prior to injection into athymic mice, indicating that no in vivo selection of cells expressing higher or lower levels of the transfected oncogene had occurred (data not shown).

Radioimmunoprecipitation analysis was also used to compare the level of v-myc p110 expression in the MSU-1.1-T24 cell strains, with that in the parental, MSU-1.1 cell strain, using as a positive control a chicken hepatoma cell line LSCC-DU72 that expresses a v-myc p110. Such analysis indicated that in the MSU-1.1-T24 cell strains, v-myc p110 was expressed at levels comparable to that found in the parental MSU-1.1 cells (Fig. 5, see arrow). As expected, no v-myc p110 was detected in the LG1 cells.

Karyotype Analysis of MSU-1.1-T24 Cell Strains. Analyses of G-banded, and conventionally stained chromosomes from MSU-1.1 fibroblasts and

MSU-1.1-T24 fibroblasts were carried out to determine karyotype and chromosome stability respectively. A detailed description of the banding patterns for MSU-1.1 fibroblast chromosomes will be reported elsewhere. The MSU-1.1-T24 cell strains analysed (strains 2 and 3), as well as cells taken from the tumors derived from them, showed the same karyotype and modal chromosome number, i.e., 45, as the parental MSU-1.1 cell strain, as well as the two marker chromosomes characteristic of this cell line.

Tumorigenic Transformation of Infinite Lifespan Human Fibroblasts Derived from Other Cell Lines. To determine whether expression of T24 H-ras in infinite lifespan, non-tumorigenic human fibroblast cell lines other than MSU-1.1 also caused tumorigenic transformation, we transfected pHO6T1, or the control plasmid, pHO6N1, into the SUSM-1 (23) and KMST-6 (24) cell lines, as well as into a cell line (GM0637B) generated following SV40 virus infection. The transfectants were selected for G418 resistance, rather than focus formation, because unlike MSU-1.1 cells, these target cell lines already exhibit a transformed morphology and aberrant growth behavior. G418-resistant colonies were pooled, and their progeny cells tested for tumorigenicity in athymic mice. Within 4.5 mo, four out of five athymic mice injected with pHO6T1-transfected KMST-6 cells (KMST-6-T24) and three out of six injected of pHO6T1-transfected GM0637B cells (GM0637B-T24) developed progressively growing malignant fibrosarcomas within that reached a 1 cm diameter. No tumors were formed in 5 mice injected with pHO6T1-transfected, SUSM-1 cells (SUSM-1-T24) generated in two separate experiments, or with any of the pHO6N1 transfected, G418-resistant controls. Because of the long time required for tumor development by these

cell strains relative to MSU-1.1-T24 tumorigenic cell strains, cells were cultured from tumors formed by these cell strains and reinjected in athymic mice to determine if an in vivo selection for tumorigenic cells had occurred. Two out of 2 mice injected with tumor-derived KMST-6-T24 cells developed 1 cm diameter tumors within 25 days, (a period similar to that of MSU-1-T24 tumorigenic strains), but no decrease in time for tumor development with tumor-derived GM0637B-T24 cells. Both KMST-6-T24 cells and GM0637B-T24 cells expressed T24 H-ras oncogene, but the SUSM-1-T24 cells did not (data not shown), which correlates with their tumorigenicity.

DISCUSSION

In an earlier study (13) we demonstrated that expression of a transfected T24 H-ras oncogene in normal diploid human fibroblasts resulted in their transformation to morphologically altered cells that form foci and exhibit anchorage-independent growth. Analysis of the relationship between expression of the transfected ras oncogene and tumorigenicity was not possible because expression of the transfected ras oncogene and the transformed phenotype were lost concomitantly during expansion of transformant cell populations. Later, we succesfully isolated several T24 H-ras transformed diploid human fibroblast populations that were stable, i.e., did not revert during expansion in culture subsequent to transfection, and showed that these cells had a finite lifespan in culture and were not tumorigenic (14). In the present study we have extended this research by demonstrating that expression of an exogenous T24 H-ras oncogene in a human fibroblast strain that has an infinite lifespan in culture resulted not only in morphological transformation, focus formation and anchorage independence, but also in tumorigenicity. The tumors formed by MSU-1.1-T24 cell strains grew progressively in athymic ice and were diagnosed as invasive fibrosarcomas. It must be noted that cell strains 4 and 5 did not give rise to tumors (Table 1) even though they expressed the T24 H-ras oncogene and exhibited the other transformed characteristics. These 2 strains were retested in a separate experiment and again proved nontumorigenic. The reasons for the lack of tumor formation by these strains are not yet understood.

Three other studies have investigated the transforming potential of ras

oncogenes in infinite lifespan human fibroblast cell lines. O'Brien et. al. (36) utilized an SV40-virus transformed human fibroblast line, Va2, and Namba et. al. (37,38) utilized the KMST-6 cell line. Athough neither of these target cell lines are tumorigenic, they exhibit many characteristics of ras-transformed rodent fibroblasts, i.e., they are morphologically transformed, exhibit aberrant growth behaviour, and are highly aneuploid. Introduction of the ras oncogene by either virus infection protocols (36,37), or by transfection (38), resulted in cells able to form tumors in athymic mice, although the tumors produced with the Va2 cells did not grow progressively (36). Our results, using cells of the KMST-6 cell line as recipients for transfection of plasmid pHO6T1, confirm the results of Namba et. al. (37,38), and our results using the SV40-transformed infinite lifespan human fibroblast cell line, GM0637B, are similar to those of O'Brien et. al. (36), although our cells formed progressively growing tumors.

Because the near-diploid MSU-1.1 cell strain exhibits no transformation-related phenotypes, other than its infinite lifespan in culture, it permitted a more complete evaluation of the spectrum of transformation characteristics induced by ras oncogenes in infinite lifespan human fibroblasts, that are predicted from studies with rodent fibroblasts. Indeed, we found that expression of the transfected T24 H-ras oncogene in MSU-1.1 fibroblasts induced several transformation-related phenotypes including malignancy. In addition, because of the chromosome stability of MSU-1.1 fibroblasts, we conclude that the transformed phenotypes that resulted following transfection of the T24 H-ras oncogene was not the result of the selection of tumorigenic

variants arising from a karyotypically unstable population of transfectants.

Each of the focus-derived MSU-1.1-T24 cell strains generated by transfection of plasmid pHO6T1 expressed a high level of T24 H-ras oncogene encoded p21. Transfection of the normal H-ras gene in the same plasmid construct, or the T24 H-ras oncogene in plasmids containing only one or no transcriptional enhancer elements did not result in focus formation. These data suggest that a sufficiently high level of T24 H-ras oncogene expression was necessary to transform MSU-1.1 fibroblasts. Thus, the potent transforming activity of the T24 H-ras oncogene appears dependent not only on mutational activation, but also on its enhanced expression.

Although the MSU-1.1 cell strain arose following transfection of finite lifespan diploid human fibroblasts with a plasmid containing a v-myc oncogene, it is not clear that expression of this oncogene was responsible for induction of the infinite lifespan phenotype since we have only one such cell strain. Likewise, it is not known whether, as proposed for rat embryo fibroblasts (4), the expressed v-myc protein acts in a cooperative fashion with the T24 H-ras oncogene to cause transformation. However, our results showing that ras oncogene expression induces tumorigenic transformation of non-v-myc-transfected infinite lifespan human fibroblasts suggest that the infinite lifespan phenotype, rather than v-myc expression, is the characteristic of MSU-1.1 cells that complemented ras oncogene expression to cause tumorigenicity. Taken together with results showing that finite lifespan human fibroblasts transfected with

ras oncogenes exhibit a transformed phenotype, but do not have an infinite lifespan in culture and are not tumorigenic (13,14,19), the results described here indicate both the fundamental and practical importance of the infinite lifespan phenotype for in vitro transformation systems that use tumorigenicity as an endpoint (21).

ACKNOWLEDGEMENTS

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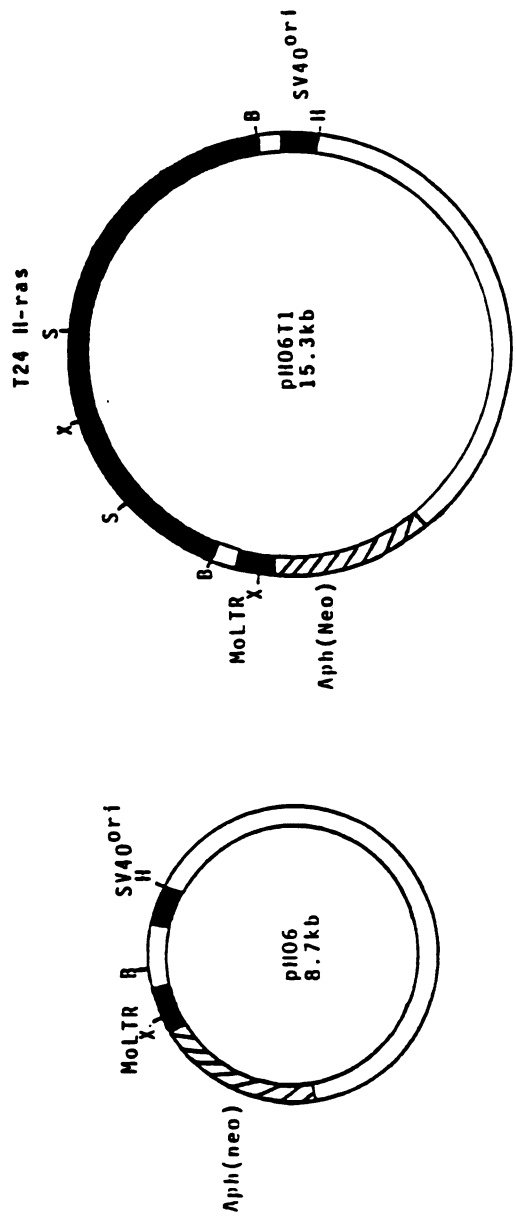
APPENDICIES

APPENDIX A



Maps of plasmids, pHO6 and pHO6T1. Plasmid pHO6N1 (not shown) contains the normal H-ras gene in the pHO6 vector. Xba I (X), Bam HI (B), Hind III (H) and Sac I (S) restriction sites are shown.

APPENDIX B

Athymic mouse with tumor formed by subcutaneous injection of MSU-1.1-T24 strain 3 cells.

APPENDIX C

Data "not shown" in Chapter III

Figure 1. Comparison of ras p21 expression in focus-derived, anchorage independent colony-derived and tumor-derived MSU-1.1-T24 cell strains. Analysis was performed as described in the Methods and Materials section of Chaptor II. T24 is the T24 bladder carcinoma cell line. 2T, 3T1 and 3T2 are were cultured from independent tumors formed by MSU-1.1-T24 cell strains 2 and 3. 3B1 and 3B2 are cell strains derived from anchorage independent colonies formed by MSU-1.1-T24 cell strain 3. The bars indicate the location of the normal p21 doublet and the arrow indicates the approximate position of T24 H-ras p21.

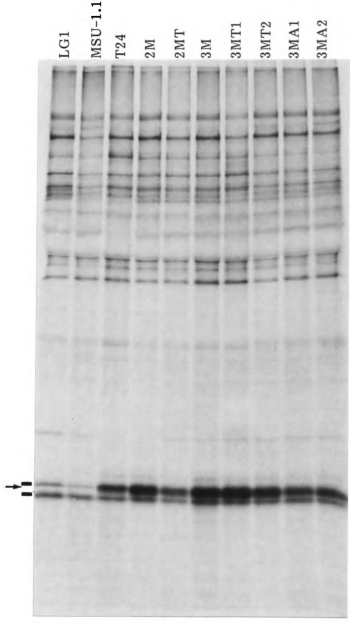

LG1
MSU-1.1
T24
2M
2MT
3M
3MT1
3MT2
3MA1
3MA2

Figure 2. Southern blot analysis of MC29 sequences in MSU-1.1-T24 cell strains. Genomic DNAs were digested with Xho I, and Bam HI, and hybridized with the Sac I fragments of the MC29 virus (Reddy et al. (1983) Proc. Natl. Acad. Sci. USA 80, 2500-2504). Hybridizing sequences specific to MC29 viral sequences are indicated.

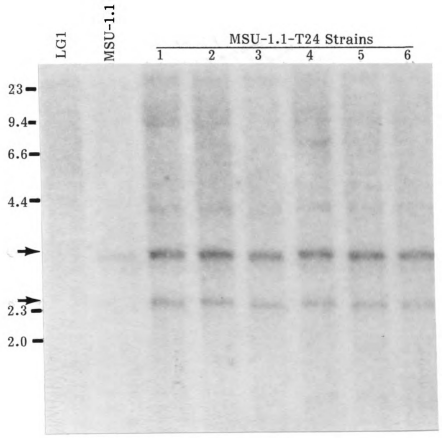
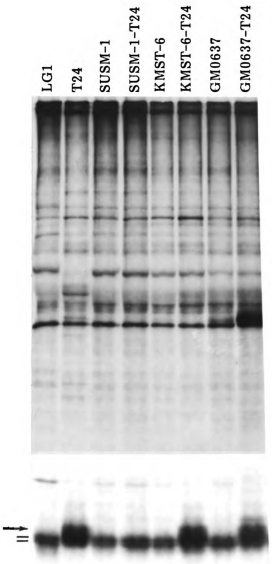

LG1
MSU-1.1
MSU-1.1-T24 Strains
1
2
3
4
5
6
23
9.4
6.6
4.4
2.3
2.0

Figure 3. Analysis of ras p21 expression in SUSM-1-T24, KMST-6-T24 and GM0637-T24 cell strains and in their parent cell lines. Analysis was performed as described in Chapter II Maaterials and Methods with the exception that immunoprecipitates were solubilized in SDS-PAGE buffer containing 8 M urea. This modification caused a shift in the mobility of the mutant p21 so that it migrated to a position above the characteristic p21 doublet (see arrow). T24 is the human bladder carcinoma cell line. The bars indicate the location of the normal p21 doublet and the arrow indicates the location of the T24 H-ras encoded p21.


LG1
T24
SUSM-1
SUSM-1-T24
KMST-6
KMST-6-T24
GM0637
GM0637-T24
II