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Malignant Transformation of Human Fibroblast Cell Strains By Transfection of the v-fes Oncogene : Evidence for Oncogene Cooperation

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MALIGNANT TRANSFORMATION OF HUMAN FIBROBLAST CELL STRAINS BY TRANSFECTION OF THE V-*FES* ONCOGENE: EVIDENCE FOR ONCOGENE COOPERATION

By

Chiencheng Lin

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ABSTRACT

MALIGNANT TRANSFORMATION OF HUMAN FIBROBLAST CELL STRAINS BY TRANSFECTION OF THE V-*FES* ONCOGENE: EVIDENCE FOR ONCOGENE COOPERATION

By

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To examine the possible role of the v-*fes* oncogene in the process of malignant transformation of human fibroblasts in culture, I transfected this oncogene into five non-tumorigenic, near-diploid, infinite life-span human fibroblast cell strains from the MSU-1 lineage and examined the transfectants for characteristics of tumor-derived cells, e.g., growth in medium without exogenous growth factors, growth in agarose, and the ability to form tumors in athymic mice. The most transformed strain, MSU-1.2, was converted from a non-tumorigenic to tumorigenic strain by expression of a transfected v-*fes* oncogene. Expression of the v-*fes* oncogene in MSU-1.1 cells, the parental cells of MSU-1.2, did not produce tumorigenic cells. However, when MSU-1.1 cells that contain a transfected v-*sis* or a c-H-*ras* oncogene expressed at a moderate level were used as target populations, the v-*fes* oncogene supplied the additional change required for malignant transformation of such cells. MSU-1.1 cells that contain a transfected c-N-*ras* oncogene expressed at a low level did not become tumorigenic with the expression of the v-*fes* oncogene.

Since the v-*fes* oncogene causes tumors in mesenchymal tissue in cats and chickens, I hypothesized that an "activated" c-*fes* gene plays a role in causing such tumors in humans. To test this hypothesis, I assayed 26 cell lines/strains derived from human mesenchymal tumors for expression of the c-fes protein. No evidence of expression of c-fes protein was found in

any of the 26 cell lines/strains assayed. Also I investigated whether a human c-*fes* proto-oncogene, transcribed from a strong viral promoter and fused with a viral *gag* sequence, has transforming ability in the MSU-1.2 human fibroblast strain. In contrast to the v-*fes* oncogene, expression of this special human c-*fes* gene did not cause malignant transformation of MSU-1.2 cells.

DEDICATED:

To my wife, Ying-ruei, for her love and support throughout the years,

and

to my parents for their love and faith in me.

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INTRODUCTION

Studies of human tumors and tumors in experimental animals have supported the hypothesis that carcinogenesis is a multistep process. Experimental induction of tumors in animals indicates that multiple genetic events are necessary for the carcinogenesis process (Scherer, 1989). Examination of clinical samples of various types of human tumors reveals that the multiple genetic changes, including point mutations, chromosomal translocations, gene amplifications, and inactivation of tumor suppressor genes, are commonly found in specific tumor cells. In human colorectal carcinomas, mutations in at least four or perhaps five genes are required before a cell can form a malignant tumor, and the number of detectable events increases as the stage of tumor become more malignant (Fearon and Vogelstein, 1990). However, the results from such studies cannot prove that any specific genetic change plays a causal role in the transformation process.

For this reason, model systems for transformation of mammalian cells in culture (mainly rodent fibroblasts) have been widely used by investigators. Among them, transfection studies have proven especially useful in understanding the molecular mechanisms of carcinogenesis, since by this technique, one can transfer oncogenes isolated from human tumors and retroviral oncogenes into cells in culture and study what properties they confer. Also these studies are useful in determining the number of

steps required for the transformation process. If one assumes that malignant transformation of cells in culture occurs by the same mechanisms as tumor development in vivo, conversion of a normal cell into a malignant cell in culture also results from a multistep process. Indeed, studies on transformation of primary rodent fibroblasts and some immortalized cell lines by transfection of viral and/or cellular oncogenes have provided support for the multistep process of malignant transformation. For example, Weinberg and colleagues (Land et al., 1983; Ruley, 1983) showed that transfection of a H-*ras* oncogene into normal diploid rodent fibroblasts cannot convert these cells into tumorigenic cells unless the cells also express a second oncogene which has a complementary function.

Protein phosphorylation on tyrosine residues has been demonstrated to be a common mechanism underlying malignant transformation by oncogene products and mitogenesis by growth factors. In fact, phosphorylation of proteins on tyrosine residues is the most common activity of oncogene products (Martin et al., 1984). Two types of protein tyrosine kinases (PTKs) have been reported: the receptor PTKs and the cytoplasmic PTKs. The involvement of some of the receptor PTKs, e.g., *neu* and v-*erbB* (Slamon et al., 1987), in human cell carcinogenesis is well documented, but the role of the cytoplasmic PTKs, e.g., *fes* and *src*, in this process is not clear. The v-*fes* oncogene used in this research is a transforming gene of the Gardner-Arnstein feline sarcoma virus (Gardner et al, 1970). It encodes a protein tyrosine kinase whose enzymatic activity is essential for both tumor induction in animals and transformation of mammalian cells in culture (Donner et al., 1980; Barbacid et al., 1981).

The objectives of the present research were (1) to define the biological characteristics of transformed cells obtained by transfection

of the v-*fes* oncogene into a near-diploid, infinite life span, growth factor independent human fibroblasts strain, MSU-1.2; (2) to determine whether the c-*fes* gene, transcribed from a viral strong promoter and fused with a truncated viral *gag* sequence, has transforming activity when transfected into the MSU-1.2 strain; (3) to define the biological characteristics of transformed cells obtained by transfection of the v-*fes* oncogene into a near-diploid, infinite life span human fibroblasts strain MSU-1.1; (4) to determine whether a second oncogene can complement the v-*fes* oncogene and cause MSU-1.1 cells to become malignant. By comparing the *in vitro* transformed characteristics and tumorigenic potential induced by the v-*fes* oncogene in five closely-related cell strains with different growth properties and genotypic alterations, i.e., MSU-1.1, MSU-1.1:*sis*A, MSU-1.1:H*ras*98, MSU-1.1:N*ras*73 and MSU-1.2, we determined the kind of changes and number of steps required for the malignant transformation of human fibroblasts in culture.

Chapter I of the thesis reviews the literature that supports the "somatic mutation" theory of carcinogenesis and the hypothesis that the process involves multiple steps. Evidence for the "somatic mutation" theory of carcinogenesis includes the recognition of inherited predisposition to cancers, the studies of specific chromosomal changes in leukemia cells, evidence that relates the mutagenic potential of chemical agents and their carcinogenicity, the connection between susceptibility to cancers and an impaired ability of cells to repair damaged DNA, and the identification of oncogenes and tumor suppressor genes. Evidence supporting the multistep hypothesis of carcinogenesis includes studies of human tumors and tumors in experimental animals, as well as cell transformation studies using rodent and human cells. Also discussed in

Chapter I is the current understanding regarding the functions of the genes involved in the carcinogenesis process and the existence of multiple signaling pathways in cells. Chapter II consists of a manuscript that had been submitted to the journal *Molecular Carcinogenesis*. It describes the research I carried out which shows that expression of a transfected v-*fes* oncogene causes malignant transformation of MSU-1.2 cells. Chapter III consists of a manuscript that will be submitted to the journal *Oncogene*. It describes my research which demonstrated that malignant transformation of the MSU-1.1 cell strain, the parental strain of MSU-1.2, required cooperation of two oncogenes, e.g., v-*fes* and v-*sis*. Expression of the v-*fes* oncogene did not cause malignant transformation of cells from the MSU-1.1 strain, the parental strain of MSU-1.2. However, when MSU-1.1-derived cell strains that contain a transfected v-*sis* or a c-*ras* oncogene expressed at a moderate level were used as targeted populations, expression of the v-*fes* oncogene could supply the penultimate change needed for malignant transformation of these cell strains.

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CHAPTER I

LITERATURE REVIEW

A. EVIDENCE SUPPORTING THE "SOMATIC MUTATION" THEORY OF CARCINOGENESIS

The hypothesis that cancer is a disease of genetic origin was first suggested from the pioneering research conducted by Boveri (1929). He observed that chromosomal abnormalities are common features in cancer cells, and he found that the entire population of cells in a given tumor usually shared the same chromosomal abnormalities, suggesting that the abnormalities were passed on from parental to progeny cells. Based on these observations, he proposed that heritable alterations in the chromosomes were responsible for such cancer. Successive generations of scientists have added to and modified this original theory. It is now well recognized that carcinogenesis is a complex process, involving multiple independent genetic events.

Evidence supporting this theory comes from several sources: (1) the recognition of inherited predisposition to certain cancers; (2) the detection of characteristic chromosomal abnormalities in leukemia cells; (3) evidence that relates the mutagenic potential of chemical agents to their carcinogenicity; (4) the connection between susceptibility to cancer and an impaired ability of cells to repair damaged DNA, and more recently, (5) the identification of cellular genes (proto-oncogenes and tumor suppressor genes) that when mutated can cause neoplastic growth.

1. Inherited Predisposition to Certain Cancers

Mendelian inheritance of predisposition to several cancers, such as retinoblastoma, Wilms' tumor, colon cancer, breast cancer, and type I neurofibromatosis, is well known in humans (Knudson, 1977). Patients with a genetic predisposition carry a very high risk of cancer. The best documented example of which, retinoblastoma, is reviewed here.

Retinoblastoma (Rb) is a rare tumor of the retinal tissue of the eye. All Rb patients develop the tumors in their childhood. Rb tumors occur in either a familial or a sporadic form. Approximately one-third of the Rb cases are familial, with the trait being transmitted in an autosomal dominant fashion with very high penetrance (Vogel, 1979). The rest of the cases represent sporadic Rb which occurs at a very low frequency in children with no family history of the disease. In the familial form of Rb, the tumors are usually bilateral and multifocal whereas the sporadic form of Rb usually occurs as a single focal lesion in one eye. Although the frequency and the number of tumors differ between the two forms of Rb cases, the genetic change responsible for Rb is the same: inactivation of both copies of a single gene in a single retinal cell.

Knudson (1971) was the first to propose that two mutational events in the *Rb* gene are involved in the development of retinoblastoma. His hypothesis was based on the statistical analysis of tumor incidence data for familial and sporadic forms of Rb. In the familial form, the affected person is born with a mutant allele, and the mutation is present in all of a person's cells. A second mutation in the other allele of any retinal cell would cause the cell to become malignant. In contrast, two mutations are required to affect both alleles of a single retinal cell to cause the

sporadic Rb. The requirement for one more mutation in the sporadic Rb is consistent with the observation that the patient usually has only one tumor in one eye as compared to multiple tumors in patients with familial form of Rb. Support for this hypothesis emerged recently when the *Rb* gene was identified and shown to be mutated in both alleles of all retinoblastoma cells. (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987).

2. Characteristic Chromosomal Abnormalities in Certain Leukemia Cells

Characteristic chromosomal abnormalities have been shown to be associated with human leukemia cells by cytogenetic analysis. These abnormalities are generally classified as structural or numerical. Structural abnormalities include translocations, inversions, deletions, insertions, and amplifications, whereas numerical abnormalities are losses or duplication of whole chromosomes. Although most abnormalities appear to be non-specific, some chromosomal abnormalities are associated with specific tumor types, and they have provided good materials for studying the mechanisms of carcinogenesis of such tumors. Studies of such tumor cells have revealed that these translocations either affect the expression of adjacent genes, resulting in structurally normal proteins being expressed at inappropriate levels, or alter the protein coding sequences, yielding structurally altered proteins that behave aberrantly. Two classic examples of such translocations are reviewed here to elucidate these two general mechanisms of genetic disruption brought about by gene rearrangement.

2.1 Burkitt's Lymphoma-A Model for Transcriptional Deregulation

Burkitt's lymphomas contain one of three characteristic chromosomal translocations: t(8,14), t(8,22) or (2,8). All three translocations join the c-*myc* proto-oncogene normally found on chromosome 8 to immunoglobulin loci on chromosome 2, 14 or 22 (Leder et al, 1983; Croce and Nowell, 1985; Cory, 1986). As a consequence, the c-*myc* is deregulated (over-expressed) by the immunoglobulin heavy-chain or light-chain λ or κ enhancer, when it should normally be transcriptionally inactive in lymphoid cells (Zimmerman et al., 1986).

Molecular analysis of these characteristic chromosomal translocations showed that the break points in the c-*myc* locus in tumor cells from various individuals are not identical (Battey et al., 1983). The c-*myc* gene has three exons, its protein being encoded exclusively in the last two (Watt et al., 1983). Break points of the t(8,14) translocation usually occur within the first intron, the first exon, or 5' to the c-*myc* gene (Neri et al., 1988), whereas break points of the t(8,22) and t(2,8) translocations are found exclusively 3' to the last c-*myc* exon. This results in transcription of the c-*myc* gene either from its normal promoter in exon one or from a cryptic promoter in the first intron if the first exon has been deleted by translocation. The translational products, however, are structurally identical to the normal c-myc proteins in all three cases. The translocation of c-*myc* into immunoglobulin loci is now recognized as one of the causative changes responsible for the formation of such tumors. This is supported by the observations that transgenic mice carrying the Burkitt's translocation developed B-cell lymphoid tumors at a high frequency (Adams et al., 1985; Harris et al., 1988).

2.2 Philadelphia Chromosome-Positive Leukemia-A Model of Protein Structure Alteration

The Philadelphia chromosome is the result of a reciprocal translocation between band q34 of chromosome 9 and band q11.2 of chromosome 22. It is present in greater than 90% of patients with chronic myelogenous leukemia (CML) and 4-5% of pediatric patients with acute lymphoblastic leukemia (ALL) (Nowell and Hungerford, 1960; Kurzrock et al., 1988). In both ALL and CML, the translocation juxtaposes the amino-terminal portion of the *bcr* gene normally located on chromosome 22 to the carboxyl-terminal portion of the c-*abl* proto-oncogene on chromosome 9. The break points within the *bcr* gene, however, are not identical and thus the proteins coded for by the hybrid *bcr-abl* genes differ in size. The translocation of the *bcr* gene results in a 210 kDa hybrid protein in CML (Ben-Neriah et al., 1986) whereas the translocation produces a shorter bcr-abl fusion protein of 185 kDa in most cases of Philadelphia chromosome-positive ALL (Clark et al., 1988). The protein sequence of the 210 kDa protein differs from that of the 185 kDa protein only in the presence of mid-*bcr* exons. Both hybrid genes contain the same c-*abl* sequence and have been shown to encode proteins which have increased levels of tyrosine kinase activity compared to the normal c-abl protein (Konopka, 1985).

3. Evidence That Relates the Mutagenic Potential of Chemical Agents to Their Carcinogenicity

Numerous chemicals have long been suspected as possible etiological agents for several types of human cancers. In 1761 Hill reported that users of tobacco snuff developed cancer of the nasal passage much more

frequently than non-users (Redmond, 1970). Subsequently, in 1775, Pott reported that males who had been employed as chimney sweeps when they were boys had a higher rate of death due to scrotal cancer than men in the general population. Based on these anecdotal reports and others, exposure to various chemicals or chemical mixtures have been considered to be associated with human cancers. Many were subsequently identified as carcinogens through animal experiments (Tomatis et al., 1989). The mechanisms underlying the carcinogenesis process, however, remained unknown until the 20th century.

In 1964 Brookes and Lawley studied polycyclic aromatic hydrocarbons with different carcinogenic activity and demonstrated a correlation between the binding of these carcinogens to mouse skin and their ability to cause tumors in such tissue. This observation provided the first clue that the carcinogenesis process is caused by a mutation or a series of mutations induced by carcinogens. However, until the late 1960s, some of the most potent carcinogens, e.g., the polycyclic aromatic hydrocarbons, were unable to cause mutations in the assays used at the time. This was because many carcinogens need to metabolized to a reactive derivative before they can be effective, and the test for mutagens, which involved use of lower organisms, did not take this into account. In 1961 Miller et al. first demonstrated that N-hydroxy-2-acetylaminofluorene, a metabolite of the carcinogen 2-acetylaminofluorene, has an increased carcinogenic activity in the rat as compared to 2-acetylaminofluorene. Since this finding, many chemical carcinogens were also found to require metabolic activation for their carcinogenicity. Subsequently, in 1968, Maher demonstrated that the active form of two carcinogens, 2-acetylaminofluorene and N-methyl-4-aminobenzene, bound covalently to donor

DNA isolated from prototroph donor *Bacillus subtilis* and when transferred into recipient *Bacillus subtilis*, the treated donor DNA produced more mutant bacteria defective in synthesis of tryptophan precursors at a higher frequency than did the untreated DNA. By demonstrating that these carcinogens can cause mutations, Maher showed that DNA is the common target of mutagenesis and carcinogenesis by these chemicals. This established a clear, although not proven, theory: that carcinogens lead to cancer through their ability to damage DNA and induce mutations.

4. Connection Between Susceptibility to Cancer and Impaired Ability of Cells to Repair Damaged DNA

Xeroderma pigmentosum, Cockayne's syndrome, ataxia telangiectasia, Fanconi anemia, and Bloom's syndrome are rare genetic disorders characterized by cellular defects in the ability to process DNA damage. These cancer-prone syndromes differ from other cancer-prone diseases, such as retinoblastoma and Wilms' tumor, in that the inherited defects raise the frequency of carcinogen-induced mutations and in this way, increase the cancer incidence.

The best example is the syndrome known as xeroderma pigmentosum (XP). This syndrome occurs worldwide in all ethnic groups with a frequency varying from one to ten patients per million. XP patients are characterized by their severe sun-sensitivity, leading to a high incidence of sunburn, pigmentation abnormalities, and malignancies in the area of skin exposed to sunlight (Robbin, 1974). Cleaver (1968) were the first to discover that fibroblasts isolated from XP patients are defective in nucleotide excision repair of DNA damage induced by ultraviolet radiation. Further research using XP fibroblasts revealed that treatment of these

cells with UV radiation resulted in less surviving cells but more azaguanine-resistant mutants per 10^5 surviving cells when compared to that of normal fibroblasts (Maher, 1976). The conclusion from these studies was that cells from xeroderma pigmentosum patients have a decreased rate of excision repair, and as a consequence, these cells have a higher mutation frequency when exposed to UV radiation than do normal cells. This higher mutation frequency can in turn lead to higher rate of accumulation of mutations in genes directly contributing to cancer and thus tumor incidence. This is consistent with the observation that a slight sunlight exposure of the skin is liable to provoke skin cancers in XP patients, but not in normal individuals.

By fusing two XP cells isolated from different patients and analyzing for the UV-sensitivity of the hybrid cells, it is possible to determine whether the two individual represent different genetic loci that lead to expression of the UV-sensitive phenotype (Hashem et al., 1980). Two cell lines that have genetic defects in different loci can complement each other, and they result in hybrid cells that attain near-normal UV-resistance. Cell lines that have genetic defects in the same locus cannot complement each other and, therefore, the hybrid cells remain UV-sensitive. A complementation group is defined as a group of cell lines that have genetic defects in the same locus. This type of genetic analysis has shown the existence of nine complementation groups, clearly demonstrating the genetic complexity of the disease. The functions of these nine "DNA repair" genes are postulated to be involved in the early steps of the excision repair process. This hypothesis is based on the observation that XP cells of different complementation groups attain near-normal UV resistance when treated with the enzyme endonuclease V (Smith

and Hanawalt, 1978). Since endonuclease V is produced in bacteriophage T4 infected *E. coli* and specifically incises UV-induced pyrimidine dimers in bacteria, this result indicates that these XP cells have genetic defects in steps prior to the incision of UV-induced pyrimidine dimers.

5. The Identification of Oncogenes

5.1 Retroviral Oncogenes and Integration Sites

Retroviruses have a distinct life cycle that allows them to convert normal cellular genes (proto-oncogenes) from the host genome into transforming genes, now known as oncogenes (Bishop, 1983). They have been shown to activate proto-oncogenes by two mechanisms, viral transduction and viral integration.

The virus can capture a proto-oncogene in its genome and bring the gene under the control of its strong promoter and enhancer, causing it to be expressed at a high level. A typical example is the Rous sarcoma virus (RSV), the first retrovirus found to contain a oncogene. This virus was isolated by Rous in 1910 from a spontaneous sarcoma that had been passaged through Plymouth Rock chickens. He subsequently established the viral etiology of the tumor by demonstrating that tumor extract remained pathogenic (transforming) after being passed through filters designed to exclude bacteria (Rous, 1911). During the period between 1911 and 1970, most studies of RSV focused on the transmissibility and pathogenicity of this virus in avian and mammalian species. It was not until the 1970s that the v-*src* oncogene was identified as the genetic entity responsible for RSV-mediated transformation. The identification of the v-*src* oncogene was made possible by the isolation and analysis of deletion mutant viruses that were defective for transformation, but competent in replication

(Kawai and Hanafusa, 1971; Bader, 1972). Vogt (1971) analyzed these deletion mutants and found that the *src* gene responsible for RSV-mediated transformation is located in the 3' region of the viral genome. Subsequent molecular analysis identified the v-*src* oncogene and demonstrated that a DNA probe complementary to the v-*src* oncogene can hybridize to normal chicken DNA (Stehelin et al., 1976). This provided the first evidence that retroviral oncogenes are normal cellular genes captured by retroviruses.

An alternative mechanism whereby retroviruses activate proto-oncogenes is by viral integration. Random insertion of DNA copies of the viral RNA into the host genome is part of the viral life cycle. When a DNA copy of the viral RNA accidentally inserts itself at sites close to, or within, a cellular gene, it can upregulate the expression of the adjacent gene by bringing the sequence under the control of the viral strong promoter and enhancer. This mechanism, denoted "insertional mutagenesis", has provided another important method to search for oncogenes since the location of these oncogenes can be easily detected by their proximity to the inserted retroviruses. Proto-oncogenes found to be activated by insertional mutagenesis often turn out to be the same as the retrovirus-captured oncogenes. Some new ones, however, were also identified in this manner. For example, the *int*-1 and *int*-2 oncogene were found to be activated by insertional mutagenesis in mouse mammary tumor virus-induced breast tumors (Nusse and Varmus, 1982; Dickson et al., 1984). The cellular *int* genes have been shown to be conserved among mammals, and they are generally considered to be involved in intercellular communication, which is important in embryonic development.

5.2 Oncogenes Identified by Gene Transfer

The gene transfer technique was first introduced by Graham and van der Eb (1973) who showed that application of DNA to cells as a co-precipitate with calcium phosphate allows stable uptake and incorporation of DNA into the genome of the recipient cells. Several groups improved and applied this technique for introducing exogenous DNA into NIH3T3 cells, a special line of mouse cells, to search for the etiology of non-viral induced tumors in animals and humans. In such studies, DNA isolated from chemically transformed cells or from tumors of different origins was shown to be capable of inducing morphological transformation of the recipient NIH3T3 cells (Shih et al, 1979). This transformed characteristic allows one to easily identify cells that take up and express the oncogenes responsible for this characteristic. Weinberg's group subsequently developed a strategy that made it possible to clone the transfected genes of human origin from DNA of the transformed mouse cells (Weinberg, 1982). By using human repetitive sequences, which are randomly distributed throughout the human genome in approximately 5 X 10^5 copies (Jelinek et al., 1980) as hybridization probes, human sequences could be readily detected and isolated from a mouse background.

Using this technique several groups independently isolated a human transforming gene from the established T24 bladder carcinoma cell line (Der et al., 1982; Goldfarb et al., 1982; Parada et al., 1982; Santos et al., 1982). This gene was found to be related to the *ras* oncogene of the Harvey strain of murine sarcoma virus. Restriction enzyme and heteroduplex analysis subsequently showed that the T24 *ras* oncogene is the active homologue of the human c-H-*ras* gene. Further molecular analysis revealed that the activation of the T24 *ras* oncogene resulted from two specific

point mutations; a mutation to modify the activity of the protein and the other to alter its level of expression. One point mutation was identified in codon 12 which changed a glycine to a valine (Reddy et al., 1982; Tabin et al., 1982; Capon et al., 1983). The other point mutation was found in the last intron of the H-*ras* gene and this mutation was shown to be responsible for the higher level of expression of the mutated H-*ras* gene (Cohen and Levinson, 1988).

Since the cloning of the T24 H-*ras* oncogene, vigorous efforts were undertaken to identify and clone additional oncogenes from human tumors (reviewed in Varmus, 1984). The results revealed that DNA isolated from many types of human tumors contained transforming genes detectable in the NIH3T3 transformation assay. These tumors included those of lung, colon, urinary bladder, gall bladder, pancreas, breast, and ovary. Hematopoietic-cell malignancies such as B- and T-cell lymphomas, lymphoid and acute myeloid leukemias, as well as neuroblastomas and sarcomas, were also positive. Most of the transforming genes isolated from these tumors were found to be activated c-H-*ras* or c-K-*ras* genes. A number of these *ras* oncogenes were sequenced and the results showed that mutations within codons 12, 13, or 59-61 were responsible for the activation (Varmus, 1984).

5.3 Oncogenes Identified by Gene Amplification

In 1984, Schimke first reported that gene amplification causes human leukemia cells to acquire resistance to methotrexate, a chemotherapeutic agent. Gene amplification is now known to be a common genetic abnormality in cancer cells and it provides one of the mechanisms by which cellular genes can be over-expressed.

High levels of gene amplification (10- to 100-fold) in cancer cells

are commonly manifested chromosomally as double-minute chromosomes or homogeneously staining regions. These cytogenetic markers have provided a useful tool for identifying oncogenes involved in many types of human tumors. Several new oncogenes were first identified in this manner. For example, the *gli* oncogene were identified from amplified DNA in a human glioma cell lines (Kinzler et al., 1987). The function of this oncogene is not yet clear.

An increased copy number and enhanced expression of cellular oncogenes frequently correlates with the more advanced and aggressive stages of malignancy and a poor prognosis (Schwab, 1990). One typical example is the amplification of the N-*myc* oncogene in various types of human tumors. Schwab et al. (1983) were the first to discover this *myc*-related oncogene in neuroblastoma by employing a viral *myc* probe under conditions of reduced stringency. Further analysis revealed that the N-*myc* gene is amplified in about 50% of late stage human neuroblastomas (Kohl et al., 1983, Schwab et al., 1984). This gene was also found to be amplified in 20% of human retinoblastomas and 20% of small cell lung cancers (Lee et al., 1984; Squire et al., 1985; Nau et al., 1986). The degree of N-*myc* amplification in these tumors ranges from 5- to 700-fold (Broudeur et al., 1984), and a higher degree of the amplification is usually associated with a poorer prognosis of the cancer.

5.4 Oncogenes Identified by Other Methods

Another useful method for identifying oncogenes is via their association with specific chromosomal abnormalities in certain human tumors. Classic examples are the c-*myc* oncogene in Burkitt's lymphoma and the *bcr-abl* gene in acute lymphoblastic leukemia and chronic myelogenous leukemia. These have been reviewed in previous sections.

The last approach to identify oncogenes is by their structural homology with genes that were previously identified. Using these genes as probes, one can identify related genes from DNA libraries derived from human tumors by Southern blot analysis. These putative oncogenes can then be examined for their transforming activity or any alteration in other human tumors. For example, the v-*myc* oncogene has been used as a probe to identify two related genes, N-*myc* amplified in neuroblastoma (Schwab et al., 1984) and L-*myc* amplified in small cell lung cancer (Nau et al., 1985). Another example comes from the c-*erb*B1 gene which encodes epidermal growth factor receptor. This gene was used to isolate the c-*erb*B2 gene (Slamon et al., 1987) which was later found to be amplified in advanced stages of breast and ovarian cancers (Slamon et al., 1989).

6. The Identification of Tumor Suppressor Genes

6.1 Somatic Cell Hybrids and Single Chromosome Transfer Experiments

The earliest indication that the carcinogenesis process may involve loss of gene functions was provided by evidence from studies of somatic cell hybrids. The basic methodology is to fuse two cells, one normal and one tumorigenic, to form hybrid cells. The hybrid cells are selected and examined for their ability to form tumors in appropriate animal hosts, usually nude mice. Early passage culture of the hybrid cells usually have the phenotype of the normal cell, i.e., they are not tumorigenic. However, with continued growth in culture these hybrid cells sometimes lose chromosomes and, as a result, the hybrid cells occasionally become tumorigenic. Subsequent cytogenetic analysis of non-tumorigenic and tumorigenic hybrids permitted the identification of chromosomes associated with the normal phenotype, which, when present, suppressed the malignant

phenotype (Benedict et al., 1984; Kaelbling and Klinger, 1986; Srivatsan et al., 1986). This phenomenon of tumor suppression suggests that a gene (or genes) from a normal cell supplies a function in the cancer cell and renders it responsive to normal regulation of cell growth.

The technique has been further refined through microcell transfer experiments in which single intact chromosome or at most a few chromosomes are transferred into tumorigenic cells. Using this method, the tumorigenicity of several human tumor cell lines has been suppressed. For example, the tumorigenicity of HeLa cells, derived from a human cervical carcinoma, was suppressed by the introduction of human chromosome 11 (Saxton et al., 1986). This phenomenon also has been observed for renal cell carcinoma and melanoma cell lines by chromosome 3p (Shimizu et al., 1990) and chromosome 6 (Trent et al., 1990), respectively. Although these techniques are useful in identifying chromosomes involved in tumor suppression, they are not useful for direct identification of genes responsible for such suppression. In fact, no genes have been directly identified through these methods.

6.2 Tumor Suppressor Genes Identified Through Studies of Hereditary Tumors

6.2.1 Retinoblastoma

On the basis of statistical analysis of tumor incidence data for familial and sporadic forms of retinoblastoma (Rb), Knudson (1971) proposed his two-hit hypothesis for the formation of such tumors. He suggested that hereditary tumors required only one mutation in the gene responsible for the development of Rb, i.e., the *Rb* gene, whereas the sporadic tumors required two mutations (Knudson, 1978).

The chromosomal location of the *Rb* gene was suggested by an investigation of a group of familial Rb patients with rare constitutional deletions on chromosome 13 (Francke, 1979). Francke analyzed these cases

and found that only one band, 13q14, was common to all the deletions. These observations provided evidence that the inherited mutation may involve loss of a small region of chromosome 13. The nature of the second mutation was subsequently suggested from studies on the DNA of retinoblastomas obtained from patients with the familial form of the disease. Most of the retinoblastomas had lost one copy of chromosome 13 as detected by restriction fragment length polymorphism technique, using polymorphic markers on chromosome 13 (Cavanee et al., 1983), and pedigree analysis showed that the remaining copy of chromosome 13 in Rb tumors from the patient and the affected parents always contained the inherited mutation. Taken together, these observations indicated that the inherited mutation in familial retinoblastoma is usually a small deletion or point mutation in one allele whereas the second event often involves complete loss of the other allele.

Dryja et al. (1986) constructed a chromosome 13 genomic library to clone the *Rb* gene. From this library, they identified a probe that was able to detect homozygous deletion of a DNA sequence at 13q14 in 3 out of 37 retinoblastomas, indicating that the probe was located extremely close to the suppressor gene. After this breakthrough, this probe was used to perform a chromosome walking that led to the eventual cloning of the retinoblastoma gene (Friend et al., 1986; Lee et al., 1987).

6.2.2 Wilms' tumors Wilms' tumor (WT) is a pediatric nephroblastoma with an incidence of 1/10,000 and accounts for 85% of childhood kidney cancer (Matsunaga, 1981). Like retinoblastomas, Wilms' tumors occur both in a sporadic and a familial form. Cytogenetic and chromosomal deletion analysis of this syndrome by several groups independently identified two predisposition loci; one at 11p13 (Francke et

al., 1979; Riccardi et al., 1980) and the other at 11p15 (Reeve et al., 1989; Koufos et al., 1989). Three groups have cloned the *WT* gene located at the 11p13 locus (Rose et al., 1990; Call et al., 1990; Gessler et al., 1990). Rose et al. generated probes from radiation-reduced somatic cell hybrids that contain small fragments of chromosome 11 as their only human component. The probes were then used to locate and construct a complete physical map of the 11p13 Wilms' tumor locus. The identity of this putative *WT* gene was supported by the studies of Cowell et al. (1991) who described a Wilms' tumor with a homozygous internal mutation in this gene, later designated as the *WT*1 gene. The locus at 11p15 has not yet been cloned. A third locus for this disease has been suggested since genetic predisposition in some pedigrees has failed to map to either of these loci (Grundy et al., 1988; Huff et al., 1988).

6.2.3 Neurofibromatosis type 1 Von Recklinghausen's neurofibromatosis (NF1) affects one in approximately every 3500 individuals, and it is inherited as an autosomal dominant trait with high penetrance (Stumpf et al., 1987). Approximately half of all NF1 cases occur in patients with a familial history (Riccardi, 1981). Affected individuals are characterized by café-au-lait spots, which are flat hyperpigmented areas on the skin, and an increased risk of neurofibromas and neurofibrosarcomas (Riccardi and Eichner, 1986). The most common cause of death in NF1 patients is cancer.

A locus on chromosome 17q was first implicated as the locus responsible for NF1 by DNA linkage analysis of affected individuals in several kindreds (Barker et al., 1987; Gordgar et al., 1989). Subsequent detection of different translocations involving chromosome 17 [t(1;17) and t(17;22)] at breakpoints 17q11.2 in two NF1 patients provided the first

suggestion that the disorder could originate by chromosomal rearrangements close to, or within, the locus responsible for the disease (Schmidt et al., 1987; Ledbetter et al., 1989). The mapping and cloning of these breakpoints resulted in the identification of the *NF1* locus (Wallace et al., 1990; Viskochil et al., 1990; Cawthon et al., 1990). The fact that this gene was either mutated, deleted or translocated in different NF1 patients implicates the involvement of this gene in causing the disease.

6.3 Tumor Suppressor Genes Identified by Loss of Heterozygosity

The inherited mutations in most familial, cancer-prone diseases are usually small deletions or point mutations in the affected suppressor genes. However, the loss of the second copy of the gene in tumor cells often involves the loss of a large segment, or even all, of the remaining normal chromosome. Such losses can be identified as loss of heterozygosity (LOH) for adjacent polymorphic alleles when DNA from the tumors is compared by Southern blot analysis to DNA from normal cells of the same individual. A consistent finding of LOH for polymorphic markers in a particular locus in DNA from tumors indicates that the locus harbors a suppressor gene.

Using this type of analysis, the loci of several putative tumor suppressor genes have been identified and in some cases the genes have been cloned, e.g. *DCC*, *p53*, and *MCC* genes (Vogelstein et al., 1988; Baker et al., 1990a). For example, Vogelstein et al. (1988) observed allelic deletions of chromosome 18q in 70% of all cases of sporadic colorectal cancer, and the deletions frequently involved the 18q21-qter region. They subsequently identified a probe from this region that detected homozygously deleted DNA in one out of 120 colorectal carcinomas, indicating that the probe was located extremely close to the suppressor

gene. Following this breakthrough, the region pinpointed by this probe was cloned and found to contain a candidate suppressor gene, designated *DCC* (deleted in colorectal carcinomas) gene (Fearon et al., 1990). To date the alterations of *DCC* gene have been found only in colorectal carcinomas. LOH on chromosome 18q, however, has been seen in other tumor types, suggesting the *DCC* gene has a broader function in human cancers.

B. CARCINOGENESIS IS A MULTISTEP PROCESS

One of the important differences between cancer cells and normal cells is that cancer cells no longer respond to normal growth controls like normal cells do. Since multiple levels of growth control exist to regulate proliferation and differentiation of cells, cancer cells very probably represent the end result of multiple changes that take years to develop. This hypothesis is now supported by in vivo and in vitro evidence.

1. In Vivo Evidence

1.1 Experimental Induction of Tumors in Animals

One advantage of studying the carcinogenesis process in experimental animals is that the type of carcinogens and/or tumor promoters used to treat the animals and the duration of the treatment can be carefully controlled. Another is that animals with specific genetic traits, e.g., a high frequency of leukemia, a low frequency of liver tumors etc., can be chosen as the target for treatment. The use of animals as a valid model for human has been established by two observations. First, the natural history of tumors arise spontaneously or induced by carcinogen treatment in animals are similar with that of human tumors. Second, the sequence of

oncogenes and the mechanism for their regulation are conserved among mammals, including humans, and oncogenes isolated from human tumors are able to transform rodent cells in DNA transfection experiments.

Studies of carcinogen-induced tumors in animals (mainly in mice and rats) have demonstrated distinct stages that divide the carcinogenesis process (Pitot, 1989). For example, induction of hepatocellular carcinomas by chemical carcinogens fed to rats has demonstrated three distinct stages in the development of such tumors (Scherer, 1989). The earliest effect of feeding the rats with liver carcinogens, such as diethylnitrosamine (DEN), is the appearance of focal lesions (foci) of morphologically altered hepatocytes, and these foci can be readily detected by histochemical examination because they are deficient in ATPase and Glucose 6-Phosphatase, and they exhibit altered metabolism, i.e., an altered carbohydrate metabolism leading to the excess storage of glycogen (Bannasch et al., 1980). The majority of these early foci are sensitive to tumor promoting agents, and treatment of the rats with these agents, e.g., phenobarbital, can lead to the appearance of more advanced lesions, such as hyperplastic nodules (benign tumors) characterized by acidophilic and basophilic cytoplasm and eventually hepatocellular carcinomas (malignant tumors). The existence of such distinct stages appears to be common to most experimental animal models, and it is consistent with the multistep nature of carcinogenesis.

The number and types of the enzyme-altered foci and the carcinomas induced in the livers of tested animals depend on the protocol used. In a protocol in which the rats are given only a single, low dose of diethylnitrosamine (DEN) 24 hours after partial hepatectomy, the rats give rise only to a small number of enzyme-altered foci which are heterogeneous

in their enzyme deficiency (Pitot et al., 1978). This treatment is termed "initiation", and normal hepatocytes are considered to be transformed during the treatment by a single genetic change into focus forming cells. In the typical initiation-promotion protocol, rats are given a single, low dose of DEN and subsequently placed on a diet containing 0.05% phenobarbital. Rats undergoing this treatment give rise to increased number of enzyme-altered foci and a small number of hepatocellular carcinomas were also observed. The few carcinomas that arose is considered the results of a second genetic change. that occur spontaneously. Evidence supporting this hypothesis was provided by Scherer et al. (1983), who developed a initiation-promotion-initiation protocol in which the initiation-promotion protocol is followed by treatment with a second carcinogen, i.e., ethylnitrosourea. Rats undergoing this protocol give rise to more carcinomas than rats undergoing the initiation-promotion protocol, and the carcinomas arose in a relatively short period of time. The effect of the phenobarbital in these protocols is limited to promoting the growth of the previously initiated cells, since the treatment of phenobarbital without the initiation treatment yields no enzyme-altered foci or carcinomas.

The progression from premalignant lesions, i.e. enzyme-altered foci and hyperplastic nodules, to malignancy is a complex process. The best example to illustrate this is the induction of carcinomas in the initiation-promotion-initiation protocol. Focal carcinomas can be induced by ethylnitrosourea within approximately 2% of the enzyme-altered foci and hyperplastic nodules. These focal carcinomas manifest themselves as secondary foci within the preexisting foci or nodules. The characteristic focus-in-focus structure is considered to reflect the progression of the

initiated cells by a second genetic change. This hypothesis was further supported by cytogenetic analysis of cells from the foci-in-foci and the pre-existing lesions. Cells in the enzyme-altered foci and hyperplastic nodules do not exhibit any visible cytogenetic abnormalities (Sargent et al., 1989), whereas cells from hepatocellular carcinomas show a relatively frequent loss of specific chromosomes, e.g., chromosome 1 and 3 (Sargent et al., 1992).

Taken together, these observations indicate that hepatocellular carcinomas are the results of multiple genetic changes, which is consistent with the multistep nature of carcinogenesis.

1.2 Studies of Human Colorectal Tumors

Examination of colorectal tumors in humans has defined distinct stages in the development of such tumors (reviewed in Sugarbaker et al., 1985). The carcinogenesis process is initiated by a widespread cellular proliferation, which can be detected in colonic tissue by in situ DNA labeling technique (Lipkin et al., 1988). The second step in the process is the clonal expansion of a small fraction of the hyper-proliferative cells to form small adenomas (benign tumors). A even smaller fraction of the adenomas gradually progress through distinct stages, recognizable by their increase in size and dysplastic characteristics. When the adenomas acquire the ability to invade through the basement membrane, they become (by definition) carcinomas. Carcinomas, in turn, can progress by acquiring the ability to metastasize to regional lymph nodes or distant sites.

Three approaches have been used to study the molecular mechanisms underlying colorectal carcinogenesis: (1) examine the methylation status of DNA from tumor cells; (2) search for genetic changes in proto-oncogenes, e.g., mutations or amplifications; (3) search for allelic

losses in DNA from tumors as compared to normal tissue which suggest lack of suppressor gene activity. First, Goelz et al. (1985) showed that DNA hypomethylation occurs as an early event in the colorectal carcinogenesis. They obtained samples of colorectal tumors and analyzed the DNA methylation status in these samples by digesting the DNA with restriction enzymes and then carrying out Southern blotting. DNA from both early adenomas and carcinomas was found to be substantially hypomethylated as compared to DNA from normal tissue. Since hypomethylation of DNA has been shown to be associated with gene activation, the results suggest that a general aberration in gene regulation could be a contributing epigenetic event.

Second, search for genetic changes in proto-oncogenes has provided insight into the mechanism underlying the progression from early adenomas to carcinomas. Vogelstein and colleagues (Bos et al., 1987) showed that mutations in the K-*ras* gene, occur in nearly 50% of colorectal carcinomas, as well as in a similar fraction of intermediate and late adenomas. In contrast, such mutations occur in less than 10% of early adenomas. Based on these observations, Fearon and Vogelstein (1990) suggest that mutations in the K-*ras* gene occur in pre-existing adenomas and are responsible for the progression of small adenomas to larger and more dysplastic adenomas. Amplification of proto-oncogenes have also been noted in colorectal carcinomas. For example, the c-*myc* and c-*erbB2*, a gene related to v-*erbB*, have been shown to be amplified in approximately 2% of colorectal carcinomas examined (D'Emilia et al., 1989; Finley et al., 1989). Amplification of the c-*myb* was also reported to be amplified in cell lines derived from colorectal carcinomas (Anitalo et al., 1984). However, their role in the progression of colorectal carcinogenesis is not clear.

Finally, studies of allelic losses in DNA isolated from tumor cells have shown loss of several tumor suppressor loci in colorectal carcinomas. Allelic loss of three loci occurs at high frequencies (35% to 75% of all carcinomas studied), and they reside in a region on chromosome 5q, a region on chromosome 17p and a region on chromosome 18q. Approximately 35% of sporadic colorectal carcinomas have allelic loss of the region on chromosome 5p which contains the familial adenomatous polyposis (*FAP*) gene (Bodmer et al., 1987; Leppert et al., 1987). Mutations in this locus are now considered to be responsible for the hereditary predisposition to colorectal cancer. Allelic deletions of chromosome 17p occur in 75% of the colorectal carcinomas (Vogelstein et al., 1989), the most common allelic loss in such tumors. Baker et al. (1989) subsequently examined the *p53* gene, a previously identified tumor suppressor gene, in seven colorectal carcinomas with detectable deletions of chromosome 17p. The results showed that one copy of the *p53* gene is deleted in all the carcinomas examined, and the remaining *p53* alleles in these colorectal carcinomas contains at least one point mutation. Furthermore, the locus on chromosome 18q is deleted is nearly 70% of colorectal carcinomas (Vogelstein et al., 1988). One gene in that region, termed deletion in colorectal carcinoma (*DCC*) gene, was later identified as a result of intensive study of the consensus region of various deletions.

Based on these observations, a model relating these genetic alterations to different stages in the tumorigenesis process has been proposed by Fearon and Vogelstein (1990). They speculated that mutations in at least four to five genes are necessary for the development of a malignant tumor, and that the total accumulation of changes, rather than their order with respect to one another, is the critical determinant of

the biological properties of the tumors.

2. Cell Transformation In Vitro

Transformation is a term used for changes in tissue culture, whereby normal cells acquire characteristics of cancer cells. One advantage of using cell culture as in vitro model for carcinogenesis is that it provides a mean to dissect the transformation process, i.e., immortalization, aberration in growth, and tumorigenicity, and to describe the molecular mechanisms underlying the process.

2.1 Oncogene-Mediated Transformation of Rodent Cells

The multistep nature of carcinogenesis implies that the transformation of cells in culture is also a multistep process. Indeed, numerous reports on oncogene-mediated transformation of rodent cells in culture have shown that transformation of normal rodent cells requires the cooperation of at least two oncogenes. Three examples have demonstrated this point. First, van de Eb and colleagues (van den Elson et al., 1983) found that both oncogenes from adenoviruses, i.e., early 1A and 1B (*E1A* and *E1B*) oncogenes, are required to transform primary rat cells into tumor cells. Second, Cuzin and collaborators reported that the polyoma middle and large tumor (*MT* and *LT*) oncogenes are both required for conversion of such cells into tumorigenic cells (Rassoulzegan et al., 1982). Finally, Weinberg and colleagues (Ruley, 1983; Land et al. 1983) showed that a cellular *ras* oncogene requires cooperation of either *myc* or *E1A* oncogene to malignantly transformed primary rat cells.

According to these results and the results of others, there appear to be two distinct mechanisms of growth control in normal rat cells. One operates in the nucleus and may be perturbed by oncogenes like *myc*, *LT*,

and *E1A*, and the other operates in the cytoplasm and may be perturbed by oncogenes like *ras*, *MT*, and *E1B* (Weinberg, 1985). The nuclear oncogenes and cytoplasmic oncogenes encode distinct functions in such cells. For example, the *ras* oncogene induce morphological alteration and growth in agarose, whereas the *myc* oncogene induce immortalization (Land et al., 1986). Similarly, the *LT* oncogene was found to initiate and maintain several transformed characteristics of such cells including an infinite life span and reduced serum requirement, whereas the *MT* oncogene conferred upon cells the ability to grow in agarose and to continue growing even when confluent (Treisman et al., 1981). The results from these studies are consistent with the multistep nature of carcinogenesis if one recognizes that oncogenes chosen for these early studies had pleiotropic effects.

Studies on oncogene-mediated transformation of Syrian hamster embryo (SHE) cells also support that malignant transformation is a multistep process. Barrett et al. (1987) attempted to malignantly transform SHE cells by transfection of the v-H-*ras* and v-*myc* oncogene, since this treatment had been shown to cause malignant transformation of rat embryo fibroblasts. They showed that SHE cells that express both the v-H-*ras* and v-*myc* oncogenes exhibit several transformed characteristic, e.g. growth in agarose (Thomassen et al., 1985), but do not acquire infinite life span and are not tumorigenic. The vast majority of the *ras*-plus-*myc*-transformed cells undergoes cellular senescence. From the senescing population, a few clones escaped senescence and gave rise to an infinite life span cell line. It is the infinite life span, *ras*-plus-*myc*-transformed cells that have the ability to form tumors when injected into athymic mice or syngeneic hamster. Subsequent cytogenetic analysis revealed that tumors produced from injection of the *ras*-plus-*myc*-transformed cells are

monoclonal and exhibit a nonrandom loss of chromosome 15 (Oshimura et al., 1985). Taken together, these observations indicate that malignant transformation of normal SHE cells required at least four genetic changes, i.e., the expression of the *ras* and *myc* oncogenes, an unidentified genetic change that contributes to the infinite life span phenotype, and loss of a tumor suppressor gene. The essential role of the loss of chromosome 15 in the transformation process is further supported by the observations that tumorigenicity of the *myc*-plus-*ras*-induced tumors was suppressed when the tumor cells were fused with normal SHE cell, and some of the non-tumorigenic cell hybrids re-expressed tumorigenicity at later passage with the loss of chromosome 15 (Barrett et al., 1987).

2.2 Studies on Human Cell Transformation

While a majority of cell transformation studies have relied on the use of rodent cells in culture, experimental models must be established using human cells to define the role of specific genetic changes in the development of human cancers. This is because rodent cells in culture have certain fundamental differences compared to human cells, e.g., genomic instability (Harris, 1987). Two lines of observations support this hypothesis. First, normal diploid human fibroblasts do not spontaneously transform in culture to either infinite life span cell lines or malignant cells (reviewed in McCormick and Maher, 1988), whereas rodent cells do so with varying frequencies. Second, treatments that successfully transform rodents cell in culture usually cannot be repeated using human cells in culture. The genetic stability of human cells make them perfect candidates for studying the process and the mechanisms by which malignant transformation occurs.

During the past several years, remarkable progress has been made in

the several human cell transformation systems. Malignant transformation has been achieved in a step wise fashion--immortalization and conversion of immortalized cells into malignant cells.

2.2.1 Transform human cells to infinite life span cells

Immortalization of human cells in culture can be achieved by infecting or transfecting the cells with genes from DNA tumor viruses, such as simian virus 40 (SV40), adenovirus 12 (AD12) or AD12-SV40 hybrid virus. For example, Reznikoff and colleagues (Christian et al., 1987) obtained several infinite life span cell lines by transfecting human uroepithelial cells with plasmid containing SV40 large tumor (*LT*) and small tumor (*ST*) oncogenes. These cell lines, termed CK/SV-HUC, are highly aneuploid and has acquired other altered growth characteristics including growth in medium without supplements for optimal growth and growth in agarose. These and other transformed characteristics are common in all SV40-transformed cells, and they reflect the pleiotropic effects of the viral oncogenes. In addition, Harris and colleagues (Reddel et al., 1988) have reported similar observations. They obtained several infinite life span cell lines by infecting normal human bronchial cells with AD12-SV40 hybrid virus. These results from these studies show that immortalization is a multistep process, i.e. required the concerted action of two or more viral oncogenes.

Immortalization can also be achieved by transfecting normal human cells with the v-*myc* oncogene. However, it occurs infrequently. McCormick and colleagues (Morgan et al., 1991) obtained an infinite life span human fibroblast cell line by transfection of normal diploid fibroblasts with the v-*myc* oncogene. All of the progeny of the clonally-derived populations expressed the same level of v-myc protein, but the vast majority of the

population went into crisis and senesced. From the senescing populations, a few clones of viable cells were seen and their progeny cells give rise to an infinite life span cell line, designated MSU-1.0. Since the *myc*-expressing siblings of MSU-1.0 in the senescing population all died, they concluded that expression of the v-myc protein is not sufficient to immortalize human fibroblasts, and at least one additional change is required to generate the infinite life span MSU-1.0 cell line. Although the change required to cause *myc*-expressing cells to acquire an infinite life span is not yet clear, these observations clearly indicate that immortalization is at least a two-step process.

2.2.2 Malignant transformation of infinite life span human cells by oncogene transfection

To date numerous investigators have achieved malignant transformation of human cells by the introduction of oncogenes into infinite life span human cells. The results of such experiments have shown that malignant transformation of infinite life span human cells in culture is also a multistep process. Reznikoff and colleagues (Christian et al., 1990; Kao et al., 1992) showed that expression of a transfected c-H-*ras* oncogene in the CK/SV-HUC cells confers upon the cells morphological alteration, but not the ability to form tumors in athymic mice. The few rare clones that progressed to become tumorigenic always show nonrandom loss of specific chromosomes, e.g., chromosome 3p, 11p, and 13q (Wu et al., 1991; Kao et al., 1992) These results indicate that malignant transformation of SV40-transformed, infinite life span human cells required not only the activation of a *ras* oncogene but probably also the inactivation of several tumor suppressor genes. Harris and colleagues (Pfeifer et al., 1989) showed that malignant transformation of a AD12-SV40

transformed human bronchial epithelial cell line required cooperation of two proto-oncogenes, the c-*raf*-1 and c-*myc*.

Recently, McCormick and colleagues (Hurlin et al., 1989; Wilson et al., 1990) showed that malignant transformation of the MSU-1.1 strain, a derivative of MSU-1.0 requires at least two genetic changes. In such studies, they showed that MSU-1.1 cells can be malignantly transformed by a transfected N- or H- *ras* oncogene in a high expression vector, but not by the same oncogenes in a low expression vector. These results showed that two genetic changes in *ras* genes are required to convert an infinite life span human fibroblast cell line to malignancy. First, the proto-oncogene has to acquire a mutation in a specific codon, and second that oncogene has to be over-expressed to a high level (McCormick and Maher, 1991).

It is clear from these studies that malignant transformation of human cells in culture is a multistep process, and at many steps on the pathway, alternative genes may be activated and/or inactivated that contribute to transformation.

C: THE FUNCTIONS OF GENES INVOLVED IN CARCINOGENESIS AND THE SIGNALING PATHWAYS IN THE CELL

1. Functions of Oncogene Products

A knowledge of the functions of proteins coded for by the oncogenes provides insight into the molecular mechanisms of malignant transformation. To date more than sixty different oncogenes of viral or cellular origin have been identified, and good progress has been made in understanding their biochemical functions. Based on the functions

elucidated so far, oncogenes can be classified into five distinct groups. I will review the best studied oncogene with each distinct function.

1.1 Protein Tyrosine Kinases

The largest group consists of oncogenes that encode protein tyrosine kinases (PTKs), enzymes that phosphorylate proteins on tyrosine residues. All PTKs have sequence homology over a stretch of approximately 260 amino acids, which has been defined as the kinase domain (Hanks et al., 1988). This domain is required for the catalytic activity of these proteins. PTKs are divided into the cytoplasmic PTKs which lack a extracellular domain and are entirely intracellular and the receptor PTKs which contain an extracellular domain and span the plasma membrane (Pawson, 1988).

1.1.1 Cytoplasmic protein tyrosine kinases

Cytoplasmic PTKs include the *src* family of genes (*src*, *yes*, and *fgr*) as well as the *fes/fps* and *abl* oncogenes (Kitamura et al., 1982; Naharro et al., 1984). All of these genes were initially identified as oncogenes of retroviruses.

The *src* oncogene, the transforming gene of the Rous sarcoma virus (RSV), is the prototype of the *src* family. The protein coded for by the v-*src* gene was the first retroviral oncogene product identified. Brugge and Erikson (1977) observed that serum from rabbits bearing RSV-induced tumors can immunoprecipitate a 60-kDa protein. This protein was subsequently found to be identical to the protein translated in vitro using RNA of the v-*src* region of RSV and rabbit reticulocyte lysate (Purchio et al., 1978). Using an in vitro kinase assay, several groups showed that the v-src protein exhibits a protein kinase activity with specificity for tyrosine residues (Collett and Erikson, 1978; Levinson et al., 1978; Hunter and Sefton, 1980). In addition, several groups showed a good correlation

between the level of PTK activity of these v-src proteins and their ability to transform fibroblasts in culture through the studies of both conditional and non-conditional mutant viruses (Weber, 1984). This indicates that such enzymatic activity is essential for the transforming activity of RSV. These observations also indicate that phosphorylation of cellular proteins on tyrosine residues is one of the mechanisms underlying the carcinogenesis process (Sefton and Hunter, 1984).

Since the finding that the v-src protein is a protein tyrosine kinase, the search for the critical targets for v-src has been an active area for investigation. Sefton et al. (1980) showed that cells infected by RSV or transfection with the v-*src* oncogene exhibited a 7- to 10-fold higher level of phosphotyrosine in cellular proteins than do uninfected cells. Further studies have revealed that many different cytoplasmic proteins are phosphorylated, including vinculin (a protein present in the focal adhesion plaques in the plasma membrane), the glycolytic enzyme enolase, phosphoglycerate mutase, and lactate dehydrogenase, p36 (a calcium-dependent actin-binding protein (Saris et al., 1986), talin (Pasquale et al., 1986), the fibronectin receptor, and a 50-kDa protein that is associated in a complex with the v-src protein and hsp90 (Brugge, 1986). This suggests that the v-src protein has several primary targets, and more than one (perhaps all) play a role in generating the transformed characteristics of RSV-infected cells (Weber, 1984). However, the role of each phosphorylated protein in cell transformation remains to be elucidated.

Another example of the cytoplasmic PTKs is the v-*fps/fes* oncogene. The v-*fps* oncogene has been identified in Fujinami sarcoma virus (FSV) and four other independent isolates of avian sarcoma viruses (Fujinami and

Inamoto, 1914; Car and Campbell, 1958; Balduzzi et al., 1981; Neel et al., 1982). The v-*fes* oncogene, identified in two independent isolates of feline sarcoma viruses, is found to be a cognate oncogene from the feline species (Franchini et al., 1981; Hampe et al., 1982; Shibuya et al., 1982). In a manner similar to v-*src*, the v-*fps/fes* oncogene encodes a protein which exhibits PTK activity, and such enzymatic activity is essential for its transforming ability (Pawson et al., 1980; Hanafusa et al., 1981). Cells infected with FSV have a higher level of phosphotyrosine in cellular proteins than do the uninfected cells (Beemon et al., 1982). The species of cellular proteins phosphorylated at tyrosine residues in FSV-transformed cells are similar to that in RSV-transformed cells (Cooper and Hunter, 1981; Erikson et al., 1981).

The expression of the c-*fps/fes* gene in mammals, unlike the c-*src* gene which is ubiquitously expressed, is limited to hematopoietic tissues, such as granulocytes and macrophages (Feldman et al., 1985; MacDonald et al., 1985). FSV-transformed myeloid cells do not require exogenous growth factors to differentiate in vitro, whereas normal myeloid cells do (Carmier and Samarut, 1986). Based on these observations, the c-*fps/fes* gene product is considered to be involved in myeloid cell differentiation and/or related to cellular responses to a growth factor specific to these cell types.

1.1.2 Receptor protein tyrosine kinases

Studies on viral oncogenes have provided several examples which show that normal counterparts of oncogenes (proto-oncogenes) encode receptor PTKs. These receptor PTKs normally function to respond to specific extracellular ligands and mediate the biological effects (Yarden and Ullrich, 1988). When activated by mutations, structural deletions or

rearrangement, they can also function as transforming proteins and play a causal role in cell transformation.

A prime example is the v-*erbB* oncogene isolated from an avian erythroblastosis virus (Graf and Beug, 1978). Waterfield and colleagues (Downward et al., 1984) were the first to show the sequence homology between the v-*erbB* oncogene product and the epidermal growth factor receptor purified from normal placenta and from a human epidermoid carcinoma cell line. The v-*erbB* oncogene product was later found to be derived from chicken epidermal growth factor receptor, containing the transmembrane and kinase domains but lacking most of the extracellular domain responsible for growth factor binding. Further nucleotide analysis revealed that the v-*erbB* oncogene contains amino- and carboxy-terminal deletions. As a consequence of the deletion, the v-*erbB* protein has a constitutively active kinase activity even in the absence of its ligand (Kris et al., 1985), and *erbB*-containing viruses has been shown to transform chicken erythroid and fibroblast cells in culture (Gamett et al., 1986).

Another example is the v-*fms* gene which was isolated from the McDonough strain of feline sarcoma virus (Besmer et al., 1986). The v-*fms* oncogene and its cellular homologue both encode the receptor for the macrophage-colony stimulating factor-1 (CSF-1) (Sherr et al., 1985). The CSF-1 receptor is primarily expressed in mononuclear phagocyte lineage cells where it functions to transduce the signal triggered by CSF-1. When the CSF-1 growth factor binds to its receptor, the receptor phosphorylates itself and various cytoplasmic proteins which have not yet been identified (Downing et al., 1988; Sengupta et al., 1988; Huhn et al., 1989). The v-*fms*-encoded protein, however, is constitutively phosphorylated on tyrosine

(Woolford et al., 1985; Tamura et al., 1986), and induces tyrosine phosphorylation of cellular proteins in the absence of exogenous CSF-1 (Morrison et al., 1988a). This difference has been attributed to the deletion of a autoregulatory tyrosine 969 in the v-*fms*-encoded protein.

1.2 Protein Serine/Threonine Kinases

Studies on viral oncogenes have provided examples of oncogenes which code for proteins that have serine/threonine kinase activity. These oncogenes code for proteins that contain a 260 amino acid region kinase domain responsible for the enzymatic activity. The proteins coded for by these oncogenes phosphorylate proteins on serine or threonine residues, instead of tyrosine residues, although their kinase domains re homologous to that of the protein tyrosine kinases.

A typical example is the v-*raf* oncogene originally identified as a transforming gene of a murine sarcoma virus (Rapp et al., 1983). The *raf*-containing retroviruses have been shown to transform rodent fibroblasts in culture and to induce tumors in mice (Rapp et al., 1986). using an in vitro kinase assay, Moelling et al. (1984) demonstrated that the v-raf gene product has protein kinase activity with specificity for serine and threonine residues. In addition, The c-*raf* proto-oncogenes have been shown to be "activated" in a variety of human tumors, including human stomach cancer (Shimizu et al., 1985), glioma (Fukui et al., 1985), breast cancer, and renal cell carcinoma (Stanton and Cooper, 1987). Nucleotide analysis of these "activated" c-*raf* oncogenes have shown that c-*raf* can be activated by amino-terminal fusions, truncations, and site-specific point mutations.

The role of the c-raf protein in the ras signaling pathway was first suggested by functional analysis of the v-raf protein. Three lines of

evidence revealed that v-raf acts downstream of the ras protein in its signaling pathway. First, Noda et al. (1983) showed that the v-*raf* oncogene can transform revertants of v-K-*ras*-transformed cells whereas the *ras* oncogenes and oncogenes of the tyrosine kinase class cannot. Second, Stacey and colleagues (Smith et al., 1986) showed that microinjection of a neutralizing antibody against ras protein does not inhibit the v-*raf*-transformed phenotype. Finally, Kolch et al. (1991) showed that the c-raf protein is required for the *ras*-induced transformed phenotype (Kolch et al., 1991).

It is now well documented that the c-raf protein plays a central role in the ras signaling pathway (Kizaka et al., 1992). Mitogenic stimuli, such as growth factors and tyrosine kinase oncogene products, can activate the ras signaling pathway by increasing the intracellular level of GTP-bound ras which in turn associates and phosphorylates the c-raf protein on its tyrosine residues (Morrison et al., 1988b,1989; Rapp, 1991). Phosphorylation of the raf protein in turn activates the c-raf kinase, and the activated c-raf kinase can transduce the signal downstream by eliciting a kinase cascade and ultimately leading to altered gene expression (Heidecker et al., 1989). Studies suggest that the v-raf protein exert its growth-promoting action by activating the kinase cascade, just like the activated c-raf does (Kolch et al., 1991).

1.3 Growth Factors

Studies on viral oncogenes have provided examples which show that normal counterparts of some oncogenes (proto-oncogenes) encode proteins that act as growth factors (Dickson and Gordon, 1987). Studies of these oncogenes have provided one of the mechanisms for carcinogenesis, i.e., uncontrolled cell growth. If tumor cells simultaneously produce growth

factors and respond to such growth factors, they would be constantly stimulated to proliferate (autocrine mechanism).

1.3.1 The v-*sis* oncogene and platelet-derived growth factor

The v-*sis* oncogene of the simian sarcoma virus represents a prototype for oncogenes that encode growth factors. The cellular homologue of this transforming gene codes for the B chain of platelet-derived growth factor (PDGF) (Doolittle et al., 1983). Cells transformed by simian sarcoma virus can proliferate in medium without exogenous growth factors because they produce their own PDGF (Johnson et al., 1986). The v-*sis*-encode protein has been shown to activate the PDGF receptors by binding and stimulating the receptors at the cell surface and in intracellular compartments (Huang et al., 1984; Keating and Williams, 1988). The activated PDGF receptor in turn mediated the biological response by triggering a complex cascade of secondary events that signal the cell to replicate (Satoh et al., 1993; Valius and Kazlauskas, 1993).

PDGF is a potent growth factor for several types of mesenchymal cells which express the PDGF receptor, i.e., fibroblasts, vascular smooth muscle cells, and glial cells. The cellular *sis*/PDGF-B gene has been shown to be over-expressed in cell lines derived from human mesenchymal tumors (Reinhold et al., 1984). The endogenously produced PDGF probably play a role in the carcinogenesis process of such tumors since cells from the tumors simultaneously express PDGF and respond to the growth factor.

1.3.2 Oncogenes related to basic fibroblast growth factor

Another example comes from oncogenes that are related to basic fibroblast growth factors, a heparin-binding growth factor. The *int*-2 oncogene was identified in murine sarcoma virus-induced breast tumors (Dickson et al., 1984; Peters et al., 1984). The *hst* oncogene was isolated

independently from a human stomach tumor and a Kaposi's sarcoma (Delli-Bovi et al., 1987; Taira et al., 1987). The amino acid sequence of the *int*-2 and *hst* oncogene products has a significant homology to that of basic fibroblast growth factor. The cellular homologues of the *int*-2 and *hst* oncogenes has been found to be amplified in about 10-20% of human mammary tumors (Lidereau et al., 1988; Zhou et al., 1988). The isolation and expression of these oncogene in tumor cells responding to such growth factor suggests that an autocrine mechanism is involved.

1.4 Ras Proteins and GTPase activity

The *ras* multigene family consists of three active members, i.e., the H-, K-, and N-*ras* genes. The H-*ras* and K-*ras* gene were identified as the transforming genes of Harvey and Kirsten strains of murine sarcoma viruses respectively (Barbacid, 1987). A third member of this family was isolated from DNA of a human neuroblastoma by DNA transfection (Shimizu et al., 1983).

Genes from the *ras* family are frequently found to be mutated in various types of human tumors. Nucleotide sequence analysis of the *ras* oncogenes isolated from human tumors and viral *ras* oncogenes have provided insight into the mechanism for "activation" of this gene. Such studies show that the most common difference between these mutant *ras* sequences and their normal counterparts is a point mutation that leads to the substitution of a different amino acid. Furthermore, site specific mutagenesis *in vitro* showed that changes in the ras protein at residue 12 or 13, or residues 59-61, almost invariably leads to a change in the transforming ability of the protein (Taparowsky et al., 1982; Bos et al., 1984).

The *ras* genes encode intracellular membrane protein of 21 kDa,

termed p21 (Langbeheim et al, 1980; Willingham et al., 1980). The very significant insight into the biochemical function of the ras protein was contributed by Shih et al. (1979), who showed that it specifically binds guanine-nucleotides. The protein was subsequently shown to have an intrinsic GTPase activity, which hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP) (McGrath et al., 1984). It is now known that the GTPase activity of the ras protein is tightly regulated by a GTPase activating protein (GAP). When cells are not stimulated by external growth factor, GAP stimulates the GTPase activity of normal ras protein, maintaining ras in its inactive, GDP-bound states (McCormick, 1989). Most *ras* oncogenes, however, encode mutant ras proteins that have a reduced ability to hydrolyze GTP or are no longer subjected to stimulation by GAP, resulting in an accumulation of GTP-bound ras. It is this GTP-bound ras which actively transduces signals for growth even in the absence of external growth stimuli, causing uncontrolled growth.

Recently, it has become clear that the ras protein serves as a common transducer within the cells for signals initiated by epidermal growth factor, platelet-derived growth factor, and a wide variety of other growth factors (Qiu and Green, 1991; Nakafuku et al., 1992; Satoh et al., 1993). Receptors for these growth factors are usually protein tyrosine kinases. When the ligand binds to the receptor, the enzymatic activity of the receptor is stimulated and it phosphorylates itself. The phosphorylated (activated) receptor can then recruit cellular factors to activate ras by causing it to exchange GDP for GTP. The GTP-bound ras in turn leads to activation of the c-raf protein, a serine/threonine kinase which in turn activates MAP kinase kinase and ultimately results in altered gene expression.

1.5 Nuclear Transcription Factors

The best characterized of the nuclear oncogene family is the v-*jun* oncogene which was isolated in 1987 as a transforming gene of an avian sarcoma virus (Maki et al., 1987). Its function was suggested by its homology with the yeast transcription factor GCN4 (Vogt et al., 1987). Vogt and colleagues (Bohmann et al., 1987) first reported that the consensus target binding sequence of GCN4 -TCACTCA- is identical to the recognition sequence of the human transcription factor AP-1, suggesting that jun is related to AP-1. Subsequently, they showed that the c-jun protein associates with the c-fos protein, another nuclear proto-oncogene product, to form the AP-1 transcription factor which is capable of regulating target gene expression (Rouscher III et al., 1988). The nature of the interaction between c-fos and c-jun was found to be through a dimerization motif termed the leucine zipper (Landschulz et al., 1988). Now it is known that the genes, *jun* and *fos*, belong to two multigene families and all of the members in the *jun* family can interact with all members in the *fos* family (Vogt and Bos, 1990). Since the discovery of specific DNA binding with *jun* and *fos*, a number of other nuclear oncogenes, including c-myc, c-myb, c-rel, have been shown also to have sequence specific DNA binding and transcription activation capability (Lucibello and Muller, 1991).

2. Functions of Tumor Suppressor Gene Products

Much less is known about the functions of these tumor suppressor genes as compared to that of the oncogenes. To date only ten tumor suppressor genes have been cloned, although many genomic regions have been recognized to contain tumor suppressor genes. The identification of four

of these tumor suppressor genes is reviewed in previous sections. Here I will review the five genes whose functions are best characterized; Retinoblastoma (*Rb*), *p53*, Wilms' tumor (*WT1*) , Neurofibromatosis type 1 (*NF1*) and *DCC* genes. Three of these five genes (*Rb*, *p53*, and *WT1*) code for proteins that function as nuclear transcription factors. The *NF1* gene product is a GTPase activating protein, and the *DCC* gene product is a cell adhesion molecule.

2.1 The Retinoblastoma (*Rb*) gene

The role of the Rb gene product in regulating cell growth was first suggested by its binding to the transforming proteins of several DNA tumor viruses, including SV40 large tumor (LT) antigen, adenovirus E1A and papillomavirus E7 (DeCaprio et al., 1988; Whyte et al., 1988; Dyson et al., 1989, 1990). The three viral oncoproteins are structurally distinct but share a common sequence in the region utilized for Rb binding, suggesting that a strong selective pressure has favored the evolution of viral proteins able to alter *Rb* gene function. Mutational analysis of the transforming proteins has demonstrated a correlation between their ability to transform cells and their ability to bind Rb protein (Whyte et al., 1989). These data suggest that the loss of the *Rb* gene function may account for some of the biological effects of these viruses, such as the ability to immortalize and transform cells in culture. Consistent with this is the finding that mutant Rb protein found in tumors fails to bind LT antigen and the mutations in these Rb proteins map to the regions required for binding in vitro.

Using an antibody specific for the Rb protein, Lee et al. (1987) demonstrated that Rb protein is a nuclear phosphoprotein with DNA-binding activity. Following this finding, several groups showed that the

phosphorylation pattern of Rb protein varies depending on the proliferative state of the cells. (Buchkovich et al., 1989; DeCaprio et al., 1989). The majority of Rb protein in rapidly proliferating human fibroblasts is the phosphorylated. In contrast, most of the Rb protein in nonproliferative fibroblasts is the underphosphorylated. Based on these observations, a hypothesis that the Rb protein regulates cell cycle progression was proposed (Buchkovich et al., 1989). This hypothesis was subsequently proven by Lee and his colleagues (Goodrich et al., 1991), who showed that injection of purified Rb protein into cells inhibits the progression of cells from G_1 into S phase, and co-injection LT antigen can block this function. Consistent with this is the finding that oncoproteins of DNA tumor viruses binds preferentially to the underphosphorylated forms of Rb protein (Ludlow et al., 1989).

Preliminary indication that the Rb protein acts as a negative regulator was contributed by Robbins et al. (1990), who showed that expression of Rb protein can suppress the expression of a reporter gene transcribed from the c-fos transcriptional promoter. In such study, they defined the element in the c-*fos* promoter that confers responsiveness to Rb suppression as the retinoblastoma control element. This element has been found also in the promoter region of the c-*myc* gene. Taken together, these observations suggest that Rb act by suppressing the expression of genes like *fos* and *myc*. However, the mechanism for such activity and the sequence specific for Rb binding is not yet clear.

2.2 The *p53* gene

The *p53* gene was initially classified as an oncogene because its over-expression resulted in immortalization of rodent cells (Jenkins et al., 1984), and in transformation of primary rat embryo fibroblasts in

concert with an activated *ras* gene (Eliyahu et al., 1984; Parada et al., 1984). However, further analysis revealed that these studies were conducted using mutant forms of the gene. When the same experiments were repeated with the wild-type *p53* gene, the transformation did not occur (Finlay et al., 1989; Eliyahu et al., 1989).

Like the Rb protein, the p53 protein has the ability to form stable complex with the transforming proteins of several DNA tumor viruses (Levine and Momand, 1990). In fact, the p53 protein was first identified as a 53-kDa cellular protein bound to SV40 LT antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). Besides SV40 LT antigen, adenovirus early region 1B (E1B) and papillomavirus E6 proteins also can bind to the p53 protein (Finlay et al., 1989). The p53 binding sites on LT antigen, E1B, and E6 are homologous to one another, and each is required for cellular transformation in cell culture by the respective virus. Thus, three different DNA tumor viruses, SV40, adenovirus, and human papilloma virus 16, have all evolved a mechanism to deal with the same negative regulator of cellular growth, p53. These data suggest that loss of *p53* gene function may account for some of the transforming properties shared by these viruses, such as the ability to immortalize and transform cells in culture (Levine and Momand, 1990).

The effect of the p53 protein on cell proliferation is well documented. Introduction of a wild-type p53 gene can suppress the growth of p53-negative human carcinoma cells in vitro, often through arrest at the G1/S boundary of the cell cycle (Baker et al., 1990b; Chen et al., 1990; Diller et al., 1990; Martinez et al., 1991). More recently, the *p53* gene has been shown to play a role in determining the programmed cell death of myeloid leukemic cells (Yonish-Rouach et al., 1991,1993). These

results are consistent with the wild-type p53 gene being a tumor suppressor. However, it is still not clear how the *p53* gene functions. Two possible mechanisms have been proposed. The p53 protein could act as a regulator of gene transcription, either by promoting or repressing messenger RNA synthesis. The p53 protein has a DNA binding motif in its carboxyl terminus (Kern et al., 1991), and the amino-terminus of the p53 protein has been shown to promote the transcription of a test gene when fused with a DNA binding region of another gene, e.g., the yeast *Gal4* gene (Raycroft et al., 1990; Fields and Jang, 1990). Alternatively, the p53 protein could regulate the assembly or function of the DNA replication-initiation complex (Braithwaite et al., 1987; Gannon and Lane, 1987). This is based on the observation that p53 can inhibit the adenosine triphosphate-dependent helicase activity of LT antigen, and thus abrogate the ability of LT antigen to bind α DNA polymerase or replicate SV40 DNA.

Mutations in the *p53* gene have been identified in many human tumors. They occur at codons throughout the whole gene but tend to be clustered in four regions that are highly conserved (Levine et al., 1991). These mutations result in mutant p53 proteins that lacks DNA binding capacity, transcriptional activator function, or inhibitory effect on LT antigen, or any combination of the three.

2.3 The Wilms' Tumor (*WT1*) Gene

The function of the *WT*1 gene product as a transcription factor was first suggested by sequence analysis of the *WT*1 gene. Based on the nucleotide sequence, the predicted *WT*1 gene product contains four zinc finger domains and a region rich in proline and glutamine (Rose et al., 1990; Gessler et al., 1990; Call et al., 1990). Subsequently, the *WT*1 gene product was shown to have sequence-specific DNA-binding activity, and the

sequence recognized are similar to that recognized by EGR-1 (Rauscher III et al., 1990), a zinc finger-containing protein that is induced by mitogenic stimuli protein. A mutation in the zinc finger domain region of the *WT1* gene product originally identified in cells from a Wilms' tumor abolished its DNA binding activity. The *WT1* gene seems to have a restricted tissue expression whereas the *Rb* and *p53* genes are expressed in virtually all tissues. No evidence currently exist to implicate the *WT1* gene in malignancies other than Wilms' tumor.

2.4 The Neurofibromatosis (*NF1*) Gene

The *NF1* gene codes for a 280-kDa protein, denoted neurofibromin (Wallace et al., 1990). The function of neurofibromin was first suggested by its sequence homology with the catalytic domain of the mammalian GTPase activating protein (GAP) protein and of the products of the *IRA1* and *IRA2* genes, inhibitors of ras in yeast (Buchberg et al., 1990; Xu et al., 1990). Subsequently, several studies provide strong evidence that the neurofibromin negatively regulates the GTPase activity of ras protein (Martin et al., 1990; Ballester et al., 1990). Based on these data, it has been suggested that neurofibromin may be involved in the control of cell growth by interacting with proteins such as ras, in a manner similar to the way GAP interacts. The role of loss of the NF1 gene function in tumorigenesis is not clear at the present time.

2.5 The Deleted in Colorectal Carcinoma (*DCC*) Gene

The function of the *DCC* gene product was suggested by its sequence homology with neural cell adhesion molecules. It contains conserved domains shared by other cell adhesion molecules, i.e., the immunoglobulin-like and fibronectin-related domains. This suggests that the *DCC* gene product may function by binding to an extracellular matrix or basement

membrane component (Fearon et al., 1990).

3. Multiple Signaling Pathways

The mitotic activity of cells is controlled by external signals that stimulate or inhibit intracellular events. The process by which an external signal is transmitted into and within a cell is referred to as signal transduction. Signal transduction is generally initiated by the interaction of extracellular ligands (growth factors, neurotransmitters and factors involved in the immune response) with their receptors on the cell surface. During the last decade, the signal transduction pathways triggered by some of the ligands have become a very active area of investigation. Results of these investigation have led to the identification of key proteins involved in these signaling pathways. New information is accumulating to reveal a complex, inter-related network of signal transduction inside the cell. Here I will review some of the well-documented examples which reflect the complexity of this network.

3.1 Ras-Dependent and Ras-Independent Signaling Pathways

Growth factors are specific to certain cell types or tissue. Typical examples are platelet-derived growth factor in fibroblasts, nerve growth factor in neurons, colony stimulating factor-1 in macrophages, and bombesin in bronchial epithelial cells. These extracellular ligands signal the cell to replicate by binding to their respective receptor on the cell surface, and activation of ras protein is a key step in these signaling pathways (Heidaran et al., 1992; Szeberenyi et al., 1992; Profrock et al., 1992; Satoh et al., 1993). When a ligand binds to its receptor, the receptor autophosphorylates which leads to the recruitment of a guanine-nucleotide releasing factor which activates ras by catalyzing the release

of bound GDP, a rate limiting reaction in the exchange of GDP to GTP. The active ras in turn transduces the signal by eliciting a kinase cascade and ultimately altered gene expression.

An additional complexity of growth factor signaling pathways has been demonstrated by recent studies showing several different growth factors can also exert their activity via a pathway independent of ras. These growth factors, including platelet-derived growth factor, epidermal growth factor, interferon-α and interferon-γ, exert their activity by inducing a nuclear transcription factor complex termed SIF (sis-inducible factor). This transcription factor complex in turn binds to a specific sequence in the promoters of several cellular genes, resulting in induction of the expression of genes, such as *c-fos* (Hayes et al., 1987; Wagner et al., 1990). A 91 kDa subunit of the SIF complex, termed p91, is responsible for the DNA binding and transcriptional activity of the SIF complex (Silvennoinen et al., 1993). Further analysis revealed that growth factors, such as PDGF, raised the level of SIF by stimulating tyrosine phosphorylation and nuclear accumulation of p91. The p91 protein is probably phosphorylated directly by the stimulated receptors since it can interact with receptor PTKs through its src homology region 2 (SH-2) domain.

3.2 Activation of Multiple Signaling Proteins by One "Activated" Receptor

As indicated above, distinct cell surface receptors function to transduce signals for growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor, nerve growth factor and insulin. The receptors share a common molecular structure, consisting of a extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase domain.

The binding of a growth factor to its receptor causes receptor dimerization and activation of the tyrosine kinase activity of the receptor. The stimulated receptor in turn phosphorylate itself at several tyrosine residues. The phosphorylated tyrosines provide a mechanism for the receptor to activate cytoplasmic proteins by binding to these proteins via their SH-2 domains and phosphorylating them. Recent studies on these cytoplasmic proteins have revealed a number of second messenger proteins whose functions are regulated by tyrosine phosphorylation.

The signaling pathway mediated through the PDGF receptor has served as the prototype for identification of substrates of the receptor tyrosine kinases. The activated PDGF receptor has been shown to physically associate with and phosphorylate several cytoplasmic proteins that can trigger various secondary events (Westermark and Heldin, 1985). These proteins include: 1) phosphatidylinositol-3 kinase (PI-3 kinase); 2) phospholipase C-γ; 3) GTPase-activating protein, which modulates the signal transducing activity of the ras protein (Molloy et al., 1989); and 4) three tyrosine kinases of the src family, c-src, c-yes, and c-fyn (Kypta et al., 1990).

The PI-3 kinase was the first cytoplasmic enzyme found to be recruited by PDGF receptor in a ligand-dependent manner. Cantley and colleagues (Kaplan et al., 1987) demonstrated that this enzyme is rapidly recruited to the plasma membrane in PDGF-stimulated fibroblasts. The recruitment of PI-3 kinase was shown to require the kinase activity of the PDGF receptor. PI-3 kinase phosphorylates the inositol ring of phosphatidylinositol (Coughlin et al., 1989). This enzyme may function in controlling cell shape and cell movement which change as a result of PDGF treatment since by the interaction of the catalytic product,

phosphatidylinositol biphosphate, can interact with profilin, a cytoskeleton protein.

Phosphorylation of phospholipase C-γ by the PDGF receptor on its tyrosine residue has been shown to increase the catalytic activity of this enzyme (Nishibe et al., 1990). This cytoplasmic enzyme functions to catalyze the hydrolysis of phosphatidylinositol biphosphate and generates two second messengers, inositol triphosphate and diacylglycerol. Inositol triphosphate in turn leads to the mobilization of intracellular Ca^{++}, whereas diacylglyceral activates protein kinase C (Whitman and Cantley, 1988). These events ultimately stimulate cellular growth via a series of cellular responses, including changes in cytosolic pH and potassium concentration, as well as the transcription of certain genes. The role of other phosphorylated proteins is less clear, but their activity is considered to be important in the PDGF-mediated signaling pathway.

Another example of a receptor that activates multiple cytosolic proteins is the EGF receptor. When bound by its ligand, the EGF receptor was shown to activate phospholipase C-γ, phosphatidylinositol-3 kinase, phosphatidylinositol-4 kinase, phosphatidylinositol-5 kinase, and GTPase-activating protein.

3.3 Signaling Pathways Mediated by Cytoplasmic Protein Tyrosine Kinases

Studies on the cytoplasmic proteins phosphorylated by cytoplasmic PTKs, such as v-src, have shown that cytoplasmic PTKs share some of the cellular substrates with receptor PTKs. For example, the v-*src* oncogene can phosphorylate phospholipase C-γ, phosphatidylinositol-3 kinase, GTPase-activating protein and the c-raf protein (Cantley et al., 1991). Furthermore, studies on revertants of *ras*-transformed NIH3T3 cells indicated that several cytoplasmic PTK oncogenes, including src, fes, and

abl, act upstream of ras in the same pathway which is triggered by receptor PTKs. This conclusion is based on the observation that these oncogenes cannot transform a revertant cell line isolated from K-*ras* oncogene transformed cells (Noda et al., 1983). This was further supported by the observations that transformation by these oncogenes can be suppressed by either microinjection of an anti-ras monoclonal antibody or introduction of a dominant negative ras mutant protein (Smith et al., 1986). Taken together, these results suggest the cytoplasmic PTKs and the receptor PTKs act in a converging signaling pathway.

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CHAPTER II

Malignant Transformation of a Human Fibroblast Cell Strain by Transfection of a v-*fes* Oncogene, But Not a *gag*-Human c-*fes* Construct

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ABSTRACT

To determine whether the human c-*fes* gene, a homolog of the feline v-*fes* oncogene, can play a role in the malignant transformation of human fibroblasts, we transfected a near-diploid, infinite life-span, growth factor independent, human fibroblast cell strain, MSU-1.2, with plasmids carrying a *fes* gene along with a selectable marker. The *fes* gene was either the v-*fes* oncogene from the Gardner-Arnstein strain of feline sarcoma virus or a chimeric construct in which 835 base pairs representing exons 10-19 from the human c-*fes* proto-oncogene had been substituted for the corresponding feline sequence in the v-*fes* oncogene. The transfected cells were selected in appropriate medium, and a number of drug-resistant clones were isolated and the progeny cells were assayed for *fes* expression by immunoprecipitation analysis. Six independent clones that expressed the v-fes protein and four that expressed the gag-c-fes protein were further characterized. The former exhibited anchorage-independence and formed progressively growing, invasive, spindle cell sarcomas in athymic mice after only a short latency period. The latter strains were not anchorage-independent and did not form tumors in athymic mice. These results show that the v-*fes* oncogene can malignantly transform an infinite life-span human fibroblast cell strain, but the human c-*fes* gene cannot.

INTRODUCTION

It is now well documented that carcinogenesis is a multistep process and that genetic changes lie at the heart of this process. It appears that there are a limited number of genes, which normally function in cell division and/or cell differentiation, that contribute to the neoplastic phenotype when altered in either function or expression. These normal cellular genes, or proto-oncogenes, become transforming cellular oncogenes because of point mutations, deletions, amplification or rearrangement of DNA. The methods available to identify the cellular proto-oncogenes are tedious and subject to failure. However, the acutely transforming retroviruses provide a natural source of mobilized oncogenes that can be recognized by their ability to cause cell transformation. The cellular counterparts (proto-oncogenes) of at least nine of the retroviral oncogenes (v-*abl*, v-*erb*B, v-*ets*, v-*mos*, v-*myb*, v-*myc*, v-H-*ras*, v-K-*ras*, and v-*sis*) have been found to be involved in human tumorigenesis [1-9]. Therefore, the retroviral oncogenes can be useful models in analyzing the mechanisms of neoplastic growth.

The v-*fes* oncogene was identified as a transforming gene of the Gardner-Arnstein feline sarcoma virus (FeSV) [10]. The identical gene was found in three independent isolates of feline sarcoma viruses. In addition, the v-*fps* oncogene, identified in five independent isolates of avian sarcoma viruses [11], was found to be a cognate gene of *fes* in avian species. The c-*fes/fps* gene is highly conserved in mammals as well as in vertebrates. For example, the overall homology between the feline and the human c-*fes* coding sequences is 94% at the amino acid level and 91% at the

DNA level [12]. The *fes/fps* oncogene encodes a cytoplasmic tyrosine kinase whose enzymatic activity is essential for both tumor induction in animals and transformation of mammalian cells in culture [13-16]. In cats [10], dogs [17], and chickens [18], the *fes/fps*-containing retroviruses principally induce fibrosarcomas and myxosarcomas. It is possible, therefore, that tumors in human mesenchymal tissues contain an activated c-*fes* gene that contributes to the malignant transformation of such cells. Consistent with this suggestion is the finding that transgenic mice that express the v-*fps* oncogene product exhibit a high frequency of tumors of mesenchymal tissues [19]. It is this question that led to the present study.

The *fes* gene in the Gardner Arnstein strain of FeSV lacks exons 5-9 of the feline c-*fes* gene and in addition contains three amino acids that do not correspond to those of the c-*fes* gene. What is more, a truncated viral *gag* sequence is linked to the amino terminal of the feline c-*fes* gene in the v-*fes* oncogene. Any one or a combination of these three structural changes could be responsible for the activation of the c-*fes* sequence. These possibilities have been studied by constructing various chimeric constructs and testing them for their ability to transform cells in culture. For example, Hanafusa and his coworkers [20] and Haseltine and his colleagues [21] showed that simple over-expression of the human c-*fes* proto-oncogene is not sufficient to cause transformation of NIH3T3 cells, whereas its fusion with FeSV *gag* gene produces a hybrid protein that is active as a kinase and can transform NIH3T3 cells. Their work indicates that the *gag* fusion is essential to confer transforming ability in the c-*fes* gene. In contrast, Foster and Hanafusa [22] presented data indicating that fusion with *gag* was not the essential change for transformation. They

showed that an avian v-*fps* oncogene that lacks the viral *gag* sequence was active in transforming chicken fibroblasts. However, the avian c-*fps* proto-oncogene in the same construct was not active, indicating that the avian c-*fps* sequence requires more than mere over-expression to become an oncogene. The v-*fps* oncogene in the construct differs from the avian c-*fes* gene in the same construct by 43 base substitutions [23]. This suggests that one or more of these mutations were involved in the activation of the c-*fes*.

Transfection studies have proven especially useful in analyzing the mechanisms of carcinogenesis, since by this technique, one can identify oncogenes and determine what transformed phenotypes they confer. The most common activity of oncogenes is the phosphorylation of protein on tyrosine residues. Two types of protein tyrosine kinases (PTKs) has been reported: the receptor PTKs and non-receptor PTKs. The involvement of some of the receptor PTKs, e.g., *neu* and v-*erb*B, in human cell carcinogenesis is well documented, but the role of the non-receptor PTKs, e.g., *src* and *fes/fps*, in this process remains less clear. To determine if v-*fes* or a *gag-c-fes* construct can cause malignant transformation of human fibroblasts and to see if this action is correlated with the PTK activity of the fes protein, we transfected these genes into an infinite life span fibroblast strain, MSU-1.2., a member of the MSU-1 lineage developed in this laboratory. The results showed that expression of the v-*fes* oncogene, but not the *gag-c-fes* construct caused MSU-1.2 cells to form very large colonies in agarose and form high grade, malignant sarcomas in athymic mice, and that this difference reflected a large difference in the PTK activity of the two gene products.

MATERIAL AND METHODS

Cell Strain and Culture Conditions

The MSU-1.2 cell strain, derived from MSU-1.1 cells [24], has an infinite life span in culture and a stable, near-diploid karyotype, composed of 45 chromosomes including two marker chromosomes. The cells were routinely cultured in Eagle's minimal essential medium supplemented with aspartic acid, serine and pyruvic acid as described (growth medium) [25]. They were maintained at 37°C in a humidified incubator with 5% CO_2. For selection of cells for histidinol resistance, we used McM medium [26], a modified version of MCDB-110 [27], lacking histidine and supplemented with 1 mM L-histidinol dihydrochloride (Sigma, ST. Louis, MO), 10% supplemented calf serum (Sterile Systems, Logan, UT), hydrocortisone (10 μg/ml), penicillin and streptomycin. The condition for selection of cells for mycophenolic acid resistance has been described previously [28]. For studies of anchorage independence, McM medium supplemented with 2% fetal calf serum was used.

Transfection Procedure

Approximately 2x 10^5 cells were seeded per 100 mm dish the day before transfection. After 16-24 h, 2 μg of plasmid DNA was transfected into the cells using the Polybrene/ DMSO method adapted for use with human cells by Morgan et al [29]. Twenty hours after transfection, the growth medium was replaced with the appropriate selection medium. The selective medium was refreshed twice weekly and the cells were observed for formation of drug-resistant clones.

Immunoprecipitation Analysis of *Fes* Gene Product

To assay the amount of *fes* expression in transfected cells, we followed the procedure described by Hurlin et al [30] with the following modifications. Ten μl of radioactive cell lysate was precipitated in 10% trichloroacetic acid (TCA) and counted in a scintillation counter. The volume of supernatant containing 10^8 cpm of TCA-precipitable counts was incubated for 4 h at 4°C on a shaker with 10 μl aliquot of antibody v-fes (Ab-1) (Clone F-113, Oncogene Science, Manhasset, NY), which reacts with the fes specific domain common to the Gardner-Arnstein and Snyder-Theilen FeSV translational products. Protein A-Agarose was coated with goat anti-rat IgM and 50 μl was added to the incubation mixture and allowed to react for 30 min at 4°C. The samples were centrifuged and the pellets were washed as previously described [30]. The pellets were resuspended in 50 μl of 1x polyacrylamide gel electrophoresis (PAGE) sample buffer, heated for 5 min at 90°C, and centrifuged. Gel electrophoresis was performed using 8% polyacrylamide gels and a Tris-glycine buffer system [31]. The samples were loaded using 20 μl per lane. After electrophoresis the gels were soaked in Resolution solution (EM Corp., Chestnut Hill, MA) for 30 min and in distilled water at 0°C for 30 min and then dried and analyzed by autoradiography.

Protein Kinase Assay

Cells were grown in 60 mm culture plates as described. They were washed twice with ice-cold phosphate buffered saline, and the culture plates were placed immediately on ice. All subsequent operations were carried out at 4°C unless otherwise noted. Cells in each plate were lysed in 1 ml of modified RIPA buffer [0.05 M Tris-HCl (pH 7.4); 0.15 M NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% sodium dodecylsulfate; 2 mM EDTA]

containing 9 μg/ml aprotinin (Sigma, St Louis, MO), 2 mM phenylmethyl sulfonyl floride (Boehring, Indianapolis, IN) and 1 mM sodium orthovanadate. The cell lysate from each plate was scraped with a rubber policeman and transferred into a 10 ml polycarbonate tube (Nalgene, Rochester, NY). After the cell lysate was vortexed vigorously for 20 sec, it was centrifuged at 35K rpm for 35 min. The resulting clarified supernatant was then assayed for protein kinase activity.

Aliquots of cell lysate containing 50 μg protein were incubated with 15 μl Protein A-Agarose coated with goat anti-FeLV p15 (Quality Biotech, Camden, NJ) and allowed to react for 2 h at 4°C. The samples were centrifuged and the pellets were washed 5 times with modified RIPA buffer, and twice with 50 mM Tris-HCl (pH 7.4). The pellets were then resuspended in 40 μl of kinase reaction buffer (10% glycerol, 50 mM Hepes, 10 mM $MnCl_2$); 20 μl of each suspension was assayed for protein kinase activity and 20 μl was subjected to Western blot analysis to determine the amount of protein precipitated in each tube. For the protein kinase assay, 20 μg dephosphorylated α-casein (Sigma, St. Louis, MO) and 1 μl [P^{32}]-γ-ATP (6,000 Ci/mmole) (Dupont, Wilmington, DE) was added to the suspension. The contents of the tube were mixed gently and incubated in 30°C water bath for 15 min. The reaction was stopped by the addition of 20 μl 2X PAGE sample buffer. The sample was then subjected to gel electrophoresis as described [31], with 20 μl of sample loaded per lane. After electrophoresis the gels were dried and analyzed by autoradiography.

Western Blot Analysis

Protein in the other 20 μl of the suspension was electrophoresed in an 8% sodium dodecylsulfate/ polyacrylamide gel and transferred onto Immobilon PVDF transfer membranes (Millipore, Bedford, MA). To assay the

amount of fes protein in the suspension, we followed the procedure described by Yang et al [28], using goat-anti-FeLV p15 as the primary antibody and horseradish peroxidase-conjugated rabbit-anti-goat antibody as the secondary antibody.

Southern Blot Analysis

The presence of the v-*fes* gene was determined using the Southern blot procedure described by Wilson et al [31]. An 8-kb *Eco*RI fragment of pGAF*his*D plasmid containing the v-*fes* gene was used as the probe.

Assay for Anchorage Independence

To assay the ability of cells to form colonies in 0.33% agarose, we followed the procedures described by Hurlin et al [25]. The agar plates were kept in incubator with 3% CO_2.

Assay for Tumorigenicity

Athymic nude mice (BALB/c background) originally purchased from Harlan Sprague Dawley (Indianapolis, IN) were bred and maintained in a high efficiency particulate air (HEPA)-filtered laminar flow hood containment system. Absorbable gelatin sponges (Upjohn Co., Kalamazoo, MI) 1 cm^3 in size were implanted subcutaneously in the subcapular region and/or rear flank region of 6-week-old mice to serve as a matrix. One week later, 10^7 cells in 0.2 ml of serum-free medium were injected directly into the sponge. Mice were examined weekly for tumor growth and tumors were removed when they reached approximately 1 cm in diameter.

Histopathology

Tumor tissue for histopathology was fixed in 10% phosphate buffered formalin, pH 7.0, embedded in paraffin, sectioned at 4-5 μm, and stained with hematoxylin-eosin using standard technique [32].

RESULTS

Choice of the Target Cell Strain

An infinite life span human fibroblast cell strain arose following transfection of a v-*myc* oncogene into finite life span human foreskin-derived fibroblasts [24]. This strain, designated MSU-1.0, is diploid, has a stable karyotype, does not form colonies in agarose, does not grow in medium without exogenous growth factors, and does not form tumors in athymic mice. A variant strain of MSU-1.0 arose spontaneously. This strain, designated MSU-1.1, has two marker chromosomes and is able to grow in medium without exogenous growth factors as rapidly as normal fibroblasts do in medium with 5% serum. MSU-1.1 cells are not tumorigenic, but can be transformed to malignancy by high expression of N- or H-*ras* oncogenes [30,31]. A spontaneous variant of MSU-1.1 cells was selected by its ability to grow in medium without exogenous growth factors. This strain, designated MSU-1.2, grows in medium without exogenous growth factors as rapidly as normal fibroblasts do in medium with 10% serum, a characteristic of malignant human fibroblasts. However, it does not form large colonies in agarose and is not tumorigenic. In contrast, malignant human fibroblasts have both characteristics [30,31,33]. It has been reported that expression of the v-*fes* oncogene confers upon NIH3T3 cells the ability to form large colonies in agarose, and such cells are tumorigenic. We, therefore, chose MSU-1.2 cells as the recipient strain for testing the ability of v-*fes* or a *gag-c-fes* construct to transform human fibroblasts to anchorage independence and to the malignant state.

Construction of pGAF*his*D Plasmid

Plasmid pGAFeSV containing the complete proviral sequence of the Gardner-Arnstein feline sarcoma virus was kindly provided by Dr. Charles Sherr, Howard Hughes Medical Institute, St. Jude Children's Research Hospital, Memphis, TN [34]. To allow selection of transfectants, we inserted a *Salmonella typhimurium* histidinol dehydrogenase (*his*D) gene [35] at the *Eco*RI site of this plasmid. The altered plasmid was named pGAF*his*D and used in our study.

Transfection of MSU-1.2 Cells by v-*Fes* Oncogene

MSU-1.2 cells were transfected with pGAF*his*D plasmid DNA, and cells containing the stably-integrated plasmid DNA were selected by their ability to grow in medium containing histidinol but lacking histidine. Histidinol-resistant clones were isolated approximately 3 wk after transfection by dislodging them from the dishes using trypsin, and transferring them into separate 25-cm^2 flasks. Thirty histidinol-resistant clones were isolated and propagated. The progeny cells were assayed for v-*fes* expression by immunoprecipitation analysis as described, using a fes-specific monoclonal antibody. Six clones out of the 30 were found to express the 110 kDa v-fes protein at detectable levels (Figure 1, Lanes 1-6).

Characterization of the Six v-*fes* Transfectants

Southern blot analysis of the location of the v-*fes* gene in the genomic DNA of the progeny of the individual clones was carried out using v-*fes* specific DNA probe to determine if the sites of integration differed. The results showed that each of these six clones represented an independent transfection (Figure 2). These six independent cell strains were then further characterized. The v-*fes*-transformed cells from each of

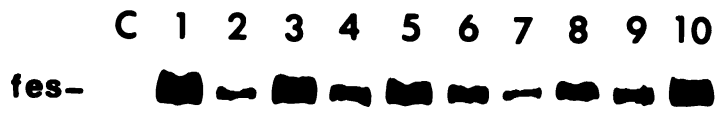

Figure 1. Relative expression of *fes*-encoded p110 in six MSU-1.2-v-*fes*-transformed cell strains (lanes 1-6) and four MSU-1.2-*gag*-c-*fes*-transformed cells (lanes 7-10). Cells were labeled with [^{35}S]-methionine for 18 h in medium lacking methionine. Cell lysis, immunoprecipitation with a fes specific antibody, electrophoresis and fluorography were performed as described in Material and Methods. Lane C, MSU-1.2 cells transfected with the control plasmid containing the bacterial *his*D gene.

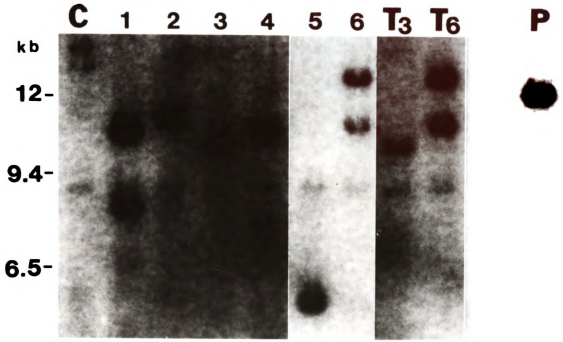

Figure 2. Southern blot analysis of DNA from six MSU-1.2-v-*fes*-transformed cell strains and tumor-derived cells. Genomic DNA was digested with *Bam*HI restriction enzyme and hybridized with the 8-kb *Eco*RI fragment of the v-*fes* oncogene from plasmid pGAF*his*D. Lane C, MSU-1.2 cells transfected with the control plasmid; Lanes 1-6, MSU-1.2:*fes*1-6 cell strains respectively; Lanes T3 and T6, cells derived from tumors formed by cell strain MSU-1.2:*fes*3 and MSU-1.2:*fes*6 respectively; Lane P, 10 pg linearized pGAF*his*D. Sizes of DNA molecular weight standards are indicated.

the six cell strains were slightly more refractile than their parental MSU-1.2 cells, but retained the fibroblastic morphology of the parental cells. The six strains were assayed for anchorage-independence, along with their parental MSU-1.2 and MSU-1.1 strains. These strains formed colonies larger than 100 μm in diameter at frequencies ranging from 4% to 56% (Table 1); the non-transformed parental MSU-1.2 cells did not form any such colonies (Figure 3). The frequency of anchorage independent colonies formation by the six strains correlated well with their levels of v-*fes* expression (Table 1).

Evidence of Malignant Transformation by v-*fes*

Cells from the six v-*fes*-transformed cell strains were injected into athymic mice to determine if they could form tumors. All six cell strains gave rise to tumors after a latency period of 7-60 days. The shortest latency was found with the cell strain that showed the highest v-*fes* expression (Table 1), but the rate of their growth for the other five strains was not highly correlated with v-*fes* expression. All tumors were composed primarily of a monomorphic population of spindle-shaped cells arranged in interdigitating fascicles and bundles (Figure 4). Based on the histological pattern, the tumors were classified as high grade, malignant spindle cell sarcomas.

Portions of several tumors arising from two of the v-*fes*-transformed strains were minced and the cells were returned to culture. All gave rise to histidinol-resistant populations of fibroblasts. Southern blot analysis of DNA from these tumor-derived cells showed identical size bands corresponding to v-*fes*, indicating that the tumors arose directly from the injected v-*fes*-transformed cells (Figure 2, Lane T3 and T6). When the tumor-derived cells were injected s.c. into mice, they produced tumors

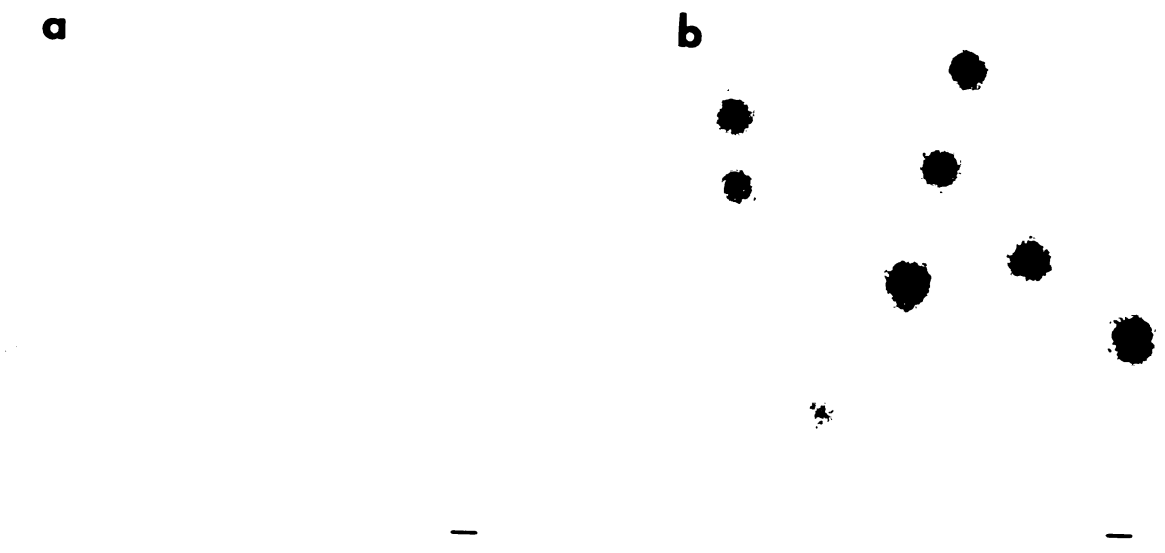

Figure 3. Representative example of growth of MSU-1.2-v-*fes*-transformed cell strains in 0.33 % agarose. (a) MSU-1.2 cells, forming only a background of small colonies in 3 wk. (b) MSU-1.2:*fes1* cells, forming large-sized colonies, 100-300 μm in diameter in 3 weeks. (Bar= 100 μm)

Table 1. Relative v-*Fes* Expression, Anchorage Independence, and Tumorigenicity of the Six MSU-1.2-v-*fes*-Transformed Cell Strains

Cell strains	Relative v-*fes* expression*	Frequency of agar colonies with diameter ≥100 μm	Tumorigenicity assay	
			Tumors/ injection sites†	Latency period (days)‡
MSU-1.1	0	< 0.01 %	0/12	-
MSU-1.2	0	< 0.01 %	0/12	-
MSU-1.2:*fes*1	16	54 %	4/4	5-9
MSU-1.2:*fes*2	1	4 %	4/4	15-24
MSU-1.2:*fes*3	8	56 %	4/4	16-24
MSU-1.2:*fes*4	2	15 %	2/4§	61-70
MSU-1.2:*fes*5	5	8 %	2/4§	50-55
MSU-1.2:*fes*6	3	15 %	4/4	26-39

* Bands corresponding to the v-fes signal on the blot were cut out and assayed by scintillation counting. The cpm counts in the MSU-1.2:*fes*2 were arbitrarily scored as 1 and the other cell strains were scored relative to it.

† Each mouse was injected at two sites, i.e., a subscapular region and in the opposite flank.

‡ Days required for a tumor to reach 1 cm in diameter.

§ Both mice were sacrificed when the tumors in the subscapular regions reached 1 cm in diameter. At that time, tumors were not apparent in the flanks, but they might have developed if we had waited to sacrifice the animals.

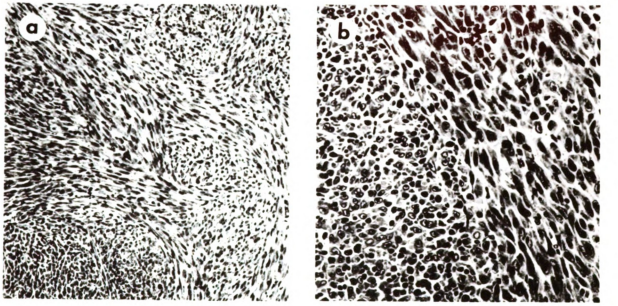

Figure 4. Representative histology showing a poorly differentiated, spindle cell sarcoma formed by MSU-1.2:*fes3* cells. (a) 40X magnification. (b) 100X magnification. A 2-cm diameter tumor, removed 4 wk after subcutaneous injection of 10^7 cells, was sectioned and stained with hematoxylin/eosin.

identical to those from which they were derived, but with a shorter latency period.

Transfection of MSU-1.2 Cells with a *gag-c-fes* Construct

Plasmid pRec*Kpn* which contains a *gag-c-fes* gene and a bacterial *gpt* gene was kindly provided by Dr. William Haseltine at Dana-Farber Cancer Institute, Boston, MA [21]. The *gag-c-fes* gene was constructed by recombining the N-terminal of the GA-FeSV v-*fes* sequence and the C-terminal of the human c-*fes* sequence at the conserved *Kpn*I site. MSU-1.2 cells were transfected with pRec*Kpn* plasmid DNA and selected in medium containing mycophenolic acid. Mycophenolic acid-resistant clones were isolated approximately 3 wk after transfection by transferring them into separate 25-cm^2 flasks, using trypsinization as described. Forty mycophenolic acid-resistant clones were isolated and the progeny cells were assayed for c-*fes* expression by immunoprecipitation analysis using a fes- specific monoclonal antibody. Four clones out of the 40, each isolated from separate dishes, were found to express the gag-c-fes protein (Figure 1, Lanes 7-10). The fusion protein product of the *gag-c-fes* construct is the same size as the product of the v-*fes* gene.

Characterization of the Four *gag-c-fes* Transfectants

The *gag-c-fes*-transformed MSU-1.2 cells from each of the four strains exhibited an fibroblastic morphology identical to the non-transfected parental MSU-1.2 strain. None of these strains exhibited the ability to form large-sized colonies in agarose, and none formed tumors when injected into athymic mice. Thus, the four *gag-c-fes*-expressing strains were phenotypically similar to their parental cells, suggesting that the human c-*fes/fps* gene cannot be activated into an oncogene by mere over-expression and fusion with a viral *gag* sequence.

Comparison of the Tyrosine Kinase Activity

One possible explanation for the inability of the *gag-c-fes*-expressing cells to become malignant is that the *gag-c-fes* construct encodes a protein that has no tyrosine kinase activity or has reduced activity compared to the v-fes protein. We, therefore, compared the tyrosine kinase activity of proteins encoded by both plasmids. The results showed that the level of ability of the gag-c-fes protein to autophosphorylate was 54-fold lower than that of the v-fes protein. Its ability to phosphorylate an exogenous substrate, α-casein, was 5-fold lower.

Assaying of c-fes Protein Expression in Human Mesenchymal Tumors

The c-*fes* proto-oncogene is not expressed in normal mesenchymal tissue [36,37]. However, our data indicate that expression of v-fes protein can contribute to the malignant transformation of human fibroblasts in culture. It is at least possible that some mesenchymal tumors have developed as the result of activation and expression of c-*fes*. To test this hypothesis, we assayed 11 cell lines derived from human mesenchymal tumors for expression of the c-fes protein using immunoprecipitation. Fifteen malignant cell strains derived from MSU-1.1 cells spontaneously or by carcinogen treatment were similarly assayed. To assure that our assay conditions were adequate to detect the c-fes protein, a rodent cell strain that expresses protein from a transfected human c-*fes* gene [38] was included with each assay as a positive control. The latter cell strain always exhibited a strong band on the gels, but c-fes protein was not detected in any of the 26 cell lines/ strains assayed (data not shown).

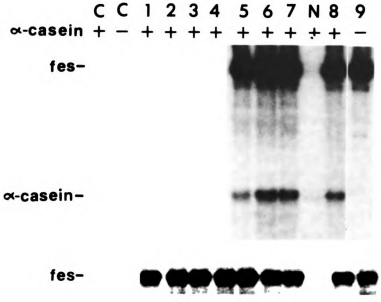

Figure 5. Protein kinase activity in immunoprecipitates containing p110. Unlabelled cell extracts prepared from v-*fes* (lanes 5-9) or *gag-c-fes* (lanes 1-4) transformed MSU-1.2 cells were immunoprecipitated with anti-FeLV p15 antibody, assayed for protein kinase activity (upper panel) or the amount of protein precipitated (lower panel). The "+" or "-" symbols indicates the presence or absence of an artificial substrate, α-casein. Lane C, MSU-1.2 cells transfected with control plasmid; lane N, cell lysate from lane 8 was precipitated with a non-specific goat antiserum first, material remaining unprecipitated was subsequently reacted with a gag-specific antibody as described.

Table 2. Human Cell Lines/ Strains Studied for c-*fes* Expression

Cell line or strain	Cell type - origin
HT1080, SHAC, NCI, 8387, VIP-FT	Fibrosarcoma-derived cell lines*
WSU-11	Fibrosarcoma-derived cell lines†
WSU-1	Malignant fibrous histiocytoma-derived cell line†
WSU-4, WSU-10	Neurofibrosarcoma-derived cell lines†
WSU-8, WSU-9	Desmoid tumor-derived cell lines†
CSV0.20	BPDE-transformed MSU-1.2 cell strain‡
DY6.16 2C1, DY6.16 3C1, DY6.16 3C10, DY6.16 4C5.	BPDE-transformed MSU-1.1 cell strains§
SD53.2AC4, 2+F/T, CS77F.2AC4	ENU-transformed MSU-1.1 cell strains‡
L55I-3T	MNNG-transformed MSU-1.1 cell strain‡
L203.3B1, L203.4B2	Co^{60}-transformed MSU-1.1 cell strains‡
L45I-B5T, L46I-5T, L46I-7T, L48I-1T	Spontaneous malignantly transformed MSU-1.1 cell strains‡

* These cell lines were obtained from the American Type Culture Collection, Rockville, MD.

† These cell lines were generated in this laboratory from fresh human tumors. The cells were introduced into culture, propagated and characterized by Jeremy Wray, a gradulate student of J. Justin McCormick.

‡ Justin J. McCormick, unpublished studies.

§ These cell strains were provided by Dr. Dajun Yang [43].

DISCUSSION

We conclude that the v-*fes* oncogene can play a role in causing human cells to become malignant, because the six cell strains that expressed the transfected gene produced spindle cells sarcomas after a very short latency period. This is the first such report using human cells. Our results are consistent with the multistep nature of the transformation process because the human cell strain we used, MSU-1.2, has already acquired an infinite life span in culture and full growth factor independence. Haseltine and his colleagues [21] have shown that v-*fes* can also malignantly transform NIH3T3 cells, a line that is immortal and has acquired some of the changes characteristic of malignant cells since it responds so readily to transfection of *ras* oncogenes.

Our results with the c-*fes* gene indicate that over-expression and *gag* fusion is not enough to convert the c-*fes* proto-oncogene into an oncogene. Even though our data showed that c-fes protein was expressed at a high level in four cell strains, they did not produce tumors. Haseltine and his colleagues [21] showed that c-*fes*, in the same construct we used, was capable of transforming NIH3T3 cells into malignant cells. This difference very probably reflects the difference in the target cells used for transfection. The NIH3T3 transfectants that expressed the gag-c-fes fusion protein in their study produced colonies in agarose and apparently it was cells derived from these colonies that proved to be malignant. In our study, the human MSU-1.2 cells that expressed the identical gag-c-fes fusion protein did not yield colonies in agarose, and the populations were not tumorigenic. As noted in the Results section, the tyrosine kinase

activity of the protein encoded by the *gag-c-fes* construct was significantly lower that of the v-fes protein. It is known that NIH3T3 cells take up significantly more exogenous DNA during the transfection process than do human fibroblasts [39]. Therefore, it is possible that NIH3T3 cells contained a higher number of copies of the transfected *gag-c-fes* gene than did MSU-1.2 cells. If so, this could account for the positive results with the NIH3T3 cells. Although the changes that have occurred in NIH3T3 cells have not been systematically analyzed, and it is possible that these cells have acquired more changes on the path toward full malignancy than MSU-1.2 cells have and that the *gag-c-fes* construct can only confer full malignancy on cells that have already acquired enough changes.

The results of animal studies during the late 70s and early 80s on the pathogenicity of the *fes/fps*-containing retroviruses, showed that these viruses induce progressively growing fibrosarcoma at the site of injection in their natural hosts, e.g., cat and chicken [40]. These data appear to contradict the multistep nature of the transformation process. The tumors in those studies formed after only a short latency period, and were attributed to a single step process. Since the changes that occurred in such virus-transformed cells have not been systematically analyzed, it is possible that the virus-infected cells that made up the tumors were not malignantly transformed. Instead, it may be that multiple benign tumors caused the massive neoplastic growth that resulted the death of the host animals. Such a situation has been reported in the case of Rous sarcoma virus-induced tumors in chickens [41]. Also, it has been shown [42] that in non-feline species, such as dogs, rabbits, fetal sheep, pigs and monkeys, three isolates of *fes*-containing FeSV induced sarcomas that

regressed. These results suggest that v-*fes* expression is insufficient by itself to cause the malignant transformation of mesenchymal cells.

The fact that no c-fes protein was detected in cells derived from any of the human mesenchymal tumors tested suggests that either the human c-*fes* gene is not involved in malignant transformation of human mesenchymal cells or the frequency of its activation in such tumors is very low. Another possibility is that the c-*fes* gene is only activated in a specific type of mesenchymal tumor which was not represented in our samples. However, the successful transformation of the MSU-1.2 cells by v-*fes* suggests that other oncogenes from the non-receptor protein tyrosine kinase family, e.g., *fgr*, *fyn*, and *yes*, may well be involved in the origin of human mesenchymal tumors.

ACKNOWLEDGEMENTS

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CHAPTER III

Malignant transformation of human fibroblast strain MSU-1.1 by v-*fes* requires an additional genetic change

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Abstract

To determine whether the v-*fes* oncogene can malignantly transform human fibroblasts that have acquired an infinite life span and are partially growth factor independent, we transfected cell strain MSU-1.1 with a plasmid containing the v-*fes* oncogene and a bacterial histidinol dehydrogenase gene. The transfectants were selected for drug resistance. Sixty independent histidinol-resistant clones were isolated and the progeny cells were assayed for v-*fes* expression by immunoprecipitation analysis using a *fes* specific monoclonal antibody. Six clones were found to express the v-*fes* protein at a detectable level, and these cells were further characterized. Three of the six clonal populations exhibited a significant increase in the ability to form medium-sized colonies in agarose, but none were not tumorigenic in athymic mice. However, when the populations were propagated for many generations, these three acquired the ability to form very large colonies in agarose, and they produced malignant tumors, suggesting that an additional genetic change had been required. To investigate the kind of genetic change involved, we transfected the v-*fes* oncogene into derivative strains of MSU-1.1 that express a transfected v-*sis*, H-*ras* or N-*ras* oncogene. The results showed that when complemented by either the v-*sis* oncogene or a *ras* oncogene expressed at a relatively high level, v-*fes* could supply the additional change required for malignant transformation.

Introduction

It has long been recognized that transformation of rodent fibroblasts in culture is a multistep process, i.e., it requires cooperation between at least two oncogenes (reviewed in Weinberg, 1985). For example, Weinberg and colleagues (Land et al., 1983) showed that *myc* and *ras* oncogenes acting in concert can convert diploid rat embryo fibroblasts into tumorigenic cells in culture, whereas neither oncogene alone has this capacity. In such studies of cooperation between oncogenes, the nuclear oncogene caused immortalization whereas the cytoplasmic oncogene conferred characteristics of tumor-derived cells, such as focus formation or ability to grow in agarose.

Less is known about oncogene-induced transformation of human fibroblasts. The number of steps involved in malignant transformation of human fibroblasts by oncogene transfection is also not known. Clearly, the target cells used must have an infinite or greatly-extended life span. This was shown by work from McCormick and colleagues (Hurlin et al., 1987; Wilson et al., 1989) who attempted to transform diploid, finite life span human fibroblasts in culture by transfecting the cells with a H- or N-*ras* oncogene in a high expression vector. The *ras* transfectants exhibited many of the characteristics of tumor-derived cells, e.g., morphological transformation, growth in medium without exogenous growth factors, and growth in agarose, but they did not acquire an infinite life span and were not tumorigenic. In contrast, when these investigators used as their target for transfection a cell strain, designated MSU-1.1, that had spontaneously acquired an infinite life span following transfection and

expression of a v-*myc* oncogene (Morgan et al., 1991), the transfectants were malignant. To achieve malignant transformation of MSU-1.1 cells, the *ras* oncogene had to be over-expressed since transfectant of the same *ras* oncogenes carried in low expression vectors did not produce malignant cells. The MSU-1.1 strain expresses a transfected v-*myc* oncogene, has two marker chromosomes, is able to grow in medium without exogenous growth factors as rapidly as normal fibroblasts do in medium with 5% serum.

The v-*fes* oncogene, a transforming gene of the Gardner-Arnstein feline sarcoma virus (Gardner et al., 1970), encodes a cytoplasmic tyrosine kinase whose enzymatic activity is essential for both tumor induction in animals and transformation of mammalian cells in culture (Barbacid et al., 1981; Donner et al., 1980). Because the v-*fes* oncogene cause tumors of mesenchymal tissue in cats and chickens (Hanafusa et al., 1988), we considered it possible that activated c-*fes* genes play a role in causing such tumors in human. If this is the case, a transfected v-*fes* oncogene might be able to cause malignant transformation of the MSU-1.1 human fibroblasts just as the over-expressed *ras* oncogenes did. If it did not, then it might at least cooperate with another oncogene by supplying one step in the multistep transformation process. To test this hypothesis, we transfected the v-*fes* oncogene into the MSU-1.1 strain and assayed the cells for tumorigenicity. The results indicated that expression of a transfected v-*fes* oncogene is not sufficient to convert these cells into malignant cells. To determine whether or not the v-*fes* oncogene could supply the penultimate change in these cells, we transfected the v-*fes* oncogene into three derivative strains of MSU-1.1 that express a transfected v-*sis* or c-*ras* oncogene. We found that the v-*fes* oncogene can supply the additional change required for malignant transformation of MSU-

1.1 cells that expressed a v-*sis* or a c-H-*ras* oncogene expressed at a moderate level.

Results

Choice of target cells

The characteristics of the target cell strains used in this study are summarized in Table 1. The parental MSU-1.1 cell strain derived in this laboratory (Morgan et al., 1991) expresses a transfected v-*myc* gene and a *neo* gene, coding for resistance to Geneticin. It has a normal fibroblastic morphology and a stable, near-diploid karyotype consisting of 45 chromosomes including two marker chromosomes, and it exhibits partial growth factor independence. The MSU-1.1:*sis*A cell strain was obtained by transfecting MSU-1.1 cells with a plasmid carrying the v-*sis* oncogene and a bacterial xanthine-guanine phosphoribosyl transferase (*gpt*) gene and selecting for drug resistance (Yang et al., 1994). The MSU-1.1:H*ras*98 cell strain was obtained by transfecting MSU-1.1 cells with a plasmid carrying the T24 H-*ras* oncogene and a *gpt* gene and selecting for drug resistance. The MSU-1.1:N*ras*73 cell strain was obtained by transfecting MSU-1.1 cells with the HT1080 N-*ras* oncogene and selecting for focus formation. The *ras* oncogenes are transcribed from their endogenous promoters and as a result, neither gene is over-expressed. None of the cell strains form large-sized (diameter $\geq$ 100 μm) colonies in agarose, a characteristic of fully malignant fibroblasts, nor do they form tumors in athymic mice.

Transfection of MSU-1.1 cells with a v-fes oncogene

MSU-1.1 cells were transfected with pGAFhisD plasmid DNA and cells containing the stably integrated plasmid DNA were selected by their ability to grow in medium lacking histidine and containing histidinol as described (Lin et al., 1994). Individual histidinol-resistant clones were

Table 1. Growth characteristics of the target cell strains

Cell strains	Oncogene transfected	Degree of growth factor independence*	Colonies in agarose per 10,000 cells	
			Diameter ≥ 60μm	Diameter ≥ 80μm
MSU 1.1	-	+ (48 h)	10	1
MSU-1.1:*sis*A	v-*sis*	++ (20 h)	150	10
MSU-1.1:H*ras*98	c-H-*ras*	++ (22 h)	370	90
MSU-1.1:N*ras*73	c-N-*ras*	+ (47 h)	10	2

* + = able to grow in medium without exogenous growth factors as rapidly as normal fibroblasts do in medium with 5% serum; ++ = able to grow in medium without exogenous growth factors as rapidly as normal fibroblasts do in medium with 10% serum. The number in parentheses is the population doubling time in the test medium.

isolated from dishes that had been transfected independently to assure that we would not be studying siblings. The cells from these clones were propagated to approximately 4 X 10^6 and assayed for v-*fes* expression by immunoprecipitation analysis using a v-*fes* specific monoclonal antibody. Of 60 clonal populations examined, six were found to express the 110 kDa v-fes protein at detectable levels (Figure 1). These clonal populations were further characterized.

Characterization of the six v-fes-transfectants

The six v-*fes*-transformed MSU-1.1 strains were slightly more refractile than the non-transfected parental MSU-1.1 strain, but they retained the fibroblastic morphology of the parental cells. When early passage cells from these six strains were assayed for anchorage independence, they formed colonies larger than 60 μm but less than 100 μm in diameter at frequencies higher the age-matched, mock-transfected parental MSU-1.1 cells (Table 2). However, when assayed in medium without exogenous growth factors, they did not replicate at rates faster than the parental MSU-1.1 strain, i.e., remained only partially growth factor independent, and they did not form tumors when injected into athymic mice.

Spontaneous progression of three MSU-1.1-v-fes-transformed strains

When late passage cells from the six MSU-1.1-v-*fes*-transformed strains were similarly characterized, they still did not grow in medium without exogenous growth factors at rates faster than the parental MSU-1.1 strain, but cells from three of the six strains (MSU-1.1:*fes*1-3) formed large-sized colonies in agarose ($\geq$ 100 μm in diameter) at high frequencies (2-17%) and gave rise to tumors in athymic mice (Table 3). The other three strains (MSU-1.1:*fes*4-6) did not form such colonies in agarose and did not form tumors in athymic mice.

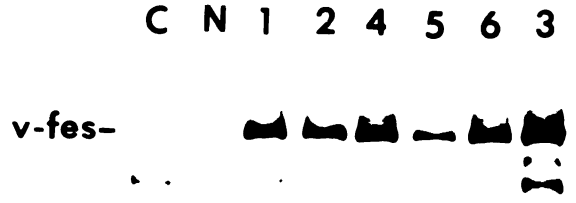


Figure 1. Relative levels of expression of v-*fes* protein in six MSU-1.1-v-*fes*-transformed cell strains. Cells were labeled with [^{35}S]-methionine for 18 h in medium lacking methionine. Cell lysis, immunoprecipitation with the v-*fes* specific antibody, electrophoresis and fluorography were performed as described in Material and methods. Lane C, MSU-1.1 cells transfected with the control plasmid; Lane N, MSU-1.1:*fes*1 cells, mock treatment without the primary antibody; Lanes 1-6, MSU-1.1:*fes*1-6 cell strains, respectively.

Table 2. Relative v-*fes* expression, anchorage independence, and tumorigenicity of the six MSU-1.1-v-*fes*-transformed strains

Cell strains	Relative v-*fes* expression*	Colonies in agarose per 10,000 cells		Tumors/total injection sites
		Diameter ≥ 60μm	Diameter ≥ 100μm	
MSU-1.1	ND	10	0	0/12
MSU-1.1:*fes*1	1.4	390	10	0/4
MSU-1.1:*fes*2	1.8	620	70	0/4
MSU-1.1:*fes*3	3.1	630	20	0/4
MSU-1.1:*fes*4	2.4	170	60	0/4
MSU-1.1:*fes*5	1.0	20	10	0/4
MSU-1.1:*fes*6	2.6	40	0	0/4
MSU-1.1:H*ras*10	ND	2200	870	†

* Bands corresponding to the v-fes signal on the blot were cut out and assayed by scintillation counting. The counts per minute in the lowest sample, i.e., MSU-1.1:*fes*5, were arbitrarily scored as 1.0 and the other cell strains were scored relative to it.

ND, Not detectable.

† The tumorigenicity of this cell strain was not re-examined in this series of experiments. However, it yielded six tumors out of eight injections when

Table 3. Relative v-*fes* expression, anchorage independence, and tumorigenicity of the late passage and tumor-derived cells of MSU-1.1:*fes* strains

MSU cell strains	Description	Relative v-*fes* expression*	Number of agar colonies with diameter ≥ 100 μm per 10,000 cells	Tumorigenicity assay: Tumors/ injection sites	Tumorigenicity assay: Latency period† (days)
1.1:*fes*1	late passage	1.6	1720	4/4	27-54
1.1:*fes*1T	tumor-derived	5.5	1820	4/4	21-28
1.1:*fes*2	late passage	1.5	800	4/4	27-75
1.1:*fes*2T	tumor-derived	3.4	2940	4/4	21-28
1.1:*fes*3	late passage	3.0	220	6/8‡	160-200
1.1:*fes*3T	tumor-derived	15.6	880	4/4	11-18
1.1:*fes*4	late passage	2.0	40	0/8	-
1.1:*fes*5	late passage	1.0	4	0/4	-
1.1:*fes*6	late passage	2.4	40	0/4	-

* Bands corresponding to the v-fes signal on the blot were cut out and assayed by scintillation counting. The counts per minute in the lowest sample, i.e., MSU-1.1:*fes*5, were arbitrarily scored as 1.0 and the other strains were scored relative to it.

† Number of days required for a tumor to reach 600 mm^3 in size (about 1 cm in diameter).

‡ Two of the four mice were sacrificed when the tumor in the subscapular region reached 1 cm in diameter. At that time, tumors were not apparent in the flank, but they may have developed if we could have waited to sacrifice the animals.

Characterization of tumors and cells derived from tumors

Mice were necropsied when the tumors reached 1 cm in diameter, and tumor specimens were prepared for histopathological examination. All tumors were composed of a monomorphic population of either spindle- or round- shaped cells (Figure 2). Based on the histological pattern, the tumors formed by MSU-1.1:*fes*1 and MSU-1.1:*fes*3 were classified as high grade, malignant spindle cell sarcomas; the tumors formed by MSU-1.1:*fes*2 were classified as round cell sarcomas. Portions of several tumors arising from the MSU-1.1:*fes*1-3 strains were minced and the cells were returned to culture. The cells derived from these tumors were histidinol-resistant as expected. They did not grow in medium without exogenous growth factors at rates faster than the parental MSU-1.1 cell strain, but they formed colonies in agarose that were even larger than those produced by the cells originally injected into the mice (Figure 3). The level of expression of v-fes protein in the tumor-derived population was 2- to 5-fold higher than that in the cells originally injected into the mice (Figure 4). When reinjected into the mice, these tumor-derived cells produced tumors more rapidly than did the cells from which they were derived (Table 3). The tumors were classified as high grade, malignant sarcomas, identical to the tumors produced by their original strains.

In vitro selection for malignant cells

Because the level of v-*fes* expression in the six original v-*fes*-transformed cell strains did not correlate with their ability to form malignant tumors (Table 3), we considered the possibility that in the case of strains MSU-1.1:*fes*1, 2 and 3, a subpopulation of tumorigenic cells that were anchorage independent had overgrown the rest of the non-tumorigenic cells in the population as these were being subcultured. If

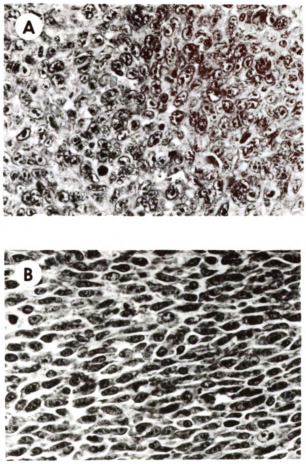
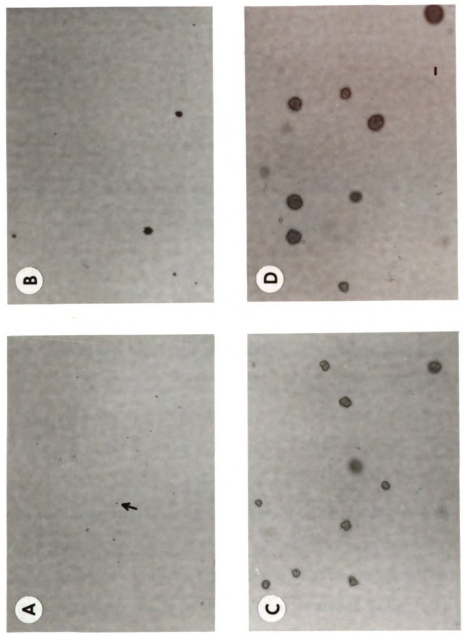


Figure 2. Tumor histology showing a poorly differentiated, round cell sarcoma (A) or spindle cell sarcoma (B). These tumors were produced from injection of late passage MSU-1.1:*fes2* and MSU-1.1:*fes1* cells, respectively. (100X magnification)

Figure

Figure 3. Colony formation by MSU-1.1-v-*fes*-transformed cell strains in 0.33% agarose. (A) Parental MSU-1.1 cells, forming only a background of very small colonies in 3 weekk; A single cell is indicated with an arrow; (B) Early passage MSU-1.1:*fes*1 cells, forming medium-sized colonies, 60-80 μm in diameter, in 3 weeks; (C) Late passage MSU-1.1:*fes*1 cells, forming large-sized colonies, 100-200 μm in diameter, in 3 weeks; (D) Cells derived from tumors formed by injection of the MSU-1.1:*fes*1 cells. Bar= 100 μm.


A
B
C
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this were the case, cells derived from large-sized agarose colonies should prove to be malignant. To test this hypothesis, we plated 10^6 early passage cells from each of the non-tumorigenic strains (MSU-1.1:*fes*4-6) in 0.33% agarose to select for any rare variant cells present in the population that could form large-sized colonies. One agarose colony with a diameter between 100-200 μm was isolated from each cell strain and propagated for further characterization. The progeny cells of these agarose colonies did not express v-fes protein higher than those of the populations from which they were derived, and did not grow in medium without exogenous growth factors at a faster rate than the parental MSU-1.1 cells. However, they formed large-sized colonies in agarose at very high frequencies (up to 26%) and produced tumors in athymic mice with a latency period of less than 28 days (4/4 for each of the agarose colonies isolated from the MSU-1.1:*fes*4-6 strains).

These results strongly suggest that at least one additional genetic change is required for v-*fes*-transformed MSU-1.1 strains to become tumorigenic. Karyotyping of tumor-derived cell strains from each of the six tumorigenic strains showed that these strains have exactly the same 45 chromosomes as the parental MSU-1.1 cells, indicating that no gross chromosomal changes were had occurred. To see if a spontaneous mutation in one of the growth-related genes were responsible for the malignant phenotype of the three strains derived from agarose colonies, or of the progression of late passage cells from each of the MSU-1.1:*fes*1-3 strains, we assayed the MSU-1.1:*fes*1-3 cells for over-expression of various growth factors that are mitogenic to such cells. We found no evidence of upregulation of *PDGF*-B or *TGF*-α genes in the six tumorigenic strains as tested by RT-PCR, or of secreted growth factors as tested by the ability

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of conditioned medium to stimulate the growth of normal fibroblasts.

Cooperation between the v-sis and the v-fes oncogene

Earlier studies showed that transfection of MSU-1.1 cells by either a N-*ras* or a H-*ras* oncogene in a high expression vector causes them to become malignant (Hurlin et al., 1989; Wilson et al.,1990). In contrast, transfection of these cells with either the H-*ras* oncogene isolated from the EJ bladder carcinoma cell line or the N-*ras* oncogene isolated form the HT1080 fibrosarcoma cell line expressed from their endogenous promoter, did not result in malignant transformation (J.J. McCormick, unpublished data). These data, along with our data with the v-*fes* oncogene suggest that malignant transformation of MSU-1.1 cells requires at least two genetic changes. To determine if the v-*sis* oncogene could supply the change required by the v-*fes* oncogene in malignant transformation of MSU-1.1 cells, we transfected the v-*fes* oncogene into non-tumorigenic MSU-1.1 cells that contain and express a transfected v-*sis* oncogene (strain designated MSU-1.1:*sis*A). The v-*sis* oncogene contains a cDNA copy of the simian c-*sis/PDGF*-B gene transcribed from simian sarcoma virus LTR promoter. The protein coded for by this oncogene have been shown to be mitogenic to human fibroblasts, presumably due to the high homology between the simian PDGF-B and the human PDGF-B proteins.

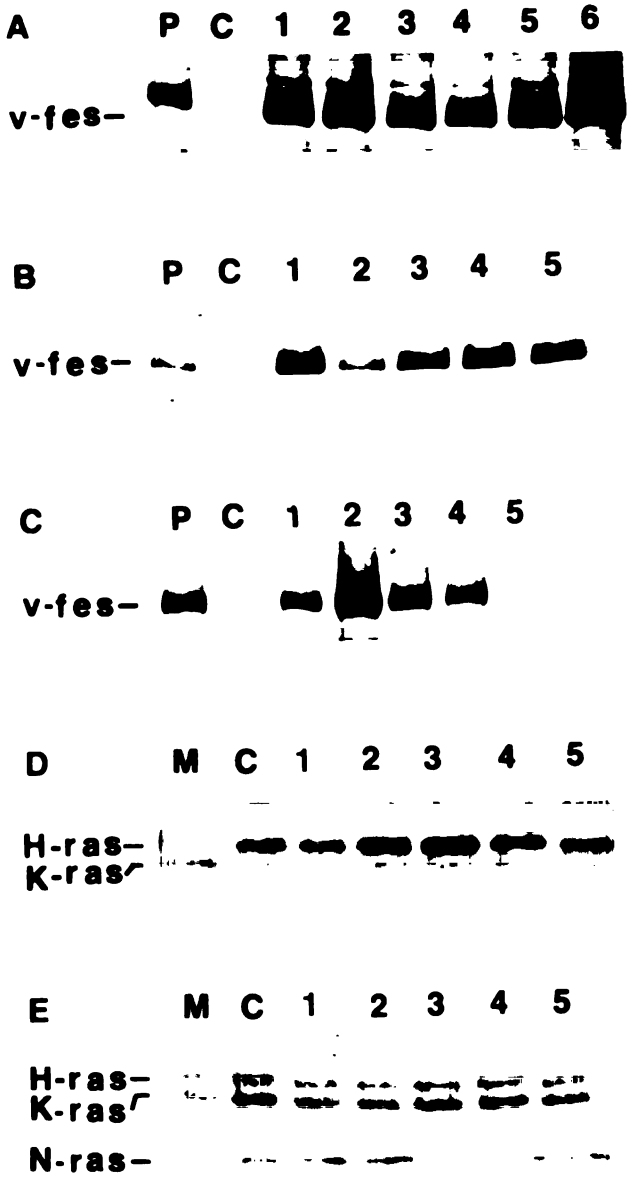
Transfection of MSU-1.1:*sis*A cells with pGAF*his*D plasmid and selection of histidinol-resistant clones were performed using the same procedure as described for the MSU-1.1 strain. Fifty individual histidinol-resistant clones were isolated and propagated. The progeny cells were assayed for v-*fes* expression by immunoprecipitation analysis as described, using a v-*fes* specific monoclonal antibody. Six clonal populations out of the 50 were found to express the v-fes protein at

detectable levels (Figure 5A). These six populations were also found to express v-*sis* mRNA at the same level as the parental MSU-1.1:*sis*A strain (Figure 6). These six cell strains were then further characterized. All six formed large-sized (≥100μm) colonies at high frequencies (2%-9%), and gave rise to tumors after a short latency period (16-48 days, Table 4). These tumors were graded as malignant spindle cell sarcomas.

*Cooperation between the v-*fes and a H-ras, *but not a N-*ras *oncogene*

To determine if a mutant *ras* oncogene can supply the change required by the v-*fes* oncogene in malignant transformation of MSU-1.1 cells, we transfected the v-*fes* oncogene into two non-tumorigenic, MSU-1.1-derived strains that contain and express a transfected T24 H-*ras* or HT1080 N-*ras* oncogene expressed from an endogenous promoter, i.e., strains MSU-1.1:H*ras*98 and MSU-1.1:N*ras*73. Following transfection, a number of histidinol-resistant clones were isolated and propagated using essentially the same procedures as described above. The progeny cells from these clones were assayed for v-*fes* expression as described. Five clones derived from the MSU-1.1:H*ras*98 strain and five from the MSU-1.1:N*ras*73 cell strain were found to express the v-*fes* protein (Figure 5B and 5C). We also assayed the amount of *ras* protein expression in the v-*fes*-transformed strains derived from either MSU-1.1:H*ras*98 or MSU-1.1:N*ras*73 strain by immunoprecipitation analysis, using two ras-specific monoclonal antibody. To assay for mutant H-ras protein, a monoclonal antibody (Ab-2) that reacts only with human H- and K-ras protein was used. The expression levels of mutant H-ras in the MSU-1.1:H*ras*98 derived cell strains were quantified by subtracting the endogenous H-ras signal found in MSU-1.1 cells from the signal in these cell strains. To assay for mutant N-ras protein, an excess amount of monoclonal antibody (Ab-2) was used to

Figure 5. Relative levels of expression of v-*fes* protein (A-C) and ras protein (D,E) in a series of independent v-*fes* transfectants derived from cell strain MSU-1.1:*sis*A (A), cell strain MSU-1.1:H*ras*98 (B,D) and cell strain MSU-1.1:N*ras*73 (C,E). Lane P, MSU-1.1:*fes*5 cells included as a positive control to correct for blot-to-blot variation; Lane C, the respective parental cell strain lacking the v-*fes* gene; Lanes 1-6, independent transfectants generated with the respective cell strain.


A
P C 1 2 3 4 5 6
v-fes-
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K-ras-
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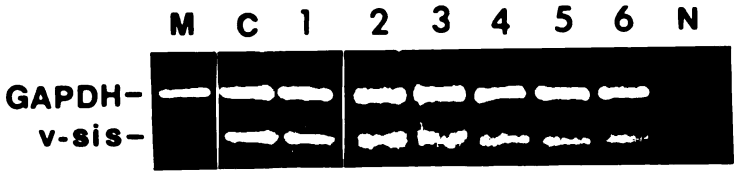

Figure 6. RT-PCR analysis of the relative levels of expression of v-*sis* mRNA. Lane M, parental MSU-1.1 cells; Lanes 1-6, MSU-1.1:*sis*A:*fes*1-6 cells respectively; Lane N, RT-PCR negative control, i.e., mock treatment without RNA.

Table 4. Relative v-*fes* expression, anchorage independence, and tumorigenicity of v-fes-transformed MSU-1.1:*sis*A strains

MSU cell strains	Relative v-*fes* expression*	Number of agar colonies with diameter ≥ 100 μm per 10,000 cells	Tumorigenicity assay: Tumors/ injection sites	Tumorigenicity assay: Latency period† (days)
MSU-1.1	ND	< 1	0/12	-
1.1sisA	ND	3	0/12	-
1.1sisA:*fes*1	1.7	300	4/4	16-25
1.1sisA:*fes*2	2.0	220	4/4	20-36
1.1sisA:*fes*3	1.1	190	4/4	26-32
1.1sisA:*fes*4	1.0	200	4/4	22-25
1.1sisA:*fes*5	2.0	930	4/4	18-48
1.1sisA:*fes*6	3.7	530	3/3	16-18

* Bands corresponding to the v-fes signal on the blot were cut out and assayed by scintillation counting. The counts per minute in the lowest sample, i.e., MSU-1.1:*fes*5, were arbitrarily scored as 1.0 and the other strains were scored relative to it.

† Number of days required for a tumor to reach 600 mm^3 in size (about 1 cm in diameter).

deplete and precipitate both ras proteins in each cell lysate. The non-precipitated cell lysate was subsequently reacted with a pan-ras monoclonal antibody (Ab-1) to precipitate N-ras protein. The expression levels of mutant H-ras in the MSU-1.1:N*ras*73 derived cell strains were quantified by subtracting the endogenous N-ras signal found in MSU-1.1 cells from the signal in these cell strains. The v-*fes*-transformed strains expressed similar amount of mutant *ras* protein as the strain from which they were derived, confirming the continued expression of the previously transfected H-*ras* and N-*ras* oncogene in all strains assayed (Figure 5D and 5E).

These ten populations were then further characterized. All five v-*fes*-transformed-MSU-1.1:H*ras*98 strains formed large colonies at high frequencies (7%-19%), and gave rise to tumors after a short latency period (18-120 days). The tumors were graded as malignant spindle cell sarcomas. When the five v-*fes*-transformed-MSU-1.1:N*ras*73 strains were similarly assayed, they did not form large-sized colonies ($\geq$100μm) in agarose and were not tumorigenic in athymic mice.

Discussion

When infinite life-span mouse NIH3T3 fibroblasts were used as target cells, expression of a transfected v-*fes* oncogene caused malignant transformation (Feldman et al., 1989; Sodroski et al., 1984). However, our data show that at least one extra event, in addition to expression of a transfected v-*fes* oncogene is required for the malignant transformation of the infinite life span MSU-1.1 human fibroblasts. Acquisition of growth factor independence can supply this requirement since expression of a transfected v-*fes* oncogene was able to transform MSU-1.1:*sis*A and MSU-1.1:H*ras*98 cells into malignant cells.

Our data also indicate that acquisition of a genetic change that confers anchorage independence on MSU-1.1 cells is also able to supply the additional change needed to cooperate with v-*fes* and allow this oncogene to convert the cells into malignant cells. We showed that when non-tumorigenic MSU-1.1:*fes* cell strains were cultured for a long period of time, some of them produced malignant tumors, and even before being injected the mice, these same strains could form very large-sized colonies in agarose (Table 3). Furthermore, if instead, we selected early passage, non-tumorigenic populations of MSU-1.1:*fes* cells for the ability to form these large-sized colonies in agarose, the progeny of cells isolated from such colonies proved to be malignant.

The failure of the c-N-*ras* oncogene to supply the additional change required for the v-*fes* oncogene to transform MSU-1.1 cells to the malignant state was not unexpected since the transfected c-N-*ras* oncogene did not confer full growth factor independence on the MSU-1.1 cells.

Immunoprecipitation analysis revealed that, although both *ras* oncogenes are driven by their respective endogenous promoters, the T24 H-*ras* oncogene in MSU-1.1:H*ras*98 cells expressed 2.5 times more *ras* protein than did the HT1080 N-*ras* oncogene in the MSU-1.1:N*ras*73 cells (Figure 6C and 6D). This difference could merely reflect a difference in the intrinsic properties of the two endogenous promoters. However, the T24 H-*ras* oncogene has been shown to contain a mutation in the fourth intron that results in increased expression of the protein (Cohen and Levinson, 1988). In any event, our data suggest that a threshold amount of mutant *ras* protein is required for the MSU-1.1 cells to be fully growth factor independent. We consider it likely that if the c-N-*ras* oncogene were expressed at a level similar to the T24 H-*ras* oncogene, it would be able to cooperate with the v-*fes* oncogene.

Ras oncogenes encode mutant proteins that have a reduced ability to hydrolyze guanosine triphosphate (GTP), resulting in an accumulation of GTP-bound ras in cells. The GTP-bound ras protein can, in turn, transduce a growth signal by eliciting a kinase cascade (ras-raf-MAP kinase) and ultimately altering gene expression. Functional analysis of the role of the v-fes protein, a non-receptor protein tyrosine kinase, has shown that it acts upstream of ras in the signaling pathway (Noda et al., 1983; Smith et al., 1986). Its critical substrate(s) has not yet been identified, but it is likely that the mutant *ras* protein cooperates with the v-fes protein by activating the ras-raf-MAP kinase signaling pathway.

The v-*sis* oncogene encodes a homologue of the B-chain of platelet-derived growth factor (PDGF). The v-*sis* oncogene product has been shown to exert its growth-promoting action by binding to the PDGF receptor, which has protein tyrosine kinase activity (Johnson et al., 1986). Upon binding

of the v-*sis* oncogene product, the PDGF receptor is "activated" and can, in turn, transduce the growth signal by activating the ras-raf-MAP kinase signaling pathway. In addition, the activated PDGF receptor has been shown to phosphorylate several cellular proteins, e.g., phosphatidylinositol-3 kinase, phospholipase c-γ, GTPase-activating protein, and a sis-inducible transcription factor (Kaplan et al., 1987; Molloy et al., 1989; Nishibe et al., 1990; Wagner et al., 1990). Either one or both of these v-*sis*-mediated responses may be involved in the cooperation between the v-*sis* oncogene and the v-*fes* oncogene in the malignant transformation of MSU-1.1 cells.

In summary, our data show that the v-*fes* oncogene can supply the ultimate change required for the malignant transformation of infinite life span human fibroblasts only if the cells have already acquired genetic changes in growth factor-related genes. This finding is consistent with the hypothesis that malignant transformation of human cells in culture is a multistep process.

Material and methods

Cell culture conditions

The cells were routinely cultured in Eagle's minimal essential medium containing aspartic acid, serine and pyruvic acid as described (Bettger et al., 1981) and supplemented with 10% supplemented calf serum (Sterile Systems, Logan, UT), hydrocortisone (10 $\mu g\ ml^{-1}$), penicillin (100 $U\ ml^{-1}$) and streptomycin (100 $\mu g\ ml^{-1}$). They were maintained at 37°C in a humidified incubator with 5% CO_2. For transfection experiments McM medium, a version of MCDB-110 (Ryan et al., 1987), lacking histidine and containing 1 mM L-histidinol dihydrochloride (Sigma, ST. Louis, MO) was substituted for Eagle's medium.

Plasmid construction and transfection

Plasmid pGAFeSV containing the complete proviral sequence of the Gardner-Arnstein feline sarcoma virus was kindly provided by Dr. Charles Sherr, Howard Hughes Medical Institute, St. Jude Children's Research Hospital, Memphis, TN (Fedele et al., 1981). To allow selection of transfectants, we inserted the *Salmonella typhimurium* histidinol dehydrogenase (*his*D) gene from plasmid pMSV*his*D (Hartman and Mulligan, 1988) into the *Eco*RI site of plasmid pGAFeSV. The altered plasmid was named pGAF*his*D and used in our study. Approximately 2 x 10^5 cells were plated per 100 mm dish. After 16-24 h, two μg of plasmid DNA was transfected into the cells by the Polybrene/ DMSO method adapted for use with human cells by Morgan et al. (1986). Twenty hours after transfection, growth medium was replaced by McM medium lacking histidine but supplemented with 1 mM L-histidinol dihydrochloride, 10% supplemented calf

serum, hydrocortisone (10 μg ml^{-1}), and antibiotics. The selective medium was refreshed twice weekly and the cells were observed for formation of histidine-resistant clones.

Immunoprecipitation analysis of oncogene products

The amount of v-fes protein expression in the v-*fes*-transformed strains was assayed essentially as described (Lin et al., 1994). The amount of p21 ras protein in cell strains derived from the MSU-1.1:N-*ras*73 or the MSU-1.1:H-*ras*98 strain was assayed following the procedure described by Wilson et al (1990) with the following modifications. For N-ras protein, radiolabelled cell lysate was first precipitated with an excess amount of ras antibody Y13-258 (v-H-ras, Ab-2, Oncogene Science, Manhasset, NY) to deplete the H-ras and K-ras protein in the lysate. The N-ras protein in the unprecipitated portion was then precipitated with a second ras antibody, Y13-259 (v-H-ras, Ab-1, Oncogene Science). The former antibody reacts only with human H- and K-ras proteins and the latter antibody reacts with all three human ras proteins.

RT-PCR analysis of v-sis mRNA

Total RNA was isolated using the method of Chomczynski and Sacchi (1989). RNA (1 μg) was reverse transcribed into cDNA using a random primer, and v-*sis* cDNA was amplified by the polymerase chain reaction for thirty cycles. The final concentration of Mg^{++} ion in each PCR reaction mixture was 2 mM. To ascertain that suitable conditions for amplification were present for each sample and to correct for the small differences in the amount of sample added to each reaction tube, the cDNA of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene in each sample was also amplified simultaneously (Noonan et al., 1990). The primers used for the v-*sis* and GAPDH genes are to be published elsewhere. The PCR products

were run on 2% agarose gels and the gels were stained with ethidium bromide.

Assay for anchorage independence

Cells were assayed for the ability to form colonies in 0.33% agarose following the procedure described by Hurlin et al. (1989). The agar plates were maintained at 37°C in a humidified incubator with 3% CO_2. MSU-1.1 cells and MSU-1.1:H*ras*10 cells which was derived from MSU-1.1 by transfection of MSU-1.1 cells with a T24 H-*ras* oncogene were included in each assay as negative and positive control respectively.

Assay for growth factor independence

Cells were assayed for the ability to grow in medium without exogenous growth factors following the procedure described by Scudiero et al. (1988) with the following modification. Cells in McM medium containing 5% serum were plated at 1000 cells per well in 96 well plates on Day 0. The next day (Day 1) the medium was aspirated off each well and replaced by 200 μl of serum-free McM medium containing 0.1 mM calcium and the serum replacement supplements of Ryan et al. (1987). The cells were then allowed to grow for 6 days with a medium change every 3 days. Cells in McM medium containing 0.1 mM calcium, but supplemented with 10% serum were run in parallel as a positive control. On Days 1, 4 and 7, the cells were stained with2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide to determine the relative cell number. This agent was dissolved in water at 1 mg ml^{-1}, and phenazine methosulfate was added to give a final concentration of 0.05 mM. Next, 50 μl of this solution was added to each well and the 96 well plate was incubated at 37°C for 30 min. The $O.D._{450}$-$O.D._{650}$ was then read, using a Molecular Devices microplate reader. The population doubling time for each cell strain was calculated

from the log of $O.D._{450}$-$O.D._{650}$ plotted as a function of days in culture.

Assay for tumorigenicity

Athymic nude mice (BALB/c background) originally purchased from Harlan Sprague Dawley (Indianapolis, IN) were bred and maintained in a high efficiency particulate air (HEPA)-filtered laminar flow hood containment system. Absorbable gelatin sponges (Upjohn Co., Kalamazoo, MI) 1 cm^3 in size were implanted subcutaneously in the subcapular region and/or rear flank region of 6-week-old mice to serve as a matrix. One week later, 10^7 cells in 0.2 ml of serum-free medium were injected directly into the sponge. Mice were examined weekly for tumor growth and the tumors were removed when they reached 1 cm in diameter.

Histopathology

Tumor tissue for histopathology was fixed in 10% phosphate buffered formalin, pH 7.0, embedded in paraffin, sectioned at 4-5 μm, and stained with hematoxylin-eosin and examined (Durfee, 1992).

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