GENETIC AND MOLECULAR STUDIES ON MALIGNANT TRANSFORMATION OF HUMAN FIBROBLAST CELL STRAINS BY CARCINOGEN TREATMENT AND/OR ONCOGENE TRANSFECTION: EVIDENCE FOR MULTISTEP CARCINOGENESIS

By

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ABSTRACT

GENETIC AND MOLECULAR STUDIES ON MALIGNANT TRANSFORMATION OF HUMAN FIBROBLAST CELL STRAINS BY CARCINOGEN TREATMENT AND/OR ONCOGENE TRANSFECTION: EVIDENCE FOR MULTISTEP CARCINOGENESIS

By

Dajun Yang

Conversion of a normal cell into a malignant cell is generally recognized to be the result of a multistep process. To study the number of steps and nature of changes involved in the process leading to the malignant transformation of human fibroblasts in culture, I treated a near-diploid infinite life-span human fibroblast strain, MSU-1.1, and a diploid strain, MSU-1.0, with direct-acting carcinogen (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE). A single exposure of MSU-1.1 cells with BPDE induced focus formation. Eight independent foci were isolated and all grew to a higher density in medium containing 1% serum than did the MSU-1.1 cells. Four of the eight grew rapidly in serum-free medium without added growth factors, formed colonies in agarose with diameters ≥ 120 μm at high frequency, and formed benign to malignant tumors when injected into athymic mice. Cell strains that

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formed malignant tumors and/or the tumor-derived cells also exhibited loss of specific chromosomes and/or rearrangements. A single treatment of MSU-1.0 cells with the same carcinogen, BPDE, also induced focus formation, and the progeny cells of these foci grew moderately well in medium without exogenous growth factors and formed colonies in agarose but were not tumorigenic. Additional treatment of the progeny cells of these transformed variants with BPDE, followed by more stringent selection conditions, produced variant cells that exhibited more transformed characteristics, but the cells were not malignant.

Transfection of MSU-1.1 cells with a plasmid containing a drug-resistant marker and the v-sis oncogene which encodes a protein homolog of platelet-derived growth factors-B, yielded many drug-resistant colonies. Progeny cells from the six drug-resistant colonies tested expressed v-sis gene mRNA and grew to a higher density than the MSU-1.1 cells. Three of these cell strains expressed the gene at relatively high levels and grew rapidly in serum-free medium without added growth factors and formed colonies in agarose at high frequency. When injected into athymic mice, these strains formed typical benign tumors which grew and then maintained a static size or regressed.

THIS WOR

THIS WORK IS DEDICATED TO:

My Wife and Daughter: Yifan Zhai and Alina Z. Yang

My Parents: Daocheng Yang and Chuanzheng Zhu

My Country: China

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my major professor, Dr. J. Justin McCormick, for all of his intellectual guidance, financial support, willingness to help me with everything I asked in and outside the laboratory and for the friendship we developed throughout my years in the Carcinogenesis Laboratory. I would like to express my sincere and deep appreciation for Dr. Veronica M. Maher for her cordial encouragement and insightful support throughout my Ph.D. program. I would also like to thank Drs. Richard Schwartz, and James Higgins for their contributions as members of my guidance committee.

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acknowledge,

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acknowledge, but space does not allow me to mention them. However, "Friendship is forever".

Finally, my deepest thanks go to my wife, Yifan, for all her love, support, and understanding, and to my daughter, Alina (JiaJia), who for me, is "the best of everything in the world". I am also very grateful to my parents, and my brothers and sister for their years of loving support and tolerance of our being thousand of miles apart. Words cannot express my gratitude to all of my family members for everything they did for me.

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BPDE

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H-*ras*

K-*ras*

LOH

LTR

MNNG

N-*ras*

NMU

4-NQO

p21

PCR

PDGF

PKC

PTK

Rb

ABBREVIATIONS

BPDE	(±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene
DMBA	7, 12-dimethylbenzan-thracene
FGF	fibroblast growth factors
G Proteins	guanine nucleotide binding proteins
H-*ras*	*ras* gene homologous to transforming gene of Harvey murine sarcoma virus
K-*ras*	*ras* gene homologous to transforming gene of Kirsten murine sarcoma virus
LOH	loss of heterozygosity
LTR	long terminal repeat
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
N-*ras*	cellular *ras* gene first isolated from neuroblastoma
NMU	N-nitro-N'-methylurea
4-NQO	4-nitroquinoline-N-oxide
p21	21,000 dalton *ras* protein
PCR	polymerase chain reaction
PDGF	platelet-derived growth fctor
PKC	protein kinase-C
PTK	protein-tyrosine kinases
Rb	retinoblastoma gene

RFLP

SHE

SV40

TGF-α

TGF-B

TPA

v-myc

RFLP	restriction fragment length polymorphism
SHE	Syrian hamster embryo
SV40	simian virus 40
TGF-α	transforming growth factor α
TGF-ß	transforming growth factor ß
TPA	12-O-tetradecanoylphorbol 13-acetate
v-*myc*	*myc* oncogene of avian myelocytomatosis virus

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INTRODUCTION

The development of cancer is now generally recognized to result from a multistep process. Although the nature of the changes involved in each step is not yet fully understood, it seems clear that multiple, independent genetic alterations are involved. Considerable evidence now exists from in vivo and in vitro studies to support a multistep model of carcinogenesis. For instance, epidemiological analysis of the frequency of cancer incidence in humans with age suggests that five or six steps may be required for the formation of a tumor (Peto, 1977). Experimental tumor induction in animals indicates that multiple genetic events are necessary for tumorigenesis (Balmain and Brown, 1988). Examination of clinical samples of a various types of human tumors reveals that the multiple genetic changes including gene mutation, chromosomal translocation, gene amplification, loss of an allele, and tumor suppressor gene inactivation are commonly present in a specific tumor (Bishop, 1991). In human colorectal carcinoma, mutations in at least four or 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). These observations suggest a requirement for multiple genetic events in tumorigenesis, but cannot prove directly that any specific genetic change was responsible for causing a specific change characteristics of tumorigenic cells.

For this reason, model systems for in vitro transformation of

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mammalian cells in culture (mainly rodent fibroblasts), have been widely used by investigators. One of the assumptions for cell transformation assay in culture is that malignant transformation induced in cells by carcinogenic agents occurs by the same mechanisms as tumor development in vivo. As one might expect, the conversion of a normal cell into a malignant cell in culture is itself a multistep process. For example, exposure of primary cultures of Syrian hamster embryo fibroblasts to carcinogen resulted in cells capable of forming tumors only after the progeny of treated-population acquired multiple cellular changes in a stepwise fashion by extended subculturing (Barrett and Ts'o, 1978; Koi and Barrett, 1986). Studies on transformation of primary early passage rodent cells and some immortalized cell lines by transfection of viral and/or cellular oncogenes have provided additional support for the multistep process of carcinogenesis as well as new insight into the molecular basis of certain steps in this process. In general, transfection of one active oncogene into a normal diploid cells cannot fully convert these cells to tumorigenic, but can cooperate with a second oncogene which has a different function to accomplish neoplastic transformation (Land et al., 1983, Ruley, 1983). In contrast, an established cell line is readily converted to neoplastic cell by a single oncogene (Newbold and Overell, 1983). Other studies suggest that neoplastic transformation of diploid rodent cells requires an additional event, such as loss of a specific chromosome (Oshimura et al., 1988).

One of the major advances in the last ten years of cancer research has been the development of in vitro transformation assays utilizing human cells as target cells to identify the role of carcinogenic agents in the

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development of human cancers and to study the genetic mechanisms of carcinogenesis. Several lines of evidence have proved that human cells in culture are more difficult to transform than rodent cells, and the malignant transformation of human cells in culture is a complex, multistep process (Chang, 1986; McCormick and Maher, 1988). For example, earlier attempts to reproduce the results obtained with rodent cells in culture transformed by oncogenes or carcinogen treatment using human fibroblasts have failed (see literature review). Induction of neoplastic transformation of normal diploid human fibroblasts by transfection of a single oncogene or one treatment of a carcinogen has never been observed. One explanation for the inability of human cells to be neoplastically transformed is that these cells have limited proliferative capacity and senesce after 50-70 population doublings in culture. Normal diploid human fibroblasts do not spontaneously give rise to an infinite life-span cell lines (McCormick and Maher, 1988). This stability makes human fibroblasts ideal candidates for studying the process and the mechanisms by which malignant transformation occurs. Moreover, the frequency of immortalization of human diploid fibroblasts by carcinogen treatment or expression of an oncogene is much lower than that routinely obtained with rodent cells. Very rarely, human fibroblast strain with infinite life-span were isolated after either repeated carcinogen treatment (Namba et al., 1986), or transfection with a v-myc oncogene (Morgan et al., 1990), or spontaneously developed from fibroblasts of patients with Li-Fraumeni cancer-prone syndrome (Bischoff et al., 1990). Subsequent transformation of these various immortalized human fibroblast strains with overexpression of ras oncogenes resulted in cells capable of producing tumors when

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Chapter I

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injected into athymic mice (Namba et al., 1986, 1988; Hurlin et al., 1988; Wilson et al., 1990; Bischoff et al., 1991). To date, neoplastic transformation of human fibroblasts in culture by carcinogen treatment or oncogenes encoding a growth factor have not been reported.

The objectives of the present research were (1) to investigate the biological properties and genetic alterations involved in the malignant transformation of a near-diploid, infinite life-span human fibroblast strain MSU-1.1 by treating these cells with a direct-acting carcinogen BPDE; (2) to determine the biological effects of BPDE exposure in a diploid infinite life-span human fibroblast strain MSU-1.0, a strain which has growth properties identical to the finite life-span diploid human fibroblasts and is the precursor of strain MSU-1.1; (3) to define the biological and biochemical characteristics of transformed cells obtained by transfection of the v-sis oncogene, encoding for a protein homolog of platelet-derived growth factor B chain (PDGF-B), into the near-diploid, infinite life-span human fibroblasts strain MSU-1.1; By comparing the in vitro transformed characteristics and tumorigenic potential induced by the same carcinogen in the two closely-related, but with different growth properties and genotypic alterations, i.e., MSU-1.0 and MSU-1.1, we hoped to learn the kind of changes and number of steps required for the malignant transformation of human fibroblasts in culture. Additional insights might also be obtained by comparing the carcinogen transformed cells with MSU-1.1 cells transformed by transfection of H-ras and N-ras oncogenes as reported by Hurlin et al., 1988 and Wilson et al., 1990.

Chapter I of the thesis reviews the literature that provides the evidence in support the hypothesis of multistep carcinogenesis model,

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including the epidemiological studies, animal models of tumorigenesis, transformation of rodent cells in culture, transformation of human cells in culture, clinical examinations of human tumors, and transgenic models of tumorigenesis. Also discussed in Chapter I is the current understanding regarding the genetic and molecular mechanisms of carcinogenesis, including chromosomal aberrations, growth factors, proto-oncogenes and suppressor genes. Chapter II consists of a manuscript published in the Proceedings of National Academy of Science USA, 89, 2237-2241, 1992. It describes the research I carried out which demonstrates that a single exposure of a near-diploid infinite life-span human fibroblast strain MSU-1.1 with a direct-acting carcinogen BPDE induced foci formation, and the progeny cells of 50% of the foci produce benign or malignant tumors when injected in athymic mice. Calvert Louden, D.V.M., assisted in the histopathology analysis of the tumors and also performed the immunohistochemical staining of $p21^{ras}$ proteins of tumor tissues which is presented in Appendix I. David S. Reinhold, Ph.D., conducted the RT-PCR analysis of PDGF-B mRNA and TGF-α mRNA with the RNA that I isolated. Additional data relevant to this study that could not be included in the published manuscript because of space considerations are shown in Appendix I. Chapter III describes my research showing that one treatment of the diploid infinite life-span human fibroblast strain MSU-1.0A1 with the carcinogen BPDE results in variant cells that grow moderately well in medium lacking serum or growth factors, just like MSU-1.1, and form small colonies in 0.33% agarose but are not tumorigenic. An additional treatment of these variant cells with BPDE followed by more stringent selection, produces transformed variant cells that are more transformed,

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but no definitive tumorigenic growths have been observed. The data suggests that neoplastic transformation of MSU-1.0 cells by carcinogen treatment needs at least three steps. The format used for Chapter IV is that of a manuscript to be submitted to the journal Cancer Research or Carcinogenesis. Chapter IV consists of a manuscript written in the style of the journal Cancer Research, which describes my research which shows that the v-sis oncogene induces benign tumor formation when it is transfectd into a near-diploid infinite life-span human fibroblast strain MSU-1.1. I also isolated one large agarose colony from a strain designated MSU-1.1-*sis* G and characterized this agarose-derived strain along with those v-sis transformed MSU-1.1 cells described in Chapter IV. These results are presented in Appendix II.

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CHAPTER I.

LITERATURE REVIEW

A. EVIDENCE IN FAVOR OF MULTISTEP CARCINOGENESIS.

1. Epidemiological Evidence.

Epidemiological analysis of the distribution of various forms of cancer has pointed to several agents as possible causes of such cancer and has also led to the development of various models of carcinogenesis. Stochastic analysis of the frequency of human cancer incidence as it varies with age indicates that five or six steps may be required for the genesis of a tumor (Peto, 1977). The exponential rise in cancer incidence with increasing age is consistent with the possibility that multiple genetic events occur over time and the cumulative effect of these changes results in cancer late in life. According to Peto's model, the multistage hypothesis is that "a few distinct changes (each heritable when cells carrying them divide) are necessary to alter a normal cell into a malignant cell, and that human cancer usually arises from the proliferation of a clone derived from a single cell that suffered all the necessary changes and then started to proliferate malignantly".

A very strong correlation has been shown between tobacco smoking and human lung cancer. The incidence of lung cancer among the regular smokers is 14 times higher than non-smokers, and is proportional to the duration of exposure and intensity of exposure (Doll, 1978). This suggests that repeated exposure may be necessary in order to produce multiple genetic

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changes. The age-specific occurrence of adult T-cell leukemia (ATL) also supports a multistep model carcinogenesis and the number of independent leukemogenic events in ATL is estimated to be five (Okamoto et al., 1989).

Neither definition of the specific genetic changes nor information on the mechanisms by which these changes occur can be obtained by epidemiological investigation. In addition, some types of cancer, such as childhood leukemia or retinoblastoma, a tumor of the eye that appears in children, do not appear to conform to the multistep model proposed to explain adult tumors. Presumably, these childhood cancers have a different etiology than adult cancers. Since tumors occurring early in life have had only a short time to accumulate genetic alterations, one or several mutations may be inherited, or fewer mutational events may be needed to initiate cancers in specific cells or tissue types. In 1971, Knudson examined the epidemiological evidence for a possible role of inherited alterations and mutational events that result in the origin of retinoblastoma. He hypothesized that retinoblastoma was caused by two mutational events. In the dominantly inherited form of the disease, one mutation is inherited via the germinal cells and the second occurs in somatic cells. In the non-hereditary form, both mutations occur in somatic cells. Although Knudson could not pinpoint the identity of the genes nor the kinds of mutations that were involved, his discovery ultimately led to the identification and isolation of the retinoblastoma (Rb) gene, which plays an important role in the development of several types of cancers (see below).

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Animals are afflicted by tumors similar to ones seen in humans, and therefore can provide model systems in which tumorigenesis can be analyzed systematically. A variety of model systems have been used to study tumor formation in animals and have revealed a multistep process. Here, I will describe those systems that have been studied in detail and in which mutationally activated oncogenes and other mechanisms have been identified.

2.1 Mouse Skin Carcinogenesis

Since the skin is easily accessible and various lesions can be easily monitored, the mouse skin model system has served as the pioneer experimental tissue for animal carcinogenesis studies. The multistage model for carcinogenesis of mouse skin originated in the 1940s from studies by Rous, Mottram, and Berenblum, who observed that various treatments are synergistic in inducing skin tumors in rabbits or mice (reviewed in Boutwell, 1974). Basically, application of a low concentration of a polycyclic hydrocarbon carcinogen to mouse skin resulted in few tumors, but when this treatment was followed by repeated exposure to croton oil, a non-carcinogenic plant extract, the mice rapidly developed a large number of skin tumors. The hydrocarbon treatment was defined as the initiating event for carcinogenesis while the croton oil was said to act as a tumor promoter. The identification of the active component of croton oil, 12-O-tetradecanoylphorbol 13-acetate (TPA) (Hecker, 1968) provided an important tool for experimental study of tumor promotion in mouse skin carcinogenesis.

The kinetics of the appearance of benign papillomas and malignant carcinomas in mouse skin differ depending on the protocol used. In the

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typical initiation-promotion protocol, a carcinogen such as 7, 12-dimethylbenzan-thracene (DMBA) is applied once to the skin of a mouse, and then multiple treatment with a tumor promoter, such as TPA, is given over a period of many weeks (Boutwell, 1974; Verma et al, 1980). This regimen primarily induces benign papillomas. After approximately 30 weeks of promoter treatment, squamous cell carcinomas also develop, but at a much lower frequency than papillomas. More than 90% of these carcinomas are located in areas where papillomas are growing. In the "complete carcinogenesis" protocol, mice are treated repetitively with DMBA alone. With this regimen, papillomas arise later and in lower yield than in DMBA-initiated mice treated with TPA, but carcinomas begin to appear at 16 weeks with a much greater incidence (Verma et al, 1982). However, only a fraction of these carcinomas arise in areas that contain papillomas. A rapid, high incidence of carcinoma resulted when the usual initiation-promotion format was subsequently followed by application of a carcinogen, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or 4-nitroquinoline-N-oxide (4-NQO), as the second genetic insult (Hennings et al., 1983). The tumor promoter TPA was far less effective in the production of carcinomas. The increased efficiency for the induction of malignant lesions by the "complete carcinogenesis" or initiation-promotion-initiation, as compared to two-stage carcinogenesis, is consistent with a requirement for two or more mutational events in the acquisition of malignant phenotypes, with one of them being involved in papilloma formation.

The reproducible induction of specific types of tumors in animals by particular carcinogens provides an ideal system to investigate the sequential molecular events associated with the different stages of

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carcinogenesis. In the mouse skin system, initiation with DMBA and promotion with TPA induced papillomas and carcinomas in which the H-ras gene was consistently activated by an A:T -> T:A transversion at the second position of codon 61 (Quintanilla et al., 1986). This mutation was not seen if MNNG, a methylating agent, was substituted for DMBA. Instead, 40% of the skin tumors investigated showed a G:C -> A:T transition at the second position of codon 12 (Balmain and Brown, 1988). Since these base substitution mutations are the kinds expected to be caused by the respective carcinogens (initiators), it is reasonable to conclude that the ras mutations occurred during initiation.

Retroviral induction of mouse skin carcinogenesis gives evidence that ras activation is involved in tumor initiation. When retroviruses containing activated H-ras genes are applied directly to mouse skin (Brown et al., 1986), some cells within the epidermis become stably infected, but do not give rise to tumors in the absence of the tumor promoter. When promotion is carried out, benign papillomas develop with a relatively short latency period (four weeks), and a proportion of these subsequently progress to carcinomas. Analysis of viral integration sites shows that the papillomas are polyclonal in origin, but the carcinomas are monoclonal, a result which suggests that the carcinomas arise after additional genetic event(s) have taken place in a single papilloma cell. These results strongly suggest that ras mutations play a causal role in carcinogenesis in mouse skin.

The conversion of benign papillomas to malignancy is complex, and represents a composite of events involving alteration in the expression of specific genes, proliferation of the initiated cell and its clonal

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descendants, and the accumulation of additional genetic alterations (Drinkwater, 1990). Cytogenetic studies have identified numerical chromosome changes in the form of progressive aneuploidy and sequential trisomization of chromosomes 6 and 7 as the primary nonrandom cytogenetic events in this model (Aldaz et al., 1987, 1989). Recent molecular evidence has demonstrated amplification of the mutated ras allele and/or loss of the corresponding normal allele via chromosomal alteration (nondisjunction or mitotic recombination) in advanced papillomas and carcinomas (Bianchi et al., 1990; Bremner and Balmain, 1990). Moreover, an increase in mutant ras gene dosage has been observed to constitute a discrete step in the progression from squamous carcinoma cells to anaplastic or spindle cell tumors (Buchmann et al., 1991a). In addition, loss of heterozygosity of other polymorphic markers in chromosome 7 has suggested that tumor suppressor genes are inactivated during conversion from papilloma to carcinoma (Bremner and Balmain, 1990). Very recently, the tumor suppressor gene p53 is reported to involve at the late stage of mouse skin tumor progression, since both homozygous and heterozygous mutations have been found in carcinomas but not in papillomas (Ruggeri et al., 1991). Additionally, Haddow et al (1991) reported that growth control by TGF-beta is lost at a late stage in mouse skin carcinogenesis independently of ras gene activation. Taken together, these results support the hypothesis that multiple and cumulative genetic alterations in oncogenes and tumor suppressor genes are responsible for the acquisition of the full expression of malignant phenotype in the mouse skin tumorigenesis model.

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Another well-studied animal tissue for multistage carcinogenesis is the liver. Experimental liver carcinogenesis in most models is characterized by the sequential development of morphologically distinct lesions. At the earliest stage after carcinogen treatment, numerous clonal proliferations (foci) of phenotypically altered hepatocytes are observed; later a more limited number of benign tumors (neoplastic nodules) appear; and eventually a few hepatocellular carcinomas arise. This sequence of events is common to most experimental systems and is thought to reflect the multistep nature of cancer development (Scherer, 1984). Analysis of the kinetics of dimethylnitrosamine-induced carcinogenesis in mouse liver suggests that at least four independent events are required for carcinoma formation (Vesselinovitch and Miahilovich, 1983).

The early focal lesions observed in livers of carcinogen-treated animals are very heterogeneous, exhibiting a variety of enzymatic and metabolic alterations, in various combinations (Peraino et al., 1984). Nodules are less heterogeneous than foci and seem to be at greater risk of developing into cancer than are simple foci. Additional phenotypic alterations are associated with the development of hepatocellular carcinoma (Huber et al., 1988). Several lines of evidence indicate that at least a subset of these enzyme-altered foci or nodules are the immediate precursors to hepatic neoplasms (Drinkwater, 1990). Scherer et al (1984) has developed a rat liver model similar to the initiation-promotion-initiation regimen in mouse skin tumorigenesis, in which focal carcinomas can be induced within pre-existing altered hepatic foci and/or nodules. Such lesions have been designated "foci-within foci".

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Various strains of rodents differ in their susceptibility to hepatic tumor formation, as demonstrated by the frequency and/or latency of spontaneous as well as carcinogen-induced tumor formation. The genetic mechanisms for the differences in susceptibility to liver carcinogenesis are not known at present. One possible link may be the mutational activation of the H-ras gene. Certain rodent strains, e.g., B6C3F1, CBA, C3H/He, are highly susceptible to hepatocarcinogenesis, either induced by carcinogens or arising spontaneously. Nearly all liver tumors arising in these strains contain a point mutation at codon 61 of the H-ras gene (Stower et al.,1988; Buchmann et al., 1989, 1991b). However, no such mutational activation of H-ras proto-oncogene is seen in any of liver tumors of the insensitive mouse strains studied (i.e., BALB/c and C57BL) or in rat liver tumors (Stowers et al., 1988; Watatani et al., 1989; Buchmann et al., 1991b). Since ras mutations are not detected in the normal liver tissue of the susceptible strains, the results strongly suggest that mutations in H-ras play an important role in the hepatocarcinogenesis in the susceptible mouse strains (Buchmann et al., 1991b).

2.3 Mammary Carcinogenesis

Exposure of female rats during sexual development (50-days old) to N-nitro-N'-methylurea (NMU) results in the induction of mammary carcinomas in 90% of the animals, with a latent period of 60 days (Gullino et al., 1975). Molecular analysis of these tumors by Barbacid and his colleagues has revealed the presence of an activated H-ras gene , with 90% of the tumors containing a G:C -> A:T transition at the second position of codon 12. This particular mutation is the predicted consequence of unrepaired

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O^6 methylguanine induced by NMU. In contrast, the ras mutations found in DMBA-induced mammary tumors are point mutations in either of two adenosine residues in codon 61 (Sukumar et al., 1983; Zarbl et al., 1985). This observation suggests that the activating mutations in H-ras genes represent direct mutagenic effect of the carcinogen. If the animals are exposed to carcinogens at a much earlier age, such as during fetal development (2-days old), rather than during sexual development, tumors develop after a longer latency period (mean latency of 3.7 months) (Kumar et al., 1990). By utilizing the PCR amplification of H-ras sequences, the activated ras genes can be found as early as 2 weeks after carcinogen exposure--long before the histological manifestation of microtumors, which are not apparent until 10 weeks later. These results clearly indicate that activated H-ras oncogene can precede the onset of neoplasia.

The long latency of tumor development suggests that activation of the H-ras gene is not sufficient for the malignant transformation of the cells in which this has occurred. Additional changes of genetic or epigenetic nature need to follow for cells to acquire malignant potential. One of the well-studied factors in mammary carcinogenesis is estrogens. Ovariectomy of pubescent rats immediately after NMU injection reduces mammary tumor development to negligible levels. When rats that were treated neonatally with NMU are then ovariectomized at 1 month of age, the incidence of mammary tumor formation is reduced from 80% to 4%. When these ovariectomized rats receive estrogen treatment later, tumors arise within 3 months of treatment and 50% of the tumors are found to contain activated H-ras genes (Kumar et al., 1990). These studies indicate that factors associated with normal sexual differentiation or others are

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3. Rodent Cell Transformation In Culture

Cell culture systems are useful experimental models for identifying environmental carcinogens and assessing potential risks of these substances to humans. The phenotypic alterations of cells in culture such as morphological changes, abnormal growth on plastic and semisolid medium can be assayed qualitatively or quantitatively, and are associated with the neoplastic transformation of the cells as determined by their ability to form tumors in susceptible animals. The use of cells in culture provides a tool to study the cellular events in carcinogenesis independent of certain host effects, such as immunological surveillance and cell-tissue interactions. One line of evidence that supports the hypothesis that carcinogenesis is a multistep process has been obtained using cells in culture to study the process of malignant transformation in vitro.

Since there is often an inaccurate use of the terminology regarding transformation of cells in culture and confusion as to the meaning of such terms, I will use the terminology as defined by the Tissue Culture Association. That group has come to consensus as follows. The term "in vitro transformation" indicates that cells have acquired altered morphological, antigenic, neoplastic, proliferative or other properties due to a heritable change occurred in culture. "In vitro neoplastic transformation" indicates that the cultured cells are able to form neoplasms, benign or malignant when inoculated into animals. "In vitro malignant transformation" or "in vitro malignant neoplastic transformation" indicates that injected cells invade surronding tissues or

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3.1 Carcinogen-Induced Transformation of Finite Life-span Cells

The transformation of cultured cells with chemical carcinogens were first reported by Berwald and Sachs in 1963. They showed that the carcinogen benzo(a)-pyrene could cause alteration in the colony growth pattern of cells derived from primary cultures of Syrian hamster embryo (SHE) in culture. These transformed cells were shown to form tumors after extended subculturing. Similar studies using ionizing radiation as the transforming agent were carried out by Borek and Sachs (1966). After extended subculturing, the progeny of cells derived from the abnormal colonies formed sarcoma-like tumors in 2-6 week old hamsters which regressed later. Dipaolo as well as others, have further elaborate the assay conditions for in vitro transformation assays using these cells, and showed they were sensitive to many carcinogens and some of the transformants were tumorigenic (Dipaolo et al., 1969, 1971; Pienta et al., 1977). However, it is not clear from those studies whether the morphologically-transformed colonies are tumorigenic per se, or only become so after further subculturing. The fact that extended subculturing necessarily preceded tumorigenisis is evidence that additional genetic events were required for neoplastic transformation.

Studies by Barrett and Ts'o in 1978 and Newbold et al. in 1982 supported this latter interpretation. These researchers treated SHE cells with carcinogen benzo(a)pyrene, and at various time during subsequent repeated subculturing, the treated cells were assayed for the various

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transformed characteristics which are frequently associated with tumors of fibroblastic origin. They observed that altered morphology, enhanced fibrolytic activity, and the ability to grow in soft agar, and tumorigenicity arose independently and at different times after treatment of the target cell population with carcinogen, benzo(a)pyrene. A small percentage of the treated cells (3%) displayed morphological alteration within one week following exposure to benzo(a)pyrene. Cells from this early stage were unable to grow in soft agar and were not tumorigenic. They estimated that their soft agar assay could detect as few as 1 anchorage independent cell per 10^5-10^6 cells plated. The ability of cells from the population to form colonies in soft agar was not seen until the ninth subculturing (46 population doublings after treatment). At the tenth and all subsequent subcultures, the frequency of cells able to form colonies in soft agar progressively increased. By this time, greater than 90% of the cells were shown to have morphologic alteration and enhanced fibrolytic activity. Populations of cells which displayed growth in soft agar formed tumors in 100% of the animals injected, whereas the cells which displayed only altered morphology were not tumorigenic. These results strongly suggest that transformation did not occur in a single step, but rather a population of cells acquired multiple cellular changes in a stepwise fashion which eventually resulted in a tumorigenic cell.

Some of these carcinogen transformed SHE cells were found to be immortal. Using cell hybrids between immortal non-tumorigenic cell lines and a highly tumorigenic, chemically transformed hamster cell line, Koi and Barrett (1986) showed that cells that had escaped senescence still retained the tumor suppression function, therefore senescence and tumor

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suppression are separable phenotypes, and the loss of a tumor-suppression function is a key step in the neoplastic transformation of these cells.

3.2 Carcinogen-Induced Transformation of Infinite Life-span Cell Lines

A number of infinite life-span rodent fibroblastic cell lines were established in the early 1970s. Because of their infinite life-span, these cell lines must be considered already transformed to some degree. Some of these cell lines are aneuploid and/or have some other transformed phenotypes, such as morphological alterations. However, none of these cell lines has the ability to grow in soft agar or to produce tumors when injected into suitable animal host systems under ordinary conditions. By definition then, these cell lines are "transformed", but not "neoplastically transformed". Studies of transformation of these cell lines should be viewed as the effects on one stage in neoplastic transformation or from "preneoplastic cells" to the neoplastic state as compared to those obtained with normal diploid cells.

Among the more commonly used cell lines are the mouse-derived C3H/10T1/2, clone 8 (Reznikoff et al., 1973a, 1973b); mouse-derived BALB/c 3T3, clone A-31 (Kakunaga, 1973); and Syrian hamster-derived BHK 21/clone 13 cells (DiMayorca et al., 1973). When treated with carcinogenic agents, the two mouse cell lines form distinct foci on top of a confluent monolayer. Cells within such foci frequently exhibit a disordered growth pattern. Progeny cells from some foci have been shown to form fibrosarcomas in athymic mice or in BALB/c 3T3 weanling mice. Exposure of the BHK-21/clone 13 cells to carcinogens induces a dose-dependent increase in the frequency of anchorage independent (AI) cells, i.e., cells able to grow in semisolid medium. Cells isolated from the foci or the anchorage

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independent colonies were able to produce malignant tumors when injected into suitable animals. Apparently, these aneuploid cell lines are capable of becoming neoplastically transformed in a single step.

Other investigators have used cell lines that have acquired an unlimited life-span but were diploid or near-diploid, and phenotypically like normal cells in culture. Smith and Sager (1982) found that treatment of Chinese hamster embryo fibroblast cell line (clone CHEF/18) by carcinogens resulted in variants capable of growing in methylcellulose or in low-serum, but these variants were non-tumorigenic. When some of these variants were remutagenized and then injected into nude mice, tumors appeared at many of the injected sites. Similarly, Perez-Rodriquez et al (1982) found that if cells of a near-diploid Chinese hamster cell line (CC139) were to form progressively growing tumors shortly after injection into nude mice, it was necessary that the cells acquire the ability to grow in an anchorage independent manner, and without addition of protein growth factors found in serum. These results suggest that neoplastic transformation of these diploid or near-diploid cell lines is a multistep process.

The aneuploid cell lines appear to be preneoplastic, since spontaneous transformation has been observed in these cell lines (Boone et al., 1976). Therefore, the changes which have occurred in these cells make them unsuitable for deliniating the number and nature of the genetic events necessary for malignant trnasformation that begins with a normal diploid cell.

3.3 Oncogene-Mediated Transformation of Infinite Life-span Cell Lines

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transformation was the discovery of DNA from a human bladder carcinoma cell line (EJ/T24) that are capable of transforming the infinite life-span mouse fibroblast cell line NIH 3T3 to form foci of morphologically altered cells (Shih et al., 1982; Krontiris and Cooper, 1981). The transfected gene responsible for the transformation was shown to exhibit a dominant effect in the cell. It was identified as the human homolog of the Harvey or Kirsten sarcoma virus transforming gene ras (Der et al., 1982; Parada et al., 1982) and was shown to contain a point mutation at codon 12 as does the viral gene. Subsequently, a number of previously unknown dominant oncogenes have been identified and implicated in human cancers by transfection studies, most of them using the mouse NIH 3T3 cell system (Barbacid, 1987).

Most studies on the transfection of rodent cells have involved ras oncogenes. When ras oncogenes are transfected into NIH 3T3 or Rat-1 cells, malignantly transformed cells are produced. As with chemical carcinogen transformation of Balb/c 3T3 cell line, it appears that a single oncogene is sufficient to induce malignant transformation. Again the apparent discrepancy between these results and the multistep model of carcinogenesis can be easily reconciled if one recongnized that NIH 3T3 and Rat-1 cells are already transformed, i.e., have infinite life-span or other unknown changes, even though they are not neoplastically transformed. It has been shown that transfection with an activated ras gene will not lead to neoplastic transformation of certain other established cell lines. For example, in contrast to NIH 3T3 cells, EK-3 cells, a cell line derived from NIH 3T3 cells, required both myc and ras genes for in vitro transformation (Katz and Carter, 1986). An established

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cell line REF52 needs collaboration of ras and adenovirus early region 1A (Franza et al., 1986). In addition, the level of activated (mutated) ras gene expression may play an important role in the determination of cellular transformation of both NIH 3T3 and Rat-1 cells. Individual transfectants display great variations in number of copies (between 1 to over 100 copies) of the integrated ras sequences by Southern hybridization. Cells expressing multiple copies of the oncogene have a more transformed phenotype than cells which express only single copy ras oncogene (Sistonen et al., 1987). The low level of ras expression is not sufficient to either deregulate the cells' cycle or override the cells requirement for growth factors. The cells with low level of ras expression require serial cultivation to become tumorigenic (Kovary et al., 1989). In Rat-1 cells, although elevated expression of normal cellular ras gene (44 fold greater than endogenous levels) cause partial transformation, the cells are not neoplastically transformed (Ricketts and Levinson, 1988). Malignant transformation of these cells seems to require at least two changes, one being the mutation of a ras gene and the other accomplished increased, unregulated expression of the mutated ras genes.

3.4 Oncogene-Mediated Transformation of Finite Life-span Cells

An initial connection between oncogenes and the multistep nature of tumorigenesis was provided by studies of two viral oncogenes, the middle T (MT) and large T (LT) genes of polyomavirus (Rassoulzadegan et al., 1982). Neither gene was able to transform rat embryo fibroblasts on its own. However, the two working in collaboration elicited a fully tumorigenic phenotype. This suggests that each oncogene was specialized

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The model was extended to a number of oncogenes of cellular origin. An example of one such study is that of Land et al. (1983), who showed that transfection of finite life-span, rat embryo fibroblasts with an activated H-ras oncogene (EJ) resulted in morphological transformation, but not tumorigenicity. Co-transfection of the H-ras oncogene with either the v-myc or polyoma virus large-T antigen oncogenes into rat embryo fibroblasts resulted in the formation of transformed foci. When the cells from the foci were isolated, expanded in culture, and injected into athymic mice, they gave rise to tumors. The tumors induced by cells containing the co-transfected ras and myc oncogenes were benign and stopped growing before becoming sufficiently large enough to kill the mouse, whereas the tumors induced by the co-transfected ras and polyoma virus large-T antigen oncogenes formed malignant tumors that killed the mice. This suggests that an additional change or changes was necessary to produce malignantly transformed cells in the former case.

Detailed studies in rodent cells showed that the ras oncogene alone can induce refractility, anchorage independence, and growth factor secretion, but can not produce immortalization in culture. Conversely, the myc oncogene or the E1A gene are reported to cause immortalization and, occasionally, they also induce anchorage independent growth and growth factor secretion (Land et al., 1986; Ruley, 1983). This distinction in function has been observed in a variety of different types of cells.

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of immortalization and transformation appear to be sufficient for the induction of malignancy, other situations obviously exist where additional steps are required. Barrett's group found that transfection of Syrian hamster embryo cells with the v-H-ras and v-myc oncogenes was necessary but not sufficient to cause malignant transformation. They reported that all tumors arising from the transfected cells showed consistent loss of chromosome 15, in addition to the expression of ras and myc (Thomassen et al., 1985; Oshimura et al., 1985). They interpret these observations as indicating that loss of a suppressor gene located on hamster chromosome 15 was essential for malignant transformation. Cell fusion experiments further supported the existence of a suppressor gene (Oshimura et al., 1988). Therefore, they proposed that transformation involves at least three steps in their system: 1)induction of immortalization, 2)activation of a transforming oncogene, and 3)loss of a tumor suppression function.

Contrary to the reports that the activation of multiple oncogenes was necessary for complete transformation of cell in culture, Spandidos and Wilkie (1984) found that transfection of early passage Chinese hamster fibroblasts or rat embryo fibroblasts with the ras oncogene cloned into high expression vectors was capable of causing malignant transformation. When the ras oncogene is regulated by an exogenous promoter and linked to transcriptional enhancers as in this case, the oncogene expression is the result of regulatory changes. When Spandidos and Wilkie used the same ras oncogene in a low expression vector, no malignant transformation was observed, indicating that overexpression as well as the mutational activation of the gene is necessary for malignant transformation. Therefore, malignant transformation in this case was caused by at least

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4. Human Cell Transformation In Culture

4.1 Differences Between Human Cell and Rodent Cell Transformation

Although it seems reasonable to assume that the mechanisms causing the malignant transformation of human cells in culture are the same as those operating in rodent cells, there may well be some species specific differences, such as genetic stability, DNA repair mechanisms, and capacity to metabolically activate chemical carcinogens (Harris, 1987). Spontaneous malignant transformation commonly occurs in mouse fibroblasts in vitro. It has been found that the principal agents responsible for such spontaneous transformation is the atmospheric concentration of oxygen the cells are exposed under common culture conditions (Sanford et al., 1979) and the DNA damage caused by the type of room lighting they were exposed to while being cared for (Parshad et al., 1978). Cells from other species, such as rat, are far less susceptible than mice to transformation by exposure to high levels of oxygen and visible light (Parshad et al., 1978). The spontaneous transformation is accompanied not only by a change from a diploid to aneuploid karyotype, but also by the expression of other typical transformation characteristics, including tumorigenicity (Sanford et al., 1979).

Normal diploid human fibroblasts do not spontaneously transform in culture to either infinite life-span cell lines or malignant cells (McCormick and Maher, 1988). This stability makes them ideal candidates

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for studying the process and the mechanisms by which malignant transformation occurs. When normal human diploid fibroblasts are passaged in culture, they reach, after a fixed number of cell doublings, a stage of no proliferation in which the cells become quiescent and cell division ceases. This phenomenon of cellular "senescence" in human fibroblasts was first observed by Hayflick and Moorhead (1961) and since then it had been seen with other types of human cells that have been cultured in vitro (Chang, 1986). Whereas spontaneous immortalization of primary rodent cells of some species has been observed in culture with a low but regularly detectable frequency, it is an extremely rare phenomenon amongst in vitro transformed human cells (Chang, 1986; McCormick and Maher, 1988). Abundant evidence now shows that the malignant transformation of human cells is a complex process, and clearly involves more than two distinct stages.

4.2 Carcinogen-Induced Transformation of Finite Life-span Cells

There are numerous reports of "partial transformation" of human fibroblasts in culture following carcinogen treatment. Many workers have used growth in soft agar to assay for transformation of human cells after treatment with suspected carcinogens. For example, Milo and Dipaolo (1978) used a variety of carcinogens to induce anchorage independent growth in human fibroblasts. The cells isolated from agar colonies in these cases had a finite life-span, were morphologically similar to normal fibroblasts, and, when injected into athymic mice, either did not form tumors or formed small nodular growths which later regressed. By definition, therefore, these cells were not malignantly transformed. Similar results were obtained by Landolph's group using carcinogenic

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metals (Biedermann and Landolph, 1987), and by McCormick and Maher and their coworkers using radiation and chemical carcinogens (Silinskas et al., 1981; Wang et al., 1986). DeMars and Jackson (1977) used focus formation to assay for transformation of human cells. These cells had been obtained by a single treatment of normal human fibroblasts with MNNG and subsequent isolation of cells from a morphologically altered focus on a uniform monolayer of confluent cells. They also observed the appearance of small regressing nodules at the site of injection of focus-derived human cells.

There are a few reports that human fibroblasts can be malignantly transformed after exposure to carcinogens. However, results of a recent study have indicated that these reports are invalid (McCormick and Maher, 1988). For example, the most widely known study on the malignant transformation of human fibroblasts is that of Kakunaga (1978) who reported the in vitro transformation of a cell line designated KD by exposure to 4-NQO or MNNG. In fact, careful analysis of the reported transformants has revealed that the transformed cells were not derived from KD cells, but were derived from a contaminating human fibrosarcoma cell line 8387 (McCormick et al., 1988). Other reports of transformation of human fibroblasts did not convincingly demonstrate that the tumors were actually malignant (Borek, 1980).

4.3 Oncogene-Mediated Transformation of Finite Life-span Cells

Induction of neoplastic transformation by transfection of a single oncogene into normal, diploid finite life-span human fibroblasts has never been observed. Newbold et al. (1983) reported that finite life-span human fibroblasts could not be malignantly transformed by transfection of the

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cloned T24-H-ras oncogene. Similarly, Sager et al. (1983) reported that diploid human fibroblasts were not transformed after transfection of the EJ-H-ras oncogene, even though the transfected oncogene was shown by Southern hybridization analysis to be incorporated intact into the genome. More recently, Hurlin et al. (1987) found that overexpression of a transfected, activated H-ras oncogene in human diploid fibroblasts induced focus formation and morphological alteration, but these focus-forming cells had a finite life-span and were not tumorigenic. Similarly, human diploid fibroblasts when transfected with an activated N-ras gene under the control of the viral long-terminal repeat produced foci composed of morphologically transformed cells (Wilson et al., 1989), but these cells also retained their finite life-span and were not tumorigenic. When transfected with the N-ras oncogene driven from its endogenous promoter, the cells expressed protein products of the oncogene but did not exhibit any transformed characteristics. Transfection of human diploid finite life-span fibroblasts with other oncogenes also did not produce cells that were immortal or tumorigenic (see Fry et al., 1986; 1988, for example).

4.4 Transformation of Human Cells to Infinite Life-span in Vitro

The failure to produce malignant tumors in those experiments may simply reflect the fact that by the time the progeny population isolated from a single colony or transfectant clone had been propagated to the size needed for testing tumorigenicity, the cloned cells were already nearing the end of the their finite life-span and could not proliferate sufficiently in vivo to form a tumor. This suggests the need for an infinite, or at least greatly extended, capability to proliferate, if an individual cell is to acquire a series of transformation-related changes

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as a result of sequential clonal selection. One way to test this hypothesis is to start with normal cells and immortalize them. Such immortal nontumorigenic cell lines would provide excellent starting material for further transformation studies. In fact, all of the in vitro malignant transformation of human cells (fibroblasts or epithelial cells) reported to date has been accomplished using cell lines with infinite life-spans.

A series of human cell transformation experiments of particular interest are those reported by Namba and associates (1981; 1985). They reported the isolation of six cell lines (only two immortal lines were proved later) that were morphologically altered and had acquired an infinite life-span in culture. These cell lines were generated by repeatedly treating the same population of fibroblasts with either 4-NQO or x-rays. These cell lines proved not to be tumorigenic in athymic mice, but were aneuploid and exhibited many characteristics of tumorigenic cells in culture. Similarly, two immortalized human mammary epithelial cell lines were recovered after long-term exposure to benzo(a)pyrene (Stampfer and Bartley, 1985). The immortalization of human fibroblasts and epithelial cells by carcinogen treatment has been achieved only rarely and has never been reproduced. The fact that these cell lines were detected only after multiple carcinogen exposures carried out over an extended period of time, suggests that generation of cells with an infinite life-span also is a multistep process.

There are several families of DNA tumor viruses, such as SV40 in the polyomavirus family, HPV 16 and 18 of the papillomaviruses, and type 5 adenovirus that increase the immortalization frequency in a variety of

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human cells and have been used successfully by many workers,(reviewed in Shay et al., 1991). Human cells infected with SV40 (Shay and Wright, 1989) express large T antigen, develop an altered morphology, have a reduced serum requirement for growth, and acquire an extended life-span in vitro. Cells infected with SV40 grow for about 20-30 population doublings beyond their normal life-span, and then go into a period called "crisis" (progressive decrease of cell numbers) and senesce. In some instances a rare focus of dividing cells appears among the senescing cells and gives rise to a cell line with infinite life-span. The frequency with which such immortal cells arise is very low, i.e., about $3x10^{-7}$. Wright et al.(1989) also showed that continued proliferation of these infinite life-span cells requires continued T-antigen expression. Similar studies on establishment of human epithelial cells by AD-12/SV40 or SV40 have been reported on human bronchial cells, keratinocytes, and uroepithelial cells, etc. (Rhim et al., 1985; Christian et al., 1987). However, cell lines that express SV40 T-antigen are highly aneuploid and exhibit other changes characteristic of tumor-derived cells. Such cells have limited usefulness in studies designed to analyze the step-wise changes required for a normal diploid human cell to become a malignant cell.

Recently, an infinite life-span human fibroblast cell strain, designated MSU-1, was isolated in this laboratory following transfection of normal diploid fibroblasts with the v-myc oncogene (Morgan et al., 1991). All of the progeny of the clonally-derived population expressed the same level of myc protein, but the vast majority of the population went into crisis and senesced. From the senescing populations, clones of viable cells could be seen and these eventually gave rise to an infinite

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life-span cell strain designated MSU-1.0. MSU-1.0 is the first apparently stable diploid infinite life-span human fibroblast cell strain ever reported. In contrast to the SV40-immortalized human cells, MSU-1.0 strain has undergone only minimal changes. These cells do not morphological alteration and do not exhibit any growth rate advantage over the finite life-span parental cells. Since the myc-expressing siblings of MSU-1.0 in the senescing population all died, it is clear that expression of the v-myc protein is not sufficient to immortalize human fibroblasts. At least one additional change is required to generate the infinite life-span MSU-1.0 cell strain. A somewhat similar result by Kinsella et al. (1990) with human fibroblasts supports the role of unregulated myc oncogene in the acquisition of an infinite life-span in human fibroblasts. However, Brondyk et al. (1991) failed to obtain immortalized human fibroblasts even using cells that expressed the N-myc oncogene at high level. These data suggest that a single oncogene like myc is not efficient in immortalizing diploid human fibroblasts or immortalization occurs at a frequency much lower than it does using DNA tumor viruses. This is consistent with the multistep nature of immortalization in human cells (Shay et al., 1991).

In contrast, fibroblasts derived from seven of eight patients with Li-Fraumeni cancer syndrome developed changes in morphology, anchorage-independent growth, and chromosomal abnormalities in culture (Bischoff et al., 1990). These fibroblast lines all escaped senescence during passaging in culture but remained nontumorigenic in athymic mice. The cells from the Li-Fraumeni families appear to progress from a normal phenotype to an altered one simply through passage in culture, suggesting

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that the cells are prone to spontaneous mutations that predispose to immortalization. Sporadic reports of spontaneous immortalization of other human cells appear occasionally (Shay et al., 1991), but there is no indication that these workers have a reproducible procedure and conditions to immortalize human cells in culture.

4.5 Malignant Transformation of Infinite Life-span Cell Lines In Vitro

There are several studies which describe the introduction of oncogenes into human cells that have acquired an infinite life-span in culture. The results of such experiments, in contrast to those of transformation studies in finite life-span human cells, show that tumorigenic cells are produced. For example, O'Brien et al. (1986) infected Va2 cells, a human fibroblast cell line that had acquired an infinite life-span after SV40 infection, with Kirsten murine sarcoma virus and used a focus assay to select transformed cells. When cells from such foci were injected into athymic mice, static tumors composed of human cells occurred at each injection site. Namba et al (1986, 1988) reported the successful malignant transformation of an infinite human cell line, KMST-6, obtained by repeated carcinogen treatment, by transfection with H-ras oncogene or infection with Ki-MSV. Similarly, immortal cell lines that arose spontaneously from fibroblasts of patients with Li-Fraumeni cancer syndrome have been transformed by an activated H-ras oncogene to form tumors in athymic mice (Bischoff et al., 1991).

In contrast to the previous work with human cells with a finite life-span in culture, which are refractory or resistant to malignant transformation, these cited studies suggest that human cells with infinite life-span in culture can be malignantly transformed in what appears to be

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a single step. However, recent studies using a derivative of MSU-1.0 cells, i.e., MSU-1.1, show that malignant transformation of immortal human cell lines actually requires two or more changes. At least two changes in ras genes are required to convert MSU-1.1 cells into malignant cells. First, the proto-oncogene has to acquire a mutation in a specific codon, and second that oncogene has to be overexpressed to a high level. The low expression of ras oncogene is ineffective in transforming MSU-1.1 cell to malignancy (Hurlin et al., 1989; Wilson et al., 1990; McCormick and Maher, 1991). Similarly, neoplastic transformation of carcinogen-immortalized human mammary epithelial cells needs SV40 T-antigen and v-H-ras oncogene (Clark et al., 1988). Expression of mutant ras in SV40 immortalized human uroepithelial cells is insufficient for tumorigenic conversion. Rare tumorigenic transformants always show loss of specific chromosomes that have been reported deleted in human bladder cancers (Wu et al., 1991; Pratt et al., 1992).

In addition, some immortal human cell lines have been transformed to malignancy by exposure to carcinogens. For example, human keratinocytes immortalized after infection with AD12-SV40 hybrid virus, and human uroepithelial cells immortalized by SV40, were converted to the malignant state after treatment, respectively, with MNNG and 4-NQO (Rhim et al., 1985; 1990) or 3-MCA (Reznikoff et al., 1988).

Although SV40 T-antigen immortalized human bronchial epithelial cells are readily transformed to tumorigenicity by v-H-ras oncogene expression (Amstad et al., 1988), concomitant expression of the c-raf-1 and c-myc proto-oncogenes under the control of retroviral promoters are required if the cells are to induce tumors in athymic mice (Pfeifer et

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al., 1989). Similarly, infection of an Ad12-SV40-immortalized human keratinocyte with a variety of retrovirus containing either H-ras, bas, fes, fms, erbB, or src oncogenes converts them into cells that can produce rapidly growing squamous cell carcinomas (Rhim et al., 1990).

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

5. Studies on Human Tumors

An examination of human tumors by a variety of methods has revealed that there are usually genetic changes in proto-oncogenes as well as tumor suppressor genes in such cells. The sequential nature of tumorigenesis can be inferred from analysis of samples obtained during tumor development. The multiple genetic changes found in specific tumors strongly suggest that such genetic alterations play a causal role in tumorigenesis and support the hypothesis of multistep carcinogenesis.

5.1 Human Colorectal Tumorigenesis

Colorectal carcinoma is one of the most extensively characterized human malignancies in terms of the molecular genetic alterations associated with its progression. This is in part because, unlike most other common human tumors, tissues representing the progressive stages from hyperplasia to small adenoma to carcinoma in situ to large metastatic carcinoma can be obtained for study from the same patient. Abundant clinical and histopathological data suggest that most malignant colorectal carcinomas arise from preexisting benign tumors, and this conversion is

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Vogelstein et al. (1988) have obtained colorectal tumors of various stages from patients, and screened the DNA from these samples for activated oncogenes and loss of restriction fragment length polymorphism (RFLP) heterozygosities that would be indicative of loss of tumor suppressor gene function. Specific events have been observed in colon carcinoma development, beginning with a hyperplastic adenoma. These include: allelic loss or mutation in a region on the long arm of chromosome 5, encompassing the familial adenomatous polyposis genes (FAP gene and APC gene) which predisposes to a high risk of colorectal cancer; an activating mutation in the K-ras gene; allelic loss on the long arm of chromosome 18 including the DCC gene (deleted in colorectal carcinoma); and allelic loss in a region on the short arm of chromosome 17 covering the p53 gene (reviewed in Fearon and Vogelstein, 1990). In addition to the genetic changes, a general hypomethylation of tumor cell DNA was also commonly found at the early adenoma stage, which through derepression of gene expression could be a contributing epigenetic event.

Approximately 50% of adenomas greater than 1 cm in size (intermediate adenoma) and a similar percentage of colorectal carcinomas have been found to have ras gene mutations. In contrast, such mutations have been detected in fewer than 10% of adenomas less than 1 cm in size (early adenoma). The fact that genetic alterations present in cells from early adenoma are retained in cells taken form more advanced adenoma or carcinoma, indicates that the conversion of a small adenoma to a larger and more dysplastic one may be due to a rare genetic change in an individual cell of one stage leading to the next more advanced stage. The

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loss of chromosomes 18q and 17p has been found in more than 70% of colorectal carcinomas, but such loss is relatively infrequent in adenomas, suggesting an association with the progression of individual tumors from adenoma to carcinoma. Furthermore, when one p53 allele has been deleted, the other allele often harbors one or more inactivating point mutations (Baker et al., 1990). The number of detectable events increases in parallel with the stage of the disease. The evidence suggests that there is not one obligatory chain of events which occurs during colon carcinoma development, but rather the accumulation of all events in a single cell is what matters.

This observation indicates that mutations in at least four or five genes are required before a cell can form a malignant tumor, while the ability to form a benign tumor appears to require correspondingly fewer alterations. Alterations in expression or activity of other proto-oncogenes have also been noted in colon carcinoma. For example, the c-*myc* gene is often overexpressed (Rothberg et al., 1985), and the protein-tyrosine kinase activity of $pp60^{c\text{-}src}$ is increased proportionately to the stage of progression (Cartwright et al., 1990). In addition to the allelic losses noted on chromosomes 5q, 17p, and 18q, many other chromosome losses have also been identified, with a median of four to five chromosome arms suffering allelic losses per tumor. Patients with more than the median number of losses in their tumor cells have a prognosis considerably worse than that of the other patients (Vogelstein et al., 1989). However, it is not yet clear whether these changes cause the malignant state or rather are a result of the malignant state.

5.2 Human Lung Carcinogenesis

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Lung cancer is the leading cause of cancer death in both sexes, representing nearly 30%. Lung tumors are carcinomas originating from the respiratory epithelium and can be divided into two groups, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), based on histological types and marker expression. SCLC most probably arises from pulmonary neuroendocrine cells and accounts for 25% of all lung tumors. Most lung cancers are NSCLC, which usually lacks histological neuroendocrine features and has a large number of subtypes classified according to their cell of origin. It has been difficult to define the progenitor cells of lung cancer and to determine whether different or common progenitor cells give rise to the various histological tumor types.

Recent studies demonstrate that lung cancer also progresses through several histopathological stages and the genetic and epigenetic changes in preneoplastic and neoplastic cells are being defined. Overexpression of the myc family of genes is common in SCLC. Mutations in ras are frequently involved in NSCLC but have never been seen in SCLC (Mitsudomi et al., 1991). Expression of multiple growth factors message has been found in NSCLC cell lines, but not in SCLC cell lines. In contrast, gastrin-releasing peptide is frequently produced by SCLC (Carney et al., 1987). Thus there seems to be a different pattern in the molecular lesions seen in dominant oncogenes in the different types of lung cancer. Several other families of activated proto-oncogenes, such as raf, jun, myb, fms, and neu/erbB-2 have also been found to be associated with human lung cancer (reviewed in Iman and Harris, 1991).

Cytogenetic data, as well as RFLP analysis to determine loss of heterozygosity (LOH) reveals frequent loss of chromosomes 3p, 13q, and 17p

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both in SCLC and NSCLC (reviewed in Buchhagen, 1991). These chromosomal losses occur early in the development of the tumor. Abnormalities in expression of the RB gene or phosphorylation of the RB protein occur frequently in SCLC and less frequently in NSCLC (Yokoda et al., 1988). Mutations in the p53 gene appear in at least 50% of NSCLC and nearly 100% of the cases of SCLC (Chiba et al., 1990). There is no apparent correlation between p53 mutations and tumor size or whether the lesion is primary or metastatic, suggesting that such mutations are involved in "early" stages in the carcinogenesis pathway.

These findings, as well as other data, strongly suggest that there are many genetic alterations associated with the pathogenesis of lung cancer. However, because of the absence of well-defined premalignant lesions and the inability to detect tumors in the lung that are at various stages of tumor development, the precise stage at which these alterations occur and the exact roles that they play in the development of the lung tumors has not been delineated.

5.3 Human Soft Tissue Tumors

Malignant soft tissue tumors (sarcomas) account for ~1% of all cancers and ~2% of cancer deaths. But these tumors have played a unique role in our understanding of the carcinogenesis process. First, studies on the induction of soft tissue sarcomas in animal models and on the transformation of fibroblasts in culture have provided many of the major conceptual advances in understanding the mechanisms of malignant transformation. Second, certain inherited disorders can predispose to the development of soft tissue tumors, and some of these genes have been identified and cloned.

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The multistep model of carcinogenesis proposes that two or more mutations or other changes are required to make a cell malignant. Inheritance of one or more of these changes allows cells to give rise to tumors at a earlier time than cells which acquire the same changes spontaneously. The best documented of these cancers is retinoblastoma, a childhood eye tumor that occurs in both hereditary and sporadic forms. In the hereditary form, the tumors are usually bilateral and multifocal, and transmitted in an autosomal dominant fashion. In contrast, sporadic retinoblastoma is seen in children having no family history of the disease, and usually presents as a single focus of tumor formation in one eye. It is now known that both forms of retinoblastoma require a common genetic change: inactivation of both copies of a single gene in a single precursor cell. This "two hits" mechanism was first proposed by Knudson in 1971 and was based on a statistical analysis of tumor incidence. In the hereditary form, one mutant allele would be inherited and would be present in all of a person's cells, including the germ line. A second mutation in the other allele of a retinal cell would cause the cell to become malignant according to Knudsoon. Conversely, in the sporadic form, both mutations occur spontaneously in the same retinal cell, resulting in unifocal, unilateral tumors. Both cytogenetic and molecular biological studies strongly suggested that it was a loss of both RB alleles located at chromosomal region 13q14 which was responsible for development of retinoblastoma (Cavenee et al., 1983). More recently, the putative RB gene has been cloned and it has been shown that the inactivation of both alleles of this gene in a single cell is likely a causative event in the genesis of retinoblastoma (Lee et al., 1987; Huang et al., 1988).

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Individuals who inherit the susceptibility to develop retinoblastoma also have a high incidence of secondary malignancies, the most frequent of which are osteosarcoma and soft tissue sarcomas. Loss of heterozygosity for chromosome 13, homozygous deletion of the RB gene and abnormalities of RB expression have been frequently found in individuals with sarcomas, whether or not they had a previous history of retinoblastoma (Weichselbaum et al., 1988). Point mutation and/or absence of transcripts of the p53 gene are also observed in sarcomas (Mulligan et al., 1990), and frequently occur together with the alterations of the Rb gene in the same tumor (Stratton et al., 1990; Copper and Stratton, 1991). The pattern of coincident alteration of Rb and p53 suggests that in some type of tumors it may be necessary to inactivate more than one tumor suppressor gene in order to allow development of the full neoplastic phenotype. Activation of ras gene families as well as other proto-oncogenes, amplification of myc genes, and overexpression of growth factors and/or their receptors have also been detected in sarcomas and cell lines derived from them (Reviewed in Copper and Stratton, 1991).

In 1969 Li and Fraumeni investigated childhood rhabdomyosarcoma patients and their family history of neoplasia, and identified four families in which siblings or cousins had a higher incidence than expected of soft tissue sarcomas and of breast cancers. Following these preliminary observations, the existence of a familial predisposition to childhood sarcomas, breast cancer and less commonly other neoplasms including brain tumors, adrenocortical carcinoma and leukemia has been described, suggesting a new familial cancer syndrome of diverse tumors, i.e., Li-Fraumeni syndrome. Individuals with this familial cancer

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syndrome appear to inherit a cancer predisposition in a dominant fashion (Williams and Strong, 1985). Li-Fraumeni patients tend to develop tumors at early ages, and multiple primary tumors are frequent. More recently, it was found that Li-Fraumeni patients inherit germline mutations in the p53 tumor suppressor gene and it is presumed that this is the lesion that predisposes such patients to cancer (Malkin et al., 1990). The fact that the cancer incidence in Li-Fraumeni patients is lower than expected suggests that several additional mutations must occur before malignancy ensues.

Soft tissue tumors are a highly heterogenous group of tumors, and are named according to the tissue they most resemble. Although each of the histogenetic categories is divided into a benign and malignant group, it is not clear that malignant soft tissue tumors originate from their benign counterparts. Sarcomas in humans rarely arise from benign soft tissue tumors, except for the occasional transformation of neurofibroma to malignant schwannoma (Enzinger and Weiss, 1988). Thus, there is a real need to investigate the genetic alterations in soft tissue tumors and to define the relationship between these changes and the development of sarcomas.

6. Transgenic Model of Tumor Development

The transfer of genetic information into mouse embryos to stably alter the genetic constitution of mice is affording new insights into the action of oncogenes and mechanism of carcinogenesis. With the use of transgenic mice, problems can be addressed that cannot be approached satisfactorily in cell culture, such as the spectrum of tissues that are

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susceptible to the transforming activity of an oncogene; target expression of the oncogene in a particular cell type; the relation between multistep oncogenesis and cooperativity of oncogenes; and the effect of oncogenes on growth and differentiation.

When different promoters or enhancers were used to direct myc or ras oncogene expression in different tissues of transgenic mice, tumor formation resulted in most cases. Oncogenes were expressed frequently in many tissues and this usually preceded tumor formation by many months. For example, when transgenic mice are generated that express the c-myc or v-H-ras expression under the control of the MMTV LTR promoter, mammary tumors occur after a long latency with variable penetrance (Leder et al., 1986). Because these tumors arise adjacent to morphologically normal epithelium, which also expresses the transgene, the expression of either c-myc or v-H-ras is not sufficient to transform mammary epithelial cells. This is consistent with the requirements for additional genetic events to convert cells expressing these oncogenes to the fully malignant phenotype. Having established strains of mice carrying the MMTV-myc and MMTV-ras, Sinn et al. (1987) interbred these lines, and the progeny were analyzed for the incidence of tumors and the spectrum of tissues with tumors. The results are dramatic in several respects. First, tumor formation is much more rapid in the F1 generation than in the parent strains carrying a single oncogene. Second, although the most common tumors observed are tumors of the mammary glands, other tumor types not previously developed were also seen in the animals, suggesting that a greater penetrance in several tissues. Finally, tumors arise stochastically and apparently are monoclonal in origin, suggesting that additional somatic events are

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necessary for cells to become malignant, even when they express activated v-H-ras and c-myc transgenes.

However, many tissues in which a given oncogene was expressed never developed tumors. Tissue-specific phenotypes of oncogene action may reflect the normal pattern of tissue-specific expression of the gene and/or the requirement of other factors in addition to oncogene expression for realizing a transformed phenotype. For example, although pancreatic acinar cells were highly susceptible to transformation by the ras oncogene, no tumors developed when myc was expressed from the same promoter (Quaife et al., 1987). The ras transformed cells were also not truly malignant as they were not tumorigenic in syngeneic animals or athymic mice, nor were they invasive or metastatic. Additional events are undoubtedly necessary for progression to a tumorigenic state. Conversely, neoplastic transformation of mammary gland cells was efficiently induced by myc expression but rarely by expression of ras (Andres et al., 1987). This strengthens the view that the consequences of oncogene expression also depend on the particular cell type and may differ dramatically between one tissue and another.

In only one case so far has one oncogene-bearing transgenic mouse been found to develop tumors apparently without the need for a second event. A strain of transgenic mouse carrying the activated neu gene under the control of MMTV LTR showed an extremely high incidence of mammary carcinoma, which developed in both females and males at a relatively early age (Muller et al., 1988). The tumors were polyclonal and developed in every mammary gland, in contrast to MMTV-myc transgenic mice, where a single monoclonal tumor develops in one gland during lactation (Leder et

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al., 1986). Other very similar MMTV-neu transgenic lines did not have this propensity to develop multiple monoclonal tumors (Bouchard et al., 1989), which indicates that there was something unique about the strain that produces polyclonal tumors.

The potential disadvantage of the transgenic model is that since all the cells in a given tissue express the oncogene, this may reduce the suppressive effects that would usually be exerted by the normal surrounding cells. Since the vast majority of models exhibit a stochastic onset of tumors, tumors of different stages are found simultaneously in the same animal allowing one to analyze the process of carcinogenesis in vivo. Thus, such transgenic mouse models provide access to preneoplastic stages and present an opportunity to define the characteristics of preneoplastic stages and the requirements for their conversion into malignancy.

B. GENETIC AND MOLECULAR MECHANISMS OF CARCINOGENESIS

1. Chromosomal Aberrations

Genetic alterations which occur during multistep carcinogenesis may be mediated through gross chromosomal changes and therefore can be detected by cytogenetic analysis. In fact, the association of consistent chromosome aberrations with particular types of cancer has led to the identification of specific genes which are causally-involved and then to the elucidation of their mechanisms of action (Dal Cin and Sandberg, 1989). Chromosome aberrations are generally classified as structural or numerical. The common tumor structural chromosome aberrations include

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translocations, inversions, deletions, insertions, and amplifications, whereas numerical abnormalities are losses or duplications of whole chromosomes. One way to analyze the chromosomal abnormalities observed in human tumors is to place them in three broad categories based on the mechanisms through which they contribute to the tumorigenesis: 1)rearrangements, including translocations, insertions, and inversions; 2)deletions, including terminal or interstitial deletions, and chromosome monosomy; 3)amplification, including trisomies, isochromosome, double-minute chromosomes and homogeneously staining regions. Loss of heterozygosity (LOH), analyzed by means of RFLPs, provides molecular confirmation of chromosomal deletions and monosomies, and reveals the loss of genetic information which can not be observed by cytogenetical techniques. Thus, LOH detected by RFLP analysis will be included under deletions.

1.1 Chromosomal Rearrangement

Recurrent chromosome changes reported in human neoplasia (mainly in leukemia and lymphoma, but also in solid tumors) support the idea that chromosome rearrangements play an important role in tumor development. Some of these nonrandom karyotypic changes have been investigated at a molecular level, and it has been demonstrated that structural chromosome rearrangements can alter specific genes located at the breakpoint either by overexpression or fusion.

Translocation may lead to the deregulation (overexpression) of oncogenes by their juxtaposition to enhancer or promotor sequences that are active in the cell type from which the tumor arises, for example, the

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immunoglobulin (Ig) and T cell receptor (TCR) enhancers in hematological systems. The first translocation of this type to be analyzed at a molecular level was the translocation between chromosomes 8 and 14 [t(8;14)] of Burkitt lymphoma. In that case, the myc proto-oncogene is translocated so that it is under the control of the Ig heavy chain enhancer or light chain enhancer (Leder et al., 1983). A more recent example is that of parathyroid adenomas, where a rearrangement, most probably an inversion between the short and long arms of chromosome 11, leads to the juxtaposition of the parathyroid hormone regulatory elements and the PRAD1 putative oncogene, resulting in dramatic overexpression of PRAD1 (Rosenberg et al., 1991).

An alternative molecular consequence of translocation is the fusion of two genes which results in a chimeric protein. For example, a reciprocal translocation between chromosomes 9 and 22 in chronic myelogenous leukemia, which is referred to as the Philadelphia chromosome (Nowell and Hungerford, 1960), resulted in the c-abl proto-oncogene (9q34) being joined at the break-point cluster region (BCR gene) on chromosome 22 (q11), leading to a BCR-ABl fusion message and protein (Groffen et al., 1984). In the translocation, a 3' portion of the c-abl gene is spliced in-frame to a 5' portion of the BCR sequence (Heisterkamp et al., 1985; Shtivelman et al., 1985), resulting in a protein product of 210 kDa (p210), which has increased tyrosine kinase activity compared to the normal c-abl product of 145 kDa (Konopka et al., 1985). The structure of p210 is similar to the gag-abl p160 of the Abelson murine leukemia virus. In both the p160 and the p210 proteins, there is a change in the amino-terminus of the abl protein product and a resulting increase in tyrosine

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kinase activity (Ben-Neriah et al., 1986). The normal BCR gene encodes a 16 kDa phosphoprotein with serine/threonine kinase activity (Maru and Witte, 1991). More recently, it has been found that the normal BCR gene encodes a GTPase-activating protein (GAP) for a Ras-related GTP binding protein, $p21^{rac}$ (Diekman et al., 1991). This connects BCR to an established signaling pathway.

Another example of chromosomal aberrations which result in gene activation is found in some cases of non-Hodgkin's lymphoma, where there is an insertion on chromosome 2, ins (2;2) (p13;p11.2-14), resulting in the fusion of two genes in a manner similar to translocation fusions. The resultant REL-NRG fusion protein retains the N-terminal DNA binding and transcriptional activation domains of REL but replaces its C-terminus with NRG sequences of unknown function (Lu et al., 1991).

Chromosomal translocation may also inactivate tumor suppressor genes. One example of such consequence was seen in case of the neurofibromatosis gene (NF1), in which the gene was interrupted by the (1; 17) and (17; 22) translocation breakpoints which appeared to fall within 17q11.2 (Viskochil et al., 1990; Wallace et al., 1990).

1.2 Deletion and Loss of Heterozygosity

Consistent and specific chromosomal deletions in tumors are usually suggestive of a gene whose loss of function is important in the initiation or progression of malignancy, and have led to the cloning of a number of tumor suppressor genes. More than 20 solid tumors have been shown to have karyotypic aberrations that implicate loss of specific chromosome material (reviewed in Sager, 1989).

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For example, the del(13)(q14) deletion is observed only in retinoblastoma (Cavenee et al., 1983), and 11p13 deletions are restricted to Wilms tumor (Koufos et al., 1984). In retinoblastoma, the use of RFLPs revealed that tumors lacking obvious chromosome aberrations lost allelic variation in the same region of 13q as was lost by interstitial deletion in a subset of patients.

Certain chromosomal aberrations are common to tumors of different cellular origin. For example, the 3p13-23 region is commonly affected by deletions in small cell carcinoma and adenocarcinoma of lung, renal cell carcinoma, and ovarian adenocarcinoma (Dan Cin and Sandberg, 1989). Because of the limitations of cytogenetic resolution, it has not been possible to determine whether these interstitial deletions are identical or if they represent distinct molecular locations. A candidate gene PTPG, encoding a receptor protein with tyrosine phosphatase activity, has been mapped to the smallest region of allele loss observed in the 3p21 region of lung carcinomas (LaForgia et al., 1991). Both neuroblastoma and glioma show deletions of the 1p32-36 region (Jerkins et al., 1989; Fong et al., 1989), suggesting that functional inactivation of a common regulatory locus might be involved in the development of neuroectodermal tumors.

Another type of deletion is the loss of a terminal fragment. Involvement of the well known tumor suppressor gene p53 in colorectal carcinoma was suggested by deletion on terminal fragment of chromosome 17's short arm and allele loss of 17p in over 75% of colon carcinoma (Baker et al., 1989). Another case is that of the DCC gene (deleted in clorectal carcinoma) on chromosome 18 which was discovered on the basis of allelic loss studies at 18q21-qter (Vogelstein et al., 1988).

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1.3.Amplification

Increase in gene dosage by DNA amplification is a common genetic abnormality of tumor cells. Amplification resulting from the selective increase of the copy number of a cellular oncogene usually leads to abnormally high levels of the protein encoded by the amplified oncogene. The increased copy number and enhanced expression of cellular oncogenes frequently correlate with more advanced and aggressive stages of malignancy and poor prognosis (Schwab, 1990).

Chromosomal abnormalities such as trisomy and isochromosome are often associated with low-level gene amplification. However, minor changes in gene dosage or gene balance brought on by the acquisition of all or part of a single chromosome will affect hundreds or thousands of genes. Hematological tumors are frequently associated with low-level amplification. In malignant melanoma, an extra copy of chromosome 7 was found to be consistently associated with expression on the tumor cells of the receptor for EGF located on this chromosome (Koprowski et al., 1985). In many cases, the presence of trisomy as well as isochromosome is observed in a number of malignancies of different histological type, suggesting that they might provide a rather non-tissue specific growth advantage.

High levels of gene amplification (10- to 100-fold) are commonly manifested chromosomally as double-minute chromosomes and/or homogeneously staining regions. They are frequently observed in solid tumors. For example, the oncogene N-myc is amplified in neuroblastoma and associated with a poorer prognosis (Schwab et al., 1983; Brodeur et al., 1984). Amplification of the epidermal growth factor receptor gene has been found

in brain tumor of glial origin (Libermann et al., 1985) and a related gene ERBB2 (HER-2/NEU) is amplified in the advanced stage of the breast and ovary (Slamon et al., 1989).

2.Growth Factors

Cell growth and differentiation are regulated in part by polypeptide factors that either stimulate or inhibit cell proliferation. The relationship between malignant transformation and cellular growth factors was first suggested by Sporn and Todaro (1980) who proposed the autocrine hypothesis to account for the production of transforming growth factors by murine sarcoma virus-transformed cells (Delarco and Todaro, 1978). They proposed that the uncontrolled growth of transformed cells results from the synthesis by these cells of certain growth factors. More than 30 different polypeptide growth factors have now been structurally and functionally characterized (Cross and Dexter, 1991). It is now generally believed that the unregulated expression of growth factors or components of their signaling pathways is one of the changes required for cell transformation (Aaronson, 1991). Here I will describe the examples of growth factors related to the proliferation of cells of mesenchymal origin.

2.1.Platelet-Derived Growth Factor

Human platelet-derived growth factor (PDGF) is a potent growth factor for a number of cell types of mesenchymal origin (Ross et al., 1986). PDGF is a member of a family of closely related proteins that are ~30 -kDa and have two disulfide-bonded peptid chains (A and B) (Hannink and Donoghue, 1990). The A and B chains are encoded by separate genes,

located on human chromosomes 7 and 22, respectively (Swan et al., 1982; Betsholtz et al., 1986). The two PDGF chains can form three different dimers, -AA,-AB, and -BB, with different functional activities and secretory behaviors. A molecular basis for the differences in functional activities between the isoforms of PDGF was elucidated by the demonstration that two different PDGF-receptor types exist, denoted type A and type B (Heldin et al., 1988; Hart et al., 1988). The type-A receptor binds all three PDGF isoforms, whereas the type-B receptor binds PDGF-BB with high affinity and PDGF-AB with lower affinity, but does not bind PDGF-AA (Westermark and Heldin, 1991). The two receptor types are structurally and functionally related, and both have an intracellular protein tyrosine kinase activity (Gronwald et al.,1988).

Growth factor signal transduction through the PDGF receptor consists of a complex cascade of events and has served as the prototype for identification of substrates of the receptor tyrosine kinases. Cytoplasmic proteins that can trigger some of the secondary events become physically associated and are phosphorylated by the activated PDGF receptor kinase (Westermark and Heldin, 1991) These proteins include: 1) phospholipase C-γ, which catalyzes phosphatidyl -inositol biphosphate and generates two second messengers (inositol-triphosphate and diacylglycerol) (Meisenhelder et al., 1989); 2) phosphatidylinositol-3 kinase (PI-3 kinase), which phosphorylates the inositol ring of phosphatidylinositol and becomes physically associated with a number of activated tyrosine kinases; (Coughlin et al., 1989); 3) GTPase-activating protein (GAP), which modulates the signal transducing activity of the Ras protein (Molloy et al., 1989); 4) the serine/threonine kinase Raf-1, which can lead to a

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four- to six fold increase in serine/threonine kinase activity of Raf-1 (Morrison et al., 1989); and 5) three tyrosine kinases of the src family, $pp60^{c\text{-}src}$, $pp62^{c\text{-}yes}$, and $pp59^{fyn}$ (Kypta et al., 1990). PDGF is also able to induce gene expression, including c-myc and c-fos proto-oncogenes, presumably at the level of transcription (Westmark and Heldin, 1991).

The transforming potential of the c-sis/PDGF-B gene through autocrine mechanisms is illustrated by its expression in many tumors of mesenchymal origin, its transforming ability in rodent and human cells in transfection experiments, and its homology with the v-sis oncogene of simian sarcoma virus (SSV) (Westermark and Heldin, 1991). There are also examples of PDGF-receptor negative cell types that produce PDGF, such as mammary carcinoma cells (Bronzert et al., 1987). Presumably such cells cannot respond to PDGF in an autocrine fashion. Rather, the evidence suggest that the PDGF produced from tumor epithelial cells contributes to the formation of connective tissue stroma via a paracrine mechanism (Cullen et al., 1991).

2.2.Transforming Growth Factor-α

In 1978, Delarco and Todaro originally reported that culture medium conditioned by murine sarcoma virus-transformed cell lines was able to stimulate colony formation in soft agar of nontumorigenic fibroblastic cells. The active agent was called transforming growth factor (TGF). It was later discovered that there were at least two different TGFs, an EGF-like molecule, TGF-α, and a structurally and functionally distinct molecule, TGF-ß (reviewed in Derynck, 1988). TGF-α is a 50 amino acid polypeptide with 40% sequence homology to epidermal growth factor (EGF) which is secreted into the medium (Marquart et al., 1983). TGF-α is

especially important in transformation since it interacts with the EGF receptor and mediates all of its biological effects through this receptor (Carpentar et al., 1983). Binding of this growth factor to the EGF receptor activates the receptor-associated tyrosine kinase. The effect of TGF-α in a variety of in vitro assays using cultured cells are essentially identical to those of EGF (Anzano et al., 1983). Among the cellular substrates phosphorylated on tyrosine by the EGFR are phospholipase C-γ, GAP, and the phosphatidylinositol-3-kinase associated protein p85 (Margolis et al., 1990; Skolnik et al., 1991).

TGF-α has been shown to be a potent angiogenic factor (Schreiber et al., 1986) and may stimulate the neovascularization observed in some tumors. TGF-α expression is commonly expressed in cell lines derived from human tumors or chemically induced animal tumors (Derynck et al., 1987; Luetteke et al., 1988) as well as in cells transformed by retroviruses, cellular oncogenes, and by treatment with carcinogen or tumor promoters (Delarco and Todaro, 1978; Salomon et al., 1987). These findings , together with the transforming effect of TGF-α cDNA transfected into fibroblasts (Watanabe et al., 1987), suggest that inappropriate expression of TGF-α may contribute to the malignant state of a variety of human cell types. This hypothesis is supported by the fact that transgenic mice with overexpression of TGF-α exhibited a high incidence of mammary and pancreatic neoplasia (Sandgren et al., 1990;). In addition, a putative Shope fibroma virus protein (Chang et al., 1987) have been found to contain sequences structurally related to EGF and TGF-α.

2.3.Fibroblast Growth Factors

Another class of important growth factors are called fibroblast

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growth factors (FGFs). They comprise a family of polypeptides that includes acidic FGF, basic FGF, FGF-5, FGF-6, the product of int-2 oncogene, the product of K-fgf/hst oncogene, and keratinocyte growth factor (KGF). These factors display mitogenic activity for a wide spectrum of cells of mesenchymal, epithelial and neuronal origin (reviewed in Gospodarowicz, 1989). bFGF was first identified by its ability to cause the proliferation and phenotypic transformation of BALB-C 3T3 fibroblasts (Gospodarowicz and Moran, 1974). aFGF was first identified by its ability to stimulate myoblast and endothelial cell proliferation (Lemmon et al., 1982). Since the cloning of both the bFGF and the aFGF gene, it has been found that over 30 growth factor activities that were isolated from a variety of tissue and cell lines of normal or tumor origin are actually identical or very similar to bFGF or aFGF. The human bFGF gene, located on chromosome 4, produces a 154-amino acid protein composed of a single polypeptide chain. The human aFGF gene is located on chromosome 5, and its protein shows 55% identical to bFGF in sequence (Esch et al., 1985; Mergia et al., 1986). The common denominator of FGF family members is their high-affinity binding to heparin (Shing et al., 1984). bFGF utilizes a dual receptor system composed of a classical high affinity protein-type receptor and a lower affinity glycosaminoglycan-type receptor (Klagsburn and Baird, 1991). Less is known about the other members of the FGF family. However, considerable evidence has shown that FGFs have the dual function of both stimulating responsive cells by an autocrine mechanism and inducing neovascularization of the tumor by paracrine mechanism. Both of these mechanisms require FGF to be secreted from the producer cell. Since both acidic and basic FGF lack a signal peptide that

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would facilitate their secretion via conventional exocytosis (Abraham et al., 1986), how this comes about is not clear.

3. Proto-Oncogenes

Proto-oncogenes were initially discovered as the cellular homologs of retroviral oncogenes. When they become activated by point mutations or by deregulated expression they can play a causal role in uncontrolled cell proliferation and/or differentiation (Bishop, 1987). Based on the function of the oncogene products elucidated to date, it is clear that proto-oncogene products are elements of a cellular signaling network that extends from external ligands and growth factors, through cytoplasmic protein kinases and GTP-binding proteins, to nuclear transcription factors (Hunter, 1991).

3.1 Growth Factors

Recent studies on oncogenes have provided several examples which show that normal counterparts of oncogenes (proto-oncogenes) code for proteins that act as growth factors.

The v-*sis* oncogene of simian sarcoma virus (SSV) represents a prototype for the class of oncogenes that encode growth factors, because the cellular equivalent of this transforming gene is the first one found to be a gene coding for the B chain of human PDGF (Waterfield et al., 1983; Doolittle et al., 1983). The structural and functional similarities between the v-*sis* product and PDGF implies that transformation by v-*sis* is exerted by a PDGF-like growth factor. Like PDGF, the v-*sis* gene product exerts its growth-promoting action via PDGF-receptors which are expressed by a variety of cells of mesenchymal origin (e.g.autocrine transformation)

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(Johnson et al., 1985). Autocrine stimulation by the v-sis gene product is mediated by activation of PDGF receptors at both the cell surface (Huang et al., 1984; Johnsson et al., 1985) and in intracellular compartments (Bejcek et al., 1989). Furthermore, SSV-transformed human fibroblasts are inhibited by PDGF antibodies (Johnson et al., 1986).

Two other oncogenes encode protein products which belong to the FGF family. The products of int-2 oncogene (Dickson and Peters, 1987) and hst/K-fgf (Zhan et al., 1988) oncogene have been identified and shown to be homologous to bFGF and aFGF. These oncogenes encode proteins that possess an amino-terminal signal peptide sequence which allow them to be secreted. The transforming potential of these oncogenes in vitro and the occurrence of these oncogenes in tumors suggest that autocrine mechanisms are involved.

3.2 Receptor and Nonreceptor Protein-Tyrosine Kinases (PTK)

It has become increasingly apparent that growth factor receptors, also can function as oncogenic proteins and thus play causal roles in cell transformation. A prime example is the discovery that the avian retroviral oncogene, v-erbB, is derived from the chicken epidermal growth factor receptor (EGFR) gene by a series of truncations and point mutations (Downward et al., 1984). Both the EGFR and v-erbB genes encode proteins that possess intrinsic tyrosine kinase activity, but the v-erbB protein has a deletion of the ligand-binding domain and signal peptide sequence which results in ligand-independent activation of the kinase activity (constitutively active). Another example is the v-fms gene, which encodes an oncogenic form of the colony stimulating factor-1 (CSF-1) receptor (Sherr et al., 1985). Very recently, it was found that the human trk gene

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There is a group of several PTKs that lack extracellular domains and are categorized as the nonreceptor PTK. Members of this group include the src family of genes (src, yes and fgr) as well as the fes/fps and abl gene families. All of these genes were initially identified as oncogenes of retroviruses. The src oncogene is by-far the best studied gene in this group. Its mechanism of action is as yet not fully understood. In brief, the src gene family has a short amino-terminal sequence required for addition of myristic acid and two additional domains named Src homology 2 and 3 (SH-2 and SH-3) not found in the receptor PTK family (Bolen, 1991). The SH-2 and SH-3 sequences are important for regulating src family TPK activity and the activity of src family can be regulated by a variety of mechanisms which could differ from cell to cell (Bolen et al., 1991). The SH-2 and SH-3 domains have also been discovered in a variety of proteins unrelated to PTK, such as GAP, phospholipase C-γ, phosphatidyl-inositol 3-kinase, and the crk oncogene product (reviewed in Cantley et al., 1991).

3.3 Membrane Associated G Proteins

Transfection experiments led to the identification of a family of activated genes which are homologous to the transforming gene of the Harvey and Kirsten murine sarcoma viruses, H-ras and K-ras respectively (reviewed in Barbacid, 1987). Another member of this multigene family, N-ras, was originally identified in a human neuroblastoma, but no viral counterpart has been found. The ras genes in mammalian cells encode a 21 kDa intracellular membrane protein called p21 (Willingham et al., 1980) which can be detected in normal, untransformed cells as well as in

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malignant cells. The third amino acid from the C-terminal end of p21 is a cysteine residue, to which a poly-isoprenoid group is attached, thus offering an anchor for intracellular plasma membrane adhesion (Willamsen et al., 1984). The $p21^{ras}$ is known to bind guanine nucleotides and shares sequence homology (Finkel et al., 1984) and similar predicted structure with a family of other guanine nucleotide-binding proteins (McCormick, 1989).

The normal mammalian $p21^{ras}$ protein has intrinsic GTPase activity (McGrath et al., 1984) which in the presence of the GAPase activating protein (GAP) is increased upto 500-fold over mutant forms of $p21^{ras}$ with point mutations resulted from specific amino acid substitutions at codons 12, 13 or 61 (Trahey and McCormick, 1987). Such mutant $p21^{ras}$ is incapable of hydrolyzing GTP to GDP, and as a consequence the GTP remains bound. It has been proposed that GAP maintains normal $p21^{ras}$ in its inactive, GDP-bound state. However, the oncogenic $p21^{ras}$ remains in the constitutive GTP-bound state, and escapes normal GAP control. It is this GTP-bound p21 which actively transduces signals for growth in the absence of a normal growth stimulus (McCormick, 1989). GAP may also function in a complex with $p21^{ras}$ as an effector of its downstream signaling functions (Yatani et al., 1990). Thus, mutations that impair interaction of $p21^{ras}$ with GAP also block the biological function of $p21^{ras}$ protein.

3.4 Cytoplasmic Protein-Serine Kinases

The raf proto-oncogenes encode cytoplasmic serine/threonine specific kinases, which function in mitogen signal transduction from the plasma membrane to the nucleus (Rapp, 1991). There are three active members in the raf family, c-raf-1, A-raf-1, and B-raf. c-raf-1 was first discovered

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as the cellular homolog of v-raf. It is related by sequence homology to the tyrosine kinase class of oncogenes that lack a extracellular domain (v-src, v-fes etc.). However it does not have any tyrosine kinase activity. The three genes are dispersed over different chromosomes and have been mapped to sites that are frequently altered in human tumors (Storm et al., 1990). raf genes are differentially expressed in tissues, such that c-raf-1 is expressed in all tissues, A-raf-1 is present in urogenital tissues and B-raf is most abundant in cerebrum and testis (Storm et al., 1990). These kinases are inactive in quiescent dells.

The c-raf-1 gene can be converted into an oncogene by N-terminal fusion, truncation or site-specific mutations. Recent studies on growth factor signal transduction in fibroblasts yielde evidence of a central role for c-raf (Rapp, 1991). There are multiple pathways that lead to receptor-initiated Raf-1 activation, such as tyrosine phosphorylation, or protein kinase C-dependent or protein kinase C-independent serine phosphorylation (Rapp, 1991). Raf-1 kinase seems to function downstream position in a converging signal transduction scheme, since ras induced proliferation is dependent on Raf-1 function (Kolch et al., 1991), and Raf oncoproteins can overcome a block in cellular ras activity (Rapp et al., 1991). Furthermore, activated c-raf translocates to the nucleus, where it directly or indirectly alters gene expression (Mihaly et al., 1990), Cells containing activated raf have an altered transcription pattern (Heidecker et al., 1989). Mitogenic stimuli originating from such different sources as growth factor treatment, tumor promoter (such as TPA) -activated protein kinase C, or tyrosine kinase oncoproteins, have been found to activate c-raf. This functional linkage seems to be a component

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of most of the signal transduction pathways that mediate proliferation of fibroblasts (Rapp, 1991).

3.5 Nuclear Transcription Factors

Although the myc gene is one of the first oncogenes discovered, its function in transcriptional regulation was revealed only recently. The myc family of nuclear oncogenes has three well-characterized members, c-myc, N-myc, and L-myc. The c-myc gene is the cellular homolog of the avian retroviral transforming gene, v-myc (Sherness and Bishop, 1979; Watson et al., 1983). The human N-myc and L-myc genes were isolated based on sequence homology to the c-myc gene and their frequent amplification in neuroblastoma (N-myc) (Kohl et al., 1983) and small cell lung carcinoma (L-myc) (Nau et al., 1985). The c-Myc protein is phosphorylated at serine and threonine residues in two different domains (Ramsay et al., 1984). The c-myc gene encodes a nuclear protein with a basic motif, a helix-loop-helix (HLH), and a leucine zipper, all of which are features typically found in DNA-binding proteins that act as transcription factors, including fos and jun (Prendergast and Ziff, 1989; Kerkhoff et al., 1991). The leucine repeat and/or the HLH is essential for the formation of c-Myc dimers and is necessary for the ability of the c-Myc protein to bind DNA specifically (Dang et al., 1989; Crouch et al., 1990). The particular arrangement of three motifs, i.e., basic domain, HLH and leucine zipper, in c-Myc is homologous to that found in two other transcription factors, TFE3 and USF, that bind the uE3 motif located in the immunoglobulin heavy chain enhancer and in the adenovirus major late promoter, respectively (Beckmann et al., 1990; Gregor et al., 1990). All three proteins bind to the same core DNA sequence, CACGTG (Blackwell et al., 1990). c-Myc can

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also form a heterodimer with Myc-associated protein, MAX, to bind DNA (Cole, 1991). Although the data suggest that c-myc is indeed a transcription factor, the genes that are specifically regulated by c-myc have not yet been identified. Among the nuclear effectors, genes of myc family appear to be among the most frequently altered, with activation by specific chromosomal translocation or amplification and overexpression (Zimmerman and Alt, 1990).

4. Tumor Suppressor Genes

Early evidence for the existence of tumor suppressor genes came from somatic cell hybridization, which showed that fusion of tumor cells with normal cells results in nontumorigenic hybrids (Stanbridge, 1990). The suppression of tumorigenicity in these hybrids suggests that the tumorigenic phenotype can be recessive which, in turn, implies the existence of tumor suppressor genes in normal cells that function to prevent or suppress tumorigenesis. Human cytogenetics provided another important clue that suggested the existence of tumor suppressor genes. A consistent, specific chromosomal loss and/or deletion in cell of a specific type of tumor suggests that a tumor suppressor gene is located on the lost chromosomal material (Sager, 1989). In fact, accumulating evidence suggests that the progression of many tumors to full malignancy requires both the activation of proto-oncogenes and the inactivation of tumor suppressor genes (Weinberg, 1991).

4.1 The Retinoblastoma Gene (RB)

Retinoblastoma has been the prototype cancer for studying tumor suppressor genes. There is evidence of a heterozygous constitutional

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deletion involving chromosomal band 13q14 in this tumor. The genetic predisposition to retinoblastoma is transmitted by mutant alleles of the retinoblastoma susceptibility gene (RB) (Knudson, 1977; Cavenee et al., 1983; Lee et al., 1987). Expression of normal RB protein or mRNA is lacking in all retinoblastomas examined to date (Benedict et al., 1990). Mutations of RB that abolish normal gene expression have also been found in carcinomas of breast, prostate, small cell lung, and bladder, as well as in soft tissue sarcomas and leukemia (Benedict et al., 1990). It is likely, then, that loss of RB function is involved in a wide variety of tumors. Replacement of the normal RB gene in cultured retinoblastoma cells as well as other RB-negative tumor cells by retrovirus-mediated gene transfer suppresses their tumorigenic potential in athymic mice (Huang et al., 1988; Sumegi et al., 1990; Bookstein et al., 1990). However, a recent study by Gallie and her coworkers was unable to confirm this result (Muncaster et al., 1992).

The RB gene encodes a phosphorylated protein of approximately 110 kDa ($p110^{RB}$ that is constitutively expressed and accumulate in the nuclei of most vertebrate cells (Lee et al., 1987). Three biochemical activities of the RB protein have been found to date. First, the carboxyl-terminal half of $p110^{RB}$ is capable of binding to DNA, although no sequence specific for this binding has been demonstrated (Wang et al., 1990). Second, the transforming proteins of several DNA tumor viruses, including SV40 T antigen and adenovirus E1A, can bind $p110^{RB}$ (Decaprio et al., 1988; Whyte et al., 1988; Dyson et al., 1989, 1990). Two regions within the carboxyl-terminal half of the protein are required for T antigen and E1A binding (Hu et al., 1990; Huang et al., 1990). Mutational analysis of the

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transforming proteins has demonstrated a correlation between their ability to transform cells and their ability to bind RB protein (Whyte et al., 1989). Mutant RB protein found in tumors fails to bind T antigen and the mutations map to the regions required for binding in vitro. Third, the transcription factor E2F as well as several unidentified cellular proteins were found to bind to RB protein, apparently within the same binding domain used by T antigen (Kaelin et al., 1991; Chellappan et al., 1991; Bagchi et al., 1991; Huang et al., 1991). These observations suggest that the T antigen-binding domain is critical to the function of RB protein.

In addition, the RB protein is differentially phosphorylated during the cell cycle and may be involved in regulation of the cell cycle (Decaprio et al., 1989; Buchkovich et al., 1989; Chen et al., 1989; Goodrich et al., 1991). SV40 T antigen preferentially binds the underphosphorylated forms of RB protein (Ludlow et al., 1989), and T antigen can block the G1 phase function of RB and prevent the cell from progressing into S phase (Goodrich et al., 1991).

4.2 The p53 Gene

p53 was first identified as a 53-kD cellular protein that binds to SV40 T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979), a property that is also shared by RB protein. Because of its nuclear localization and increased expression in SV-40 transformed cells, it was thought initially to function in cell transformation like the oncogene-encoded Myc protein. Indeed, the p53 gene could transform primary rat embryo fibroblasts in concert with an activated *ras* gene (Eliyahu et al., 1984; Parada et al., 1984). The p53 gene could also act on its own to immortalize rodent cells (Jenkins et al., 1984). Therefore, the p53 gene

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had been classified as an dominant oncogene. It was later found out that these studies were conducted with mutant versions of the gene, and, when the same experiments were repeated with the wild-type p53, this gene was found to suppress the transformation induced by several oncogenes (Finlay et al., 1989; Eliyahu et al., 1989).

The situation is obscured further by the observation that mutant p53 alleles appear to be phenotypically dominant to the wild-type allele in the transfection assays. Since the mutant p53 and wild-type p53 proteins can associate in vitro, a model that accounts for this observation is that the mutant protein acts in a dominant-negative fashion by binding to and inhibiting wild-type protein (Lane and Benchimol, 1990). The molecular basis of the dominant negative effect of p53 mutation is apparently due to the fact that mutant protein can form an oligomer with a wild-type subunit to produce an inactive oligomeric complex (Kraiss et al., 1988; Milner et al., 1991). More recently, it has been found that mutant p53 inactivate the suppressor function of the wild-type protein by driving the wild-type p53 into the mutant conformation (Milner and Medcalf, 1991). The wild-type conformation of p53 is directly linked to the ability of p53 to suppress cell proliferation (Milner and Medcalf, 1990). Many of the mutant proteins have a much longer half-life than wild-type and form stable complex with a constitutively expressed member of the heat shock family proteins, hsc70 (Finlay et al., 1988).

The p53 protein shares another property with the RB protein: the ability to form stable complexes with the transforming proteins of several DNA tumor viruses (Levine and Momand, 1990). Whereas simian virus 40 T antigen, adenovirus E1A protein, and human papillomavirus E7 protein bind

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to the RB protein, on the other hand, SV-40 T antigen, adenovirus E1B, and papillomavirus E6 bind to p53. The RB binding sites on T antigen, E1A, and E7 are homologous to one another, and all are required for cellular transformation in cell culture. This is also true for the p53 binding sites, but its binding sites on the T antigen are different from the RB binding sites. Thus, three different DNA tumor viruses, SV-40, adenovirus, and human papilloma virus 16, have all evolved a mechanism to deal with the same negative regulators of cellular growth, p53 and RB. It appears that these viruses need to eliminate both proteins from the cell's growth-suppression regulation for full viral transformation to occur (Levine and Momand, 1990). Not surprisingly, the mutant forms of p53 fail to bind SV-40 T antigen (Levine et al., 1991). The notion that p53 and RB serve distinct, even complementary functions in growth regulation is supported by observations that both p53 and RB genes are affected by mutations in many human soft tissue sarcomas (Cooper and Stratton, 1991). Recent evidence also showed that the immortalization of human fibroblasts requires loss of both RB and p53 function to overcome the cellular senescence (Shay et al., 1991b).

The inactivation of p53 gene has been implicated in the genesis or progression of a wide variety of tumors, including those involving the hematopoietic cells, colon, breast, brain, bladder, liver, lung and soft tissue sarcoma (reviewed in Hollstein et al., 1991). In fact, mutations in the p53 gene are documented as the most common genetic alterations in human cancers (Levine et al., 1991). In addition, germline mutations of p53 gene predisposes individuals to a multiple cancers in the Li-Fraumeni syndrome (Malkin et al., 1990; Sirvastave et al., 1990). Introduction of

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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., 1990; Chen et al., 1990; Diller et al., 1990; Martinez et al., 1991). This is consistent with the wild-type p53 gene being tumor suppressor. Mutant forms of p53 are not limited to human tumors, as they often occur in mouse erythroleukemia induced by Friend virus (Munroe et al., 1987).

4.3 Other Candidate Suppressor Genes

a.The Wilms' Tumor Gene

Wilms' tumor is a pediatric nephroblastoma with an incidence of 1/10,100 and accounts for 85% of childhood kidney cancer (Matsunaga, 1981). Like retinoblastoma, Wilms' tumor may occurs both sporadically or be familial and dominantly heritable. Genetic analysis had implicated chromosome region 11p13 as the most likely locus of the Wilms' tumor (WT-1) gene (Koufos et al., 1984). Subsequent cDNA cloning and sequence analysis of WT-1 gene shows that it codes for a protein with four zinc finger domains and a region rich in proline and glutamine, a protein that may be a transcription factor involved in regulation of gene expression (Rose et al., 1990; Gessler et al., 1990; Call et al., 1990). The WT-1 has the sequence sequence-specific DNA-binding activity (Rauscher et al., 1990). The amino acid sequence also shows significant homology with a mammalian immediate-early protein EGR-1 (Lee et al., 1987). The WT-1 gene seems to have a restricted tissue expression whereas RB and p53 are expressed in virtually all tissues. No evidence currently exist to implicate the WT-1 gene in malignancies other than Wilms' tumor. There is some evidence that the WT-1 gene represents only one of two or three loci

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b.The Neurofibromatosis Gene

von Recklinghausen neurofibromatosis (NF1) is one of the most common autosomal dominant disorders, affecting 1/3500 individuals (Stumpf et al., 1987) with peripheral neufibromas and café-au-lait spots and an increased risk of multiple tumors (Riccardi and Eichner, 1986). DNA linkage analysis has implicated a locus at the chromosome 17q as the responsible locus for NF1 (Barker et al., 1987; Gordgar et al., 1989). The report of two chromosome translocations involving 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 disease locus (Schmidt et al., 1987; Ledbetter et al., 1989). These studies had lead to eventual cloning and identification of the NF1 gene in 1990 (Wallace et al., 1990; Viskochil et al., 1990; Cawthon et al., 1990). This transcript was either mutated, deleted or translocated in different NF1 patients, which would imply that the loss of this gene product was causative of the syndrom. Further sequence analysis of the NF1 gene transcript (Xu et al., 1990) has revealed a striking similarity to the catalytic domain of the mammalian GAP and of products of IRA1 and IRA2, the inhibitor of Ras in yeast. Several recent studies provide strong evidence that the NF1 gene product regulates the GTPase activity of the Ras protein (Martin et al., 1990; Ballester et al., 1990). The NF1 gene is widely expressed in many different cell types (Wallace et al., 1990), and may be involved in the control of cell growth by interacting with the proteins such as the Ras gene product. However, the role of NF1

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c.The DCC Gene

The frequent loss of chromosome 18q sequences in human colorectal cancer imply that a candidate suppressor gene might be located in this region. The DCC (deleted in colorectal carcinomas) gene was discovered through the use of RFLP markers that showed a loss of heterozygosity of the long arm of chromosome 18 in the 18q21.3 region (Vogelstein et al., 1988). The isolated and partially cloned DCC gene now shows that it encodes a 190-kDa transmembrane phosphoprotein, including four immunoglobulin-like domains of C2 class and a fibronectin type III-related domains similar to the structure of cell adhesion molecules (CAMs), and suggesting binding to an extracellular matrix or basement membrane component (Fearon et al., 1990). It is likely that the DCC protein is a signal transducing receptor whose loss confers a growth advantage on tumor cells, such as those which promote metastasis. To date the alterations of DCC gene have been found only in colorectal carcinomas. However, LOH on chromosome 18q is seen in other tumor types (Devilee et al., 1991), suggesting a possible broad function of the DCC gene in human cancer.

d.The Krev-1 Gene

Another class of genes that can inhibit tumorigenesis is exemplified by the Krev-1 gene (also known as rap 1A and smg p21) (Noda, 1990). This gene was cloned from a human fibroblast cDNA expression library based on its ability to induce the phenotypic reversion of NIH 3T3 mouse cells that had been transformed by the K-ras oncogene (Kitayama et al., 1989). The human Krev-1 gene has been mapped to chromosome 1p12-p13 (Yamada et al., 1990). The Krev-1 encodes a guanine-nucleotide binding protein with a

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molecular weight of 21 kD whose amino acid sequence had 50% homology to the ras family genes. Krev-1 has the ability to suppress only the ras-transformed cells. The Krev-1 protein binds to GAP with a far higher affinity than ras p21 itself (Frech et al., 1990). Krev-1 mutants with a reduced GTPase activities exhibit higher rates of reversion (Kitayama et al., 1990), suggesting that the amount of GTP-bound Krev-1 might regulate signal transduction through the ras pathway by a competitive inhibition for the ras effector (French et al., 1990). All these suggest that Krev-1 (and perhaps other similar proteins) might indirectly affect the ras pathway by regulating the GDP/GTP ratio in cells.

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CHAPTER II

Malignant transformation of human fibroblast cell strain MSU-1.1 by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene

Key Words: chemical carcinogenesis/infinite life-span human cells/ focus formation/loss of chromosomes/sarcomas

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ABSTRACT

Treatment of MSU-1.1 cells, a near-diploid, karyotypically-stable, infinite life span human fibroblast strain, with (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene induced focus formation. Eight independent foci were isolated and the cell strains developed from them were examined for characteristics of malignant cells. Each grew to a higher density in medium containing 1% serum than did the MSU-1.1 cells. Three of the eight grew rapidly in serum-free medium without added growth factors, formed colonies in agarose with diameters of ≥ 120 μm at a frequency of 5- 19%, exhibited loss of genetic material, and, when injected into athymic mice, formed sarcomas that reached 6 mm in diameter within 2-3 wk. One produced high-grade sarcomas (progressively growing, invasive tumors exhibiting high mitotic activity); the other two produced low-grade sarcomas (tumors with a lower degree of mitotic activity) that developed focal areas of high grade malignant cells if left in the animals for >4 wk. A fourth cell strain formed high- grade sarcomas only after 2.5-3 mo, but the tumor-derived cells analyzed showed the same growth properties as the three malignant cell strains described above, exhibited loss of genetic material, and, when reinjected into athymic mice, produced high-grade sarcomas with a short latency period.

INTRODUCTION

Although epidemiology indicates that cancer results from exposure to carcinogens, including those resulting from life-style (1), normal human cells in culture have never been transformed to malignancy by carcinogen treatment (2). This is not surprising in view of the accumulating evidence that cancer develops as the result of a multistep process. Current hypotheses suggest that a cell acquires some change associated with tumor-derived cells that confers a growth advantage so that by clonal expansion it gives rise to numerous progeny exhibiting that phenotype. This increases the chances that an additional change related to carcinogenesis will occur in a cell that has already acquired the previous change (2,3). This process is considered to continue until by successive clonal expansion a cell acquires all the changes needed for the malignant state. Such repeated clonal expansions require that the cell possess or acquire an infinite, or greatly extended life-span. Until recently, the majority of studies of the changes involved in carcinogen-induced *in vitro* mammalian cell transformation relied on rodent fibroblasts which, unlike diploid human cells in culture, spontaneously give rise to infinite life-span cells and can yield malignant cells after extended subculturing, even without carcinogen treatment (4). Human cells in culture very rarely spontaneously acquire an infinite life-span, and no one has observed normal, finite life span human cells in culture become transformed into malignant cells, either spontaneously or after being treated with carcinogens (2). This may well reflect the inability of such cells to

undergo sufficient clonal expansions to allow a rare variant cell to acquire sufficient transformation-related genetic changes. But if nonmalignant human cells in culture have been "immortalized" --e.g., by repeated exposure to carcinogen (5,6) or after infection with a DNA tumor virus (7-9), they can be induced to become malignant by transfecting them with oncogenes expressed at high levels (9,10) or by carcinogen treatment (11,12).

However, it is usually not possible to use cell lines immortalized by either of these two methods to examine the nature of the transformation-related changes that a normal diploid finite life-span cell acquires in the process of becoming malignantly transformed. This is because these methods of immortalizing human cells yield highly aneuploid cells that already exhibit many of the characteristics of malignant cells, e.g., morphological alteration, ability to form foci, ability to form colonies in agarose, growth factor independence, spontaneous chromosome losses, etc.

Recently, we and our colleagues (13,14) succeeded in obtaining an infinite life-span diploid human fibroblast strain, after transfection of a foreskin-derived normal cell line with a plasmid carrying a v-*myc* oncogene and a selectable marker. The progeny cells from the drug-resistant clone expressed the v-*myc* gene, but virtually all entered crisis at the expected time and eventually senesced. But a cell strain with an infinite life-span and a stable diploid human karyotype arose from among the progeny. This has been designated MSU-1.0 (14).

A derivative of this diploid strain spontaneously developed a stable near-diploid karyotype, composed of 45 chromosomes including two marker

chromosomes. Its origin and characteristics have re- cently been described in detail (14). The cells, which express a transfected v-*myc* and *neo* gene, have a normal morphology, are partially growth factor independent, but do not form foci, form only very small colonies in 0.33% agarose at low frequency, and do not produce tumors in athymic mice. Using this strain, designated MSU-1.1, we generated an *in vitro* model of malignant transformation by using transfection of activated (mutated) oncogenes (15-17), and we obtained highly malignant cells that form progressively growing sarcomas in athymic mice. Cells derived from these tumors exhibit the same karyotype as the MSU-1.1 cells with no additional chromosome changes.

Since MSU-1.1 cells can be transformed to malignancy by transfection of various oncogenes, we wanted to determine whether they could also be transformed into malignant cells by exposure to carcinogens. We report here that a single exposure of the cells in exponential growth to (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), a direct-acting ultimate metabolite of benzo[a]pyrene, caused them to produce foci composed of malignant cells. BPDE was chosen as the first carcinogen to be tested in MSU-1.1 cells because we and our colleagues showed previously that it induces mutations in the *HPRT* gene of normal diploid human fibroblasts (18-20) and can induce intrachromosomal homologous recombination (21), and others had shown that it induces mutations in the *HRAS* gene of human fibroblasts (22) and chromosomal damage (23).

MATERIALS AND METHODS

Cell Culture. The cells were cultured in modified McM medium (24), a derivative of MCDB110 basic medium (25), prepared with Earle's salts and containing 10% supplemented calf serum (SCS) (HyClone, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml), and hydrocortisone (1 μg/ml) (culture medium).

Treatment of Cells with BPDE and Selection of Focus-Forming Cells. Cells in exponential growth were plated 8 X 10^5 cells per 150-mm-diameter dish (two to five dishes per determination). The next day, the medium was replaced with serum-free medium, and BPDE dissolved in dimethyl sulfoxide or the solvent alone was added by micropipette. After 1 h, the cells were washed twice with serum-free medium and given fresh culture medium. The cells in one dish for each dose were assayed immediately for survival of colony-forming ability (18). The rest were allowed to continue replicating for 7-10 days (expression period) and then 2 X 10^6 cells were assayed for focus formation by plating at 2 X 10^5 cells per 100-mm-diameter dish in McM medium containing only 1% SCS. The medium was renewed weekly, and when distinct foci developed after 4-6 wk, representative foci were isolated and the cells were recloned by plating them at cloning density to eliminate any nontransformed background cells that might have been included during the isolation. The rest of the dishes were stained, the foci were counted, and the frequency of foci was expressed per number of cells plated.

Saturation Density Test. Cells were plated at 5 X 10^3 cells per cm^2 in McM medium containing 1% SCS. The next day, the number of attached cells was determined (>90%), and the medium was changed to McM medium

containing either 1% SCS or 10 % SCS. The cells were fed twice a week with the appropriate medium and were counted weekly. The number of cells per cm^2 3-4 wk after plating, when no further increase in numbers was observed, was designated saturation density.

Growth Factor Independence Assay. Cells were assayed for requirements for growth factors and Ca^{2+} as described (14), using McM medium containing either 0.1 mM or 1 mM Ca^{2+} and the serum replacement supplements of Ryan *et al.* (24), but lacking epidermal growth factor (SR_2) (16) or McM medium containing 0.1 mM Ca^{2+} and 10% SCS.

Anchorage Independence Assay. Cells were assayed as described (15) for the ability to form colonies in 0.33% agarose with a diameter of $\geq$ 120 μm.

Tumorigenicity Test. Cells (1 X 10^7) in 0.2 ml of serum-free medium were injected s.c. into the subscapular region of BALB/c athymic mice. Animals were monitored weekly for tumors at the site of injection. Tumors were removed when they reached 1-2 cm in diameter. Portions were returned to culture and the cells were grown in medium containing G418 to eliminate any contaminating mouse cells. Sections of tumors were stained with hematoxylin-eosin and graded according to published criteria (26).

Chromosome Analysis. Chromosome preparation and G-banding was carried out as described (16).

Multiplex PCR Analysis of Platelet-Derived Growth Factor (PDGF) and Transforming Growth Factor α (TGF-α). RNA was isolated as described (27) and its integrity was determined by using a formaldehyde/1% agarose gel (28). mRNA was reverse transcribed into cDNA with 1 μg of total RNA, and the cDNA of the gene of interest was amplified by PCR as described (29) but with a final Mg^{2+} concentration of 1.88 mM in the reaction mixture.

RESULTS

BPDE-Induced Focus Formation MSU-1.1 cells that had undergone ~50 population doublings since the strain was established (14) were exposed to 0.13 or 0.18 μM BPDE or to the solvent alone, using conditions described in *Materials and Methods*. These concentrations were chosen because at that cell density they reduce cell survival to between 35% and 25% of the solvent-treated control. After an expression period of 7 days, the cells were assayed for focus formation as described. After several weeks, foci (three to five per dish) were seen on top of the lawn of confluent cells in the dishes containing progeny of BPDE-treated cells, but not in the control dishes. Phase-contrast microscopy showed that the cells in the majority of the foci were spindle-shaped and formed dense, criss-crossed multilayers. In some, the multilayers were well-ordered. A few foci showed less piling up of cells, but the cells were multinucleated. One or two foci from each dish were isolated for further characterization and recloned.

As soon as we saw that BPDE induced foci, we carried out a second experiment using the same BPDE concentrations to obtain additional unequivocally independent foci. This time the target cells had undergone 24 population doublings, and they were assayed for focus-formation 10 days after BPDE treatment instead of 7. The number of foci observed per dish for 0.13 μM was 12-16; for 0.18 μM, it was 17-23. Foci were not seen in the control dishes (Fig. 1A and 1B). Again, representative foci were isolated and recloned. [The fact that focus-forming cells have a doubling time slightly shorter than that of nontransformed MSU-1.1 cells (see

below) could account for the higher frequencies observed in the populations allowed to grow for 10 days rather than 7.) The frequencies for the two experiments averaged 45×10^{-6} for 0.13 μM and 60×10^{-6} for 0.18 μM.

The majority of the colonies obtained after recloning the focus-derived cells contained cells that divided rapidly and grew piled-up on top of each other or appeared morphologically transformed. Eight of these clones were chosen for in-depth characterization, two from each of the four independent BPDE treatments, for a total of eight focus-derived cell strains. With foci derived from the same original populations, we chose clones that differed from each other in colony growth pattern or in cell morphology to avoid studying siblings. Clones from the low dose in the first experiment were designated 1C1 and 1C4, and those from the high dose were designated 2C1 and 2C2. Clones from the low dose in the second experiment were designated 3C1 and 3C10 and clones from the high dose were designated 4C4 and 4C5.

Characterization of BPDE-Transformed Cell Strains

Cell Morphology. All cell strains were more refractile than the parental MSU-1.1 cells (Fig. 1C) and grew in an irregular pattern. Most had either a long-spindle or short-spindle morphology (Fig. 1D). Strain 1C4 was composed of epithelioid cells that grew as nonoverlapping round to polygonal cells (Fig. 1E). Strain 4C4 was derived from a focus composed of multinucleated cells and at each subculture showed the presence of giant multinucleated cells.

Evidence of Growth Factor Independence. The saturation density of the cell strains was determined and compared with that of the parental cells.

All eight showed higher saturation densities than MSU-1.1 cells at 1% or 10% serum, with the differences being most apparent at 1% (Table 1).

Oncogene-transformed MSU-1.1 cells proliferate rapidly in McM medium lacking growth factors and containing only 0.1 mM Ca^{2+}, but MSU-1.1 cells show only a modest rate of growth under the same conditions (14, 16). We therefore compared the 8 cell strains with MSU-1.1 cells for growth under these stringent conditions, as well as two other conditions: McM medium with the normal amount of Ca^{2+} (1 mM), but no exogenous protein growth factors, and McM medium with 0.1 mM Ca^{2+}, but 10% SCS. Strains 1C1, 2C1, 2C2, and 4C4 replicated at the same rate as MSU-1.1 cells under all three conditions and, therefore, are considered *partially* growth factor independent. A representative set of growth curves for this group is shown in Fig. 2B. In contrast, the other four strains replicated equally rapidly in all three media, and so are considered *completely* growth factor independent. A representative set of curves for this group is shown in Fig. 2C.

Anchorage Independence. The strains were examined as described for the ability to form large-sized colonies in agarose. The results are listed in Table 1. The frequencies for cell strains 3C1, 3C10, and 4C5 were equal to or higher than those found previously with MSU-1.1 cells transformed to the malignant state by oncogenes (15-17). Strain 1C4 showed a much lower frequency of colonies, and the frequencies for the other strains were insignificant.

Tumorigenicity Test. Only four of the eight strains formed tumors in athymic mice (Table 1). Strain 4C5 produced progressively growing tumors that attained a diameter of 6 mm in 2 wk. Histological examination of

Fig. 1. Characteristics of MSU-1.1 cells and BPDE-induced focus-forming derivatives. (*A*) Monolayer of progeny of untreated MSU-1.1 cells stained with crystal violet after 6 wk of growth in medium containing 1% SCS. (*B*) Foci on a monolayer of progeny of BPDE-treated cells similarly stained. (*C*) Phase-contrast photomicrograph of untreated MSU-1.1 cells. (*D*) Phase-contrast photomicrograph of progeny of focus-derived cells showing spindle-shape. (*E*) Phase-contrast photomicrograph of progeny of focus-derived cells showing epithelioid morphology. (C-E, X80.)

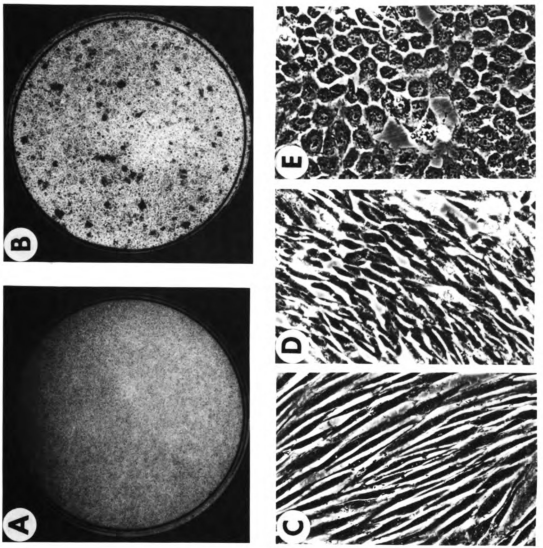

Figure1

nduced focus-
of untreated
5 wk of growth
monolayer of
. (C) Phase-
cells. (D)
focus-derived
hase-contrast
cells showing

FIG. 2. Growth factor requirements of MSU-1.1 cells and cell strains 2C2 and 3C1. Cells were plated in McM medium with 1% SCS. The next day, the number attached was determined, and the medium was replaced with McM medium supplemented with SR_2 (○), or modified to contain only 0.1 mM Ca^{2+} and supplemented with SR_2 (▲) or 10% SCS (△). Cells were refed on Day 4 and counted on the days indicated. Dashed line shows lack of growth of diploid human cells in medium with 0.1 mM Ca^{2+} and SR_2.

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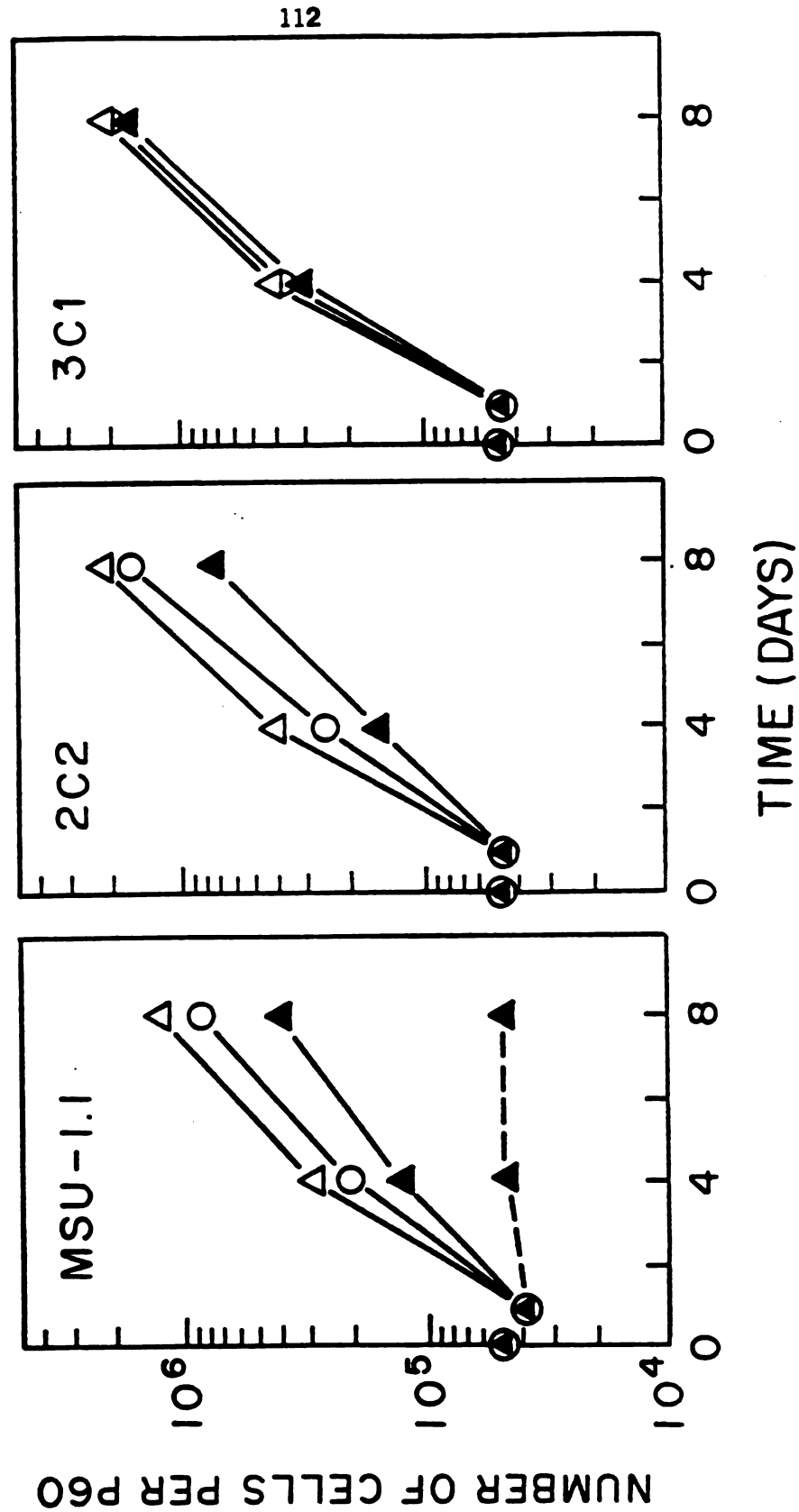

Figure 2

these tumors showed that they consisted of highly cellular, densely packed, round cells with a high mitotic index (Fig. 3A). They gave evidence of invasion of surrounding tissue. Within the tumor masses were large areas of necrosis, hemorrhage, and numerous randomly interspersed blood vessels. These tumors were classified as high-grade malignant sarcomas.

Tumors formed by strains 3C1 and 3C10 attained a diameter of 6 mm in 3 wk and after reaching 1 cm in diameter, remained at this size for several months. They were characterized as low-grade malignant sarcomas because analysis showed that they were moderately cellular with abundant collagenous stroma separating the spindle-shaped neoplastic cells, with little atypia, and mitotic figures appearing much less frequently than in the high-grade tumors. The slides did not show evidence of invasion or necrosis. The histology of the tumors removed from the animals 3 wk or 3 mo after injection was similar, but in the latter specimens a few small focal areas consisting of high grade, round cell sarcomas were observed. An example from strain 3C1 is shown in Fig. 3B.

Cell strain 2C1 did not produce detectable tumors for almost 3 mo. when a small nodule was found in three of four mice. These tumors then grew rapidly and progressively and were classified as high-grade spindle-cell sarcomas (Fig. 3C). Cells cultured from such tumors were propagated and injected into mice to determine whether the tumors had been composed of highly malignant cells. This time, tumors formed rapidly at four of four injection sites in two mice, attaining a diameter of 6 mm within 2 wk. This suggests that the original tumors had been formed by a small population of malignant cells within the population of strain 2C1

injected.

Chromosome Analysis. Analysis of the eight cell strains ~30 population doublings after isolation showed that, as expected, all contained the two marker chromosomes characteristic of MSU-1.1 cells (14). No additional gross structural or numeric chromosome changes were found in strains 1C1, 1C4, 2C1, or 2C2 (Table 1). Cell strain 4C5, the most malignant strain, exhibited a modal chromosome number of 42, with a new chromosome marker, M3, and was monosomic for chromosomes 5, 10, 13, and 17. Marker 3 resulted from translocation between the segment distal to band 10q22 and chromosome 17 at band 17p13. The rest of chromosome 10 with its centromere was lost, along with the terminal segment distal to band 17p13. Cell strains 3C1 and 3C10 had a modal chromosome number of 43, with chromosome 19 and Y lost in 3C1, and with 13 and Y missing in 3C10. The changes for a given strain were consistently found in essentially all the cells examined from that strain. Strain 4C4, which was originally isolated from a focus containing some multinucleated cells, had cells that were either hypoploid or polyploid, and the latter had additional abnormal chromosomes of undetermined origin. These cytogenetic data confirmed the clonal original and independent derivation of the latter four cell strains.

Cells derived from tumors formed by the latter strains were similarly examined. Those from the three frankly malignant strains (3C1, 3C10, and 4C5) were the same as described above, except for a slight increase in the frequency of triploid and tetraploid cells. But the karyotype of the cells from two tumors derived with strain 2C1, which appeared only after a long latency, were not identical to those seen in the original strain

FIG. 3. Photomicrograph of formalin-fixed, hematoxylin/eosin-stained tissue sections of s.c. tumors formed by BPDE-transformed cell strains. (*A*) High-grade round cell sarcoma produced by strain 4C5. Arrows indicate blood vessels formed in the tumor mass. Note the high mitotic index. (*B*) Portion of tumor removed 3 mo after injection of strain 3C1, showing part of a low grade spindle cell sarcoma (L) and part of focal aggregates ("pearls") of round cells with features of high-grade malignant sarcomas (H). (*C*) High grade spindle cell sarcoma removed 3 mo after injection of strain 2C1. (X180.)

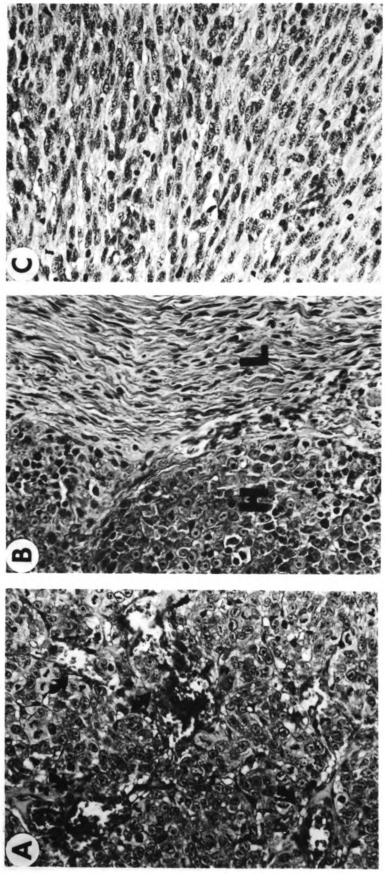

Figure 3

2C1 analyzed before injection. Instead, 90% of the cells from one tumor had lost chromosome 14 and showed a new marker chromosome, M3--i.e., a segment of unknown origin added to band 7p12. In addition, chromosome 13 or Y was missing in 30% of the cells examined. The changes seen in the chromosomes of cells from the second tumor analyzed differed from those in the first, suggesting that the further alterations in karyotype occurred after strain 2C1 had been injected into the mice.

Expression of PDGF and TFG-α mRNA. Because four of the eight cell strains could grow without exogenous growth factors, we examined the strains for expression of PDGF(B) and/or TGF-α mRNA. Cell strain 1C4 expressed an increased level of PDGF(B) mRNA similar to that found in human fibrosarcoma-derived cell line HT1080. The other strains showed only a slight or no increase in PDGF(B) mRNA expression above the MSU-1.1 control. None expressed TGF-α mRNA.

Table 1. Characterization of clonally derived cell strains isolated from independent foci induced in MSU-1.1 cells by BPDE

Cell strain	Saturation density in 1% SCS, cells per cm^2 X 10^{-4}	Degree of growth factor independence*	Colonies with diameters $\geq$ 120-μm per 10^2 cells plated into agarose†	Expression of PDGF(B) mRNA‡	Common additional chromosome changes§	Tumor incidence¶	Tumor histology	
							Type of sarcoma	Grade of malignancy
MSU-1.1	8	+	< 0.01±0.01	-	-	0/20	-	-
1C1	15	+	0.05±0.02	±	NC	0/4	-	-
2C2	25	+	< 0.03±0.02	±	NC	0/3	-	-
2C1	18	+	< 0.03±0.01	-	NC	3/4 (75)	Spindle	High
4C4	24	+	0.05±0.02	±	NA	0/4	-	-
1C4	23	+++	0.30±0.06	++	NC	0/3	-	-
3C1	27	+++	5.0 ±0.50	-	-19, -Y	3/4 (24)	Spindle‖	Low‖
3C10	35	+++	9.0 ±0.7	-	-13, -Y	3/3 (20)	Spindle‖	Low‖
4C5	28	+++	19.0 ±0.9	-	-5, -10 -13 -17, +M3	4/4 (15)	Round	High

*Estimated by rate of growth in McM medium containing 0.1 mM calcium and SR_2; +, partial independence; +++, complete independence.

†5 X 10^3 cells were plated per dish and duplicate dishes were counted at 3 wk.

‡-, Not more than the residual background amount found in MSU-1.1 cells; ±, just detectable above the control; ++, at least 2-3 times more than in control cells.

§Changes observed by G-banded analysis in addition to the two marker chromosomes found in MSU-1.1 cells (14). NC, No change; NA, not applicable(multinucleated cells).

¶Ratio of mice with tumors to number of mice injected. Numbers in parentheses indicate number of days for tumors to reach 6-mm diameter.

‖Tumors removed after 3 mo had minor focal aggregates of round cells with features of high-grade sarcomas (see Fig. 3B).

DISCUSSION

In the present study, BPDE, a well-studied, direct-acting mutagen, caused the malignant transformation of MSU-1.1 cells, a nonmalig-nant, near-diploid, karyotypically stable, infinite life-span human cell strain derived from a normal diploid fibroblast line. The frequency of focus formation induced was similar to the frequency of thioguanine resistance induced in diploid human fibroblasts by these doses of BPDE in previous experiments, i.e., ~100 X 10^{-6} and 160 X 10^{-6} (20). This result is consistent with induction of focus formation resulting from a mutational event. We also consider it likely that a mutation(s) in various gene(s) or element(s) accounts for the differences we observed in the biological properties of the various focus-derived cells, including the alterations in karyotype. This is because no such chromosome alterations beyond the two marker chromosomes that characterize MSU-1.1 cells (14) were found when the latter cells were transformed into malignant cells by transfection of various *RAS* oncogenes (15-17). In those cases, the transfectants could be recognized because they formed distinct foci composed of morphologically altered cells. The focus-derived cell strains were growth factor independent, formed very large colonies in agarose at high frequency, expressed the transfected oncogenes at high levels, and produced malignant tumors. However, the vast majority of these tumors did not show any additional chromosome changes.

In the case of BPDE-induced transformation, the traits that correlated highly with malignancy were complete independence of growth factors, the

ability to form large-sized colonies in soft agarose at a high frequency, and loss of genetic material beyond the stable changes already found in the nonmalignant MSU-1.1 cells (Table 1). The three strains that exhibited these characteristics before being injected into mice produced sarcomas rapidly, suggesting that these three characteristics are essential for malignant tumor formation. Additional support for this hypothesis comes from the behavior of cell strain 2C1. The cells did not exhibit these traits before being injected and failed to produce tumors for 2.5 mo, but, once the tumors appeared, the tumor-derived cells exhibited all three of these characteristics.

The origin of the chromosomal changes that occurred in the 2C1 cells is not certain, but they appear to have taken place after injection into the mice, since cells from the two tumors analyzed did not show the identical loss of genetic material. What matters more than the origin of the additional changes is that the tumor-derived cells, just like cell strains 3C1, 3C10, and 4C5 before injection, exhibited all three of the characteristics that we consider to be required for malignant transformation of MSU-1.1 cells by BPDE.

Loss of heterozygosity for chromosomes 13 and 17p, and alterations in the Rb-1 and/or p53 gene have been observed frequently in human sarcomas (30-32). It is interesting to note that a single exposure to BPDE resulted in a loss of a copy of chromosome 13 in malignant cell strains 4C5 and 3C10, and, as expected, both cell strains expressed reduced amounts of Rb protein (data not shown). Strain 4C5 also acquired a new marker chromosome involving chromosome 17 in the region of the p53 gene.

Whether the p53 gene has been lost is not yet known. Strain 3C1 lost other genetic material, but the relevance of these changes to malignant transformation reamins to be determined.

The nature of the changes that allowed malignant cell strains 4C5, 3C1, and 3C10 to grow rapidly in medium without growth factors and form large colonies in agarose are as yet unknown. Schilz (33) in this laboratory showed that cells from a series of human fibrosarcomas synthesized large amounts of PDGF(B) and/or TGF-α mRNA, but analysis of expression of these mRNAs in our malignant cell strains showed that they did not. The synthesis of PDGF(B) mRNA by strain 1C4, however, is sufficient to account for its growth factor independence. Presumably, this occurred as a result of a mutational event induced by BPDE.

Our use of a focus assay to identify cells that have become malignantly transformed is similar to that of Kakunaga and Kamahara (34) with a BALB/c 3T3 mouse cell line. Our results agree with the findings of Rhim and his colleagues (35, 36), who work with a non-tumorigenic aneuploid cell strain originally derived from a human osteosarcoma (HOS cells). They found that HOS cells treated with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (35) or dimethylbenz [a] anthracene (36) underwent morphological alterations and became tumorigenic. Similarly, human keratinocytes immortalized after infection with an adenovirus type 12-simian virus 40 (SV40) hybrid virus and human uroepithelial cells immortalized by SV40 were converted to the malignant state after treatment, respectively, with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and 4-nitroquinoline 1-oxide (11) or 3-methylcholanthrene (12). However, unlike MSU 1.1 cells, the human cell target populations

used in the studies described above were highly aneuploid. The availability of an infinite life-span human fibroblast cell strain that exhibits a stable karyotype and normal or quasi-normal growth control, so that variants lacking such control can be selected, makes it possible to determine what oncogenes and/or suppressor genes play a role in converting human fibroblasts to malignancy.

Note Added in Proof. Eight mo after injection, strain 1C4, which was growth-factor independent, expressed PDGF (B) mRNA, and formed colonies in agarose at 0.3%, formed small nodules (5 mm in diameter) in two of three mice. These tumors persisted for four mo at the same size. Histology showed they were typical benign fibroblastic tumors.

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CHAPTER III

Sequential transformation of an infinite life-span diploid human fibroblast cell strain, MSU-1.0, to growth factor independence and anchorage independence by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene

Key Words: chemical carcinogenesis/diploid infinite life-span human cells/focus formation/growth factor independence/anchorage independence

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ABSTRACT

We recently showed that cells of a near-diploid karyotypically stable, infinite life-span human fibroblast strain, designated MSU-1.1, form foci after a single exposure to a direct-acting carcinogen (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo-[a]pyrene (BPDE) and that the progeny cells derived from majority of the foci tested produced malignant tumors in athymic mice. MSU-1.1 cells exhibited two marker chromosomes and partial growth factor independence. Their parental cell strain, MSU-1.0, is diploid and has normal growth factor dependence. For the present study, we treated the infinite life-span MSU-1.0 cell strain with BPDE. After treatment, the cells formed distinct foci and colonies in 0.33% agarose in the presence of 10% fetal bovine serum. Cell strains were isolated from five independent foci. The cell strains had either a short-spindle morphology or an epithelioid morphology. Like MSU-1.1 cells, they grew moderately well in the absence of exogenously added growth factors, and formed colonies with diameters $\geq$ 80 μm in 0.33% agarose supplemented with only 2% serum at frequency higher than MSU-1.0 or MSU-1.1 cells, but they did not form tumors in athymic mice. These five cell strains were treated a second time with BPDE, and selected for focus formation and anchorage independent growth using more stringent conditions. Cell strains derived from these foci or agarose colonies were more transformed than cells derived from the first BPDE-treated cells, as demonstrated by the ability to replicate in medium without exogenously added growth factors and in 0.33% agarose containing 2% serum. Some of them produced small nodules at the injection sites, but these regressed,

and could not be clearly identified as neoplastic growths. Four of the BPDE-transformed cell strains derived from the second treatment were treated third time with BPDE and selected for colony formation in 0.66% agarose contianed only 2% serum. Representative agarose colonies were isolated and pooled. Cell strains derived from these clones did not form tumors. These results support the hypothesis that in vitro transformation is a multistep process and that the MSU-1.1 cell strain, which arose spontaneously from MSU-1.0 cells, has undergone one or more genetic changes essential for malignant transformation by BPDE.

INTRODUCTION

Abundant evidence shows that the malignant transformation of human cells in culture is the result of a multistep process (1-6). The same conclusion has been reached regarding in vivo carcinogenesis (7-9). To investigate the number and kinds of changes involved in the malignant transformation of human fibroblasts in culture, we and our colleagues (10-11) succeeded in obtaining an infinite life-span diploid human fibroblast strain, following transfection of a foreskin-derived normal cell line with a plasmid carrying a v-myc oncogene and a selectable marker neo gene. The progeny cells from a drug resistant clone expressed the v-myc gene, grew normally, entered crisis at the expected time. Eventually the vast majority of the cells senesced, but a cell strain with an infinite life-span arose from among the progeny of this clone. This strain which has a stable diploid human karyotype has been designated MSU-1.0 (10). Since the v-myc-expressing siblings of MSU-1.0 in the senescing population all died, it is clear that expression of the v-myc protein is not sufficient for immortalize human fibroblasts. At least one additional, spontaneously occurring event was required to generate this infinite life-span cell strain. This cell strain does not have any growth advantage over the parental LG1 cells and does not exhibit any transformed properties.

A derivative of this diploid strain with a stable near-diploid karyotype, composed of 45 chromosomes including two marker chromosomes was also found. Since the two cell strains have the same integration site for the transfected v-myc and neo containing plasmid and since spontaneous immortalization is an extremely rare event, it is very likely that this

strains is derived from the MSU-1.0 cell strain. It has, therefore, been designated as MSU-1.1 (11). The MSU-1.1 cells have a normal morphology, grow moderately well in serum-free medium, but do not form foci, form only very small colonies in 0.33% agarose at low frequency, and do not produce tumors in athymic mice. When MSU-1.1 cells were exposed to (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo-[a]pyrene (BPDE) and selected for foci, the majority of the focus-derived cell strains formed tumors when injected into athymic mice (12).

Since MSU-1.1 cells are partially growth factor independent, we wanted to determine if their presumed precursor, i.e. MSU-1.0 cell strain, which is diploid and growth factor dependent and presumably closer in characteristics to normal diploid fibroblasts than is MSU-1.1, could also be transformed into malignant cells by exposure to BPDE. We report here that after one treatment of MSU-1.0 cells with BPDE, variant cells were isolated that grew moderately well in medium lacking serum or growth factors, just like MSU-1.1, and formed small colonies in 0.33% agarose. However, these cells were not tumorigenic. An additional treatment of these variant cells with BPDE followed by more stringent selection, produced transformed variant cells that were more transformed but no growths that we could unequivocally define histologically as tumors were observed. Treatment of the the latter cells with BPDE and selection of large colonies from agarose still did not yield tumorigenic cells, indicating that further changes are required.

MATERIALS AND METHODS

Cells and Cell Culture. The derivation of the MSU-1.0 cells used in this study has been described (10). In the present study, the MSU-1.0 cells were subcloned twice and the progeny of a single colony designated MSU-1.0A1 was used. The cells have a stable diploid karyotype and a normal fibroblastic morphology. They do not grow in medium without exogenous growth factors and do not produce tumors in athymic mice. The cells were cultured in modified McM medium (13), a derivative of MCDB110 base medium (14), prepared with Earle's salts and containing 10% fetal bovine serum (FBS) or 10% supplemented calf serum [SCS] (HyClone, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml) and hydrocortisone (1 μg/ml) [culture medium]. They were maintained at 37°C in a humidified incubator with 5 % CO_2.

Isolation of the Subclones. Cells from exponentially growing cultures were trypsinized briefly, suspended in culture medium, and plated at cloning densities (i.e., 200 to 1000 cells in a 100-mm diameter dish). The medium was changed once a week. After three weeks, single large colonies were trypsinized and replated into a 35-mm diameter dish and expanded. The derivation of these subclones is described in "Results".

Transformation Protocol. *Treatment of Cells with Carcinogen and Cytotoxicity Assay.* The details of carcinogen treatment and the cytotoxicity assay have been described (12,15). Briefly, cells in exponential growth were plated 8 X 10^5 cells per 150 mm-diameter dish (2-5 dishes per determination). The next day the medium was replaced with serum-free medium, and BPDE dissolved in dimethylsulfoxide or the solvent

alone was added by micropipette. After 1 h, the cells were washed twice with serum-free medium and given fresh culture medium. The cells in one dish for each dose were assayed immediately for survival of colony-forming ability (15). The rest were allowed to continue replicating and were refed once with culture medium.

Selection of Focus-Forming Cells. On day 7 or 8 following BPDE treatment, the cells in approximately one-half of the dishes for each dose and for the DMSO controls were pooled and 2 X 10^6 cells were assayed for focus formation by plating at 2 X 10^5 cells per 100-mm diameter dish in McM medium containing the designated amount of SCS. One week later, the cells were given McM medium containing a half of the original amount of SCS. They were fed with this medium once a week and when distinct foci developed after 4-6 weeks, representative foci from some of the dishes were isolated. The rest were stained and counted, and the frequency of foci was expressed per cells plated. The progeny of the isolated foci were recloned by plating them at cloning density to eliminate any nontransformed background cells that might have been included during the isolation. A typical transformed colony derived for the progeny of each individual focus was selected and expanded for further study.

Selection of Anchorage Independent Cells in Soft Agarose. At the time the cells were assayed for focus formation as described above, the cells in other half of the dishes for each determination and for the DMSO controls were pooled, and a total of 1 X 10^6 cells/dose were assayed for ability to form colonies in agarose as described (16) with the following modifications. Cells (5 X 10^4 per dish) were plated in 0.33% or 0.66% SeaPlaque agarose (FMC, Rockland, ME) in McM medium supplemented with the

designated amount of fetal bovine serum. Agarose-free McM medium (3.5 ml) containing the designated amount of serum was added to the top agarose layer the following day and every 7 days thereafter. After 3-4 weeks, the colonies in two or three dishes with a diameter larger than 40 μm (composed of 60 or more cells) were counted and sized electronically. Recovery of cells exhibiting anchorage independent growth were accomplished by gently isolating a single colony from agarose with a micropipette and plating each into a 35-mm diameter dish containing 3 ml culture medium. In some instances, a large number of colonies were removed and pooled into a 60-mm diameter dish. After 2 to 3 days, the colonies attached to the plastic, and the cells began to grow out. These cells were propagated in flasks for further study.

When the cells isolated from the foci or from agarose colonies were retested for anchorage independence, 5 X 10^3 cells were plated per 60-mm diameter dish in 0.33% agarose and McM medium containing 2% FBS as described (16).

Assay of Growth Factor Requirements. Cells were assayed for growth factor requirements essentially as described (10,12) with the following modifications. For the majority of the studies, in addition to the three conditions used previously, cells were also assayed for growth with a reduced concentration of serum, i.e., in the McM medium containing 0.1 mM Ca^{2+} and 1% SCS, or in the McM medium containing 0.1 mM Ca^{2+} , the serum replacement supplements [SR_2], and 3 ng/ml of EGF (BRL, Gaitherburg, MD). An aqueous solution of purified human serum albumin (5 mg/ml) was prepared and used to make EGF stock solution (3 μg/ml). The cells were fed with the appropriate medium every 3 days and the cell number in duplicate dishes

for each condition was determined 4 and 7 days after plating. All experiments were carried out at least twice.

Assay for Tumor Formation. To examine the ability of cells to form tumors, 1 X 10^7 cells in 0.2 ml of serum-free medium were injected s.c. into the subscapular region of Balb C athymic mice. Animals were monitored weekly for tumors at the site of injection for 10 to 12 months. Sections of tumors were stained with hematoxylin-eosin and subjected to histopathological examination.

RESULTS

Growth Properties of MSU-1.0A1 Subclone. In preliminary studies, we found that the colony-forming efficiency of the MSU-1.0 cells was < 0.1 % in culture medium containing 10% FBS. In the same medium, the MSU-1.1 cells clone with about 80% efficiency and the parental LG1 cells at about 30%. To improve the colony-forming efficiency, the MSU-1.0 cells were plated at cloning density and several large clones were isolated and grown to large populations. One of the best growing clonal population was re-cloned and cells were isolated from the largest clone and the derived population was designated MSU-1.0A1. The colony-forming efficiency of the MSU-1.0A1 cells was about 30% in culture medium with 10% FBS.

We examined the ability of MSU-1.0A1 cells to grow in medium without exogenously added growth factors, as well as under various other growth conditions, and compared their growth with MSU-1.1 cells grown under the same conditions. A spontaneously derived, completely growth factor independent variant of the MSU-1.1 cells, designated as MSU-1.2 (McCormick et al., unpublished data), was used as the positive control. As shown in Fig. 1, the MSU-1.0A1 cells do not grow in McM medium containing 0.1 mM calcium without the addition of a protein growth factor or serum. These results are identical to those obtained with MSU-1.0 cells and LG1 cells (10). When tested for their response to the addition of 1 % FBS or epidermal growth factor (EGF), the MSU-1.0A1 cells responded only minimally. In contrast, the MSU-1.1 cells grew very rapidly with the addition of EGF and somewhat slower with 1 % FBS (Fig. 1). As expected, the MSU-1.2 cells grew very rapidly under all growth conditions. At the

same time, we examined these three cell strains for the colony-forming efficiency on plastic under the various growth conditions. The results are shown in Table 1. The MSU-1.0A1 cells were much more dependent upon exogenous growth factors than the other two cell strains.

We also compared the anchorage independent growth of MSU-1.0A1 cells with the parental LG1 cells, MSU-1.1 cells and one of the H-ras transformed MSU-1.1 cell strains in 0.33% agarose with 2% or 10 % FBS. The ras transformed MSU-1.1 cells were included as a positive control. The results are shown in Table 2. The MSU-1.0A1 cells did not exhibit anchorage independent. The morphological appearance of the MSU-1.0A1 cells was identical to that of the LG1 cells. MSU-1.0A1 cells were found to exhibit a typical diploid karyotype. As expected, the cells were not tumorigenic when injected into athymic mice.

Experimental Design. Since the MSU-1.0A1 cell strain is much closer to normal finite life-span diploid cells than is the MSU-1.1 cell strain, we did not expect to be able to transform MSU-1.0A1 cells into malignant cells by a single exposure to BPDE. Instead, our stragety was to treat them with carcinogen and isolate cell strains that had acquired one or other characteristics of tumor-derived cells, such as growth factor independence (focus formation) or anchorage incependence, and then exposed the latter strains to carcinogen and select cells that were even more altered. Figure 2 diagrams the approach we took.

Selection and Characterization of the Transformed Variants after BPDE Treatment I. In BPDE Treatment I, MSU-1.0A1 cells were exposed to 0.13 μM and 0.18 μM BPDE or to the solvent alone. These concentrations gave an average cell survival of 30% and 15%, respectively. The cells were grown

Figure 1. Proliferation of MSU-1.0A1 cells, MSU-1.1 cells and MSU-1.2 cells under different culture conditions. Cells were plated in McM medium with 1% SCS. The next day, the number of cells attached was determined, and the medium was replaced with the appropriate medium. Cells were refed on day 4 and counted on the days indicated.

Figure 1

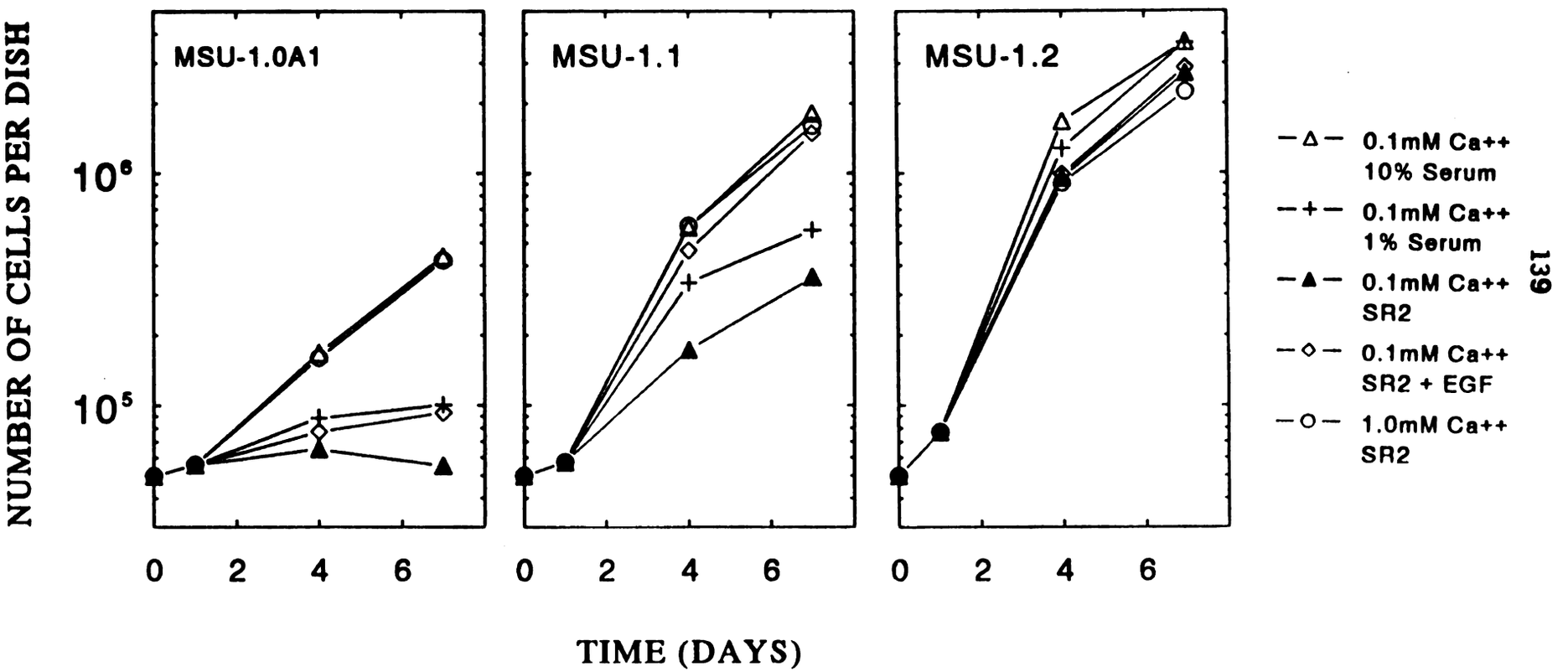
MSU-1.0A1
MSU-1.1
MSU-1.2
NUMBER OF CELLS PER DISH
10^6
10^5
0
2
4
6
TIME (DAYS)
0.1mM Ca++ 10% Serum
0.1mM Ca++ 1% Serum
0.1mM Ca++ SR2
0.1mM Ca++ SR2 + EGF
1.0mM Ca++ SR2

Table 1. Colony-forming efficiency of MSU-1.0A1, MSU-1.1 and MSU-1.2 cell strains under various culture conditions

Cell strain	Colony-forming efficiency (%)[a]				
	0.1 mM Ca^{2+} 10% FBS	0.1 mM Ca^{2+} 1% FBS	1.0 mM Ca^{2+} SR2	0.1 mM Ca^{2+} SR2 + EGF	0.1 mM Ca^{2+} SR2
MSU-1.0A1	29 (2+)	7 (+)	10 (+)	0	0
MSU-1.1	90 (3+)	72 (2+)	41 (2+)	42 (2+)	20 (+)
MSU-1.2	65 (4+)	73 (3+)	61 (3+)	73 (3+)	67 (3+)

[a]100 cells were plated into 100-mm diameter dish with appropiate medium and were refed weekly. After three weeks, the cells were stained with crystal violet, and the number of colonies composed at least 50 cells were counted in triplicate dishes. The relative size of the colonies formed under these conditions is indicated by the number of plus within the parenthesis.

Table 2. Anchorage Independent Growth of LG1, MSU-1.0A1, MSU-1.1 and MSU-1.1-H-*ras* Cell Strains from the MSU-1 Lineage

Cell strain	Cells per dish	Amount of serum	Colonies formed per 10^4 cells with diameters[a] ≥ 40 μm	≥ 80 μm	≥ 120 μm	≥ 160 μm
LG1	$5X10^4$	2 %	5	< 1	0	0
MSU-1.0A1	$5X10^4$	2 %	4	< 1	0	0
MSU-1.1	$5X10^4$	2 %	89	1	< 1	< 1
MSU-1.1-H*ras*	$5X10^3$	2 %	2544	1536	688	184
LG1	$5X10^4$	10 %	262	7	0	0
MSU-1.0A1	$5X10^4$	10 %	205	5	< 1	0
MSU-1.1	$5X10^4$	10 %	532	71	1	0
MSU-1.1-H*ras*	$5X10^3$	10 %	2995	2485	1498	992

a. Cells were plated in 0.33% agarose. After three weeks, the number of colonies of a given size in 40% of the area of triplicate dishes was determined and these values were used to estimate the total number of colonies formed per 1 X 10^4 cells using an automated image analyzer.

Figure 2. Schematic of experimental design of stepwise carcinogen treatment and selection conditions used in transformation of MSU-1.0A1 cells.

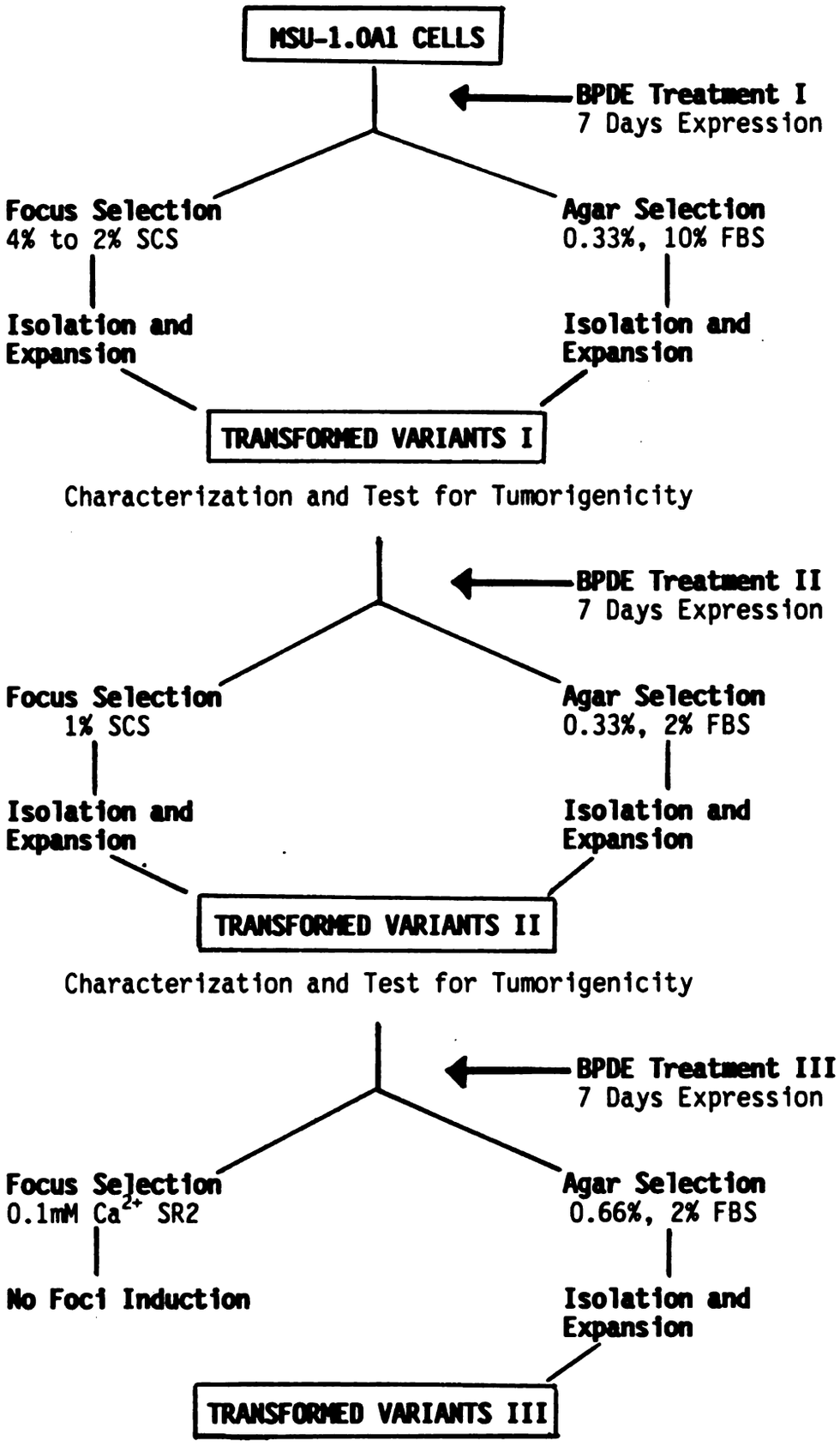
MSU-1.0A1 CELLS
BPDE Treatment I
7 Days Expression
Focus Selection
4% to 2% SCS
Agar Selection
0.33%, 10% FBS
Isolation and
Expansion
Isolation and
Expansion
TRANSFORMED VARIANTS I
Characterization and Test for Tumorigenicity
BPDE Treatment II
7 Days Expression
Focus Selection
1% SCS
Agar Selection
0.33%, 2% FBS
Isolation and
Expansion
Isolation and
Expansion
TRANSFORMED VARIANTS II
Characterization and Test for Tumorigenicity
BPDE Treatment III
7 Days Expression
Focus Selection
0.1mM Ca^{2+} SR2
Agar Selection
0.66%, 2% FBS
No Foci Induction
Isolation and
Expansion
TRANSFORMED VARIANTS III
Characterization and Test for Tumorigenicity

Figure 2

for a 7 day expression period, and then 2 X 10^5 cells were plated into 100-mm diameter dishes in medium containing 4 % SCS for selection of focus-forming cells. The amount of SCS was reduce to 2 % after one week. After 3 to 5 weeks, clear focal areas of clonal overgrowth (foci) were observed in the dishes containing the progeny of BPDE-treated cells, but not in the control cultures (Fig. 3A, 3B, and 3C). The average number of foci observed per dish was 3.4 for 0.13 μM BPDE treatment, and 4.5 for 0.18 μM BPDE treatment. Phase-contrast microscopy showed that the cells in the majority of foci were spindle-shaped, and formed well-ordered multilayers (Fig. 3D and 3E).

At the same time that focus selection was being carried out, 5 X 10^4 cells were plated into 60-mm diameter dishes in 0.33 % agarose with 10 % FBS for selection of anchorage independent colonies. Colonies between 120 μm and 180 μm diameter were scored, and the frequency was 11 X 10^{-6} for 0.13 μM BPDE and 31 X 10^{-6} for 0.18 μM, respectively. No colonies larger than 120 μm in diameter were seen in the control cells.

Individual foci were isolated and recloned as described. Cells obtained after recloning or isoated from the largest agarose colonies were expanded into large populations. These clonally-derived cell strains were then assayed for growth in medium without exogenous growth factors as well as growth in 0.33% agarose in the presence of 2% FBS. A total of twelve independent cell strains were assayed. Cell strains from five of the focus-derived cells were chosen for further characterization, because they grew significantly better than the MSU-1.0A1 cells or the other cell strains derived from foci or agarose colonies. The five cell strains were designated I-F1, I-F2, I-F3, I-F4, and I-F5. Cells strains I-F1, I-F2,

and I-F4 had a short spindle morphology, while cells from I-F3 and I-F5 had an epithelioid morphology. All five cell strains divided rapidly, were highly refractile, and grew in irregular patterns, rather than the highly oriented pattern of the parental MSU-1.0A1 cells. Representative examples of cells from strains I-F1 and I-F3 are shown in Fig. 4 and compared with MSU-1.0A1 cells.

The five cell strains were compared for their ability to replicate under different growth conditions. The results are shown in Fig.5. In contrast to the growth factor dependence of their parental MSU-1.0A1 cells shown in Fig.1, all five strains grew moderately well in medium containing 0.1 mM calcium without the addition of a protein growth factor or serum (closed triangle). Their growth rate under those conditions was similar to or faster than that of the MSU-1.1 cells (Table 3). These cell strains responded to 10% serum or medium containing 1.0 mM calcium by replicating even faster than the parental MSU-1.0A1 cells (compare Fig. 1 and Fig. 5). Cell strain I-F2 was not stimulated to replicate significantly faster by addition of 1% serum or EGF, whereas the other four strains responded to 1% serum or EGF by an increased rate of replication.

When tested for ability to grow in 0.33% agarose with 2% FBS, these cell strains formed colonies larger than 80 μm diameter with efficiencies from 28 X 10^{-4} to 2713 X 10^{-4} cells (Table 3). There was variation among the cell strains in the frequency and the size range of colonies formed . However, the frequencies for all five strains were significantly higher than the parental MSU-1.0A1 cells or MSU-1.1 cells.

Chromosome analysis was carried out for all five strains. One strain, I-F5, was found to have 47 chromosome, the other four had 46

Figure 3. The focus formation of MSU-1.0A1 cells after BPDE treatment I. A week after BPDE treatment, 2 X 10^5 cells were replated into 100-mm-diameter dish and allowed to grow in medium containing 4% for a week and then 2% SCS for 4 weeks before being stained with crystal violet. (A) Confluent monolayer of untreated MSU-1.0A1 cells; (B) Foci generated in a dish containing progeny of cells treated with the 0.13 μm BPDE; (C) Foci generated in a dish containing progeny of cells treated with the 0.18 μm BPDE; (D) and (E) Phase-contrast photomicrograph of a focus formed after 0.13 μm BPDE treatment at a magnification 50X and 100X, respectively.

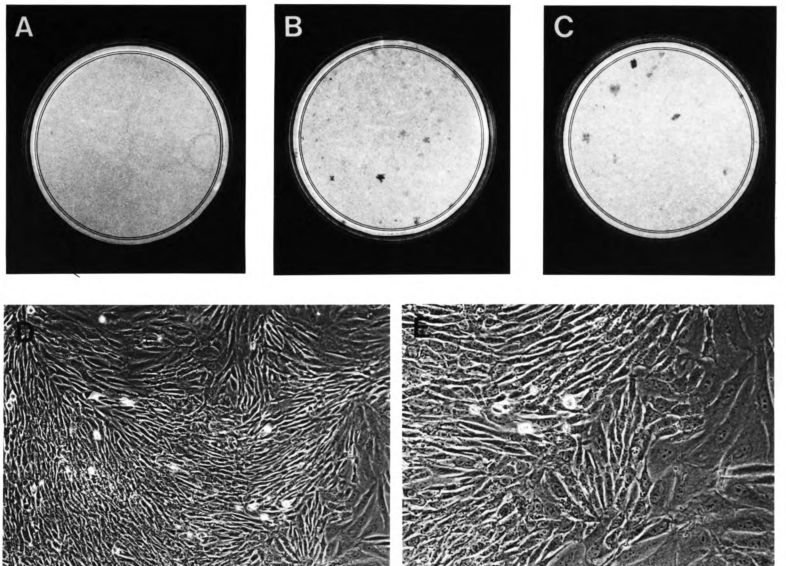

Figure 3

Figure 4. Comparison of morphology of MSU-1.0A1 cells and transformed variant cells after BPDE treatment I. (A) Phase-contrast photomicrograph of parental MSU-1.0A1 cells. (B) Phase-contrast photomicrograph of strain I-F1 cells showing spindle shape. (C) Phase-contrast photomicrograph of strain I-F3 cells showing epithelioid morphology. Magnification 100X.

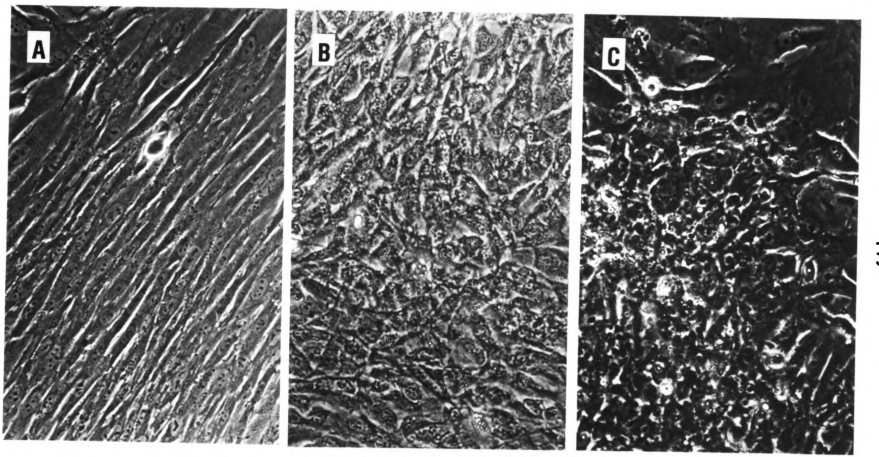

Figure 4

Figure 5. Proliferation of MSU-1.0A1 transformed variants after BPDE treatment I under different culture conditions. Cells were plated in McM medium with 1% SCS. The next day, the number of cells attached was determined, and the medium was replaced with the appropriate medium. Cells were refed on day 4 and counted on the days indicated.

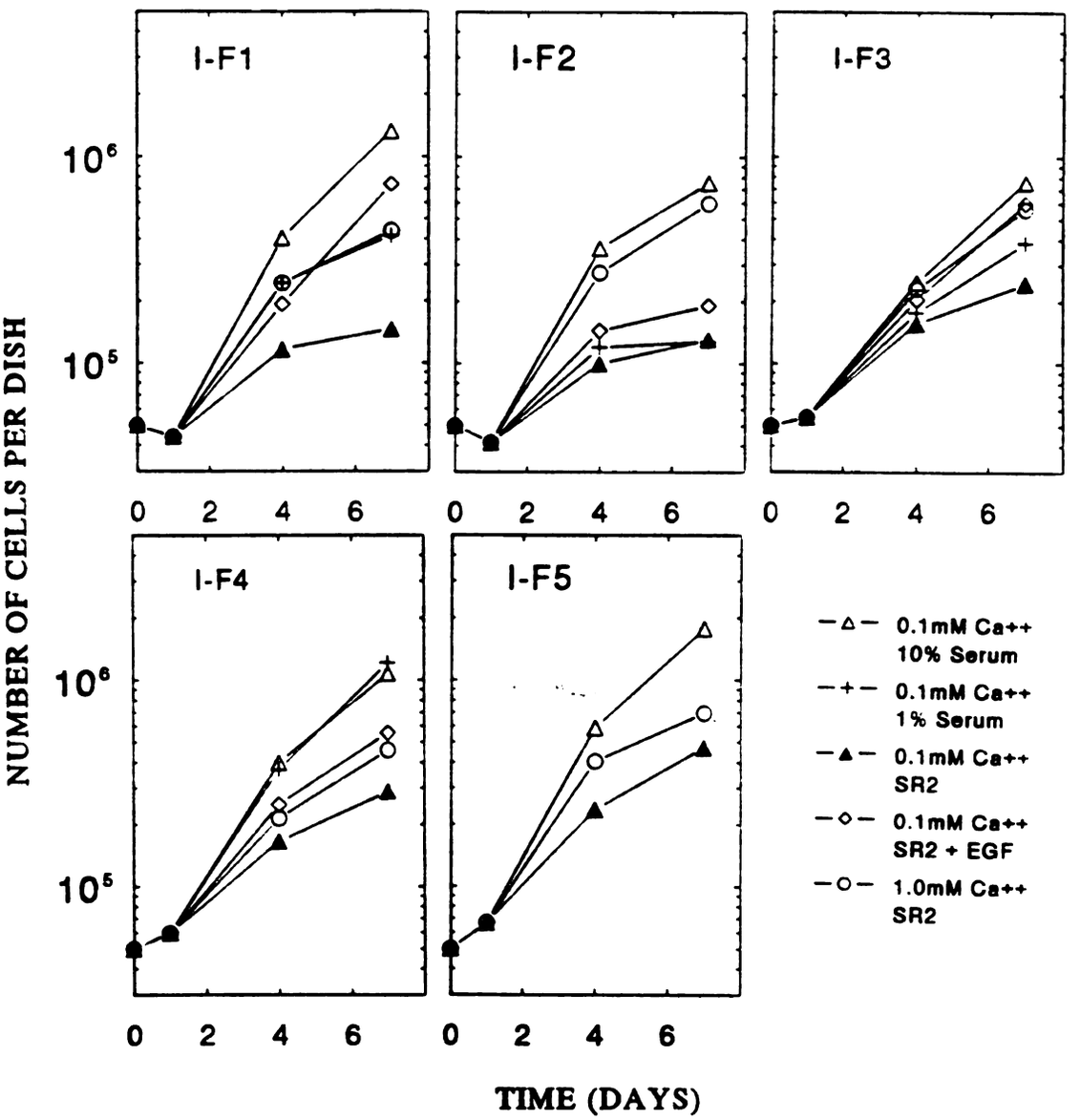
I-F1
I-F2
I-F3
I-F4
I-F5
10^6
10^5
0 2 4 6
NUMBER OF CELLS PER DISH
TIME (DAYS)
0.1mM Ca++ 10% Serum
0.1mM Ca++ 1% Serum
0.1mM Ca++ SR2
0.1mM Ca++ SR2 + EGF
1.0mM Ca++ SR2

Figure 5

Table 3. Growth Properties of MSU-1.0A1 Transformed Variants from BPDE Treatment I

Cell strain	Cell morphology[a]	Growth factor independence[b]	Colonies formed per 10^4 cells with diameters[c] ≥ 80 μm	≥ 120 μm
MSU-1.0A1	F	0.67	< 1	0
I-F1	F	1.73	413	33
I-F2	F	1.66	278	8
I-F3	E	2.16	28	5
I-F4	F	2.28	135	38
I-F5	E	2.83	2713	988
MSU-1.1	F	2.32	1	0

[a]"F" indicates spindle-shape morphlogy; "E" indicates epitheloid-like morphology.

[b]Population doublings of cells obtained after 6 days culture in serum-free McM medium with SR_2 containing only 0.1 mM Ca^{2+} and no exogenous growth factors. Duplicate dishes were counted after plating 5 X 10^4 cells per dish.

[c]5 X 10^3 cells were plated per dish and duplicate dishes were counted at 3 weeks.

chromosomes like the parental MSU-1.0A1 cells. The I-F5 strain also exhibited the fastest growth in medium without exogenous growth factors and the highest frequency of colonies formed in 0.33% agarose. The G-banded karyotype of these strains has not yet been analyzed.

All five strains were assayed for tumorigenicity in athymic mice by subcutaneous injection, and no tumors have been observed over a period of greater than one year.

Selection and Characterization of the Transformed Variants after BPDE Treatment II From the above studies, it was clear that additional changes were required to convert these cell strains into the malignant state. Therefore, all five strains were exposed to 0.18 μM BPDE (BPDE Treatment II, Fig.2), or to the solvent alone as a control. After an expression period of 7 days, the cells were assayed for focus formation as well as anchorage independent growth using more stringent conditions, i.e., 1% SCS medium for focus assay and 2% FBS medium for agarose selection. After several weeks, very distinct, dense foci (15-20 per dish) composed of highly refractile, irregularly shaped cells (Fig. 6B and 6C) were seen on top of the lawn of confluent cells in the dishes containing progeny of the BPDE-treated I-F2 strain, but the other four BPDE-treated strains showed no focus induction (Fig. 6A). No foci were observed in any of the control dishes. Several foci from the BPDE-treated I-F2 cells were isolated, and clonally expanded in culture medium. A spindle-shaped clonally derived strain, designated IF1-IIF1, and an epithelial-like clonally derived strain, designated IF2-IIF2, were chosen for further study.

Large-sized colonies in agarose ranging from 200 μm to 400 μm in diameter were observed in the BPDE-treated progeny of cell strains I-F1,

I-F3, and I-F5 with frequency of 100 X 10^{-6} to 240 X 10^{-6}. Most of the colonies in agarose were round-shaped, whereas a few colonies were irregular shape (Fig. 7). Colonies induced in the BPDE-treated progeny of strain I-F4 were ranged from 160 μm to 280 μm with average frequency of 140 X 10^{-6}. No colonies in this size range were seen in the control dishes. There was no significant induction of agarose colonies in the BPDE-treated progeny of strain I-F2, and therefore no colonies were isolated.

Colonies of BPDE-treated I-F1, I-F3, I-F4, and I-F5 were isolated from agarose and clonal populations as well as multi-clonal populations composed pools of agarose-derived colonies were created. For each cell strain, a rapidly proliferating clonal population and a pooled population were chosen for further study. These clonal population were named IF1-IIA1, IF3-IIA1, IF4-IIA1 and IF5-IIA5. The pooled populations were designated IF1-IIAP, IF3-IIAP, IF4-IIAP, and IF5-IIAP. The derivation and designation of cell strains generated from each BPDE treatment are summarized in Fig.8. Six of the ten strains isolated from foci or agarose, i.e., IF1-IIA1, IF1-IIAP, IF2-IIF1, IF2-IIF2, IF3-IIA1, and IF3-IIAP, grew more rapidly than their respective parental cell strains in medium containing 0.1 mM calcium without addition of serum or growth factors (compare Table 4 and Table 3). The other four cell strains in Table 4, IF4-IIA1, IF4-IIAP, IF5-IIA1, and IF5-IIAP, grew at a similar rate as their respective parental cells. However, all ten of these cell strains were still responsive to addition of serum or calcium, i.e., they replicated more rapidly after its addition. Representative examples of growth factor requirements of five strains are shown in Fig. 9.

As shown in Table 4, these cell strains also exhibited much better growth in 0.33% agarose in the presence of 2% FBS than their respective parental strains (*Note*: the frequencies for strains IF4-IIA1, IF4-IIAP, IF5-IIA1 and IF5-IIAP were much higher than their parental strains I-F4 and I-F5, assayed in the same experiment). The frequency of large-sized colonies ($\geq$ 120 μm) ranged from 23 X 10^{-4} to 2820 X 10^{-4}. Only strain IF1-IIA1 produced more colonies of diameter $\geq$ 120 μm than did a strain derived from a focus of MSU-1.1 cells induced by BPDE (MSU-1.1 2C1/T).

All of these cell strains have been tested for tumorigenicity in athymic mice. A number of animals given injections of the cells from strains IF3-IIA1, IF5-IIA1 and IF5-IIAP produced palpable nodules approximately 0.6 to 1.2 cm in diameter within 2 to 3 weeks. Most of these nodules stopped growing after reaching this size and then regressed after one to two months. Some of the nodules were excised before they could regress and were examined histologically by several pathologists. No definitive evidence of malignant cells was be observed. Infiltration of numerous lymphocytes and/or macrophages could be seen in most nodules. No growths have been seen from injections of the other strains during a period of 10 months.

Selection and Characterization of Transformed Variants after BPDE Treatment III Although the ten transformed variant cell strains described above expressed increasingly transformed characteristics as a result of BPDE treatment of the five focus-derived strains, followed by more stringent selection, and clonal expansion, they were not neoplastically transformed. Therefore, cell strains, i.e., IF1-IIA1, IF1-IIAP, IF2-IIF1, and IF2-IIF2, were exposed to 0.18 μM BPDE (BPDE Treatment III, Fig. 2),

Figure 6. Focus forming ability of I-F1 and I-F2 cells after BPDE treatment II. A week after BPDE treatment, 2 X 10^5 cells were replated into 100-mm-diameter dish and allowed to grow in medium containing 2% SCS for a week and then 1% SCS for 4 weeks before being stained with methylene blue. (A) Confluent multilayers in a dish containing 0.18 μm BPDE treated I-F1 cells; (B) Foci generated in a dish containing progeny of I-F2 cells treated with the 0.18 μm BPDE; (C) Phase-contrast photomicrograph of a focus formed in I-F2 cells after 0.18 μm BPDE treatment. Magnification 100X.

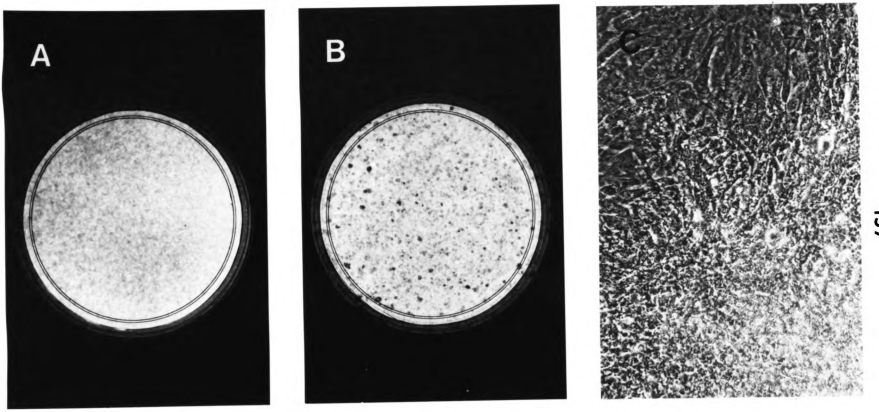

Figure 6

Figure 7. Colony formation in 0.33 agarose with 2% FBS by strain I-F2 after BPDE treatment II. A week after BPDE treatment, 5 X 10^4 cells were plated into 60-mm-diameter dishes with 0.33% agarose containing 2% FBS and allowed to grow for 3 weeks. (A)Large spherical colony and other smaller spherical colonies; (B)Large irregular-shape colony and smaller spherical colonies. Magnification 100X.

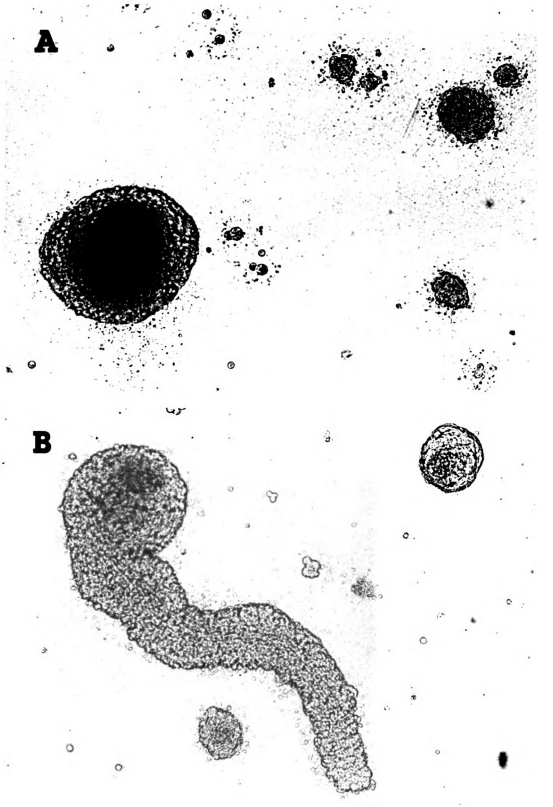

Figure 7

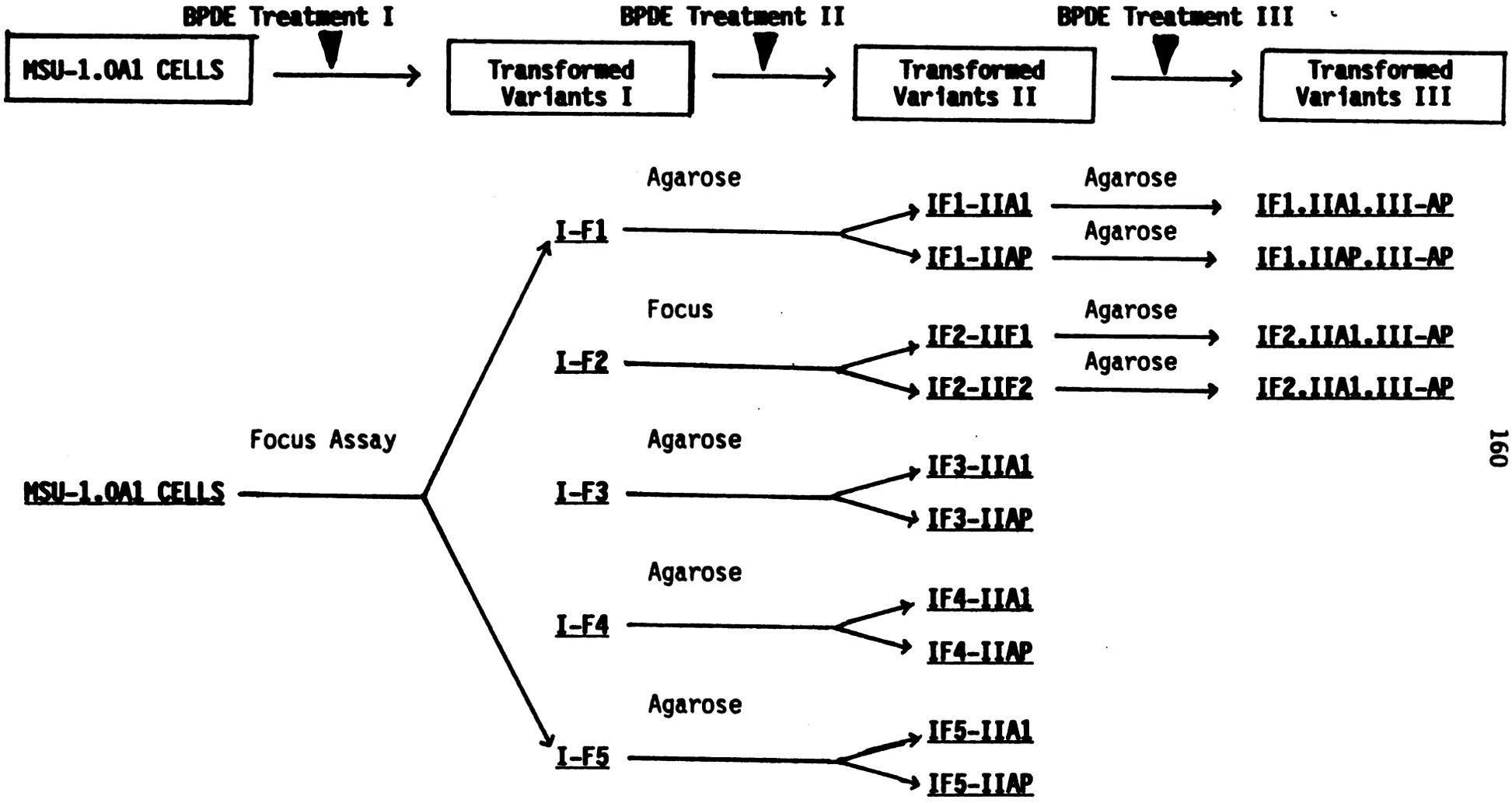

Figure 8. Diagrammatic representation of derivation MSU-1.0A1 transformed variants after sequential treatment with BPDE. F = focus-derived cells; A1 = single agarose colony-derived cells; AP = pooled agarose colonies-derived cells. See Fig. 2 for details of selection conditions used in each assay.

Figure 9. Proliferation of MSU-1.0A1 transformed variants after BPDE treatment II under different culture conditions. Cells were plated in McM medium with 1% SCS. The next day, the number of cells attached was determined, and the medium was replaced with the appropriate medium. Cells were refed on day 4 and counted on the days indicated.

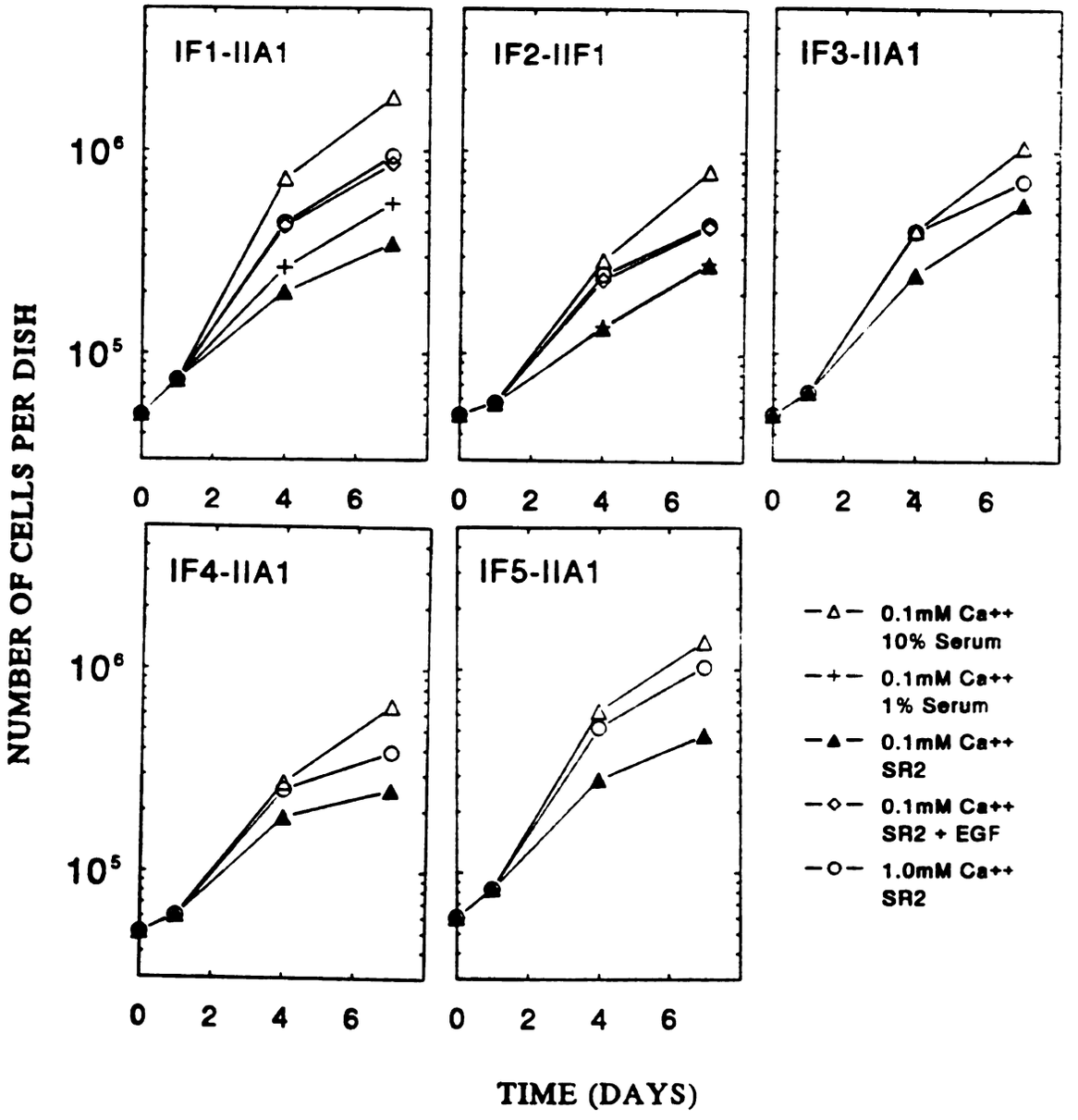
IF1-IIA1
IF2-IIF1
IF3-IIA1
IF4-IIA1
IF5-IIA1
NUMBER OF CELLS PER DISH
10^6
10^5
0 2 4 6
TIME (DAYS)
0.1mM Ca++ 10% Serum
0.1mM Ca++ 1% Serum
0.1mM Ca++ SR2
0.1mM Ca++ SR2 + EGF
1.0mM Ca++ SR2

Figure 9

Table 4. Growth Properties of Transformed Variants from BPDE Treatment II of the Five Focus-derived Strains from BPDE Treatment I

Cell strain	Selection	Growth factor independence[a]	Colonies formed per 10^4 cells with diameters[b] ≥ 80 μm	≥ 120 μm
IF1-IIA1	Agarose,clone	2.25	4552	2820
IF1-IIAP	Agarose,pool	2.26	2120	793
IF2-IIF1	Focus,clone	1.91	740	260
IF2-IIF2	Focus,clone	2.39	140	23
IF3-IIA1	Agarose,clone	3.14	215	130
IF3-IIAP	Agarose,pool	3.11	105	53
IF4-IIA1	Agarose,clone	2.07	88	30
IF4-IIAP	Agarose,pool	ND	95	48
IF5-IIA1	Agarose,clone	2.53	743	128
IF5-IIAP	Agarose,pool	2.78	805	298
MSU-1.1 2C1/T[c]		4.44	1753	1035

[a]Population doublings of cells obtained after 6 days culture in serum-free McM medium containing only 0.1 mM Ca^{2+} and supplemented with SR2. Duplicate dishes were counted after plating 5 X 10^4 cells per dish.

[b]5 X 10^3 cells were plated per dish and duplicate dishes were counted at 3 weeks.

[c]Tumor-derived cells from a focus induced in MSU-1.1 cells by BPDE (12).

and to solvent as a control. After an expression period of 7 days, the cells were assayed for selection of focus-forming cells as well as anchorage independent colonies using still more stringent conditions, i.e., McM medium containing 0.1 mM calcium without serum or growth factors for focus assay and 0.66% agarose system for anchorage independence assay (see Fig. 2). This focus assay was used because most of transformed variants derived after the second BPDE treatment did not grow as rapidly in the medium containing 0.1 mM calcium without serum or growth factors as did malignant derivatives of BPDE-treated MSU-1.1 cells (12). Selection in 0.66% agarose was used because only highly transformed cells are reportedly able to form colonies with this concentration or higher (17-18).

No induction of foci were detected with any BPDE-treated cell strains, because when the cells become confluent they detached and floated away. However, there was good induction of agarose growth in these BPDE-treated cell strains (Table 5). The largest colonies formed in 0.66% agarose from each BPDE-treated cell strain were isolated and pooled (see Fig. 8) and tested for the tumor formation in athymic mice. None of them proved tumorigenic. However, when we re-assayed these cell strains in 0.66% agarose with 2% FBS, the frequency of colonies larger than 120 μm was between 0 to 38 X 10^{-4}, lower than expected. This study will be repeated.

Table 5. Induction of Colony Formation in 0.66% Agarose by BPDE Treatment of Transformed Cell Strains derived from Agarose Colonies and Foci Previously Induced by BPDE

Cell strain	Treatment	Colonies formed per 10^6 cells with diameters[a] ≥ 120 μm	≥ 200 μm
IF1-IIA1	DMSO	2550	50
IF1-IIA1	BPDE	4326	325
IF1-IIAP	DMSO	75	0
IF1-IIAP	BPDE	200	0
IF2-IIF1	DMSO	50	0
IF2-IIF1	BPDE	1100	100
IF2-IIF2	DMSO	850	75
IF2-IIF2	BPDE	2100	275

a. 5×10^4 cells were plated per 60-mm diameter dish with 0.66% agarose containing 2% FBS in 7 days after treatment of 0.18 μm BPDE. 1×10^6 cells were assayed for each determination. Duplicate dishes were counted at 3 weeks.

DISCUSSION

This study was designed to determine if treatment with BPDE, a well-studied, direct-acting mutagen could transform the diploid, karyotypically stable, infinite life-span cell strain MSU-1.0 into malignant state as was done with MSU-1.1 cells (12). The MSU-1.0 cells have in vitro growth properties identical to the parental finite life-span diploid fibroblasts LG1 (10). Therefore, MSU-1.0 cells should be ideal for detecting the phenotypic changes associated with carcinogen exposure and genetic alterations required to make human fibroblasts tumorigenic. After a single BPDE treatment, five cell strains isolated from independent foci grew moderately well in the 0.1 mM calcium without addition of a growth factor or serum, just as the MSU-1.1 cells do, and appeared to be more transformed than the MSU-1.1 cells, since they showed morphological alterations and an increased frequency of colonies in 0.33% agarose with 2% FBS. However, they were not tumorigenic, indicating that a single BPDE treatment of MSU-1.0 cells was not sufficient to convert them into the malignant state.

Some of the transformed variants that expressed increased anchorage independent growth and growth factor independence obtained after the second BPDE treatment and subsequent selection of foci and agarose colonies under more stringent conditions, produced nodular growth in athymic mice immediately after injection which persisted for one to two months and then regressed. Although some tumor-like cells could be seen in slides from such growths, all showed overwhelming evidence of lymphocytic infiltration so that it was not clear if true neoplasms were

present. Further studies will be needed to analyze this situation. The fact that nodular growths have never seen in any untreated control cells or other transformed variants injected may indicate that these cells have some tumor forming potential.

The various BPDE-transformed variants isolated from either foci or agarose colonies varied significantly in their ability to replicate in medium without the addition of growth factors and to produce anchorage-independent colonies in semisolid medium. Since carcinogen-induced mutations are random, mutations in a common gene and/or mutations in various gene(s) or element(s) could account for the differences we observed in the biological properties of the various strains. The present study indicates that transformation of MSU-1.0A1 cells to variants capable of proliferating rapidly in medium without exogenous growth factors requires at least two steps.

Growth factor independence and anchorage independence are generally regarded as the phenotypes that correlate best with the acquisition of tumorigenicity in vivo (4-6, 16, 19-20). Supporting this conclusion is the fact that in the case of BPDE-induced transformation of MSU-1.1 cells (12), the traits that predicted malignant transformation were the ability to replicate rapidly in medium lacking exogenous growth factors and to form large-sized colonies in soft agarose at a high frequency. Only the strains that exhibited these characteristics before being injected into mice produced sarcomas rapidly, suggesting that these two characteristics are essential for the formation of malignant tumors. When compared the transformed variants of MSU-1.0A1 cells after BPDE treatment II and the tumor-derived cells from BPDE-transformed MSU-1.1 cells for these two

features (Table 4), it was clear that only the strains IF1-IIA1 and IF1-IIAP were able to form a higher or similar frequency of ≥ 120 μm colonies compared to the BPDE-transformed MSU-1.1 cells (12, and Table 4). On this basis, one would expect only these two strains to form tumors. However, these strains were not tumorigenic. These results suggest that additional carcinogen treatment and appropriate selection is required to obtain neoplastically transformed cells. If the MSU-1.0A1 derivatives are induced to acquire these transformed traits to same extent as the BPDE transformed MSU-1.1 cells but still prove not to be tumorigenic, one would have to assume that the MSU-1.0 cells need to acquire additional changes not yet selected for, such as inactivation of tumor suppressor gene(s).

Unlike the diploid MSU-1.0 cell strain, MSU-1.1 cells have two marker chromosomes (10) which may result in imbalance of oncogenes and/or tumor suppressor genes since some chromosome fragments have been lost or duplicated. Similar changes have been frequently found in human cancer cells and may have important function in regulation of cell growth, such as short arm and/or long arm of chromosome 1 (21-25), or short arm of chromosome 11 (26-28). There is also good evidence (29-33) for the existence of tumor suppressor gene(s) located at the breakpoint of chromosome 11p15.5 which was involved in the formation of marker chromosome 1 in MSU-1.1 cells (10). This part of chromosome 11 has frequently found to have been deleted in a various types of human cancer (26-29). Preliminary results of chromosome analysis of transformed variants after a single BPDE treatment revealed that, except for strain I-F5, the cell strains have no detectable chromosome changes (i.e., they remain diploid). However, whether the chromosome alterations noted in

MSU-1.1 cells are required for the malignant transformation by oncogene transfection or carcinogen exposure remains to be elucidated.

MSU-1.0 cells represent the first example of a phenotypically normal, diploid human fibroblast strain with an infinite life-span. A similar rodent cell strain was reported, viz., Chinese hamster embryo fibroblast (CHEF) cell line clone 18 (34) which is immortal, karyotypically stable, diploid and has growth properties similar to primary diploid CHEF cells. Neoplastic transformation of this cell line is also a multistep process, since one single carcinogen treatment produced variants that are either anchorage independent or grow in low-serum condition but are non-tumorigenic. An additional treatment of variants with carcinogens, but without selection, yielded tumor producing cell populations (34). The fact that in all cases, when in vitro malignant transformation of human cells has been reported, the cell lines used were aneuploid and/or exhibited other transformed characteristics (4-6, 35-36), makes it difficult to rule out the possibility that the malignant transformation in those cell lines resulted from co-operation of introduced oncogene(s) and unknown pre-existing changes that existed in these cell lines. Therefore, MSU-1.0 cells provide unique material for examining the number and kind of changes required for malignant transformation of human fibroblasts in culture.

The results of present study strongly suggest that the carcinogen-induced malignant transformation of MSU-1.0 cells will require more than three steps. Since the variants acquired various transformed properties as a result of carcinogen insults, but were still not tumorigenic, additional carcinogen treatment and appropriate selection are required to

obtain neoplastic transformation. The nature of the steps involved in the process is as yet unknown. Further studies with these BPDE-induced derivatives of MSU-1.0A1 cells are in progress.

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CHAPTER IV

v-*sis* oncogene-induced transformation of human fibroblasts into cells capable of forming benign tumors

Key Words: Autocrine Transformation/Neoplastic Transformation of Human Fibroblasts/PDGF-B Expression/Suramin/v-*sis* Oncogene

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ABSTRACT

Normal human fibroblasts do not synthesize platelet-derived growth factor (PDGF), but respond to it. However, cells derived from several human fibrosarcomas express PDGF-B. To investigate the role of PDGF in the neoplastic transformation of human fibroblasts, we transfected a plasmid carrying the *v-sis* oncogene, coding for a protein homolog of PDGF-B, into a non-tumorigenic infinite life span cell strain, MSU-1.1, and selected for drug resistance. Six clonal populations that were shown by Southern blotting to be independent transfectants were selected for study. All of them expressed v-*sis* gene mRNA and grew to a higher saturation density than the parental cell strain. Three expressed the gene at relatively low levels and did not exhibit growth factor independence. The other three expressed the v-*sis* gene at a high level, grew rapidly in medium lacking exogenous growth factors, and formed very large colonies in 0.33% agarose. These latter two characteristics were eliminated by suramin, an inhibitor of growth factor binding. When injected into athymic mice, these three cell strains formed benign tumors that histologically are identical to human fibromas. The tumors came up in less than five weeks, and then the majority grew more slowly and eventually stopped growing or regressed. But among the tumors held in the mice for six months, focal areas of malignant cells developed. These experiments provide a unique model for benign tumor formation in tissue of mesenchymal origin in humans.

INTRODUCTION

One of the properties of tumor cells, particularly those in sarcomas, is that they replicate in the body while their nontransformed counterparts are quiescent. In the late 1970's and early 1980's, DeLarco and Todaro and others found that in contrast to normal cells, many tumor-derived cell lines, as well as other transformed cells, secrete specific proteins which they referred to as transforming growth factors (1,2). This led Sporn and Todaro (3) to propose the autocrine hypothesis, namely that tumor cells can induce their own proliferation by secretion of specific protein growth factors for which they have receptors

The finding in 1983 (4,5) that the v-*sis* gene of the simian sarcoma virus is structurally similar to the platelet-derived growth factor B chain gene (PDGF-B) and was, in fact, almost surely derived from it provided further evidence that the expression of various protein growth factors can play a causal role in cell transformation. The PDGF-B gene and the v-*sis* gene code for proteins of similar molecular weight that cross-react immunologically (6). Both proteins are potent mitogens for a variety of cells of mesenchymal origin, including human fibroblasts (7,8). Normal human fibroblasts do not ordinarily express PDGF-B, whereas many human cell lines derived from tumors of fibroblastic origin (fibrosarcomas) (9-11) and others of mesenchymal origin (derived from osteosarcomas) (12,13) express PDGF-B/c-*sis* mRNA and/or protein. These findings suggest that inappropriate expression of a PDGF-B-related gene plays a key role in transforming such cells.

There is considerable experimental evidence with animals or animal

cells in culture to support this hypothesis. For example, newborn marmosets injected intravascularly with SSV develop benign fibroblastic tumors (fibromas), as well as malignant fibrosarcomas (14). Similarly, a retrovirus engineered to express the normal human PDGF-B gene causes fibrosarcomas when injected into newborn mice (15). SSV-transformed rodent mesenchymal cell lines have been shown to secrete into the culture medium a 28 kDa protein identical to or closely related to PDGF-B (16-18), and the level of secreted protein is highly correlated with the ability of the transformed cells to form tumors in athymic mice (18). Transfection of NIH 3T3 mouse cells, an established cell line, with a plasmid containing the cDNA of the human c-*sis* gene (19,20) or a genomic human PDGF-B sequence (21) under the control of viral promoters efficiently transforms these cells into focus-forming cells. Cells derived from these foci can form tumors in athymic mice within three weeks after inoculation (22).

Transfection of the v-*sis* oncogene into finite life span diploid human fibroblasts makes it possible for the cells to grow in medium lacking exogenous growth factors and to form colonies of diameter $\leq$ 80 μm in 0.33% agar (22,23). Addition of exogenous PDGF to normal human fibroblasts seeded in 0.33% agar also permits the cells to form such colonies (24). Nevertheless, these finite life span human fibroblasts that express the v-*sis* gene product do not form tumors, and do not acquire an infinite life span in culture (23,25).

Because transfection of infinite life span NIH 3T3 cells with the human PDGF-B/c-*sis* or the v-*sis* sequence causes neoplastic transformation of NIH 3T3 cells, it was of interest to determine whether such DNA sequences

could cause the neoplastic transformation of an infinite life span human cell strain. To test this hypothesis, we made use of an infinite life span, non-tumorigenic, human fibroblast strain developed in this laboratory (26) and designated MSU-1.1. This cell strain does not express PDGF-B mRNA, grows only moderately in medium without exogenous growth factors, and forms only very small colonies (<80 μm in diameter) in 0.33% agarose and at a low frequency. McCormick and his colleagues showed previously that the MSU-1.1 cells can be malignantly transformed by transfection of activated *ras* genes (27,28).

We transfected the v-*sis* oncogene along with a drug resistance marker into MSU-1.1 cells and selected for drug resistance. The progeny cells of six independent clones that expressed the v-*sis* mRNA were chosen for in-depth study. Three showed high levels of v-*sis* mRNA, grew rapidly in medium without exogenous growth factors, formed large-sized colonies in 0.33% agarose (diameter, 80 to 200 μm) at high frequency and formed tumors in athymic mice. The other three cell strains showed a lower expression of v-*sis* mRNA, had growth characteristics more similar to those of the parental cells, and were not tumorigenic.

MATERIALS AND METHODS

Cells and Cell Culture. The derivation of the MSU-1.1 cell strain has been described (26). These cells express a transfected v-*myc* gene and *neo* gene coding for resistance to Geneticin. They have a normal fibroblastic morphology, a stable, near-diploid karyotype consisting of 45 chromosomes with two marker chromosomes, and are non-tumorigenic (26). The cells were routinely cultured in Eagle's minimal essential medium containing 10% SCS (HyClone, Logan, Utah), penicillin (100 units/ml), streptomycin (100 μg/ml) and hydrocortisone (1 μg/ml)[culture medium]. They were maintained at 37°C in a humidified incubator with 5% CO_2.

RT-PCR Analysis of v-*sis*/PDGF-B mRNA. RNA was isolated using the method of Chomczynski and Sacchi (29) and the integrity of the RNA was determined by electrophoresis using formaldehyde-1% agarose gels. The gels were stained with ethidium bromide. RNA was reverse transcribed into cDNA using 1 μg of purified total RNA, and the cDNA was amplified by the polymerase chain reaction using a modification of the procedure of Yang *et al.* (30) with a final magnesium concentration of 1.88 mM in the PCR reaction. The cDNA of the GAPDH gene was amplified simultaneously. Since the GAPDH gene has a constant expression in these cells, it serves as an internal control to ascertain that conditions for amplification were present in each sample and to correct for small differences in the amount of sample added to each reaction tube (31). The primers used for the v-*sis* and GAPDH genes have been described (32). The number of amplification cycles is noted in each experiment. The PCR products were analyzed on 2% agarose gels and stained with ethidium bromide. The densitometry of UV-

induced fluorescence bands from ethidium bromide stained gels was performed using an Ambis Image system. Primers were synthesized by the Macromolecular Protein Structure Facility at Michigan State University.

DNA Transfection and Selection for Drug Resistance. A recombinant plasmid designated pSSVgpt was used. It contains the v-*sis* gene of SSV provirus inserted into the *Eco*RI site of the pSV2gpt vector (23). The pSV2gpt plasmid was used as a control (33). Cells in exponential growth were transfected with these plasmids using the dimethylsulfoxide/Polybrene method optimized by Morgan et al. (34). The cells were selected in F10 medium supplemented with 10% SCS, mycophenolic acid (25 μg/ml)(Gibco, Grand Island, NY), aminopterin (2 μg/ml), xanthine (250 μg/ml), and hypoxanthine (15 μg/ml). Drug resistant colonies were isolated using trypsin, transferred to individual dishes, and propagated.

Growth Factor Independence Assay. Studies of growth factor requirements were carried out essentially as described (26). The serum replacement supplements used, referred to as SR_3, were those specified by Ryan et al. (35), but lacking EGF and with reduction of the BSA concentration from 5 μg/ml to 0.25 μg/ml to avoid interference with suramin (36). Cells were plated into a series of 60 mm diameter at 5 X 10^4 cells/dish in McM medium (a modified version of MCDB 110) (35) containing 0.1 mM calcium, instead of the usual concentration, i.e., 1.0 mM. The medium was supplemented with 1% SCS. After 24 hr, the number of attached cells was determined in two representative plates, and the medium in the rest of dishes was changed to McM medium containing 0.1 mM or 1 mM calcium and SR_3, or McM medium containing 0.1 mM calcium and 10% SCS. The cells were fed with appropriate medium every 3 days and the number of

cells in duplicate dishes for each condition was determined 4 and 7 days after plating. All experiments were carried out at least twice.

Anchorage Independence Assay. Cells were assayed as described (27) for the ability to form colonies in 0.33% agarose in McM medium containing 2% SCS. All experiments were carried out at least twice.

Suramin. Suramin (Mobay Chemical, New York, NY) was prepared as a stock solution of 100 mg/ml in distilled water and stored at -20°C. The appropriate volume of suramin was added directly to dishes by pipette.

Tumorigenicity Test. BALB/c athymic mice 3-5 weeks of age were injected subcutaneously in the subscapular and/or flank region with 0.2 ml of serum-free medium containing 10^7 cells. Alternatively, absorbable gelatin sponges (Upjohn Co., Kalamazoo, MI) 1 cm^3 in size were implanted subcutaneously in the subscapular region to serve as a matrix, and one week later cells were injected directly into the sponge. Mice were monitored weekly for tumor growth. Tumor diameters were measured weekly using a vernier caliper. In calculating the volume of the tumors, using the formula for the volume of a sphere, we estimated the radius from the average of two diameters measured perpendicular to each other. Portions of the tissue were returned to culture and the cells were grown in medium containing Geneticin to eliminate any mouse cells. Sections of tumors were stained with hematoxylin-eosin and subjected to histopathological analysis.

RESULTS

Transformation of MSU-1.1 Cells by Transfection of a v-*sis* Oncogene. MSU-1.1 cells were transfected with pSSVgpt or pSV2gpt plasmids and selected for mycophenolic acid resistance. The frequency of drug-resistant colonies averaged 5 X 10^{-5} for pSV2gpt and 4 X 10^{-5} for pSSVgpt. The colonies were examined microscopically after three weeks. The majority derived from pSSVgpt transfection were of the same size, morphology, and growth pattern as the colonies derived with the control pSV2gpt plasmid. A few (~13%) were larger in diameter and showed a "star-like" morphology. The cells in both types of colonies had a typical spindle-cell morphology, but in the "star-like" colonies, they were more densely-packed. Cells were isolated from random pSSVgpt-derived colonies and expanded in selective medium. Seven clonal populations, designated A-G, were chosen for Southern blot analysis to determine if the site of integration of the plasmid DNA differed in each population, indicating that they were derived from independent colonies. The results showed that strain C and strain E which were from the same culture dish had identical integration patterns, suggesting they were sibs. Therefore, strain E was eliminated, and six cell strains were studied in greater depth. Three, designated MSU-1.1-*sis* A, B, and C, were derived from the "star-like" colonies. The other three, designated MSU-1.1-*sis* D, F, and G, were derived from normal-appearing colonies. An additional cell strain, designated MSU-1.1-gpt 1, was isolated from a control colony derived from pSV2gpt-transfected cells.

Expression of v-sis mRNA in MSU-1.1-*sis* Cell Strains. We compared the

level of expression of mRNA for the v-*sis* gene using a quantitative assay based on RT-PCR technique (32). As controls, the level of v-*sis*/PDGF-B gene cDNA in the parental MSU-1.1 cell strain and the MSU-1.1-gpt 1 cell strain were also analysed. On the agarose gels after 30 cycles of amplification, all six v-*sis*-transformed MSU-1.1 cell strains exhibited bands of similar intensity indicative of v-*sis*/PDGF-B and GAPDH. No band for v-*sis*/PDGF-B was detected in the parental MSU-1.1 cells or the control cell strain MSU-1.1-gpt 1. To determine the relative level of v-*sis*/PDGF-B mRNA in each of the six MSU-1.1-*sis* cell strains, we sampled the amplified PCR products after 20 and 25 cycles. After 25 cycles of amplification, the GAPDH band was identical in intensity for all cell strains. However, at 20 cycles, the bands were clearly less intense and varied slightly for each cell strain tested, indicating that the amplification of this gene had been interrupted in the exponential phase. For the v-*sis* gene, no bands were visible after 20 cycles of amplification, but bands of varying intensity were seen for each of the six MSU-1.1-*sis* cell strains after 25 cycles of amplification. The relative expression of v-*sis* mRNA after 25 cycles was normalized using the GAPDH control of the same cell strain which was sampled after 20 cycles (Fig. 1). The results were then normalized by giving a v-*sis* level of 1.0 to the cell strain with the lowest expression (MSU-1.1-*sis*D). The MSU-1.1-*sis* strains differed significantly in the relative levels of expression of v-*sis*/PDGF-B mRNA, ranging from the 1.0 lowest (MSU-1.1-*sis* D) to 3.7 the highest (MSU-1.1-*sis* C) (Table 1, column 2).

Fig 1. Detection of PDGF-B mRNA in v-*sis*-transformed MSU-1.1 cells by RT-PCR. PCR was carried out as described in the Materials and Methods. PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. The number of PCR cycles of amplification is noted. Lane 1, parental MSU-1.1 cells; lane 2, MSU-1.1-gpt 1 cells; lane 3, MSU-1.1-*sis* A cells; lane 4, MSU-1.1-*sis* B cells; lane 5, cells derived from one of their tumors; lane 6, MSU-1.1-*sis* C cells; lane 7, cells derived from one of their tumors; lane 8, MSU-1.1-*sis* D cells; lane 9, MSU-1.1-*sis* F cells; lane 10: MSU-1.1-*sis* G cells.

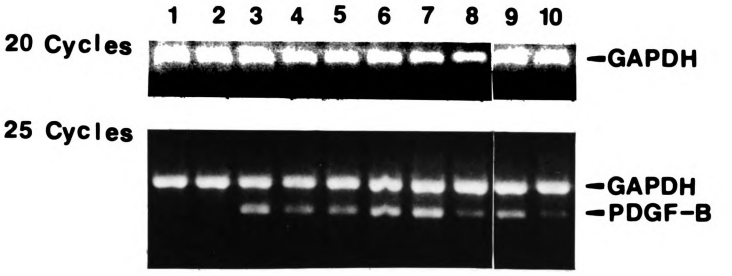

Figure 1

Growth Characteristics of the Six v-*sis*-Transformed Cell Strains. Micro-scopic observation of the MSU-1.1-*sis* cell strains revealed that all six maintained the spindle morphology of the parental MSU-1.1 cells, but the cells isolated from the "star-like" colonies were more refractile. A representative example is shown in Figure 2B. Cells from each strain were compared for the ability to grow to a higher saturation density than the parental MSU-1.1 cells. Cells were seeded into 60-mm diameter dishes and grown to confluence, and the number of cells at saturation was determined electronically. The results are summarized in column 3 of Table 1. Cell strains MSU-1.1-*sis* A-C grew more rapidly and reached a saturation density at least five-fold higher than that of the parental MSU-1.1 cells or of the pSV2gpt-transfected control cells. Figure 2D shows a representative example. The saturation density of strains MSU-1.1-*sis* F and G was only two- to three-fold higher than the parental cells. All six pSSV-transformed cell strains exhibited a normal growth pattern even though they reached a much higher saturation density than the control strain.

To investigate whether the v-*sis*-transformed cell strains could form foci when allowed to grow in a background of parental MSU-1.1 cells, we carried out a reconstruction assay. A total of 200 cells from each of four strains, MSU-1.1-*sis* A-D, along with 33,000 cells of the parental MSU-1.1 cells, were plated into a series of dishes and allowed to grow to confluence. The cultures were refed weekly and stained after three weeks. In the dishes seeded with cells from strains MSU-1.1-*sis* A, B, and C, darkly-stained clones of densely-packed cells (foci) could be seen growing on a lawn of the parental MSU-1.1 cells. Fig 2F gives a representataive example. The number of focus-forming transfectants per dish corresponded

to the number of cells able to form colonies on plastic in the absence of a feeder layer of 33,000 cells. In contrast, MSU-1.1-*sis* D cells formed small, less distinct foci (Fig 2E), while no foci were observed in the dishes seeded with the MSU-1.1 cells alone (data not shown).

Growth Factor Independent Proliferation of MSU-1.1-*sis* Cell Strains. Proliferation of normal diploid human fibroblasts in culture depends on the mitogenic stimulation of exogenous growth factors. Previous studies from this laboratory have shown that in McM medium lacking growth factors and modified to contain only 0.1 mM Ca^{++} instead of 1 mM, MSU-1.1 cells show a modest rate of growth, and respond to the addition of serum or 1 mM Ca^{++} since by replicating more rapidly (26). We compared the six v-*sis*-transformed cell strains for their ability to proliferate in the McM medium containing 0.1 mM Ca^{++} and SR_3, as well as two other conditions, viz., McM medium with 1 mM Ca^{++} in the presence of SR_3, and McM medium containing only 0.1 mM Ca^{++}, but supplemented with 10% SCS. Under all three conditions, cell strains MSU-1.1-*sis* D, F, and G showed a rate of growth similar to that of the parental MSU-1.1 cells or to the control cell strain derived from a pSV2gpt-transfected cell. An example of the growth of these cells is shown in Fig. 3B. Cell strains MSU-1.1-*sis* A, B and C were able to replicate in McM medium containing only 0.1 mM Ca^{++} but supplemented with SR_3 as rapidly as the parental MSU-1.1 cells did in the same medium supplemented with 10% SCS, or in McM medium containing the normal 1.0 mM Ca^{++} concentration with SR_3. They responded only minimally to the addition of serum or calcium. A representative example of this group is shown in Fig. 3C. As a control, we also tested a cell strain previously shown to be growth factor independent, but not to express PDGF-

B mRNA[4]. This cell strain, designated MSU-1.1-H-*ras* 10, was derived from an H-*ras* oncogene-transformed MSU-1.1 cell. The results of these comparsions are summarized in column 4 of Table 1.

Effect of Suramin or Proliferation. Recent investigations have demonstrated that a polyanionic drug, suramin, can disrupt growth factor-receptor interactions and thereby inhibit the transformed phenotypes dependent on autocrine function (7,25,36). To determine whether the transformation of MSU-1.1-*sis* cell strains to growth factor independence was dependent on such interaction, we exposed the six cell strains, the parental MSU-1.1 cells, the H-*ras* oncogene-transformed MSU-1.1 cell strain, and the pSV2gpt control cell strain, to medium containing suramin at 100 μg/ml or 200 μg/ml and compared their ability to replicate in McM medium containing only 0.1 mM Ca^{++} and SR_3. Suramin had no effect whatsoever on the morphology of any of these cell strains (data not shown). Representative results are shown in Fig.3, dashed lines. In the presence of 100 μg/ml suramin, the parental MSU-1.1 cells and MSU-1.1-*sis* G and B cells underwent only 1 to 2 population doublings in seven days in McM medium containing only 0.1 mM Ca^{++} and supplemented with SR_3. Only partial inhibition of proliferation was observed in the MSU-1-1-H-*ras* 10 cells. A concentration of 200 μg/ml of suramin was even more effective for the parental MSU-1.1 cells, MSU-1.1-*sis* G and B cells, but did not affect these H-*ras*-transformed MSU-1.1 cells (data not shown). The growth-inhibiting effect of suramin was fully reversible by removing the drug (data not shown). These results suggest that suramin specifically blocks the action of growth factors and does not act as a nonspecific toxic agent.

Fig. 2. Growth characteristics of v-*sis*-transformed MSU-1.1 cells. Morphology of the parental MSU-1.1 cells (A) and the MSU-1.1-*sis* B cells (B) under the phase-contrast microscope (microscopic magnification 50X). Appearance of confluent cultures of the parental MSU-1.1 cells (C) and MSU-1.1-*sis* B cells (D). The cell strains were plated into 60-mm-diameter dishes, grown to saturation, stained with crystal violet, and photographed. Focus formation by MSU-1.1-*sis* D cells (E) and MSU-1.1-*sis* B cells (F). A total of 33,000 parental MSU-1.1 cells were seeded into 100-mm--diameter dishes, and then 200 v-*sis* transformed cells of either strain were added to the dishes. The cells were allowed to grow for three weeks, and stained with methylene blue, and the dishes were photographed.

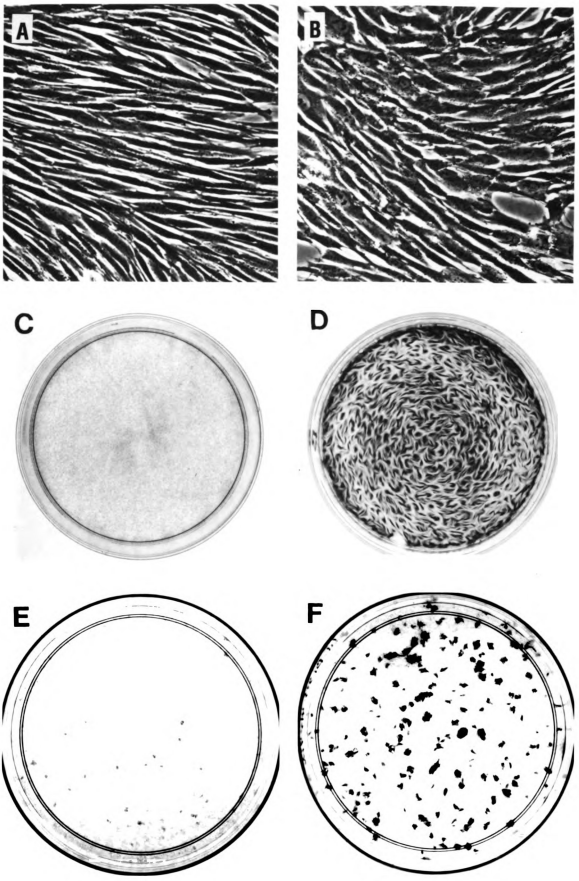
A
B
C
D
E
F

Fig. 3. Growth factor independence of v-*sis*-transformed MSU-1.1 cells, and the effect of suramin on their proliferation in medium lacking exogenous growth factors. Parental MSU-1.1 cells (A), MSU-1.1-*sis* strains G (B), MSU-1.1-*sis* B (C), and MSU-1.1-H-*ras* 10 cells (D). Cells were plated in McM medium with 1% SCS. The next day the number of cells attached was determined, and the medium was replaced with either McM medium modified to contain only 0.1 mM Ca^{++} and supplemented with SR_3 in the absence of suramin (●) or presence of 100 μg/ml of suramin (○); or into McM medium containing 1 mM Ca^{++} and supplemented with SR_3 (▲); or into McM medium with 0.1 mM Ca^{++} but supplemented with 10% SCS (▪). Cells were refed with the appropriate medium on day 4 and counted in duplicate on the days indicated.

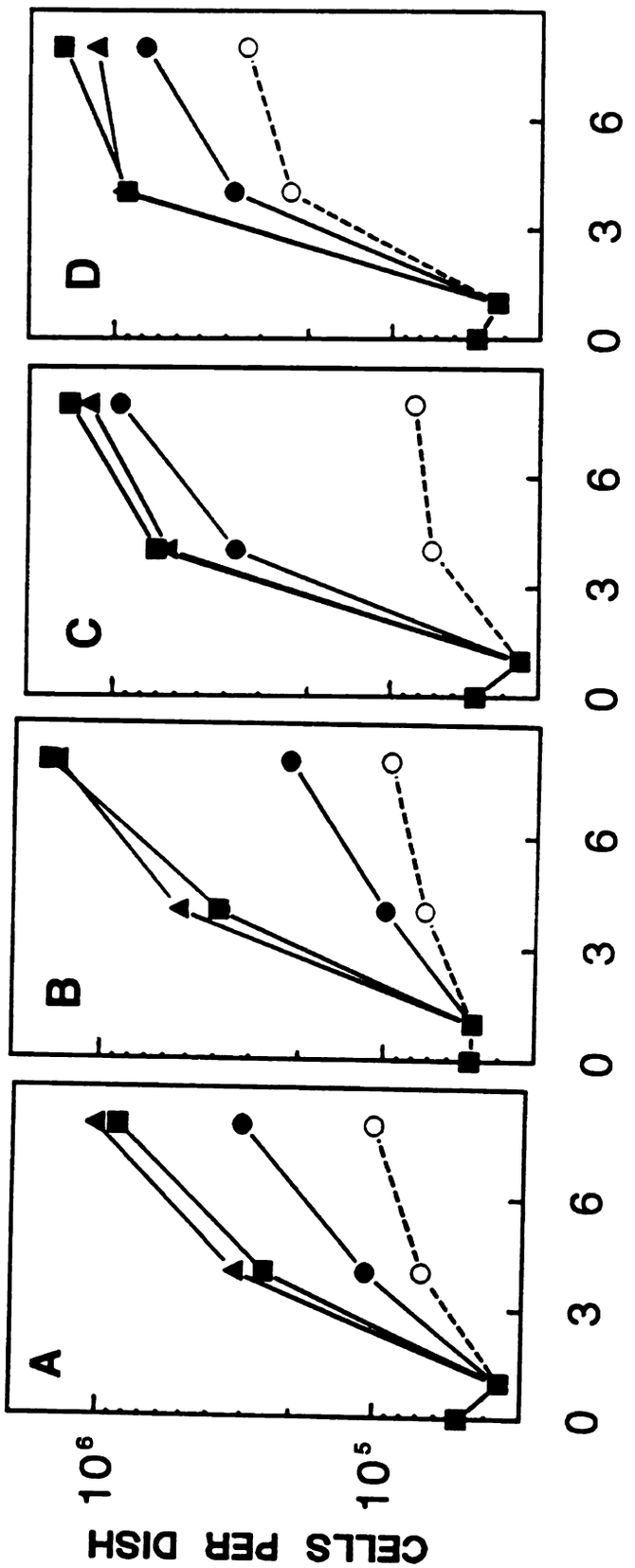

Figure 3

Fig. 4. Effect of suramin on anchorage independent growth of H-*ras*- or v-*sis*-transformed MSU-1.1 cells. A total of 5 X 10^3 H-*ras* cells (A and C) or MSU-1.1-*sis* B cells (B and D) were plated per dish in 0.33% agarose containing 2% FCS and supplemented with 50 μg/ml of suramin (C and D) or not supplemented (A and B). Dishes were refed weekly by removing 1 ml of the medium that overlaid the agarose and adding fresh medium. The dishes were photographed after three weeks of colony growth.

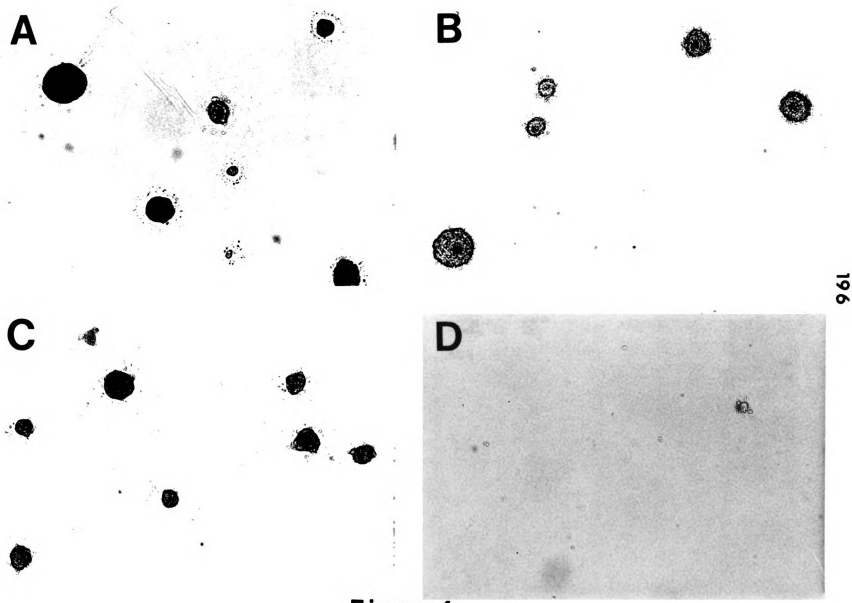

Figure 4

Anchorage Independent Growth of MSU-1.1-*sis* Cell Strains. Anchorage independence, the ability to form colonies in semisolid media, is a characteristic of many tumor-derived cell lines and has been demonstrated to be induced by PDGF in diploid human fibroblasts (24). Therefore, we tested these various cell strains for the ability to form colonies in 0.33% agarose. Cell strains MSU-1.1-*sis* D, F, and G did not grow significantly better than control strain MSU-1.1-gpt 1 or the parental MSU-1.1 cells. Strain MSU-1.1-*sis* A formed small colonies at a low frequency, but strains MSU-1.1-*sis* B and C, as well as the H-*ras*-transformed cell strain, formed large colonies at high frequency (Table 1).

The effect of suramin on agarose growth was also assayed. The results are shown in Table 1, columns 6 and 8. Suramin completely inhibited the ability of MSU-1.1-*sis* strains A, B and C to grow in an anchorage-independent manner, but failed to inhibit colony formation by the MSU-1.1-H-*ras* 10 cells in agar. A representative example of such data is shown in Figure 4.

Tumor-Forming Ability of MSU-1.1-*sis* Cell Strains. To see if a correlation existed between the growth properties and expression of v-*sis* mRNA in the six cell strains and their ability to form tumors, the strains were injected subcutaneously into athymic mice or into gelatin sponges implanted subcutaneously into athymic mice. We showed elsewhere that the presence of sponges does not affect the frequency or the histology of tumors in these animals[4]. Therefore, the data were combined except where noted. The parental MSU-1.1 cell strain has been tested extensively for tumorigenicity by subcutaneous injection of 10^7 cells into athymic mice

Table 1 Characteristics of v-*sis*-transformed MSU-1.1 cell strains

Cell strain	Relative expression of PDGF-B mRNA[a]	Saturation density X $10^{-4}/cm^2$	Growth factor independence[b]	Anchorage independence[c] (colonies per 10^4 cells) ≥ 80 μm		≥ 120 μm	
					+suramin		+ suramin
MSU-1.1	0	11	+	2	10	0	0
MSU-1.1-gpt1	0	15	+	2	8	0	0
MSU-1.1-*sis* D	1.0	--	+	4	0	0	0
MSU-1.1-*sis* F	2.4	23	+	15	20	0	6
MSU-1.1-*sis* G	1.3	32	+	10	5	0	2
MSU-1.1-*sis* A	3.3	65	+++	35	10	0	5
MSU-1.1-*sis* B	3.2	>75	+++	428	0	193	0
MSU-1.1-*sis* C	3.7	>73	+++	336	0	208	0
MSU-1.1-Hras 10	0	>50	+++	288	338	38	16

[a]The relative density of the bands of cDNA in a 2% gel were measured by AMBIS image system and normalized by comparison with the GAPDH internal control. The values were then expressed as a ratio normalized to the amount obtained in strain MSU-1.1-*sis* D.

[b]Duplicate dishes were counted after 7 days culture in serum-free McM medium containing only 0.1 mM Ca^{++} and supplemented with SR_3 .

[c]5 X 10^3 cells per dish were plated in 0.33% agarose. After three weeks, the number of colonies of a given size in 40% of the area of dupicate dishes was determined and these values were used to estimate the total number of colonies in the two dishes using an automated image analyzer.

(26) or injection into sponges implanted on athymic mice and has never given rise to tumors (0 out of >40 injections). The pSV2gpt-transfected MSU-1.1 control strain also did not give rise to tumors in the mice, nor did cell strains MSU-1.1-*sis* D, F, and G (Table 2). In contrast, cell strains MSU-1.1-*sis* A, B, and C readily formed tumors.

The mean tumor volume taken from data on tumors that developed after subcutaneous injection into athymic mice without sponges was plotted against time. The data are shown in Figure 5. The MSU-1.1-*sis* A strain formed small nodules (≤ 5 mm in diameter) which regressed within 4-6 weeks after injection (Fig. 5A). The MSU-1.1-*sis* B and C strains developed medium-sized tumors (7-8 mm in diameter), most of which (62 and 65%) did not continue to increase in size, but also did not regress (Fig. 5B). The time until to the first appearance of tumors among the injections varied from 1 to 4 weeks.

Histopathology of the Tumors. Histopathologic analysis revealed that all tumors exhibited low cellularity with abundant stromal matrix, little or no mitotic activity (< 1 per 10 high-power fields), and no evidence of necrosis, invasion, or angiogenesis. Representative data are shown in Figure 6A and 6B. These characteristics are typical of human benign tumors of fibrous tissue (37). Most tumors produced from strains MSU-1.1-*sis* B and C were moderately differentiated.

Two approaches were used to test if the benign tumors would retain their growth pattern and histology. In the first, some of the tumors formed by cells of strains MSU-1.1-*sis* B and C were kept in mice for a long period of time. In these cases, a few tumors started to grow progressively after more than six months (Table 2). Histopathologically,

Fig. 5. Growth kinetics of tumors formed by v-*sis* transformed MSU-1.1 cells in athymic nude mice. Mean tumor volume was calculated by measuring the tumors weekly and calculating the volume as described in the Materials and Methods Section. (A) A small regressing nodule ($\leq$ 5 mm in final diameter) formed by the MSU-1.1-*sis* A cell strain; (B) Slow growing, static tumors produced by the cell strains MSU-1.1-*sis* B (▲) and MSU-1.1-*sis*-C (+); Note the different scale for tumor volume (mm^3) used in each graph.

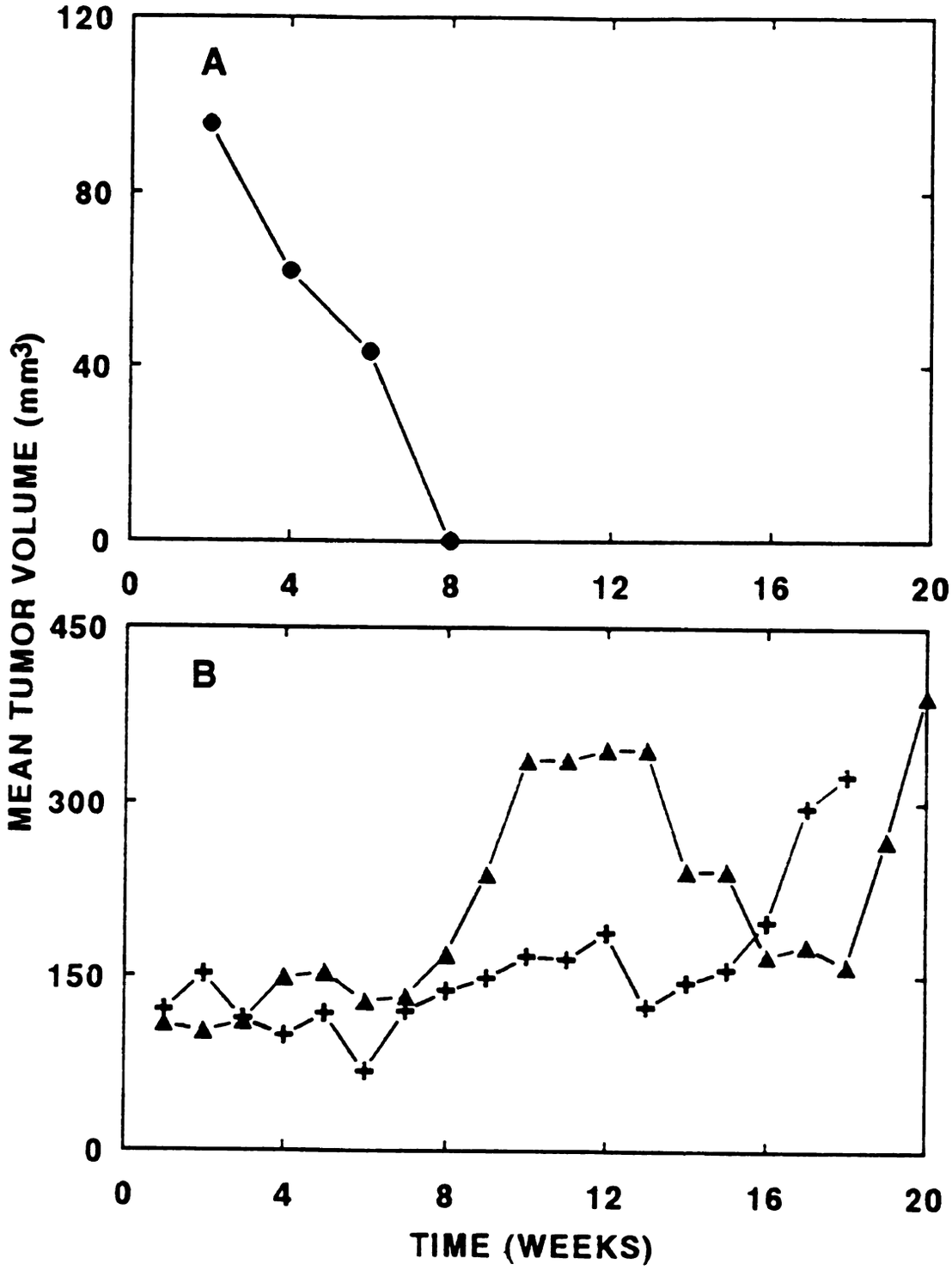
A
B
120
80
40
0
450
300
150
0
0
4
8
12
16
20
MEAN TUMOR VOLUME (mm3)
TIME (WEEKS)

Figure 5

Fig. 6. Histology of stained-sections from tumors formed by v-*sis*-transformed MSU-1.1 cells. Typical spindle cell fibroma formed by MSU-1.1-*sis* B strain (A and B). Typical low-grade spindle cell sarcoma formed by MSU-1.1-*sis* B removed 10 months after first appearance (C and D). Typical benign tumor formed by MSU-1.1-*sis*-C strain and removed three months after injection of late passage cells. Note the focal aggregates, "pearls", of round cells typical of high grade sarcomas. Magnification 100X in A, C, and E, and 200X in B, D, and F.these tumors were characterized by increased cellularity with some stromal matrix, increased anaplasia, and some mitotic activity, but exhibited no evidence of necrosis or invasion. (Examples are shown in Fig. 6C and 6D.) These tumors were classified as low grade spindle cell sarcomas.

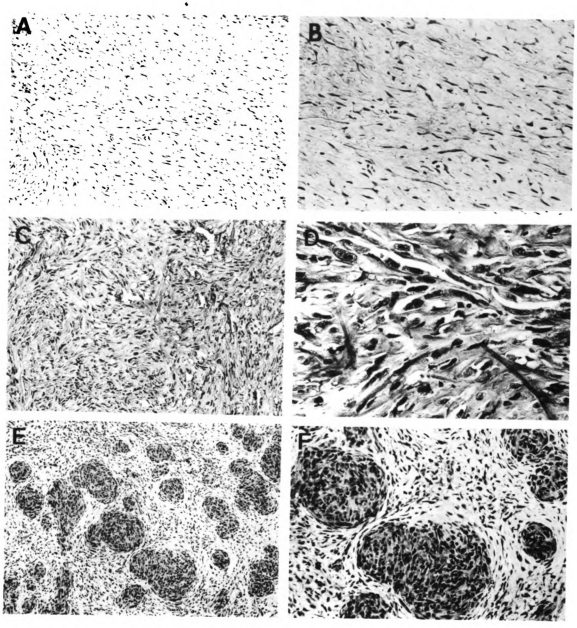

Figure 6

these tumors were characterized by increased cellularity with some stromal matrix, increased anaplasia, and some mitotic activity, but exhibited no evidence of necrosis or invasion. (Examples are shown in Fig. 6C and 6D). These tumors were classified as low grade spindle cell sarcomas.

The second approach used cells of later passage. When cells from strains MSU-1.1-*sis* B and C were passaged for an additional 20 to 30 population doublings before injection (i.e., 55 to 65 population doublings since clonal isolation), four tumors formed by strain MSU-1.1-*sis* B in 16 injection sites and eight tumors formed by strain MSU-1.1-*sis* C in 24 injection sites were found to be hetero- geneous (Table 2). Either large areas or focal aggregates of cells showing features of poorly differentiated, low or high grade spindle or round cell sarcomas presented in those predominantly benign tumors. (Examples are shown in Fig. 6E and 6F). Such heterogeneity was found even if the tumors were removed shortly after injection (Table 2). The rest of tumors formed by each strain were identical to those tumors formed by injection of early passage cells. The spontaneous development of low or high grade malignant tumors was related to the length of time the cell strain was propagated *in vitro* after clonal isolation or the length of time the animal was held after the tumor was observed.

PDGF-B Expression in the Cells Derived from Tumors. RT-PCR analysis of PDGF-B mRNA was also carried out on some of the tumor-derived cells that were obtained by culturing portions of the tumors in medium containing the appropriate selective agent (Geneticin or mycophenolic acid). The level of PDGF-B mRNA expression of cells taken from one benign tumor derived from strain MSU-1.1-*sis* B, one benign tumor derived from strain MSU-1.1-

sis C as well as two low grade spindle cell sarcomas was determined. Examples are shown in Fig. 1, lanes 5 and 7. The PDGF-B mRNA levels in these tumor-derived cells were comparable to those in their respective mycophenolic acid-resistant cell strains, indicating that *in vivo* selection of cells expressing higher (or lower) levels of the v-*sis* oncogene had not occurred.

Table 2 Tumorigenicity of v-*sis*-transformed MSU-1.1 cell strains

Cell strain	Tumor incidence[a]	Pattern of tumor growth	Population doublings of cells injected	No. of tumors with specific histology and in parenthesis, the time of removal[b]				
				B	B+FL	B+L	L	B+FH
MSU-1.1-*sis* D	0/5	-	25-65	-	-	-	-	-
MSU-1.1-*sis* F	0/13	-	25-65	-	-	-	-	-
MSU-1.1-*sis* G	0/13	-	25-65	-	-	-	-	-
MSU-1.1-*sis* A	9[c]/10	Small nodules that regressed	25-65	1 (52 wk)	-	-	-	-
MSU-1.1-*sis* B	6/8	Slow/static	25-35	3 (6-9 wk)	-	-	-	-
			25-35	-	1 (29 wk)	2 (45-52 wk)	-	-
MSU-1.1-*sis* B	9/16	Slow/static	55-65	5 (6-16 wk)	-	-	-	-
			55-65	-	-	3 (16 wk)	1 (15 wk)	-
MSU-1.1-*sis* C	7/13	Slow/static	25-35	4 (7-26 wk)	-	-	-	-
			25-35	-	2 (40-56 wk)	1 (37 wk)	-	-
MSU-1.1-*sis* C	17/24	Slow/static	55-65	9 (5-29 wk)	-	-	-	-
			55-65	-	-	2 (4wk)	1 (17wk)	5 (13-29wk)

[a]Ratio of sites with tumors to sites of injection. 10^7 were injected subcutaneously into sites containing a sponge or not containing a sponge implant. Animals were examined for tumor formation weekly for up to 12 mo. after injection.

[b]Tumors were classified as benign (B); benign containing small focal aggregates of low grade malignancy (B+FL); distinct adjacent areas of benign and low grade malignancy (B+L), low grade malignant tumor (L), and benign containing small focal aggregates of high grade malignancy (B+FH).

[c]Cells were also injected into nine additional sites containing sponge implants. However, only the data from sites lacking sponge implants were used for this Table because the presence of the sponges, which took six to eight weeks to disintegrate, made it difficult to detect small nodules that later regressed.

DISCUSSION

Although the transforming ability of the PDGF-B/*sis* gene is well established in experimental animal and animal cell culture systems, its role in the transformation of human cells has not been clear. In the present investigation we demonstrate for the first time the neoplastic transformation of human fibroblasts by the v-*sis* oncogene. The v-*sis* transformed cells that expressed the highest levels of v-*sis* mRNA, i.e., strains A, B and C, readily formed benign tumors in athymic mice, and some of these tumors spontaneously progressed to malignant tumors. Those that expressed v-*sis* mRNA at a lower level did not form tumors. The data suggest that the expression of the PDGF-B gene can play a causal role in the transformation of human fibroblasts and other cells with receptors for the PDGF-B protein, and the experiments provide a unique model for the study of benign tumors in humans. Further support for the conclusion that expression of PDGF-B in MSU-1.1 cells causes them to form benign tumors comes from recent results in this laboratory in which MSU-1.1 cells were exposed to a single dose of a chemical carcinogen and selected for focus formation. One of the cell strains produced was found to express mRNA of the PDGF-B gene at a high level and also to produce characteristic benign tumors (38). It is, perhaps, not surprising that strains MSU-1.1-*sis*-D, F and G, which exhibited the lowest level of expression of v-*sis* mRNA, were not tumorigenic and exhibited *in vitro* characteristics intermediate between the parental MSU-1.1 cells and their tumorigenic counterparts, strains A, B and C.

In previous experiments in which the v-*sis* oncogene was transfected

into finite life span human fibroblasts (22), no tumors were produced even though the cells were expressing the v-*sis* gene and showed growth factor independence. One possible explanation for the failure to obtain tumors in those experiments is that by the time the progeny population isolated from an individual v-*sis* transfected clone had been propagated to the size needed for testing tumorigenicity , the cloned cells were already nearing the end of their finite life span and could not proliferate sufficiently *in vivo* to form a tumor, as suggested earlier (26). If so, then an infinite life span target cell is required. This conclusion is supported by results of Hurlin et al. (39) and Wilson et al. (40) who showed that transfection of finite life span human fibroblasts with *ras* oncogenes produced cell strains that exhibited many of the characteristics of tumor-derived malignant cells, but senesced at the usual time and did not form tumors. When they transfected the same *ras*-containing plasmid into the infinite life span MSU-1.1 cells, the cells became malignant (27, 28).

It must be noted, however, that H-*ras*- and N-*ras*-containing plasmids that are able to transform MSU-1.1 cells to malignancy show the following qualities: they have a mutation in codon 12 or 61, a substitute-promoter that is not physiologically regulated, and a combination of promoters and enhancers engineered for high expression (27,28). Transfection with plasmids carrying mutated *ras* oncogenes expressed at a low level from their endogenous promoters does not yield malignant MSU-1.1-transfectants (McCormick et al., unpublished data). This fact indicates that in order to be malignantly transformed MSU-1.1 cells require two, or more likely, three additional genetic changes (41).

From our present studies with the v-*sis* oncogene we conclude that if

MSU-1.1 cells were to be transformed into cells capable of forming benign tumors by a method other than pSSVgpt transfection, they would require one, or more likely, two changes in the control of expression of their endogenous PDGF-B gene, one to cause expression and one to upregulate the expression level. This conclusion is based on the fact that mRNA from the transfected v-*sis* oncogene had to be synthesized at a critical level in order for the strains to be able to form benign tumors.

It should also be pointed out that MSU-1.1 cells have a stable karyotype, but have undergone several chromosome changes and differ in several ways from the diploid infinite life span cell strain, MSU-1.0 from which they were derived (26). Unlike MSU-1.0 cells, they are able to grow slowly in medium lacking exogenous growth factors. The results of the present study in which the moderate growth of MSU-1.1 cell strains in growth factor-free medium could be inhibited by suramin (Fig. 3A) suggests that they synthesize some growth factor that is secreted.

In the present experiments, the benign tumors came up quickly in the athymic mice (reached 5 mm in diameter in less than 5 weeks). If the cells that were injected had undergone more than 55 population doublings after clonal isolation, or if the tumors were held in the animal more than six months, there was a good chance of finding areas of malignancy developing within the benign tumor. This was not the case with early passage cells or tumors removed earlier, suggesting that an additional event (change) is required if MSU-1.1 cells expressing PDGF-B at a level high enough to form benign tumors are to become malignant, and that the chance of this occurring increases as the cell population doublings increase.

A spectrum of soft tissue lesions ranging from reactive to benign to

malignant tumors are found in humans. A comparison of the expression of growth factors and receptors in 69 human soft tissue tumors shows that expression of both single and multiple growth factors, as well as growth factor receptors, is significantly more frequent in malignant than in benign tumors (13). The data presented here support those results since we found that synthesis of PDGF-B at a high enough level is sufficient to cause transformation to cells capable of forming benign tumors, and these transformed cells, in turn, can become malignant after undergoing an additional spontaneous change(s).

To date there have been only few reports of experimental induction of benign tumors. Examples include benign tumors (fibromas and gliomas) seen after injection of SSV in newborn marmosets (14), and the fibromas induced in rabbits by the Shope fibroma virus (SFV) (42), a tumorigenic poxvirus which encodes gene products homologous to the family of EGF and TGF-α (43). In transgenic mice, expression of the *int*-2 gene, which encodes a growth factor of the FGF family, leads to benign hyperplasia of the mammary and prostate glands (44). Transplants of normal mouse keratinocytes infected in culture with the Harvey murine sarcoma virus result only in benign tumors (papillomas) (45). In the case of a spontaneously immortalized human skin keratinocyte cell strain (HaCaT), expression of H-*ras* oncogene resulted in varying degree of growth potential *in vivo*, ranging from benign to malignant tumors (46).

Benign tumors are frequently the precursors of malignant tumors. For example, individuals with polyposis coli have multiple benign colonic polyps, one or more of which gives rise to a malignant tumor if the colon is not removed (47). Another example is individuals with dysplastic nevus syndrome (DNS) that have a high frequency of benign melanotic skin lesions

that frequently give rise to cutaneous malignant melanomas (48). The present study clearly indicates that sarcomas can also arise from benign tumors. It will be of interest to isolate the spontaneously developed malignant cells from the heterogenous tumors we obtained composed of benign and malignant cells, and characterize them for the additional change(s) that have occurred. It will also be important to expose the benign tumor cells we obtained to various carcinogens to induce malignant conversion and analyze the nature of the events that are responsible. Such studies are in progress in this laboratory.

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APPENDIX I

Additional Results for Cell Strains described in Chapter II

The results of RT-PCR analysis for PDGF-B and of the detection of $p110^{Rb}$ protein by immunoprecipitation in the BPDE-transformed MSU-1.1 cell strains were briefly described in the paper of Chapter II, but the figures could not be presented in the paper published in PNAS because of the severe space limitation of the Journal. The eight BPDE transformed MSU-1.1 cell strains were analyzed for PDGF-B and TGF-α mRNA using PCR technique described in the Chapter II and the result of PDGF-B mRNA expression is shown in Fig.1A. One cell strain (lane 5) expressed an increased level of mRNA for PDGF-B. The amount expressed is similar to that of the human fibrosarcoma derived cell line HT1080 (lane 2). Four cell strains (lanes 4, 6, 7, 10) had a slight increase in mRNA expression for PDGF-B. The other three strains (lanes 8, 9, 11) did not express PDGF-B mRNA. The parental MSU-1.1 cells (lane 3) also did not express it. [The primers used for PDGF-B were: sense primer 5'-GAAGGAGCCTGGGTTCCCTG-3' and anti-sense primer 5'-TTTCTCACCTGGACAGGTCG-3'. Primers used for amplification of TGF-α specific sequences were GTATTGTGTTGGCTGCGTGC (residues 75-94;sense strand) and CACTGTTTCTGAGTGGCAGC (residues 492-511;antisense strand). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a gene whose mRNA level is consistent in the cells, was amplified by using the sense-strand primer GGTGAAGGTCGGAGTCAACG (residues 324-343) and the antisense-strand primer CTTCTGCATGGTGGTGAAGA (residues 2470-2489)].

Each of the eight focus-derived cell strains, as well as cells derived from tumors formed by the strains 3C1, 3C10 and 4C5 were tested

for level of expression of $p110^{Rb}$ protein using immunoprecipitation, and results are shown in Fig. 1B. Normal sized 110 Kd Rb protein was seen in most of the focus-derived strains and tumor-derived strains, at a similar level to those of the parental MSU-1.1 cells (lane 1) or the T24 H-ras transformed MSU-1.1 cells (lane 15). However, a reduced expression of $p110^{Rb}$ protein was observed in strain 3C10 and its tumor-derived cells (lanes 9 and 10), as well as in strain 4C5 and its tumor-derived cells (lanes 12 and 13). This is consistent with the loss of a copy of chromosome 13 that harbors the tumor suppressor gene Rb in these two focus-derived strains. Further studies to determine whether the remaining Rb gene product is functional in these strains are in progress.

To see if BPDE caused a change in the expression of a ras proto-oncogene and whether such an activation played a role in tumor formation, we utilized the McAb Y13-259, which reacts efficiently with the product of all three human ras genes, to detect the expression of $p21^{ras}$ proteins using immunoprecipitation and to localize $p21^{ras}$ proteins in histological sections from tumor tissues using immunohistochemical staining. As shown in Fig. 1C, most of the strains did not exhibit alterations in $p21^{ras}$ proteins expression. However, there was an extra band (lane 6, see arrow) that migrated to same position as the overexpressed T24 H-ras oncogene transfected MSU-1.1 cells (lane 15), which expressed at much lower level than that of the latter cells. The expression of $p21^{ras}$ proteins in tumor-derived cells from strain 2C1 (lane 5) appeared slightly higher than other strains and MSU-1.1 cells. To examine whether there were higher expression of $p21^{ras}$ in tumor tissues, I carried out immunohistochemical staining of $p21^{ras}$ with McAb Y13-259 (Fig.2). Cells malignantly

transformed by H-ras oncogene stained strongly for $p21^{ras}$ (Fig. 2B) while those malignantly transformed by another oncogene (non-ras gene) were negative (Fig. 2A). Among the carcinogen-transformed strains, the most extensive and intensive staining with antibody was found with a high grade round cell sarcoma from strain 4C5 (Fig. 2C). The pattern of staining in low grade malignant tumors from strains 3C1 and 3C10 was patchy; the focal, nodular aggregates of round cells with features of high grade malignancy stained more strongly than the majority of spindle cells in the fibrous matrix (Fig. 2D). The majority of sections of a high grade spindle cell sarcoma from strain 2C1 also stained strongly for $p21^{ras}$ (data not shown). Titration experiments showed positive staining in high grade malignant tumor sections, including focal areas, with as little as 0.01 ug of Y13-259 antibody, whereas sections of low grade sarcomas stained weakly or not at all at this dilution. No staining was observed with rat IgG in place of Y13-259. The preferential staining for $p21^{ras}$ in these tumors correlates with the grade of malignancy.

However, immunoprecipitation of extracts from the same cells did not exhibit significant qualitative or quantitative alterations in $p21^{ras}$ protein expression (fig. 1C). The reason for this inconsistency is not known. One possibility is the difference in the sensitivity of the technique applied. Alternatively, the positive staining by the anti-Ras antibody could have resulted from a reaction with Ras-related or associated proteins. Further studies regarding Ras expression and the nature of changes which occurred in the focal areas of these tumors are needed.

Figure 1. (A) Detection of PDGF-B mRNA in the BPDE-transformed MSU-1.1 cells by RT-PCR. PCR amplification of PDGF-B was done as described in the Materials and Methods of Chapter IV. PCR products were run on a 2% agarose gel with a 123 base pair DNA ladder and stained with ethidium bromide. Thirty cycles of PCR amplification was carried out.

(B) and (C) Analysis for $p110^{Rb}$ protein and $p21^{ras}$ proteins expression. [^{35}S] Methionine-labeled cell extracts from MSU-1.1 cells, eight focus-derived cell strains, cells derived from tumors formed from strains 3C1, 3C10 and 4C5 (3C1/ST2, 3C10/ST2, and 4C5/T3), and one of the T24 H-*ras* transformed MSU-1.1 cell strain (MSU-1.1-H-*ras* 10) were immunoprecipitated using anti-Rb monoclonal antibody Ab-1 (B) or anti-p21 monoclonal antibody Y13-259 (C) (Both antibodies were from Oncogene Science, Inc., New York). Immunoprecipitates for Rb protein were separated on 8% SDS-polyacrylamide gel, while it was separated on a 12% SDS-polyacrylamide gel for Ras proteins, and then followed by autoradiography. Lane 1, parental MSU-1.1 cells; Lane 2, 1C1 cells; Lane 3, 1C4 cells; Lane 4, 2C1 cells; Lane 5, cells derived from one of their tumors; Lane 6, 2C2 cells; Lane 7, 3C1 cells; Lane 8, cells derived from one of their tumors; Lane 9, 3C10 cells; Lane 10, cells derived from one of their tumors; Lane 11, 4C4 cells; Lane 12, 4C5 cells; Lane 13, cells derived from one of their tumors; Lane 14, T24-H-*ras* transfected MSU-1.1 cells.

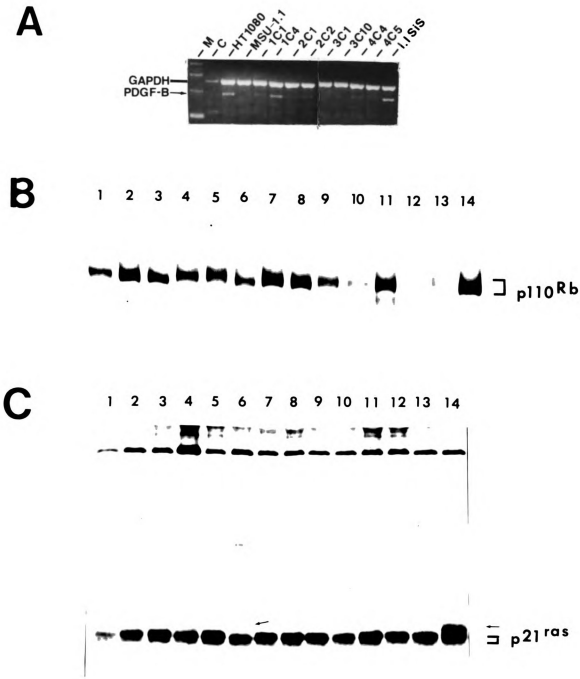
A
M
C
HT1080
MSU-1.1
1C1
1C4
2C1
2C2
3C1
3C10
4C4
4C5
1.1SIS
GAPDH
PDGF-B
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
p110Rb
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14
p21ras

Figure1

Figure 2. Reactivity of monoclonal antibody Y13-259 with tumor tissues tested by the avidin-biotin complex system. (A) Tumor produced by the malignant MSU-1.1 cells transformed by an oncogene not of the ras family, serving as negative control; (B) MSU-1.1 tumor transformed by high expression of an H-ras oncogene; (C) High grade round cell sarcoma formed by the strain 4C5; (D) Tumor removed 3 months after injection of strain 3C1 exhibiting strong reactivity of high grade round sarcoma cells (left), weak staining of scattered spindle cells, and no staining of intervening stroma (right). Magnification was 200 X.

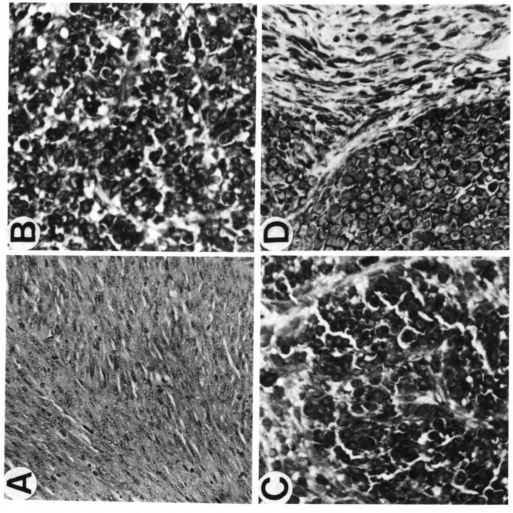

Figure 2

Appendix II

Characterization of agarose colony-derived strain MSU-1.1-*sis* G.A1

A few large agarose colonies (< 1 X 10^{-4} cells) were found in strain MSU-1.1-*sis* G. Several large agarose colonies were isolated from one assay which used 0.66% agarose with 2% FBS and the cells were propagated into large populations. One single colony-derived strain was used for further study and was named as MSU-1.1-*sis* G.A1. Characterization of this cell strain was carried out in the same experiments as shown in Chapter IV, and the data are presented together here for easy comparison. In contrast to its parental strain MSU-1.1-*sis* G, these agarose-derived cells formed a very high frequency of large colonies in 0.33% agarose with 2% FBS and also grew rapidly in serum-free medium without added growth factors (Table 1, Fig.1). Suramin did not completely inhibit the proliferation of agarose-derived strain MSU-1.1-*sis* G.A1 in 0.1 mM Ca^{++} McM medium and SR_3 (Fig. 1). However, suramin inhibited significantly the colony formation of these cells in agarose (Table 1).

All sites injected with strain MSU-1.1:*sis* G.A1 formed tumors that reached a 10 mm-diameter within 3 weeks after injection (Table 2). Unlike the strain MSU-1.1-*sis* A which formed small nodules that regressed (Fig.2A), or the slow and static growth of tumors formed by strains MSU-1.1-*sis* B and C (Fig. 2B), the tumors formed by strain MSU-1.1:*sis* G.A1 grew to 15 mm-diameter, then stopped growing, and maintained that size (Fig. 2C). The latent periods to the first appearance of tumors was about 1 to 2 weeks.

Table 1 Characteristics of MSU-1.1-*sis* G.A1 and other v-*sis*-transformed MSU-1.1 cell strains

Cell strain	Relative expression of PDGF-B mRNA[a]	Saturation density X $10^{-4}/cm^2$	Growth factor independence[b]	Anchorage independence[c] (colonies per 10^4 cells) ≥ 80 μm		≥ 120 μm	
					+suramin		+ suramin
MSU-1.1-*sis* G	1.3	32	+	10	5	0	2
MSU-1.1-*sis* G.A1	2.4	>55	+++	738	75	628	20
MSU-1.1-*sis* A	3.3	65	+++	35	10	0	5
MSU-1.1-*sis* B	3.2	>75	+++	428	0	193	0
MSU-1.1-*sis* C	3.7	>73	+++	336	0	208	0
MSU-1.1-Hras 10	0	>50	+++	288	338	38	16

[a]The relative density of the bands of cDNA in a 2% gel were measured as described in Chapter III.

[b]Duplicate dishes were counted after 7 days culture in serum-free McM medium containing only 0.1 mM Ca^{++} and supplemented with SR_3 .

[c]5 X 10^3 cells per dish were plated in 0.33% agarose. After three weeks, the number of colonies of a given size in 40% of the area of duplicate dishes was determined and these values were used to estimate the total number of colonies in the two dishes using an automated image analyzer.

Figure 1. Growth factor independence of MSU-1.1-*sis* G.A1 and one of its tumor-derived cells, and the effect of suramin on their proliferation in medium lacking exogenous growth factors. Cells were plated in McM medium with 1% SCS. The next day the number of cells attached was determined, and the medium was replaced with either McM medium modified to contain only 0.1 mM Ca^{++} and supplemented with SR_3 in the absence of suramin ( ) or presence of 100 μg/ml of suramin ( ); or into McM medium containing 1 mM Ca^{++} and supplemented with SR_3 ( ); or into McM medium with 0.1 mM Ca^{++} but supplemented with 10% SCS ( ). Cells were refed with the appropriate medium on day 4 and counted in duplicate on the days indicated.

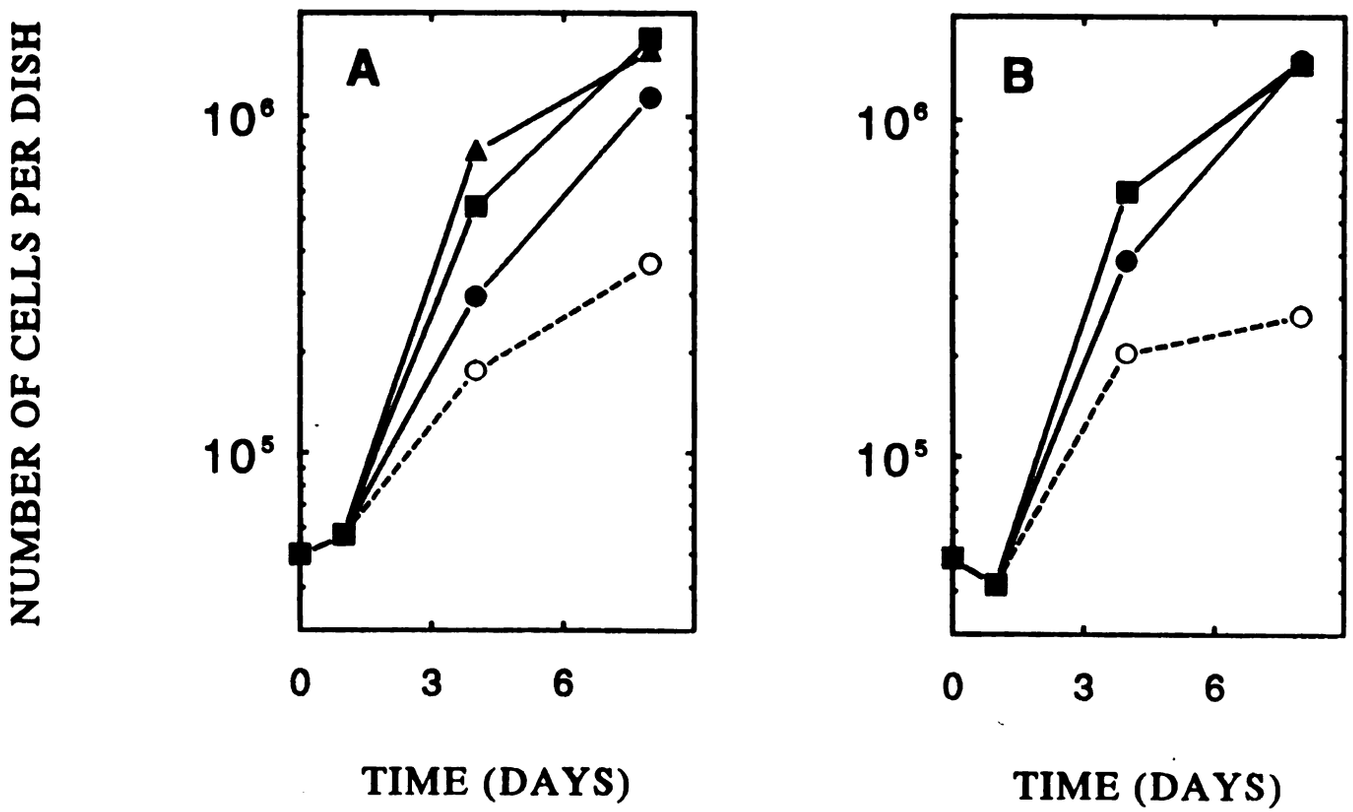

Figure 1

Histopathologic analysis revealed that all tumors exhibited low cellularity with abundant stromal matrix, very rare mitotic activity (< 1 per 10 high-power fields) and no evidence of necrosis, invasion, or angiogenesis. These characteristics are similar to the typical benign tumors produced by strains of MSU-1.1-*sis* B and C (Fig.3). The tumors produced from strain MSU-1.1:*sis* G.A1 were well differentiated. Some of the tumor-derived cells that were obtained by cultures of portions of tumors in medium containing Geneticin and/or mycophenolic acid were reinjected subcutaneously into athymic mice. As shown in Fig. 3 F and G, tumors generated from reinjection of tumor-derived cells from strain MSU-1.1-*sis* G.A1 made up of two distinctive areas. One consisted of the well differentiated spindle cell fibroma identical to the tumor shown in Fig. 3A and B and the other a high grade spindle cell sarcoma with no stromal matrix and high mitotic activity (lower part, Fig. 3G and H).

The RT-PCR analysis for PDGF-B mRNA was also carried out on MSU-1.1-*sis* G.A1 strain and cells obtained from one benign tumor derived from this strain. The PDGF-B mRNA level in the MSU-1.1-*sis* G.A1 was higher than that of the parental MSU-1.1-*sis* G (Table 1). However, the tumor-derived cells were comparable to that of MSU-1.1-*sis* G.A1 cells, indicating that *in vivo* selection of cells expressing higher (or lower) levels of the v-*sis* oncogene did not occur (data not shown)(Note that the reinjection of this tumor-derived cell gave rise to mixed benign and malignant tumors.).

These results suggest that a spontaneous event or events occurred in the strain MSU-1.1-*sis* G.A1, which make this strain capable of producing a large benign tumor in athymic mice. In addition, some of these tumor-

derived cells seem to have acquired another change(s) allowing them to produce malignant tumors. These data suggest that treatment of v-*sis* transformed MSU-1.1 cells with carcinogen or transfection of suitable oncogene will cause malignant transformation. Such experiments are in progress.

Table 2 Tumorigenicity of strain MSU-1.1-*sis* G.A1 and its parental strain MSU-1.1-*sis* G strains

Cell strain	Tumor incidence[a]	Pattern of tumor growth	Passage of cells injected[b]	Frequency of tumor with specific histopathology[c]				
				B	B+FL	B+L	L	B+FH
MSU-1.1-*sis* G.A1	4/4	Fast/Progressive	Early	4 (7-29W)	-	-	-	-
MSU-1.1-*sis* G.A1	8/8	Fast/Progressive	Late	6 (8-9W)	-	-	-	-
			Late	-	1 (8W)	-	-	1 (9W)
MSU-1.1-*sis* G	0/13	-	Early/late	-	-	-	-	-

[a]Ratio of sites with tumors formed to sties of animals injected subcutaneously in the presence or absence of sponge implantation. Animals were examined for tumor formation at least 12 months after injection.
[b]The early passage is between passage 25 to 35 after transfection, whereas the late passage is between 55 to 65.
[c]Histopathology of tumors was classified as benign (B), benign with focal aggregates of low grade malignant cells (B+FL), benign with discrete areas of low grade malignant cells (B+L), low grade malignant tumor (L), and benign with focal aggregates of high grade malignant cells (B+FH).

Figure 2. Growth kinetics of tumors formed by v-*sis* transformed MSU-1.1 cells and MSU-1.1-*sis* G.A1 cells in athymic nude mice. Mean tumor volume was calculated by measuring the tumors weekly and calculating the volume as described in the Chapter III. (A) A small regressing nodule (< 5 mm in final diameter) formed by the MSU-1.1-*sis* A cell strain; (B) Slow growing, static tumors produced by the cell strains MSU-1.1-*sis* B ( ) and MSU-1.1-*sis*-C (+); (C) large, progressively-growing tumors produced by MSU-1.1-*sis* G.A1 cell strain. Note the different scale for tumor volume (mm^3) used in each graph.

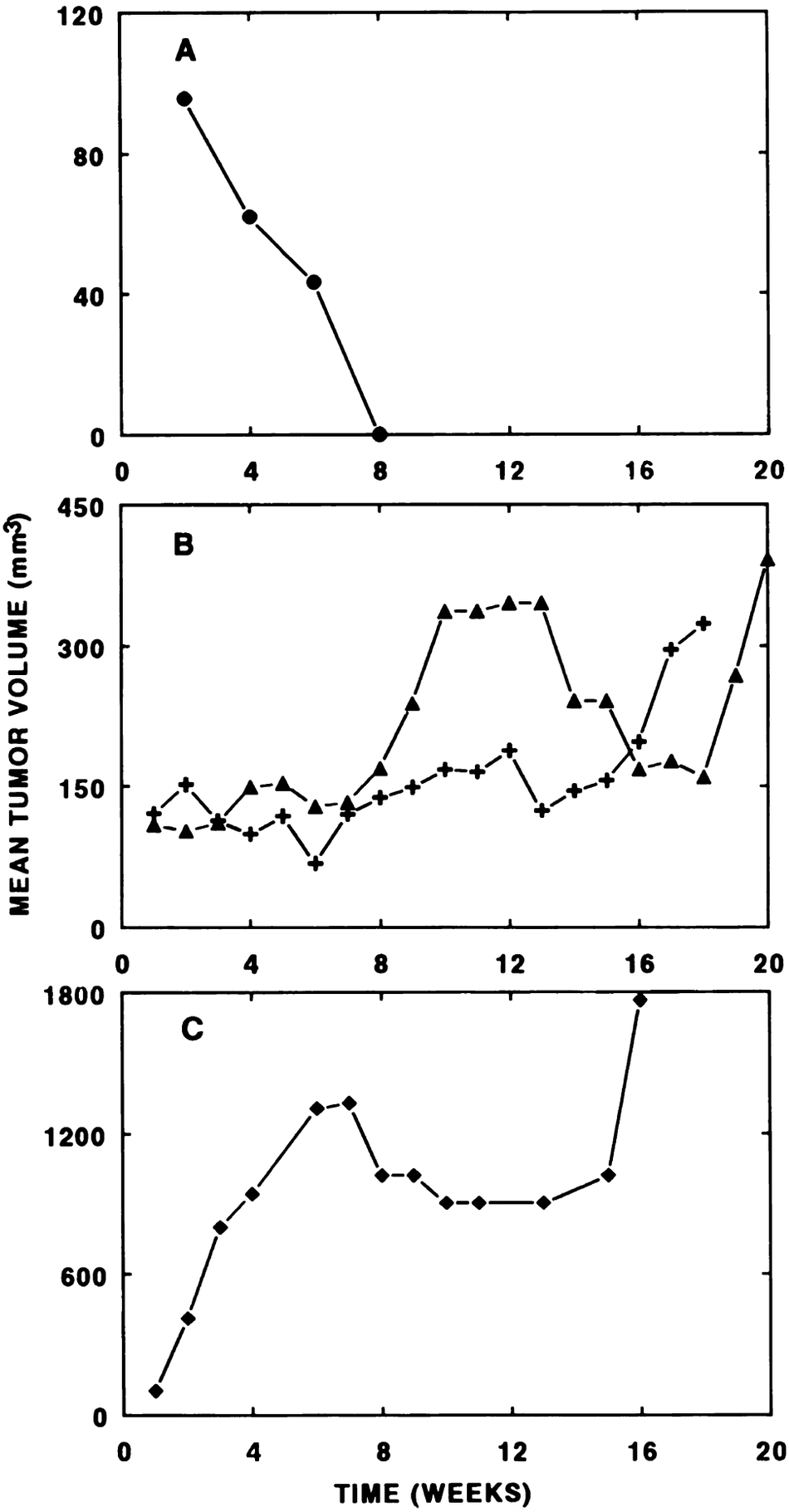
A
B
C
120
80
40
0
450
300
150
1800
1200
600
0
4
8
12
16
20
MEAN TUMOR VOLUME (mm3)
TIME (WEEKS)

Figure 2

Figure 2. Growth kinetics of tumors formed by v-*sis* transformed MSU-1.1 cells and MSU-1.1-*sis* G.A1 cells in athymic nude mice. Mean tumor volume was calculated by measuring the tumors weekly and calculating the volume as described in the Chapter III. (A) A small regressing nodule (< 5 mm in final diameter) formed by the MSU-1.1-*sis* A cell strain; (B) Slow growing, static tumors produced by the cell strains MSU-1.1-*sis* B ( ) and MSU-1.1-*sis*-C (+); (C) large, progressively-growing tumors produced by MSU-1.1-*sis* G.A1 cell strain. Note the different scale for tumor volume (mm^3) used in each graph.

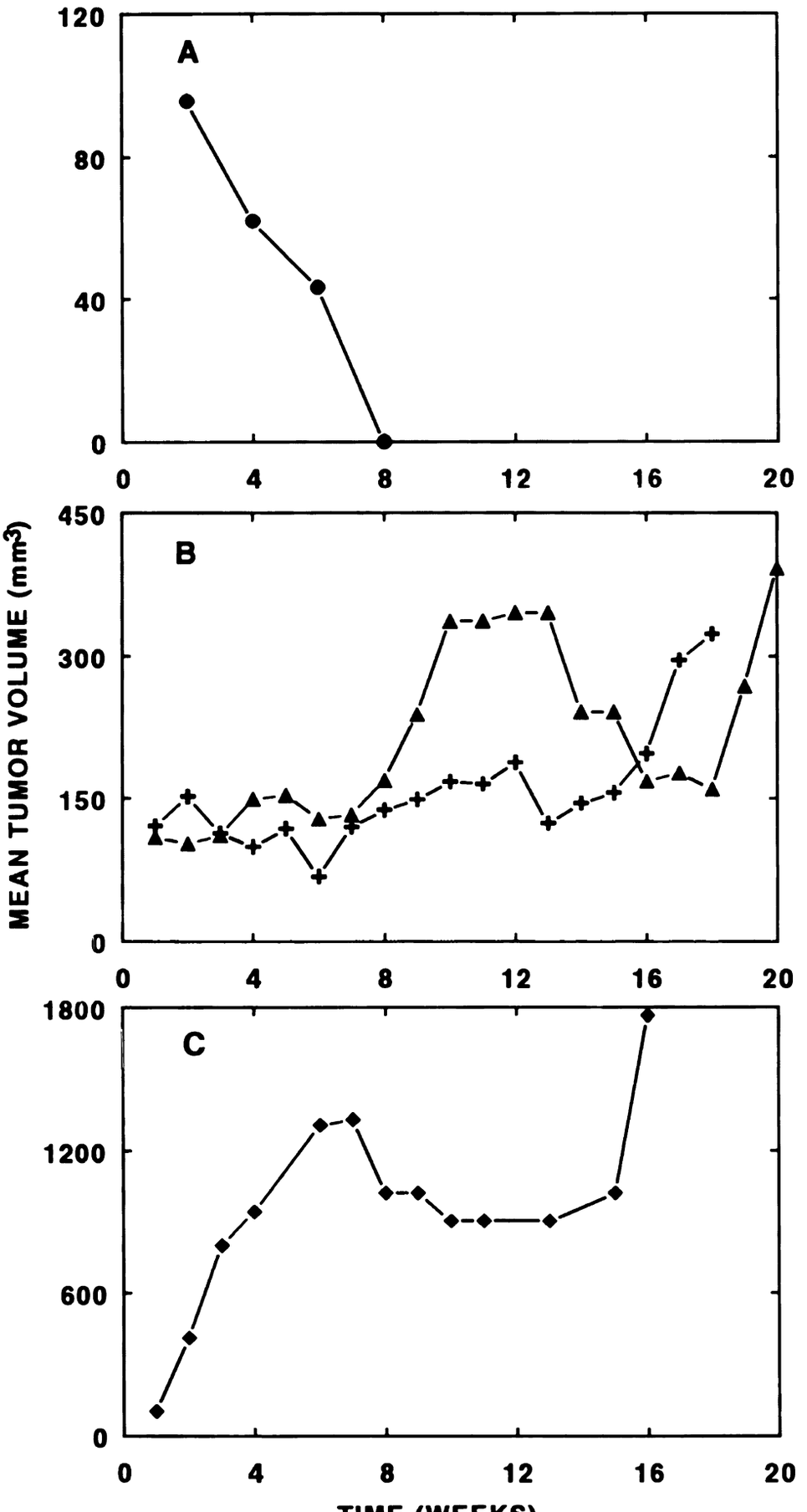
A
B
C
120
80
40
0
450
300
150
0
1800
1200
600
0
0
4
8
12
16
20
MEAN TUMOR VOLUME (mm3)
TIME (WEEKS)

Figure 2

Figure 3. Histology of stained-sections from tumors formed by v-*sis* transformed MSU-1.1 cells and MSU-1.1-*sis* G.A1 cell strain. Typical spindle cell fibroma formed by MSU-1.1-*sis* B strain (A and B). Typical low-grade spindle cell sarcoma formed by MSU-1.1-*sis* B removed 10 months after first appearance (C and D). Typical benign tumor formed by MSU-1.1-*sis*-C strain and removed three months after injection of late passage cells. Note the focal aggregates, "pearls", of round cells typical of high grade sarcomas. A tumor formed after reinjection of tumor-derived cells from cell strain MSU-1.1-*sis* G.A1 which shows a distinct bi-phasic pattern, consisting of a well differentiated spindle cell fibroma (upper portion of photomicrograph) and a high grade spindle cell sarcoma (lower portion of photomicrograph). Magnification 100X in A, C, E, and G, and 200X in B, D, F, and H.

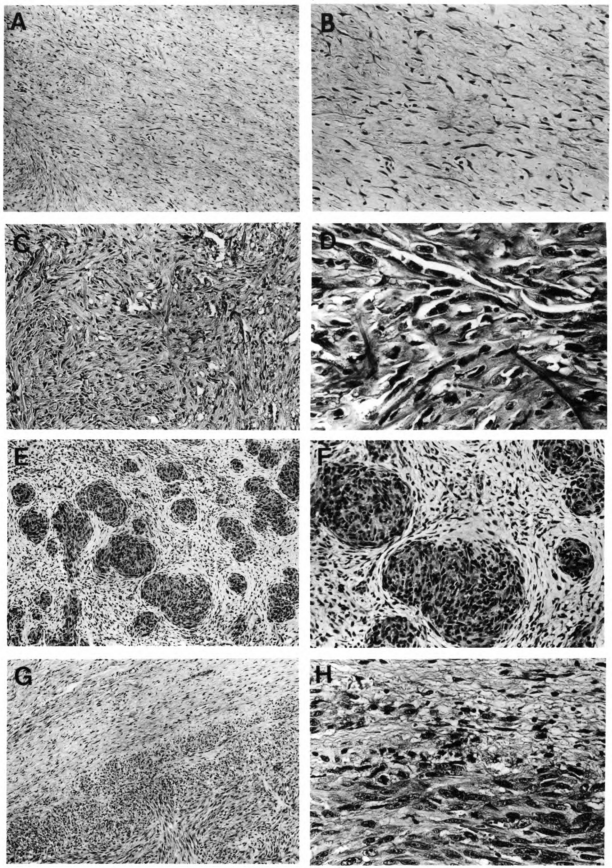

Figure 3