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P. Ann Ryan

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ANALYSIS BY CELL FUSION OF THE LOSS OF TUMOR SUPPRESSOR FUNCTIONS AT SPECIFIC STAGES IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS IN CULTURE

By

P. Ann Ryan

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ABSTRACT

ANALYSIS BY CELL FUSION OF THE LOSS OF TUMOR SUPPRESSOR FUNCTIONS AT SPECIFIC STAGES IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS IN CULTURE

By

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The observation that fusions of infinite life span cells with finite life span cells produce hybrid cells with finite life spans led to the conclusion that an infinite life span in culture is a recessive trait resulting from loss of the function of suppressor genes. Furthermore, finding that certain pairs of infinite life span cells can complement each other to yield finite life span hybrids allowed 19 infinite life span cell lines to be assigned to four immortality complementation groups (1). We fused a near diploid, morphologically normal, infinite life span cell strain, designated MSU-1.1, with its finite life span precursor cell strain and obtained finite life span hybrids, as expected if infinite life span in culture is a recessive trait. However, 14 of the 14 hybrids from our fusions of MSU-1.1 cells with representative cell lines from each of the four immortality complementation groups, and 38 of the 39 hybrids from our fusions of infinite life span cells that have been reported to complement each other, failed to exhibit finite life spans. This result suggests that infinite life span cells cannot complement each other to yield finite life span hybrids. We obtained evidence that long-term dual drug selection can be deleterious to hybrid cells, indicating that the cell death of such hybrids observed in other studies may have resulted from the cytotoxic effect of long-term drug selection, rather than from senescence.

Cell fusion studies designed to demonstrate suppression of *ras*-induced tumorigenicity must control for suppression of infinite life span since loss of

infinite life span will necessarily result in loss of tumorigenicity. Such controlled studies have previously been performed with rodent cells but not with human cells. We fused infinite life span MSU-1.1 cells with *ras*-transformed malignant cells that were derivatives of MSU-1.1 cells. The degree of suppression of tumorigenicity varied among the hybrid strains and did not correlate with the levels of expression of *ras* protein, which increased when the cells were grown in athymic mice and decreased when they were grown in tissue culture dishes.

1. Pereira-Smith, O.M., and Smith, J.R. (1988). Genetic analysis of indefinite division in human cells: identification of four complementation groups. Proc. Natl. Acad. Sci. USA 85:6042, 1988

This work is dedicated to...

Will, my son, who throughout my eight years in the Medical Scientist Training Program has helped to keep me in touch with the bright side of life, from its simplest delights to its deepest joys.

Mary, whose love and companionship have brought me a renewed sense of hope and direction.

Linda, whose nurturing has made this accomplishment possible for me.

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INTRODUCTION

Epidemiologic analysis of the frequency and age of occurrence of various cancers, genetic analysis of animal and human tumors, and experimental induction of malignant cells both in vivo and in vitro have all contributed to our understanding of the genetic basis of carcinogenesis. The results of numerous and varied studies indicate that carcinogenesis is a multistep process in which a cell accumulates, in a stepwise fashion, at least five or six genetic changes that confer upon the cell the various altered growth properties that together allow it to form a malignant tumor. This process is thought to occur by a series of sequential mutations and clonal selections. According to this theory, a normal cell acquires a mutation that gives it a proliferative advantage over surrounding cells. Within the resulting population of altered cells, a single cell incurs a second mutation that further enhances its proliferative potential. Among the progeny of this doubly mutated cell, a third advantageous transforming mutation occurs, and so on. As a result of this process, a cell that has gained the combination of altered growth characteristics necessary for it to form a malignant tumor eventually arises.

In discussions of the multistep process by which cells become malignant, it is necessary to carefully define the terms referring to the different stages. In this thesis, the term "transformed" will refer to cells that have taken on one or more characteristics of cancer cells. "Transformation" will refer to a change from the normal to the transformed phenotype. "Tumorigenic" or "malignant" will refer to the ability of cells to form progressively growing tumors that typically

are found to invade normal tissues.

Two main genetic mechanisms contribute to the process of carcinogenesis. One is the activation of dominantly acting oncogenes, which are genes that promote cell proliferation; the other is the inactivation of tumor suppressor genes, which are genes that normally act to limit cell proliferation. Numerous genes of both types have been identified and characterized. The relative contributions to human carcinogenesis of these two classes of genes and the interactions among these genes are currently the subject of intense study.

One method for assessing the relative contributions of these two classes of genes is illustrated by the work of Vogelstein and his colleagues on the genetic alterations that occur during colon carcinogenesis (Vogelstein et al., 1988). Colorectal tumors progress through a series of five easily recognizable clinical stages: from hyperplastic epithelial cells; through three stages of benign adenomas of increasing size, dysplasia, and villous content; to malignant carcinomas. Using tissue taken from human hosts, Vogelstein and his colleagues analyzed the genetic alterations of cells at each of these stages. They found that the *ras* oncogene was activated in a large percentage of colorectal neoplasms, and that this change usually occurred at an early stage of transformation. They also found evidence for the inactivation of tumor suppressor genes on chromosomes 5, 18, and 17, with the losses occurring, respectively, at early, intermediate, and late stages of transformation.

Another way to study the genetic alterations that contribute to carcinogenesis is by inducing this process in cells in culture and analyzing the phenotypic and genetic changes that occur during individual steps of transformation. The study of cells that have been transformed in culture has many advantages over the study of cells that have been obtained from tumors. Transformation of cells in culture allows the direct observation of the temporal

sequence of the transformation stages, whereas the sequence of the changes in vivo can only be inferred. Transformation of cells in culture also allows the comparison of an altered cell with its immediate precursor cell. When one examines cells that have become transformed in an animal, however, one cannot know for sure which cell gave rise to a particular population of cells. Furthermore, transformation of cells in culture has the obvious value of allowing introduction of a specific altered gene, e.g., transfection of a known oncogene, in order to study the transforming effects of the gene.

In our laboratory we are studying the phenotypic and genetic changes that occur during the transformation, in culture, of normal foreskin-derived human fibroblasts, through at least two partially transformed intermediates, to the fully malignant state (McCormick and Maher, 1991). The normal fibroblast LG1 was transfected with the *myc* oncogene. One of the *myc*-transfected strains gave rise to the morphologically normal, diploid, immortal strain designated MSU-1.0. This strain spontaneously gave rise to a partially growth factor independent , near diploid variant harboring two marker chromosomes. This second intermediate, designated MSU-1.1, has given rise to numerous malignant derivatives. Some of these have arisen spontaneously. Others have been induced by transfection with oncogenes or have arisen following carcinogen treatment.

The goal of my doctoral studies was to determine if inactivation of tumor suppressor genes contributed to transformation at specific steps within the MSU-1 lineage. I used the method of cell fusion between cells from separate stages of the lineage to make this determination. When a cell having a particular transformed characteristic is fused with a cell lacking this characteristic, the phenotype of the hybrid cell provides information about the genetic alteration that induced the transformed phenotype. If the hybrid cell exhibits the transformed phenotype, one assumes that the activation of a dominantly acting

oncogene brought about the transformed phenotype. The oncogene is supplied to the hybrid by the transformed parental cell and still acts in a dominant fashion to maintain the transformed phenotype in the hybrid. If the hybrid cell exhibits the non-transformed phenotype, one assumes that transformation occurred as a result of inactivation of both copies of a tumor suppressor gene. A functional tumor suppressor gene is supplied to the hybrid by the non-transformed parent cell causing the suppression of the transformed phenotype in the hybrid.

Chapter I of this thesis gives a brief overview of the discovery of the dominantly acting oncogenes. The mechanisms by which these genes become activated are discussed and are illustrated by an example of a prototypic gene from this class. The primary focus of this chapter, however, is on the second class of genes - tumor suppressor genes. The early evidence for the existence of these genes is presented, and the identification and characterization of several specific suppressor genes is discussed in detail. Particular attention is devoted to contributions to this area of cancer research that have come from cell fusion studies, since this methodology served as the basis of many of the experimental studies I carried out during the research described.

Chapter II is a manuscript by P. Ann Ryan, Veronica M. Maher, and J. Justin McCormick which was published in **Journal of Cellular Physiology** *159*, 151-160 (1994). The manuscript describes the results of my cell fusion studies showing that the infinite life span of MSU-1.1 is a recessive trait, as has been found for all other infinite life span cells studied (Bunn and Tarrant, 1980; Muggleton-Harris and DeSimone, 1980; Pereira-Smith and Smith, 1983), but that, contrary to earlier reports (Pereira-Smith and Smith, 1988), infinite life span cells are not able to complement each other to yield finite life span hybrids. The discussion section of Chapter II is a somewhat longer version than that included in the published manuscript.

Chapter III describes the results of cell fusion studies suggesting that *ras* oncogene-induced transformation of MSU-1.1 to the malignant state requires loss of a suppressor function that is present in MSU-1.1 cells. Examination of the *ras* protein expression of the hybrid cells and the malignant parent cells suggested that *ras* expression is increased by the growth of cells in athymic mice and decreased by the growth of cells in tissue culture dishes. Further studies will be undertaken by others to confirm these preliminary results.

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CHAPTER I

LITERATURE REVIEW

A. Dominantly Acting Oncogenes - a Brief Overview

1. Oncogenes in acutely transforming retroviruses

A major contribution to our understanding of the genetic basis of cancer comes from studies of acutely transforming retroviruses. These are RNA viruses capable of rapidly inducing tumors in infected animals. Examples are avian leukemia viruses, murine leukemia viruses, mouse mammary tumor viruses, feline leukemia viruses and feline sarcoma viruses (reviewed by Weiss et al., 1982). The RNA tumor virus first identified was the Rous sarcoma virus (RSV). Rous (1911) found that a cell-free, bacteria-free extract from a spontaneous chicken sarcoma was capable of inducing tumors when inoculated into chickens. This finding established a virus as the etiological agent of the tumors. The infecting particle was identified as a virus that contained RNA (Claude et al., 1947; Gaylord, 1955; Crawford & Crawford, 1961). Martin (1970) isolated a temperature sensitive mutant RSV that replicated normally at both restrictive and permissive temperatures but did not transform infected cells at the nonpermissive temperature. This showed that a transforming protein was encoded by the mutated RSV gene, which was named "*src*", for sarcoma. A c-DNA fragment bearing *src* alone, and no other RSV genes, was found to be capable of transforming fibroblasts in culture (Martin, 1970). In a similar manner, the genes responsible for the transforming properties

of other acutely transforming RNA tumor viruses were identified. Examples of such genes are *fps*, *yes*, and *ros* from avian sarcoma viruses; *myc*, *erb*, and *myb* from avian leukemia viruses; and *mos*, *ras*, *fes*, *rms*, and *sis* from various mammalian sarcoma viruses (reviewed in Bishop and Varmus, 1982).

Subsequently, homologs of these viral cancer genes were found in the genomes of various animals, including species as diverse as fish, birds, mammals, and flies (Stehelen et al., 1976; Spector et al., 1978; Shilo and Weinburg, 1981). Convincing evidence suggested that these transforming genes were endogenous cellular genes that had been acquired by viruses from the animal hosts, rather than being viral genes that had become integrated into the animal genome. For example, if a homolog of a particular viral transforming gene was found in an animal genome, it was present in all members of the species, whereas the transforming gene was usually restricted to a single viral strain. Each cellular homolog was found to reside at a constant genetic locus within an animal species, whereas retroviruses were found to integrate at diverse positions within a host genome. The cellular homologs were found to contain introns, whereas the viral transforming genes lack such material and, therefore, probably represent cellular cDNA that has become integrated into the viral genome.

2. Protooncogenes

As more viral cancer genes were identified and the cellular origins of each gene was shown, it became clear that normal cells contain numerous genes that can contribute to cellular transformation when they are altered in specific ways. The normal cellular forms of these genes became known as protooncogenes, and are designated by the prefix "c-" for cellular,

e.g., c-*src*. The altered viral forms capable of transforming cells are called oncogenes and are designated by the prefix "v-" for viral, e.g., v-*src*.

Although retroviral transformation is not a common mechanism of human carcinogenesis, the identification of virally-activated oncogenes gave researchers a valuable tool with which to study human cancer because, with few exceptions, sequences homologous to the viral oncogenes were also found in the human genome. Moreover, cells from many human tumors were found to contain activated forms of these genes. For example, Eva et al. (1982) used the technique of Northern Blotting to demonstrate that a human homolog to the chicken v-*myc* oncogene is overexpressed in a human sarcoma cell line and in two human carcinomas, and that a human homolog to the wolly monkey v-*sis* oncogene is overexpressed in certain human sarcomas and glioblastomas. Additional oncogenes were found to reside at common chromosomal translocations seen in specific types of tumors, e.g., *bcl*-1 at the translocation site in a lymphocytic leukemia cell line (Tsujimoto et al., 1984), or to be amplified in the genomes of tumor cells, e.g., N-*myc* in neuroblastomas and retinoblastomas (Schwab et al., 1983).

Another widely used means of identifying activated oncogenes utilizes transfection of tumor cell DNA into established rodent fibroblasts, most commonly mouse NTH/3T3 cells. Neoplastic transformation of the recipient cell occurs upon acquisition of a DNA fragment containing an activated oncogene, allowing isolation and analysis of the transfected gene (Shih et al., 1979a). The human N-*ras* oncogene was identified in this manner (Marshall et al., 1982; Hall et al., 1983) As was true for the oncogenes that were first identified in viruses, human oncogenes discovered by this method were also found to be altered forms of normal

human genes.

The discovery of protooncogenes prompted intense study of their function. Why would cells carry genes predisposing them to cancer? As the genes were cloned and their protein products were analyzed, it became clear that, rather than representing hapless sequences waiting for the chance to become destructive, protooncogenes code for essential proteins that have specific cellular functions (reviewed in Hunter, 1991). For example, the oncogene v-*src* was found to code for a protein that phosphorylates tyrosine (Brugg and Erickson, 1977; Hunter and Sefton, 1980). It is now known that this is only one in a family of more than a dozen oncogenes, including *neu* and *erb*B (Downward et al., 1984), that code for receptor and nonrecepter membrane associated protein-tyrosine kinases. These oncogenes, along with a family of oncogenes coding for membrane associated G-proteins [e.g., *ras* (Hurley et al., 1984)], and another group coding for cytoplasmic protein-serine kinases, function as signal transducers in growth factor signalling pathways. Other oncogenes code for protein growth factors [e.g., *sis*, which produces an altered form of the B-chain of platelet-derived growth factor (Doolittle et al., 1983)]. Finally, another group of oncogenes encodes nuclear proteins. Many of these (e.g., *jun*, *fos*, and *myb*) act as transcription factors that are induced when resting cells are treated with mitogens. They have been shown to initiate expression of genes involved in cell replication (reviewed in Seemayer and Cavenee, 1989; Hunter, 1991).

Each of these groups of oncogenes (i.e., growth factors, signal transducers, and transcription factors) plays a critical role in the complex process of cell replication. Therefore, controlled expression of the normal forms of these genes results in normally regulated cell

proliferation. However, when these genes are expressed in excessive amounts (see discussion of v-*myc* and v-*sis*, p.9) or in mutated forms (see discussion of *ras*, pp.12-13), transformation can result.

3. A prototypic oncogene: *ras*

A description of the discovery of a prototypic oncogene, the isolation of its protein product, and the determination of its function will illustrate these concepts. In the 1960's, two closely related defective viruses, which became known as murine sarcoma viruses, were isolated from sarcomas that developed in rats following inoculation with mouse leukemia viruses (Harvey, 1964; Kirstin & Mayer, 1967). The sarcoma viruses, although clearly derived from the leukemia viruses that had been injected into the rats, differed substantially from the leukemia viruses. The only protein found to be produced by these viruses was a 21,000 dalton phospho-protein (Shih et al., 1979b).

The proteins from these two viruses were antigenically related (Scheinberg and Strand, 1980) and had guanine nucleotide-binding activities (Scolnick et al., 1979). A temperature dependent mutation in the gene encoding for the protein demonstrated that it was responsible for the transforming properties of the viruses (Shih et al., 1979c). Fluorescent and ferritin-tagged antisera were used to localize the protein to the inner surfaces of plasma membranes in cells transformed by the virus (Willingham et al., 1980).

The oncogenes encoding the transforming proteins were named Ha-*ras* and Ki-*ras*, after the scientists who isolated the viruses from the rat sarcomas. DNA probes specific for the *ras* genes hybridized to single-copy DNA from uninfected rat cells (Langbeheim, 1980). This led to the

discovery that Ha-*ras* is derived from one of two closely related normal rat cellular genes, and that Ki-*ras* is derived from a third gene that is partially homologous to the other two (DeFeo et al., 1981; Ellis et al., 1981).

The NIH/3T3 cell assay identified transforming genes from human tumors that shared homology with the viral H- and K-*ras* oncogenes (Krontris and Cooper, 1981; Shih et al., 1981; Pulciani et al., 1982; Der et al., 1982). A third transforming *ras* gene, named N-*ras*, was also identified by this method (Hall et al., 1983; Shimizu et al., 1983a,1983b). As expected, these three human oncogenes were found to be mutated forms of normal cellular genes (Chang et al., 1982; Davis et al., 1983). The oncogenic mutations occurred only at a few restricted sites, most commonly in codons 12, 13, 59, 60, 61, and 117 (reviewed in Varmus, 1984; Barbacid, 1987).

In humans, as in rats, the *ras* protein (p21-*ras*) was found to be located on the inner plasma membrane surface and to have the ability to bind guanine nucleotides. This suggested that p21-*ras* participates in signal transduction from the cell surfaces. Investigations along these lines revealed that p21-*ras* belongs to a family of G-proteins that transduce signals from activated receptors of extracellular growth factors to secondary intracellular messenger systems (reviewed in Haubruck and McCormick, 1991; Valencia et al., 1991). GTP-bound p21-*ras* is the active form of the protein, which is subsequently transformed into the inactive GDP-bound form by hydrolysis of GTP.

The transforming mutated forms of *ras* yield proteins that are insensitive to GTP-ase activating regulator proteins. Because the GTP is not hydrolyzed, the oncogenic *ras* proteins remain in a permanently active

conformation (Trahey and McCormick, 1987). As a result, a cell containing an oncogenic *ras* continues proliferating even in the absence of exogenous growth factors.

4. Mechanisms of activation

The *ras* gene is only one of many protooncogenes for which the normal gene function and oncogenic activation have been explored in detail. The protooncogenes are of two types that differ in their general mechanisms of activation (reviewed in Weinburg, 1989). Protooncogenes of one type, exemplified by *ras*, are expressed in constant amounts. The protein products of these protooncogenes, however, oscillate between an active and an inactive state. The transitions between the active and inactive states are induced by other biochemical regulators, e.g., phosphatases and kinases. The oncogenic forms of these genes generally produce proteins that are locked in the active state. Because these proteins participate in the growth factor signalling pathway, their constitutive activity causes cell replication to proceed unchecked.

Protooncogenes of the other type, on the other hand, generally are regulated by modulatable expression. Oncogenic mutations in these genes, e.g., *sis* and *erb*B, usually uncouple them from their normal regulators, leading to constitutive expression of the gene. Elevated amounts of their protein products, usually transcription factors, enhance the expression of genes whose products are critical to cell growth and differentiation. Increased production of these critical proteins leads to excessive cell replication.

Despite the differences in activation between these two types of oncogenes, they have a very important feature in common. For both, the

oncogenic form of the gene acts in a dominant fashion over the wild type alleles. Both constitutively active and constitutively expressed oncoproteins promote unbridled cellular proliferation even when accompanied by their normal counterparts, except in certain instances of abnormally high expression of the wild type allele. Therefore, these genes are called dominantly acting oncogenes.

B. Tumor Suppressor Genes

1. Early evidence

In the excitement over the discovery of dominantly acting oncogenes and the fervor with which the identities and functions of their protein products were pursued, relatively little attention was paid to indications that an entirely different kind of cancer gene existed. In 1969, a year before the first dominantly acting oncogene was identified, Henry Harris and George Klein published results from cell fusion experiments that suggested that some cancer genes were recessive. These scientists fused highly malignant mouse cells with mouse cells that were low in malignant potential and discovered that the resultant hybrids were suppressed in their ability to form tumors (Harris et al., 1969; Wiener et al., 1974). The suppressed hybrids, however, rapidly reverted to the malignant state, a phenomenon that made it difficult to observe suppression in earlier investigations (Barski and Cornefert, 1962). It was noted that this reversion was accompanied by loss of substantial numbers of chromosomes.

The results of these cell fusion experiments and of similar

corroborating studies (Silagi, 1967; Kao and Hartz, 1977; Sager and Kovac, 1978) led to the following conclusions: The mutant genes responsible for the tumorigenic phenotype in the malignant cells are recessive. Therefore, mutation of both of the normal alleles of these genes is required for malignant transformation. Fusion of a malignant cell with one that still possesses a normal copy of the gene results in suppression of the tumorigenic phenotype. The chromosomal instability of the rodent cell hybrids allows rapid loss of the chromosomes bearing the normal suppressing alleles. When this happens, tumorigenicity is again expressed.

Because expression of tumorigenicity was overridden by the presence of the normal genome in the tetraploid hybrids, the mutant genes responsible for the tumorigenicity in the malignant parent were labeled "recessive oncogenes". "Recessive" as used here is similar but not identical to the term "recessive" as used in Mendalian genetics. The normal alleles, having the ability to suppress the malignant phenotype, were dubbed "tumor suppressor genes" or "anti-oncogenes".

The idea that some genes act to suppress tumorigenicity was not new. In 1964, Stoker reported that the growth of polyoma-transformed cells was suppressed by surrounding normal cells, suggesting that normal cells produce tumor suppressor molecules that they transfer to neighboring cells. This same phenomenon was later reported for rodent fibroblasts transformed by transfection with both the *myc* and the *ras* oncogenes (Land et al., 1986). In studies aimed at identifying these molecules, growth regulatory polypeptides were found that inhibited the replication of certain cells. examples include TGF-β, tumor necrosis factor, tumor inhibitory factors 1 and 2, interferons, interleukins, and oncostatin

(Resnitzky et al., 1986; Yardin and Kimchi, 1986; Zarling et al., 1986; Newmark, 1987; Takehare et al., 1987; Sporn and Roberts, 1988). These polypeptides could be thought of as products of tumor suppressor genes.

While scientists were struggling to understand the phenomenon of tumor suppression in vertebrates, rapid progress was being made in identifying specific tumor suppressor genes in a less complex organism, i.e., the fruit fly *Drosophila melanogaster*. By 1982, 25 recessive *Drosophila* genes had been implicated in cancer of flies (Gateff, 1982). In 1985, the most extensively studied of these, the *lethal(2) giant larvae* gene [*l(2)gl*], was cloned. Recessive mutations in this gene resulted in uncontrolled cell proliferation and death of the animal (Melcher et al., 1985). Introduction of the cloned gene into cells that were deficient at this locus suppressed malignancy, confirming the hypothesis that *l(2)gl* is a tumor suppressor gene (Opper, 1987).

2. A prototypic human tumor suppressor gene: RB1

Closely following the cloning of the *l(2)gl* gene came the cloning of the most extensively studied human tumor suppressor gene, the retinoblastoma gene. Retinoblastoma is a pediatric ocular cancer. As is characteristic of several other kinds of human cancers, retinoblastoma occurs both sporadically and as an inherited disease. In individuals with the hereditary form of the disease, multiple tumors form, commonly in both eyes, and the tumors usually arise prior to birth. The sporadic form of retinoblastoma differs from the hereditary form in that affected individuals usually develop only one tumor, and the tumors are not present at birth, but appear during the first few years of life.

To explain the basis for this difference, Knudson (1971) proposed

that two mutations are required for transformation of the proliferating retinoblast to the malignant state. In hereditary retinoblastoma, one mutation occurs in the germline, making every cell in the body a candidate for malignant transformation by the second hit. Retinoblasts proliferate during the first few years of life until there are approximately 10^7 cells in the retina. Because the somatic mutation rate is approximately one per 10^5 to 10^6 cell divisions, retinal development provides ample chance for inheritants of a germline mutation to incur a second mutation. In sporadic retinoblastoma, however, both mutations are somatic, with the second occurring in one of the progeny of the cell that acquired the first mutation. This accounts for the relative rarity of sporadic tumors, their occurrence in only one eye, and the longer length of time required for their development.

When scientists examined the genetic alterations of retinoblastoma cells, they found strong evidence in support of Knudson's hypothesis. Karyotypic analysis revealed frequent deletions in band 14 of the long arm of chromosome 13 in retinoblastoma cells from both sporadic and congenital tumors (Franke, 1978; Balaban, 1982; Benedict, 1983). This implicated 13q14 as the location of one of the disease-associated mutations and raised the possibility that the mutation inactivates the gene and leads to loss of some critical growth suppressing function. Restriction fragment length polymorphism studies showed that the second mutation leading to the development of retinoblastomas is loss of the remaining wild type allele at the 13q14 locus (Cavenee, 1983; Sparkes, 1983). This verified that the locus contains a tumor suppressor gene, and that loss of the function of both alleles leads to tumorigenicity. Chromosome walking from a known cloned, tightly linked gene allowed cloning and sequencing of the

retinoblastoma susceptibility gene, RB1 (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987a).

The RB1 gene encodes a 105 kiloDalton nuclear phospho-protein (p105-RB1) that appears to be expressed in all human tissues (Friend et al., 1986; Lee et al., 1987b). Consistent with the homozygous alteration of the gene in retinoblastoma cells is the fact that all retinoblastoma cell lines and short term cultures lack production of p105-RB1 (Horowitz et al., 1990). Introduction of a functional RB1 gene into retinoblastoma cells suppressed tumorigenicity, providing confirmation that the gene functions as a tumor suppressor (Huang, 1988; Bookstein, 1990).

Investigations into the phosphorylation of p105-RB1 led to clues regarding its function. The phosphorylation status of the protein fluctuates regularly during the cell cycle (Chen et al., 1989; Buchkovich et al., 1989; DeCaprio et al., 1989; Ludlow et al., 1990). The p105-Rb of cells in the G0- and G1-phase is unphosphorylated. It becomes phosphorylated, possibly by cdc-2 kinase, when the cells are stimulated to divide, remains phosphorylated throughout S-phase, and is dephosphorylated during mitosis (M-phase). This suggests that the unphosphorylated form of p105-Rb inhibits cell division, and that phosphorylation of the protein inactivates it, thereby allowing cell division. The unphosphorylated protein apparently blocks passage from the G1-phase to the S-phase of the cell cycle by complexing with and inhibiting the function of the transcription factor E2F, which normally can activate important proliferation genes (Chellappan et al., 1991; Bagchi et al., 1991; Chittenden et al., 1991; Hamel et al., 1992; Hiebert et al., 1992).

Evidence indicates that inactivation of the RB1 gene also participates in the genesis of cancers other than retinoblastoma.

Patients with a germline alteration in an RB1 allele are also predisposed to developing osteosarcomas and other soft tissue sarcomas in their adult years (Derkinderen et all, 1988). Examination of these tumors and a wide variety of tumors from patients that do not have a constitutional defect in RB1 showed that homozygous alterations in this gene are found with a high frequency in osteosarcomas, soft tissue sarcomas, small cell lung carcinomas, and breast cancers. They also are found in bladder cancers, glioblastomas, leukemias, and squamous cell carcinomas, but with a lower frequency (reviewed in Cavenee et al., 1989). This list by no means encompasses the full range of tissues in which the RB1 gene is expressed. It is not yet understood why the RB1 gene's oncogenic potential is limited to certain cell types, whereas its expression is ubiquitous, and this question is the subject of intense study. More precise elucidation of the function of p105-Rb may help answer this question.

3. Identification through the study of familial cancers

In the 1980's, using Knudson's hypothesis as a paradigm and encouraged by the rapid progress made in identifying and cloning the retinoblastoma susceptibility gene, cancer researchers began searching for tumor suppressor genes involved in the genesis of other familial cancers. For common hereditary cancers, linkage analysis and occasional large constitutional deletions enabled assignment of the susceptibility genes to specific chromosomal locations. In rarer cancers with more subtle genetic alterations, loss of heterozygosity at specific genetic loci in tumor tissues, which was detected by restriction fragment length polymorphism, pinpointed the locations of candidate suppressor genes.

Careful systematic application of these techniques in the genetic

analysis of numerous hereditary cancers allowed rapid identification of several tumor suppressor genes. By 1989, chromosomal sites of putative suppressor genes were identified in Wilms' tumors (11p), familial adenomatous polyposis coli (5q, 17p, 18q), multiple endocrine neoplasia type 2 (1p), renal cell carcinoma and von Hippel-Lindau disease (3p), von Recklinghausen neurofibromatosis (17q), and acoustic neurofibromatosis (22q) (reviewed in Friend et al., 1988; Marx, 1989; Sager, 1989; Weinburg, 1989). Tumor suppressor genes have been identified at most of these sites and at a few additional ones. To date, eight tumor suppressor genes, including RB1, have been cloned through study of familial cancers, and germ-line mutations have been identified for all but one of these genes (reviewed in Knudson, 1993). Two extensively studied familial cancers and their associated susceptibility genes are discussed below.

3.1 Wilms' tumor

Wilms' tumor, a pediatric renal tumor, is similar to retinoblastoma in that it occurs both sporadically and as part of a congenital condition. Congenitally affected individuals usually develop multiple tumors in both kidneys during early childhood. In contrast, individuals with the sporadic form of the disease develop a single renal tumor and these sporadic tumors arise two or three years later in childhood than those in congenital cases. This suggests that Wilms' tumor, like retinoblastoma, occurs as the result of homozygous inactivation of a tumor suppressor gene by two somatic mutations in sporadic cases, and by a germ-line mutation followed by a somatic mutation in congenital cases.

The genetic analysis of Wilms' tumors has revealed that this prediction is generally accurate, but that the genetic basis of Wilms'

tumor is much more complex than that of retinoblastoma. Only 10% of Wilms' tumors are bilateral compared with 40% of retinoblastomas. Furthermore, only 1% of Wilms' tumors occur through familial transmission, in contrast to the relatively frequent familial inheritance of susceptibility to retinoblastoma (Matsunaga, 1981). This indicates that new germline mutations are primarily responsible for the development of bilateral Wilms' tumor.

Another important difference between Wilms' tumor and retinoblastoma is that bilateral Wilms' tumors are sometimes associated with congenital abnormalities. These congenital abnormalities define two distinct clinical syndromes. In one, which was given the acronym WAGR syndrome, Wilms' tumors occur along with aniridia (i.e. absence or malformation of the iris), genitourinary abnormalities, and mental retardation (Miller et al., 1964). The second, named the Beckwith-Wiedemann syndrome after the authors who first described it in the literature, is characterized by microglossia, gigantism, earlobe pits or creases, abdominal wall defects, and an increased risk for the development of Wilms' tumor, rhabdomyosarcoma, hepatoblastoma, and abnormal carcinomas (Beckwith, 1969; Sotelo-Avila and Gooch, 1976).

Both syndromes are associated with bilateral (i.e. congenital) Wilms' tumor, but only the Beckwith-Wiedemann syndrome has been reported to be passed by autosomal dominant inheritance in families (Best and Hoekstra, 1981; Nükawa et al., 1986). Some familially transmitted Wilms' tumors do not show the clinical symptomatology of either the WAGR or the Beckwith-Wiedemann syndrome. Although it was not initially apparent, the reason for the variety of presentations of Wilms' tumors lies in the multiplicity of genetic loci contributing to formation of these neoplasms.

The first clue to the location of a gene involved in Wilms' tumor came from karyotypic analysis of cells from WAGR syndrome patients. These patients show a high frequency of gross constitutional chromosomal deletions on the short arm of chromosome 11, at band 13 (Riccardi et al., 1978; Franke et al., 1979), suggesting that the tumor suppressor gene responsible for Wilms' tumor was located in this band. Further evidence for this site as the location of a Wilms' tumor suppressor gene came from studies demonstrating loss of heterozygosity at polymorphic markers on chromosome 11p13 in sporadic Wilms' tumors (Fearon et al., 1984; Koufos et al., 1984; Orkin et al., 1984; Reeve et al., 1984).

Genetic analysis of patients with Beckwith-Wiedemann syndrome pointed to a different locus for the Wilms' tumor susceptibility gene. These patients show constitutional chromosomal abnormalities, generally duplications, of 11p15 (Waziri et al., 1983; Koufos et al., 1989). Furthermore, 15-20% of sporadic Wilms' tumors show loss of heterozygosity at this locus rather than at 11p13 (Reeve et al., 1989; Koufos et al., 1989). It is possible that genes at these two loci may interact, because some tumors arising in WAGR syndrome patients, who are constitutionally affected at the 11p13 locus, have been shown to have loss of heterozygosity within 11p15, but not 11p13 (Henry et al., 1989).

The genetic analysis of Wilms' tumor became even more complex when it was found that familial predisposition to Wilms' tumor that is not associated with WAGR or Beckwith-Wiedemann syndromes is linked to neither 11p13 nor 11p15 (Grundy, 1988). This indicates that a third gene, and conceivably more, can contribute to the genesis of this neoplasm. No location for this third Wilms' tumor gene has yet been suggested.

Though multiple genes evidently contribute to the development of

Wilms' tumor, only one has been cloned. At 11p13, within the 400 kilobases smallest overlapping region of a number of WAGR deletions (Rose et al., 1990), a transcription unit was identified, cloned, and characterized (Call et al., 1990; Gessler et al., 1990). The gene, which was named WT1, encodes a 46-49 kilodalton protein. This protein contains four zinc-finger domains that bind to the same recognition sequence as do the early growth response (EGR) zinc-finger proteins (Rauscher et al., 1990). Recently, the WT1 protein has been shown to inhibit transcription of several positive regulators of cell growth, including EGR-1, insulin-like growth factor II, and the platelet-derived growth factor A-chain gene (Madden et al., 1991; Drummond et al., 1992; Wang et al., 1992). Conversely, the WT1 protein activates transcription of other genes, including the tumor suppressor p53 (Wang et al., 1993; Maheswaran et al., 1993). Both of these actions are consistent with the hypothesis that the WT1 gene functions as a tumor suppressor gene.

The expression pattern of the WT1 gene in normal tissue and in Wilms' tumors is compatible with its reputed involvement in the WAGR syndrome. In genetically normal individuals, expression of WT1 is limited to the embryonic kidneys, the fetal gonads, and some hemopoietic cells (Pritchard-Jones et al., 1990). This restricted range of expression offers an excellent explanation for the participation of this gene in renal neoplasms and genitourinary anomalies, and for the lack of effect of this gene on other tissues. As predicted, Wilms' tumors that have homozygous deletions of 11p13 show no expression of WT1 (Gessler et al., 1990). Most other Wilms' tumors, however, show a high expression of an inactive form of the gene (Cowell et al., 1991).

In summary, the epidemiology of Wilms' tumor suggests that

homozygous inactivation of a tumor suppressor gene is the genetic basis for this neoplasm. Although genetic analysis has suggested that three or more chromosomal loci are involved in Wilms' tumor formation, only one candidate gene, WT1, which is associated with the WAGR syndrome, has been cloned. This gene has a cellular function appropriate for a tumor suppressor, and an expression pattern befitting a gene participating in genitourinary abnormalities.

3.2 Familial adenomatous polyposis coli

One of the best known hereditary predispositions to cancer is familial adenomatous polyposis (FAP) in which a susceptibility to colorectal cancer is inherited as an autosomal dominant trait. Individuals with this disease develop hundreds, sometimes thousands, of benign adenomatous polyps on their colonic mucosa. The polyps begin to appear as early as the first decade of life, and a small proportion of them inevitably progress to carcinomas. Adenomas from FAP patients arise independently from single stem cells, i.e., are monoclonal, in contrast to normal colonic epithelium which arises from numerous stem cells and therefore is polyclonal. Similar monoclonal precancerous polyps also occur sporadically in individuals who do not have the disease.

Following Knudson's idea on the origin of retinoblastoma, researchers hypothesized that a germline defect in a tumor suppressor gene, followed by a somatic mutation in the second allele, is responsible for the formation of polyps in FAP patients, and that somatic mutations in both alleles results in the formation of sporadic polyps. The study of constitutional deletions in FAP patients, chromosomal linkage in FAP family pedigrees, and loss of heterozygosity in colorectal tumors provided

the clues required for the localization of the FAP susceptibility gene, but the pattern of inactivation of the gene in colorectal adenomas and carcinomas suggested that the prevailing theories regarding recessive oncogenes needed to be expanded.

The first definite clue to the location of the FAP susceptibility gene was the discovery of a patient with a constitutional deletion of chromosomal band 5q21 (Huerra et al., 1986). Analysis of FAP pedigrees showed that markers on this band were tightly linked to the development of adenomas (Bodmer et al., 1987; Leppert et al., 1987). Detection of loss of heterozygosity of 5q21 markers in adenoma tissue also implicated this locus in the susceptibility to this disease, but the results were puzzling in that loss of heterozygosity was detected in sporadic adenomas but not in adenomas from FAP patients (Solomon et al., 1987; Vogelstein et al., 1988).

Yeast artificial chromosome vectors and chromosome walking techniques enabled isolation of several candidate cDNAs within the critical band, three of which fell within two newly discovered constitutional FAP deletions (Joslyn et al., 1991; Kinzler et al., 1991). One of these three genes was found to be mutated in the germline DNAs of FAP patients and in DNAs from sporadic colon carcinomas, establishing it as the FAP susceptibility gene (Nishisko et al., 1991). This gene was named APC for adenomatous polyposis coli.

The APC gene encodes a cytoplasmic protein with features suggesting a potential for interaction with other proteins. As expected, the mutations in this gene that are found in FAP germlines and in somatic colon carcinomas appear to inactivate the gene. The majority are frameshift or point mutations that result in premature stop codons and

lead to the synthesis of truncated proteins. Others involve total deletion of the gene. Recent evidence suggests that variations in the positions of the truncations may account, in part, for the phenotypic differences observed among FAP patients. For example, FAP patients with ocular fundus lesions were found to have truncating mutations after exon 9 (Olschwang et al., 1993) and patients with an attenuated form of FAP were found to have truncating mutations located in a discrete region of the 5' end of the APC gene (Spirio et al., 1993).

It is possible that some mutant APC genes are not true null alleles and that there are important differences in the activities of the mutant proteins produced. Su et al. (1993) reported that most truncated APC peptides can associate with the wild-type APC in vivo, perhaps inactivating it in a dominant negative manner. This could explain why polyps from APC patients generally do not show evidence of a further change on chromosome 5q21 (Solomon et al., 1987; Vogelstein et al., 1988). Alternatively, the inactivation of only one of the two alleles may lead to polyp formation by reducing the production of the suppressor protein below a critical threshold concentration.

Vogelstein (1988) hypothesized that the FAP susceptibility gene normally acts as a negative regulator of colonic epithelium proliferation, and that loss of a single FAP allele leads to ineffective control resulting in hypertrophy of the colonic epithelium. Loss of the remaining wild-type APC allele is not the event that promotes transition from the hyperplastic epithelium to the adenomatous state. Loss of the second APC allele does appear, however, to be instrumental in the progression of adenomas to carcinomas. Support for this hypothesis comes from the observation of frequent loss of heterozygosity at the APC locus in

colorectal carcinomas from FAP patients (Sasaki et al., 1989) and from the finding that the cells of 81% of colorectal carcinomas are totally devoid of normal, full-length APC protein (Smith et al., 1993).

These findings highlight the need for a clearer understanding of the function of the APC gene and of the phenotypic effects of heterozygous and homozygous alterations at this locus. Furthermore, they suggest that theories regarding the oncogenic effect of inactivated tumor suppressor genes need to be expanded to include the possible dominant negative and dosage effects of these genes.

4. Identification by loss of heterozygosity in tumor cells

4.1 Colon carcinomas

As discussed in section A1.2, the progression of the colon cells from hyperplastic epithelium, through several stages of adenomas, and finally to invasive carcinomas, suggests that colon carcinogenesis requires multiple genetic changes. Inactivation of one FAP allele apparently results in hyperplasia of the colonic epithelium. Proliferation of these cells then provides the opportunity for further mutations to occur. Because tumors of all stages, from very small adenomas to large metastatic carcinomas, can be obtained for study, colorectal carcinogenesis is an excellent system in which to study the number and kinds of somatic mutations required for malignant transformation in human cells.

Although early studies of allelic losses in colon cancer patients focused on chromosome 5q because it was known to carry the locus segregating with this disease, it was also known from cytogenetic and molecular studies that portions of chromosomes 17 and 18 are frequently

absent in colorectal carcinomas (Reichmann et al., 1981; Muleris et al., 1985; Fearon et al., 1987). Vogelstein and his colleagues (1988) used restriction fragment length polymorphisms to detect the frequency of loss of heterozygosity on chromosomes 5, 17, and 18 in 172 colorectal tumor specimens representing various stages of neoplastic development. They found that specific regions of these three chromosomes were lost, respectively, in 35, 73, and 75 percent of colon carcinomas.

They determined the prevalence of these chromosomal losses in adenomas at various stages of neoplastic development, and found that allelic deletions of 5q occur at an early stage, allelic deletions of 17p occur at a late stage, and those of 18q occur at an intermediate stage. The allelic losses at 17p and 18q were interpreted as evidence that these regions encoded additional tumor suppressor genes.

a. The DCC gene

Vogelstein et al. (1988) determined that the colorectal tumor suppressor gene on chromosome 18 resided between 18q21.3 and the telomere. Within this region, they identified a large transcriptional unit which they named DCC for *Deleted in Colon Cancer* (Fearon et al., 1990). The DCC gene is expressed in most normal tissues, including colonic mucosa, but its expression is greatly reduced or absent in most colorectal carcinomas.

The predicted amino acid sequence encoded by the DCC gene specifies a protein with considerable homology to neural cell adhesion molecules, suggesting that DCC may play a role in cell-cell interactions. The types of mutations, mostly insertions and deletions, that have been found within in the DCC genes of tumor cells usually produce termination codons (Fearon et al, 1990). The proteins produced by these mutated genes are truncated

and, presumably, non-functional.

Confirmation of the DCC gene's function as a tumor suppressor requires analysis of the phenotypic effect of its introduction into tumor cell lines lacking a functional DCC gene. Microcell transfer of a normal chromosome 8 into colon carcinoma cells suppressed the tumor forming ability of these cells (Tanaka et al., 1991; Goyette et al., 1992). Introduction of the cloned DDC gene into such cells, an experiment that would be more definitive, has not yet been done.

b. The p53 gene

After determining that loss of heterozygosity on the short arm of chromosome 17 commonly occurs at a late stage in colon carcinogenesis, Vogelstein and his colleagues used additional 17p markers to further define the region of loss. The affected region contains a gene, p53, that had already been implicated in cellular transformation (Baker et al., 1989). The p53 protein was isolated in 1979 as a protein bound to SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). Later, mutant forms of the cloned p53 gene were shown to cooperate with *ras* in transformation cells in culture (Hinds et al., 1989).

When the p53 coding regions of colorectal tumors with 17p allelic losses were analyzed, the remaining p53 alleles were found to contain mutations in highly conserved regions of the gene (Baker et al., 1989; Nigro et al., 1989). This suggested that the wild-type allele acts as a colorectal tumor suppressor gene. Transfection of the wild-type gene into colorectal carcinoma cells suppressed cell growth, offering strong support for this hypothesis (Baker et al., 1990). Furthermore, the wild-type p53 was found to inhibit *ras* oncogene-mediated transformation to focus

formation of rat embryo fibroblasts (Finlay and Hinds, 1989; Eliyahu et al., 1989).

The finding that p53 functions as a suppressor gene in colorectal tumors prompted a search for p53 mutations in a wide assortment of other human tumors, many of which were already known to have frequent losses of chromosome 17p alleles. Over the past few years, p53 has been found to be mutated, usually homozygously, in lung, breast, esophageal, liver, bladder, ovarian, and brain tumors, and in lymphomas and leukemias (reviewed in Hollstein et al., 1991). This list is still growing and the accumulating data show p53 to be the most frequently involved gene in human oncogenesis. The December 24, 1993, issue of *Science* dubbed p53 the "gene of the year" owing to the great frequency with which it appears in the scientific literature and the enormous impact it has had on our understanding of the role of tumor suppressor genes in carcinogenesis.

The p53 story presents a reverse chain of events relative to those of the retinoblastoma paradigm. For retinoblastoma, the search for a germline mutation that was associated with the familial cancer revealed chromosome deletions that pinpointed the location of the susceptibility gene. This, in turn, allowed cloning of the gene and characterization of the protein product. For p53, discovery of the protein in a complex with the SV40 large T antigen allowed cloning of the gene long before its role as a tumor suppressor was revealed. After p53 was recognized as a tumor suppressor gene, the gene was examined as a candidate for the susceptibility gene in a rare familial cancer known as Li-Frammeni syndrome.

Li-Frammeni syndrome (LFS), like retinoblastoma and Wilms' tumor, is inherited in an autosomal dominant fashion. Affected individuals are

predisposed to the development of diverse mesenchymal and epithelial neoplasms at multiple sites, including breast carcinomas, soft tissue sarcomas, brain tumors, osteosarcomas, leukemia and adrenocortical carcinomas. The rarity and high mortality of LFS precluded genetic analysis by the techniques used to find the retinoblastoma and Wilms' tumor susceptibility genes. As an alternative approach, p53 was evaluated as a candidate for the LFS susceptibility gene because the spectrum of cancers in which it was known to be mutated included those malignancies found in LFS patients. Malkin et al. (1990) and Srivastava et al. (1990) detected germline p53 mutations in all six of the LFS families analyzed, indicating that inherited p53 mutations are responsible for transmission of this disease.

The p53 gene is expressed at low levels in all cells, and the protein product has a brief half-life. The protein functions as a transcriptional regulator (Fields and Jang, 1990) that activates expression of an inhibitor of the cyclin dependent kinases (El-Deiry et al., 1993; Harper et al., 1993). Inhibition of cyclin dependent kinases prevents passage through the cell cycle. By disruption of this cascade of events, inactivation of p53 leads to uninhibited cell replication.

Most mutations in the p53 gene extend the half-life of the protein and cause it to accumulate in the cell (Levine and Momand, 1990). Some mutant proteins appear to exert a dominant negative effect (Herskowitz, 1987; Eliyahu et al., 1988) possibly by binding to the wild-type protein in tetramers (Stenger et al., 1992). Evidence that some p53 mutant proteins may have inherent transforming properties unrelated to their ability to bind the normal p53 protein comes from the report that introduction of a particular mutant p53 gene into a cell line lacking both

p53 alleles enhanced the cell's tumorigenicity (Wolf et al., 1984). The mechanisms by which these different mutations exert their various phenotypic effects are not yet understood.

4.2 Other malignancies

Loss of heterozygosity has been used to study the role of tumor suppressor gene inactivation in numerous malignancies other than colorectal cancer. Among the malignancies studied are hepatic, breast, and bladder cancer. A large percentage of human hepatocellular carcinomas exhibit loss of the function of the p53 and RB1 genes. Frequent loss of heterozygosity on chromosomes 4 and 16 in these tumors indicates that additional tumor suppressor genes may be inactivated (Buetow et al., 1989; Fujimoto et al., 1994). Analyses of primary human breast tumors show loss of heterozygosity on chromosome 11 and in multiple regions of chromosomes 1 and 17 (Ali et al., 1987; Bieche et al., 1993; Cropp et al., 1993). Allelic losses in human bladder cancers give evidence for suppressor loci on chromosomes 3, 11, and 17, and at two distinct loci on chromosome 9 (Tsai et al., 1990; Klingelhutz et al., 1992; Ruppert et al., 1993). The cumulative data from these and numerous other studies indicate that the inactivation of tumor suppressor genes may be a more common mechanism in human carcinogenesis than is the activation of dominantly acting oncogenes.

C. Contributions from Cell Hybrid Studies

Although the methodology available for finding and characterizing

tumor suppressor genes is continually expanding, the technique of cell hybridization, which initially brought these genes to our attention, still contributes significantly to the accomplishment of this task. This technique is particularly well suited for obtaining certain kinds of information. Examples of such applications, in particular those related to carcinogenesis, are discussed below.

1. Chromosomal assignment of tumor suppressor genes

Although the detection of loss of heterozygosity in tumor cells is currently the most widely used method of locating tumor suppressor genes, this is an arduous task that is eased greatly if a tumor suppressor gene can first be assigned to a specific chromosome. Sometimes a particular tumor type is associated with common gross deletions that point to the location of an inactivated tumor suppressor gene. In the absence of localizing deletions, cell hybridization experiments may enable chromosomal assignment of the tumor suppressor gene.

As was briefly discussed in section B.1., the hybrid cell resulting from fusion of a tumor cell with a normal cell nearly always is non-tumorigenic. In rodent cell hybrids, rapid chromosomal loss from the tetraploid fusion product allows a high rate of reversion to the malignant phenotype, presumably by loss of the chromosome(s) bearing the suppressor allele(s) (reviewed in Harris, 1988; Klein, 1988; Sager, 1989). When a human cell is fused with another human cell (i.e., *human X human* cell hybrids) and also in the case of some *rodent X human* cell hybrids, greater chromosomal stability allows determination of the chromosomes associated with the suppressed phenotype. Preferential retention of certain chromosomes in suppressed hybrids and non-random chromosomal loss in

malignant revertants are often observed.

1.1 Hamster cell X human cell hybrids

Although very few *tumorigenic mouse cell X normal human cell* hybrids are suppressed for tumorigenicity (Klinger et al., 1978; Kucherlapati and Shin, 1979), a high proportion (30-50%) of *tumorigenic hamster cell X normal human cell* hybrids are suppressed (Klinger et al., 1978; Stoler and Bouck,1985; Wynford-Thomas et al., 1989). The hybrids are chromosomally stable, allowing comparison of the chromosome complement of the suppressed hybrids with those of the non-suppressed hybrid populations. Furthermore, the parental origin of the chromosomes can be determined by cytogenetic and biochemical gene marker methods.

a. CHO cell X HDF hybrids

Klinger and his colleagues fused tumorigenic Chinese hamster ovary (CHO) cells with human diploid fibroblasts (HDF). They found that retention of human chromosomes 2, 9, 10, 11, and 17 was associated with suppression of tumorigenicity in the hybrids (Klinger et al., 1978;Klinger and Shows, 1983). Chromosome 2 was never found in tumorigenic hybrid cells, and the other four chromosomes listed were found only at very low frequencies. The multiplicity of the chromosomes implicated in suppression of this tumor type may reflect the multistep nature of carcinogenesis. If the additive effect of several tumor suppressor gene losses is required for the expression of tumorigenicity, then one would expect that the retention of any one of these genes would suppress tumor growth.

Additional human chromosomes are associated with tumor suppression

in the *CHO cell X HDF* hybrids if pairs of chromosomes are considered (Klinger and Shows, 1983). For example, chromosomes 7 and 13 were found together in only 1% of tumors. Considered singly, they were found, respectively, in 17% and 24% of tumors. Evidently the combination of these chromosomes is an effective tumor suppressor in CHO cells, whereas one of them alone is not. In this manner, cell fusion studies can help to identify tumor suppressor genes that require the cooperation of a second tumor suppressor gene.

Most of the suppressor chromosomes identified by Klinger and Shows (1983) are now known, or have been implicated by other studies, to contain tumor suppressor genes. As discussed above, chromosome 17 contains p53, chromosome 11 contains WT1, chromosome 13 contains RB1. Chromosome 10 has been implicated through loss of heterozygosity as the location of a suppressor gene involved in malignant melanomas (Rempel et al., 1993). The chromosomal region 9p13-p22 is a frequent site of allelic loss in leukemias, melanomas, malignant mesotheliomas, brain tumors, and lung and bladder carcinomas (Diaz et al., 1990; James et al., 1991; Fountain et al., 1992; Cairns et al., 1993; Center et al., 1993; Cheng et al., 1993; Olopade et al., 1993). The strong suppressive effect of human chromosome 2 in the CHO cell hybrids suggests that it, too, is the location of a tumor suppressor gene, although no role for this gene has yet been suggested by the study of human tumors.

b. BHK cell X HDF hybrids

Cell fusion studies indicate that the genes responsible for suppression may vary with different cell types. Stoler and Bouck (1985) fused anchorage independent baby hamster kidney (BHK) fibroblasts with normal human diploid fibroblasts. Human chromosome 1 was retained in all

hybrids that were suppressed for anchorage independence and was lost when these hybrids reverted to the transformed phenotype. This suggests that a gene or set of genes on chromosome 1 mediates suppression of anchorage independence in BHK cells.

Chromosome 1 was not among the chromosomes associated singly or in pairs with suppression of tumorigenicity in CHO cells (Klinger and Shows, 1983). Perhaps this discrepancy reflects the different embryological origins of the two cell types. BHK cells are mesenchymal, whereas CHO cells are epithelial. A second possibility is that the phenotypes of anchorage independence and tumorigenicity, although closely associated, are under separate genetic controls.

1.2 Human cell X human cell hybrids

The effectiveness of human chromosomes as suppressors of transformation in hamster cells reflects the highly conserved nature of many tumor suppressor genes and their fundamental role in basic cellular processes. Some tumor suppressor genes, p53 for example, are ubiquitously expressed in normal tissues and are inactivated in a wide variety of tumors from many animals. Others, such as WT1, have a narrow range of expression and limited oncogenic potential. Human genes of the latter type may not be easily identified through interspecies cell hybridization studies. For the identification of the chromosomes carrying these genes, intraspecies human cell hybridization is likely to be more useful.

a. HeLa cell X HDF hybrids

Successful identification of chromosomes associated with tumor suppression in intraspecies human cell hybrids has been limited to one

hybrid cell system, namely human cervical carcinoma-derived HeLa cells fused with human diploid fibroblasts. All hybrids from this cross failed to form tumors when inoculated into nude mice (Stanbridge, 1976; Klinger et al., 1978; and Klinger, 1980). Karyotypic analysis revealed that the hybrids cells maintained nearly complete complements of chromosomes from both parent cell lines during extensive massaging in culture.

Rare tumorigenic segregants arose within hybrid cell populations only after prolonged periods of culture or through specific selection for variants (Klinger, 1980; Stanbridge et al., 1981). These segregants exhibited loss, primarily from the genetic material contributed by the normal diploid parent, of ≤5% of their original chromosome complement. Restriction fragment length polymorphism analysis revealed that every non-tumorigenic hybrid contained four copies of chromosome 11, two from each parent cell. All but one of 57 tumorigenic segregants, however, lacked one or both copies of chromosome 11 from the normal parent (Kaelbling and Klinger, 1986; Klinger and Kaelbling, 1986; and Srivatsan et al., 1986). This suggests that a suppressor gene or set of genes on the normal chromosome 11 is involved in the control of tumor formation in these hybrids.

Support for this hypothesis came from the detection of loss of heterozygosity on the short arm of chromosome 11 in Hela cells, an indication that HeLa cells may be deficient in a suppressor function at this locus (Kaelbling et al., 1986). In the non-tumorigenic hybrids, the normal chromosome 11 may complement this deficiency. Microcell transfer of a normal chromosome 11 into HeLa cells, or into tumorigenic segregants of *HeLa cell X HDF* hybrids, suppressed the tumorigenicity of these cells, confirming the suppressive function of chromosome 11 in this cell type

(Saxon et al., 1986).

b. HT1080 cell X HDF hybrids

Stanbridge and his colleagues attempted to analyze suppression of malignancy in other *human tumor cell X HDF* hybrids (Stanbridge et al., 1982). When they fused human fibrosarcoma-derived HT1080 cells with normal human fibroblasts, most of the hybrids retained the tumorigenic phenotype, a finding that agreed with that of Croce et al. (1979). Karyotypic analysis revealed that all the tumorigenic hybrids were hexaploid, containing a tetraploid complement of HT1080 chromosomes and a diploid complement of HDF chromosomes.

Only two non-tumorigenic hybrids were obtained from these fusions. Both were tetraploid with one complement of chromosomes from each parent cell (Benedict et al., 1984). From one of these non-tumorigenic hybrids, rare tumorigenic segregants arose. Reversion to tumorigenicity was associated with loss of chromosomes 1 and 4, suggesting a role for these chromosomes in the suppression of the tumor phenotype of HT1080 cells. The difficulty in obtaining larger numbers of tetraploid, non-tumorigenic *HT1080 cell X HDF* hybrids precluded further analysis of tumor suppression in this system.

c. Other malignant cell X normal cell hybrids

From most fusions of malignant human cells with normal human fibroblasts, Stanbridge et al. (1982) were unable to obtain long-term hybrid cell populations. Although hybrid clones arose, the populations senesced after a limited number of population doublings. Similar observations were reported by Bunn and Tarrant (1980), Muggleton-Harris

and Desimone (1980), and Pereira-Smith and Smith (1981). On the basis of these observations, it was postulated that the infinite life span of transformed cells in is acquired through loss of a growth suppressor function, and that the normal cell supplies this function in *malignant cell X normal cell* hybrids, causing them to senesce. This hypothesis altered the direction of suppressor gene research. Investigators began to think in terms of suppression of specific transformed phenotypes, rather than of malignancy per se.

2. Suppression of specific transformed phenotypes

In *CHO cell X HDF* and *HeLa cell X HDF* hybrids, many specific transformed phenotypes segregate independently of tumorigenicity (Stanbridge and Wilkinson, 1978; Klinger, 1980; Stanbridge, et al., 1982; Klinger and Shows, 1983). Although the hybrid cells are unable to form tumors in nude mice, they retain several other transformed characteristics, including infinite life span, growth in soft agar, growth factor independence, and altered cellular morphology. This suggests that the genetic mechanisms controlling tumorigenicity differ from those controlling these other phenotypes.

2.1 Anchorage independence

Although anchorage independence was not suppressed when CHO or HeLa cells were fused with normal human cells, this phenotype was suppressed when anchorage independent BHK cells were fused with normal BHK cells (Bouck and di Mayorca, 1982) or with normal human fibroblasts (Stoler and Bouck, 1985). As already mentioned, the human chromosome that was associated with suppression of anchorage independence was not among those

reported by Klinger and Shows (1983) to suppress CHO cell tumorigenicity, suggesting that these two phenotypes may be under separate genetic control.

2.2 Growth factor independence

Strauss and Mohandas (1987) found that hybrids between mouse melanoma cells and mouse embryo fibroblasts, or between mouse L cells and normal human fibroblasts, are suppressed for growth factor independence. In the *mouse L cell X HDF* hybrids, suppression of growth factor independence correlates with retention of human chromosomes 5, 22, and X. This group of chromosomes differs from those reported to suppress either agar growth or tumorigenicity.

2.3 Infinite life span

As described above (section C.1.2c, pp. 38-39), the phenotype of infinite life span can also be suppressed in cell hybrids. The fusion of infinite life span cells with finite life span cells yields finite life span hybrids. Immortal hybrids are frequently obtained, however, when rodent cells are used in such fusions, because the chromosomal instability of rodent cell hybrids allows frequent reversion to infinite life span by loss of the chromosomes that carry the suppressor genes.

Although Stanbridge and his colleagues and Klinger and his colleagues reported that *HeLa cell X HDF* hybrids have infinite life spans (Stanbridge, 1976; Klinger et al., 1978; Stanbridge and Wilkinson, 1978; Klinger, 1980), Pereira-Smith et al. (1990) concluded otherwise after closely charting the doubling rates of numerous hybrid populations. Out of the 39 clonal *HeLa cell X HDF* hybrid populations that they examined, 28

senesced after about 25 doublings. In another six of the hybrid populations, cell division slowed after about 25 population doublings, remained slow for several weeks, then returned to the original rapid rate. Pereira-Smith and Smith interpreted this pattern as an indication that the hybrid cells had an infinite life span and that infinite life span revertants arose that outgrew the senescing hybrid populations. The five remaining hybrid populations showed no decrease in their doubling rates throughout 100 population doublings. Pereira-Smith and Smith hypothesized that infinite life span revertants arose in these populations soon after cell fusion, before the cells of the hybrid population began to senesce.

2.4 Other transformed phenotypes

Hybrids from most fusions of infinite life span human cells with finite life span human cells are chromosomally stable. Reversion to the infinite life span phenotype in these hybrids is a rare occurrence (Pereira-Smith and Smith, 1981, 1983). This explains why Stanbridge et al. (1982) were unable to obtain long-term cultures of most *human tumor cell X HDF* hybrids. To circumvent this problem, suppression of tumorigenicity and other transformed phenotypes can be tested in hybrids from fusions of tumor cells with infinite life span non-tumorigenic cells. For example, Zajchowski et al. (1990) fused MCF-7 human breast cancer cells with immortalized non-tumorigenic human mammary epithelial cells. They successfully obtained long-term cultures of hybrid cells and reported that tumorigenicity, growth factor independence, tumor necrosis factor sensitivity, and pS2 breast cancer marker expression were suppressed in these hybrids.

Moroco et al. (1990) performed fusions among hamster buccal pouch

keratinocytes at various stages of transformation. These researchers documented that the phenotypes of angiogenic activity, infinite life span, anchorage independence, and tumorigenicity, which arose sequentially in these cells and were therefore hypothesized to be under independent genetic controls, are all linked to functional loss of suppressor genes. Other examples of specific transformed characteristics that have been shown to be suppressed in cell hybrids are the interleukin 3 expression of mouse mastocytoma cells (Diamantis et al., 1989) and the high frequency of gene amplification of HT1080 cells (Tlsty et al., 1992).

3. Complementation analysis

3.1 Complementation of tumorigenicity

Considering the evidence described above that various tumor types may arise by the inactivation of separate tumor suppressor genes, it is reasonable to predict that certain pairs of tumor cells may be able to complement the genetic defects in one another to yield non-tumorigenic hybrid cells. Wiener and Harris (1974) tested this hypothesis using intraspecies mouse cell hybrids. Only one of twelve crosses among various tumor cells generated hybrid cells with reduced tumorigenicity. These researchers concluded that the genetic lesions determining the malignant phenotype, although recessive, are unable to complement each other.

Stanbridge et al. (1982) hypothesized that the apparent inability of mouse tumor cells to complement each other resulted from the same phenomenon that obscured tumor suppression when mouse tumor cells were fused with normal mouse cells. Rapid loss of the suppressor chromosomes may have allowed the hybrids to revert to the tumorigenic phenotype. To eliminate this confounding variable, Stanbridge and his colleagues tested

the complementation hypothesis in human intraspecies hybrids. They reported that tumorigenicity was suppressed when carcinoma cells were fused with melanoma or sarcoma cells, but not when they were fused with lymphoblastoid cells or other carcinoma cells (Stanbridge at al., 1982; Weissman and Stanbridge, 1983). They concluded that complementation among tumor cells can occur, that at least two complementation groups exist, and that possibly a distinct gene or set of genes controls the expression of tumorigenicity for each somatic cell type.

3.2 Complementation of infinite life span

Because multiple phenotypes contribute to a cell's tumorigenic potential, it is of interest to know which specific phenotypes were suppressed in the non-tumorigenic hybrids from Stanbridge's *malignant cell X malignant cell* crosses. The studies referenced above did not report this information. Since infinite life span is a recessive phenotype, it may be that these hybrids lacked the ability to form tumors because complementation of the genetic defects associated with infinite life span yielded hybrids that had finite life spans that were too short to allow tumor formation.

Pereira-Smith and Smith (1983) investigated complementation of the infinite life span phenotype by fusing several pairs of infinite life span cells and assaying the proliferative potentials of the hybrids. They reported that infinite life span was suppressed in the hybrids from some fusions, but not in others. In extensions of these studies, they reported that 30 different immortal human cell lines could be assigned to four immortality complementation groups (designated A, B, C, and D), with no cell line belonging to more than one group (Pereira-Smith and Smith, 1988;

Ning and Pereira-Smith, 1991). These results suggest that four different genes or sets of genes contribute to the program for senescence in human cells. Support for the validity of the immortality complementation groups came from the finding that chromosome 4 suppressed the immortal phenotype when introduced into cells from complementation group B, but had no effect when introduced into cells from the other three groups (Ning et al., 1991).

Whitaker et al. (1992) fused the SV40-immortalized human cell line BET-1 to representative cells from each of the four complementation groups established by Pereira-Smith and Smith. They obtained immortal hybrids only from fusions with cells in group D, indicating that BET-1 belongs to group D. When they later applied this method to three other SV40-immortalized cell lines, however, each of the cell lines were found to belong to more than one complementation group (Duncan et al., 1993). These results indicate that complementation among immortal cells is a more complicated phenomenon than the studies of Pereira-Smith and Smith suggest.

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CHAPTER II

Failure of Infinite Life Span Human Cells from Different Immortality Complementation Groups to Yield Finite Life Span Hybrids

P. Ann Ryan, Veronica M. Maher, and J. Justin McCormick

Carcinogenesis Laboratory-Fee Hall

Department of Microbiology and Department of Biochemistry

Michigan State University, East Lansing, MI 48824-1316

ABSTRACT

The observation that fusion of infinite life span cells with finite life span cells produces hybrid cells with finite life spans led to the conclusion that an infinite life span in culture is a recessive trait resulting from loss of the function of a gene or genes that contribute to an active program for cellular senenscence. Furthermore, finding that certain pairs of infinite life span cells, when fused to one another, can complement each other to yield finite life span hybrids allowed 30 infinite life span cell lines to be assigned to four immortality complementation groups (Pereira-Smith and Smith, 1988, Proc. Natl. Acad. Sci. USA, 85:6042). In the present study, we fused a chromosomally stable, near diploid, morphologically normal, infinite life span cell strain, designated MSU-1.1, with its normal, finite life span, precursor cell strain and obtained finite life span hybrids, as expected if infinite life span in culture is a recessive trait. However, 14 of the 14 hybrids from our fusions of MSU-1.1 cells with representative cell lines from each of the four immortality complementation groups, and 38 of the 39 hybrids from our fusions of infinite life span cells that have been reported to complement each other, failed to exhibit finite life spans. This result suggests that infinite life span cells cannot complement each other to yield finite life span hybrids. In examining this unexpected result, we obtained evidence that long-term dual drug selection can be deleterious to hybrid cells even though they carry resistance markers for both drugs, indicating that the cell death of such hybrids observed in other studies may have resulted from the cytotoxic effect of long-term drug selection, rather than from senescence.

INTRODUCTION

Normal diploid human fibroblasts in culture have a limited proliferative potential (Hayflick and Moorhead, 1961; Hayflick, 1965). However, many human tumor-derived cells can proliferate in culture indefinitely. Studies by Bunn and Tarrant (1980), Muggleton-Harris and DeSimone (1980), Pereira-Smith and Smith (1981, 1983), and Pereira-Smith et al. (1990) indicate that cellular senescence is a genetically programmed active process and that escape from cellular senescence is the result of recessive genetic alterations in this program. These investigators fused a variety of immortal human cells, including tumor-derived and simian virus-40 (SV40)-transformed strains, to finite life span human fibroblasts. The hybrids obtained from these fusions had a limited life span. This phenomenon was interpreted as indicating that immortality results from loss of function of one or more of the genes responsible for senescence in normal cells and that, when an infinite life span cell is fused with a finite life span cell, the hybrid cell senesces because the latter parent supplies the missing function.

Further support for the theory that immortality results from loss of function of the gene(s) responsible for senescence comes from the work of Pereira-Smith and Smith and their colleagues. These investigators fused various immortal human cell lines with one another and determined the life span of the hybrid cells. Certain hybrids senesced, indicating that the cells used in these fusions were able to complement the genetic defects in each other (Pereira-Smith and Smith, 1983). Through life span analysis of hybrids obtained from such cell fusions, 30 different immortal human cell lines were assigned to four immortality complementation groups (designated

A, B, C, and D), with no cell line belonging to more than one group (Pereira-Smith and Smith, 1988; Ning and Pereira-Smith, 1991). Ning et al. (1991) reported that introduction of chromosome 4 into cell lines from complementation group B resulted in loss of proliferation and reversal of the immortal phenotype but had no effect on the proliferative potential of representative cell lines from the other three complementation groups. They attributed this result to complementation, by genes on chromosome 4, of the genetic defect shared by the cell lines belonging to group B.

It has been difficult to determine the genetic and biochemical changes responsible for acquisition of an infinite life span, because the majority of the cell lines available for study not only exhibit an infinite life span, but also have many other abnormal characteristics. Many are derived from tumors, others arose following SV40 transformation (reviewed in Sack 1981), and two arose in populations of cells exposed repeatedly to carcinogen treatment (Namba et al., 1981; McCormick and Maher 1988). The majority of these cell lines are highly aneuploid and chromosomally unstable. To study the genetic changes specific to the process of immortalization, it is very useful to have infinite life span cells that maintain a stable diploid or near-diploid karyotype and do not have abnormal characteristics other than being immortal. Recently, we (Morgan et al., 1991) successfully derived such cells by transfecting a foreskin-derived, normal, diploid human fibroblast cell line, designated LG1, with a plasmid carrying a v-*myc* oncogene and a drug resistance marker, selecting for drug resistant transfectants, and expanding individual clones to the end of their life span. An infinite life span diploid cell strain, designated MSU-1.0, spontaneously arose in a v-*myc*-expressing clonal population. The MSU-1.0 cell strain gave rise to a stable, near-

diploid cell strain, designated MSU-1.1. MSU-1.1 cells have a normal morphology, are only partially growth factor independent, do not form foci, form only very small colonies at low frequency in 0.33% agarose, and are not tumorigenic (Morgan et al., 1991).

Because of their stable near-diploid karyotype and their near normal phenotype, we are using MSU-1.1 cells to study the genetic changes involved in the process of immortalization. We began by determining whether their infinite life span resulted from activation of a gene (i.e., was a dominant trait) or inactivation of dominant alleles (i.e., was a recessive trait). We did so by fusing MSU-1.1 cells with finite life span cells and determining the length of the life spans of the hybrids. The results indicated that an infinite life span is a recessive trait. We then fused MSU-1.1 cells with representative cell strains from each of the four immortality complementation groups established by Pereira-Smith and Smith (1988) to determine whether the MSU-1.1 cells belong to one of the four groups. All of these fusions yielded hybrids with infinite life spans, indicating that MSU-1.1 cells belong to all four complementation groups (i.e., had lost function of all the genes or sets of genes represented by these groups). These results called into question the existence of the four immortality complementation groups. To confirm the existence of the four groups, we repeated a select series of fusions among representatives of each of the four complementation groups, using cell strains supplied by Pereira-Smith and Smith, and analyzed the life span of the hybrids. These fusions consistently yielded immortal hybrids (i.e., they failed to confirm the existence of the four groups). The results of additional studies of the effects of drug selection indicated that the cell death reported by Pereira-Smith and Smith (1988) for the hybrids from

immortal cells fused with immortal cells probably resulted from the toxic effect of long term drug selection.

MATERIALS AND METHODS

Cells used

The human cell lines and strains used and their origin and source are listed in Table 1.

Cell culture

The cells were routinely cultured in Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, NY) supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, and 1.0 mM sodium pyruvate, or in McM medium (Ryan et al., 1987) modified by substituting 0.884 mM monobasic sodium phosphate for 3.0 mM dibasic sodium phosphate, adding 0.054 mM KCl, and adjusting the NaCl content so that the osmolarity was 285 $\pm$ 5 mOsm/kg H_2O. The medium was supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), hydrocortisone (10 µg/ml), 5% supplemented calf serum (Sterile Systems, Logan, UT), and 5% fetal bovine serum to make "complete" medium. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were subcultured by removing the medium from the cells, washing with phosphate buffer saline, dislodging them by brief exposure to 0.25% trypsin, and diluting them appropriately in the designated medium. Cell counts were performed electronically on a Coulter counter (Coulter Electronics, Inc., Hialeah,

TABLE 1. Human cells used in fusion experiments

Cell line or strain	Cell type - origin	Source
WI-38	Finite life span diploid fibroblast	ATCC[1]
LG1	Finite life span, foreskin-derived diploid fibroblasts	--[2]
MSU-1.1	Infinite life span derivative of LG1 cells	--[2]
MSU-1.1-CHAT^{s}Hg^{r}[3]	Clonally-derived from MSU-1.1	This study
VA13	SV40-immortalized lung fibroblasts	R. A. Schultz[4]
T24 (EJ)	Bladder carcinoma-derived cell line	ATCC[1]
HT1080	Fibrosarcoma-derived cell line	ATCC[1]
HT1080-CHAT^{s}Oua^{r}[5]	Clonally-derived from HT1080	Pereira-Smith
A1698	Bladder carcinoma-derived cell line	Pereira-Smith
A1698-CHAT^{s}Oua^{r}[5]	Clonally derived from A1698	Pereira-Smith
143BTK^{-}	Clonally derived from an osteo-sarcoma-derived cell line	NIGMS[6]
HeLa	Cervical carcinoma-derived cell line	Ward Peterson[7]
CT-1	Infinite life span cell derived from AD837 fibroblast cells[8]	Namba

[1]ATCC: American Type Culture Collection (Rockville, MD).

[2]See Introduction.

[3]CHAT^{s}Hg^{r}: Cells lacking a functional hypoxanthine(guanine)phosphoribosyl-transferase (*HPRT*) gene and, therefore, unable to survive in medium containing 2 X 10^{-5} M deoxycytidine, 10^{-4} M hypoxanthine, 4 X 10^{-7} M

TABLE 1 (cont'd)

aminopterin, and 3 X 10^{-5} M thymidine (CHAT) and resistant to 125 units/ml hygromycin B (Hg) (Calbiochem).

[4]John Sealy Center for Molecular Science, University of Texas, Galveston, TX

[5]CHAT^{s}Oua^{r}: *HPRT*$^{-}$ cells unable to survive in medium containing 2 X 10^{-5} M deoxycytidine, 10^{-4} M hypoxanthine, 4 X 10^{-7} M aminopterin, and 3 X 10^{-5} M thymidine (CHAT) and resistant to 10^{-7} M ouabain (Oua) octahydrate (Sigma).

[6]NIGMS: National Institute of General Medical Sciences Human Genetic Mutant Cell Bank (Camden, NJ).

[7]Children's Hospital of Michigan (Detroit, MI).

[8]Studies by McCormick and Maher (1988) showed that CT-1 cells are identical to SUSM-1 cells and are very probably cells derived from AD837 cells, not from WI-38 cells as originally reported by Namba et al. (1981).

FL). Frozen cultures were stored at -135°C in medium containing 15% serum and 10% dimethyl sulfoxide.

Transfection

The plasmid pHT containing the bacterial hygromycin resistance gene was provided by Dr. Norman Drinkwater (Univeristy of Wisconsin, Madison, WI). MSU-1.1 cells were transfected using the dimethylsulfoxide/polybrene method adapted for use with human fibroblasts (Morgan et al., 1986). Cells were plated at 2 X 10^5 cells per 100 mm diameter dish and transfected 18 h later with 1 µg/ml plasmid DNA. Hygromycin (Calbiochem, LaJolla, CA), at a concentration of 125 units/ml, was added to the medium 24 h post-transfection. The cells were refed with selective media weekly, and macroscopic drug-resistant colonies formed in 2-3 weeks.

Selective media

For every cell fusion, one parent cell was a universal fuser, that is, carried a dominant drug (hygromycin or ouabain) resistance marker, but lacked a functional dominant gene coding for the enzyme hypoxanthine (guanine) phosphoribosyl transferase (*HPRT*), and, therefore, was unable to survive in CHAT medium. The other parent cell lacked the drug resistance marker, but could survive in CHAT medium because it carried a functional *HPRT* gene. Three universal fuser strains were used: MSU-1.1-CHAT^{s}Hg^r, HT1080-CHAT^{s}Oua^r, and A1698-CHAT^{s}Oua^r.

For fusions with MSU-1.1-CHAT^{s}Hg^r cells and for selection, expansion, and life span analysis of the hybrids, McM medium (with the modification listed above) was used. Selection and expansion of hybrids took place in complete medium to which were added CHAT and hygromycin. For fusions with

HT1080-CHAT^{s}Oua^r cells or A1698-CHAT^{s}Oua^r cells, and for selection, expansion, and life span analysis of the hybrids, Eagles MEM (with the modification listed above) was used. Selection and expansion of hybrids took place in complete medium supplemented with CHAT and ouabain. When CHAT selection was discontinued, CHT (CHAT minus aminopterin) was added to the complete medium for 1-2 weeks.

Cell fusion, selection, and initial expansion

Cell fusion and selection were performed according to the procedures described by Pereira-Smith and Smith (1981, 1982) with the modifications noted below. Cells (1×10^5) of each of two parental lines were seeded in complete medium into a 35-mm diameter tissue culture dish. Eighteen to 24 h later the medium was replaced by medium lacking serum and containing 45% polyethylene glycol (1,000) (Sigma, St. Louis, MO). After another 24 h, the cells were harvested and reseeded into 100 mm diameter tissue culture dishes at 10^3 and 10^4 cells per dish in selective medium. The cells were incubated for 2-3 weeks, with weekly renewal of the selective medium. At the end of the incubation period, the dishes were scanned to locate hybrid clones. Each clone was transferred into a 35-mm diameter tissue culture dish. When the cells had filled the dish, they were transferred to a 25 cm^2 flask and then to a 75 cm^2 flask to expand the clonal population to approximately 4×10^6 cells (i.e., about 22 population doublings ([pdls]). During this expansion, selective medium was renewed twice weekly. At this point, a portion of the progeny cells from each hybrid were seeded into two 25 cm^2 flasks, and the remaining cells were stored frozen.

Determination of the life span of the hybrids

To determine the life span of the cells in a population derived from a hybrid clone, the progeny cells were serially subcultured in complete medium in two 25 cm^2 tissue culture flasks, with medium changes twice weekly. Each time the cells reached confluence, they were subcultured at a 1:4, 1:8, 1:16, or 1:32 cell dilution: the higher dilutions were used for the cells that exhibited shorter doubling times. At the time of subculture, the cells were recorded as having completed 2, 3, 4, or 5 population doublings, respectively, since the last subculture. Following the criteria of Pereira-Smith et al. (1990), a hybrid was considered to be at the end of its life span if the cells did not achieve at least one population doubling within 3 weeks. Following the criteria of Pereira-Smith and Smith (1988), a hybrid was considered to exhibit an infinite life span if it achieved a total of 100 population doublings after fusion.

DNA flow cytometry

To confirm that isolated clones were hybrids, the DNA content of parental cells and isolated hybrid clones was determined. Propidium iodide stained cells were analyzed for DNA content using an Ortho Diagnostics 50H Cytofluorograph with Acqcyte software supplied by Phoenix Flow, Inc. (San Diego, CA).

RESULTS

Life span analysis of hybrids formed by fusions with MSU-1.1 cell strains

To allow selection of hybrids between MSU-1.1 cells and various other cell strains, we developed a clonal strain of MSU-1.1 which carries both a dominant and a recessive drug resistant marker. First, MSU-1.1 cells were transfected with plasmid pHT (which carries a bacterial gene for hygromycin resistance), and a hygromycin resistant clone was isolated. Secondly, this hygromycin resistant clone was expanded and treated with 6-thioguanine to isolate an *HPRT*$^-$ CHAT sensitive subclone. This doubly-marked (Hg^r CHAT^s) subclone of MSU-1.1 was used in fusions with various other human cell lines, as listed in Table 2.

To determine whether the infinite life span phenotype of the MSU-1.1 cell strain resulted from a dominant or a recessive genetic change, we fused MSU-1.1 cells to the finite life span, diploid fibroblast line LG1 from which they were derived, and also to another finite life span, diploid human fibroblast line, WI-38. As a control, we fused MSU-1.1 cells that carried both markers to MSU-1.1 cells lacking the markers (Hg^s CHAT^r). Representative hybrid clones (i.e., cells able to grow in medium that selected against both sets of parental cells) were isolated, expanded, and assayed for the length of their life span, as described in Materials and Methods. In addition, LG1 parental cells were plated at cloning density in non-selective medium, and eight clones were isolated and assayed for the length of their life span in that medium.

All of the eight LG1 clonal strains ceased division between 7 and 27 population doublings following cloning. Similarly, all five hybrids

TABLE 2. Proliferative potential of hybrids formed by fusion of various cell strains with MSU-1.1 CHAT^{s}Hg^{r} cells

Cell strain	Finite life span	Immortality complementation group[1]	Hybrids that senesced per total hybrid clones
LG1	+	NA[2]	5/5[3]
WI-38	+	NA	4/4[3]
MSU-1.1	-	--[4]	0/6
VA13	-	A	0/1
T24 (EJ)	-	A	0/2
HT1080	-	A	0/2
HeLa	-	B	0/4
143BTK	-	C	0/4
CT-1	-	D	0/1

[1]Specified by Pereira-Smith and Smith (1988).

[2]NA, Not applicable.

[3]The progeny of these nine hybrid clones senesced at population doubling 7-16 after fusion.

[4]To be determined in these fusion experiments.

between MSU-1.1 and LG1 cells and all four hybrids between MSU-1.1 cells and WI-38 cells exhibited a finite life span, achieving only 7 to 16 population doublings before senescing. However, as expected, none of the six MSU-1.1 self hybrids senesced, demonstrating that cell hybridization itself does not cause cells to cease proliferation. These results indicate that the infinite life span of MSU-1.1 cells resulted from a recessive genetic change.

To determine to which of the four established immortality complementation groups of Pereira-Smith and Smith (1988) the MSU-1.1 cells belonged, we fused them with representative cell strains from each of the four groups and assayed the life span in culture of the resulting hybrid clonal populations. The inability of MSU-1.1 cells to complement a particular immortal cell line (i.e., to yield hybrids with finite life spans) would indicate that MSU-1.1 cells cannot supply the genetic information that is lacking in that particular cell line. In this case MSU-1.1 cells would be assigned to the complementation group of that cell line. We succeeded in obtaining hybrid clones from fusions with cell lines from all four groups. Very unexpectedly, all 14 hybrids proliferated to greater than 100 pdls, a life span that Pereira-Smith and Smith (1988) use as their criterion for immortality. These results indicate that MSU-1.1 cells were not able to complement any of these immortal cell lines (i.e., could not supply the missing genetic function of any line). In other words, the MSU-1.1 cell strain would have to be categorized as belonging to all four complementation groups.

Determining the ability of representatives of the established complementation groups to complement each other

Before concluding that MSU-1.1 cells were unable to complement immortal cells from all four groups, we needed to confirm that fusions between cells from different complementation groups would yield hybrids with finite life spans. To do this, we used cell lines supplied by Dr. Pereira-Smith to carry out some of the same fusions reported by Pereira-Smith and Smith (1988) as yielding finite life span hybrids. Seven cell fusions were carried out: two self-fusions (i.e., A1698 cells X A1698 cells and HT1080 cells X HT1080 cells) and five fusions between cell strains reported to belong to separate complementation groups (e.g., cells from group A X cells from group B, A X C, etc). Table 3 lists our results. In the experiments of Pereira-Smith and Smith (1988), self-fusions yielded immortal hybrids, whereas fusions between cells from different groups yielded hybrids that senesced by population doubling (pdl) 65 or earlier. In our experiments, self-fusions yielded immortal hybrids as expected, but 38 out of 39 of the hybrids that were expected to senesce failed to do so. Instead, such hybrids proliferated rapidly to greater than 100 pdls.

When we did not observe the expected senescence in this set of hybrids, we first considered the possibility that our culture conditions fostered longer life spans of finite life span cell strains than expected and that our hybrids would eventually senesce if grown for a longer time. Therefore, we continued to passage nine hybrid clones beyond pdl 100. All nine continued to replicate vigorously (data not shown) and were stored frozen in liquid nitrogen after a total of 146-156 pdls.

Testing the hypothesis that parental cells survived selection

We next considered that, perhaps, our selection conditions were not sufficiently rigorous and had, therefore, allowed infinite life span parental cells to survive and overgrow any senescing cells in our life span assay. Our selection system differed from that of Pereira-Smith and Smith (1988) in that after progeny cells from a hybrid clone had been expanded in selective medium from a small colony to more than 4 X 10^6 cells (i.e., had undergone $\geq$ 22 pdl), we discontinued drug selection. Pereira-Smith and Smith maintained drug selection throughout the entire life span assay. To verify that the cells growing vigorously in our immortal populations were progeny of hybrid cells, we used flow cytometric analysis to compare the amount of DNA per cell of representative hybrids with that of their respective parental cells (data not shown). For 16 of the 17 hybrids analyzed at population doublings 25-30 post-fusion, the amount of DNA per cell was virtually equal to the sum of that of the two parental cell lines. The remaining hybrid had somewhat less DNA per cell than the total amount from the two parental strains, but the amount of DNA per cell was significantly higher than that of either parent. Six of these hybrids were also analyzed at pdls greater than 100 post-fusion. Two of the six had lost some DNA during this extensive passaging, but the amount of DNA per cell for each of the six was still higher than that of either parental cell, indicating that they were hybrid cells.

To obtain further evidence that our immortal populations were the progeny of hybrid cells, rather than parental cells, we tested six of them at population doublings greater than 100 for the ability to grow in the selective medium. Five of the six doubled in selective medium at the same rate as they did in parallel flasks in the absence of selective pressure.

TABLE 3. Proliferative potential of cell hybrids formed by fusions among cells from the four immortality complementation groups

Cells fused[1]	Hybrids expected to senesce	Hybrids that senesced per total hybrid clones
HT1080 (A) X HT1080 (A)	No	0/6
A1698 (D) X A1698 (D)	No	0/5
HT1080 (A) X HeLa (B)	Yes	1/13
HT1080 (A) X 143BTK$^-$ (C)	Yes	0/15
A1698 (D) X HT1080 (A)	Yes	0/5
A1698 (D) X HeLa (B)	Yes	0/5
A1698 (D) X 143BTK$^-$ (C)	Yes	0/1

[1]For each cross listed, the parent cell line on the left was CHAT^s and Oua^r. The letters in parentheses indicate the complementation group to which the cell line has been assigned by Pereira-Smith and Smith (1988).

The sixth doubled somewhat more slowly in selective medium than in its absence, but the cells appeared perfectly healthy, indicating that they were resistant to the selective agents and, therefore, were hybrid cells. Therefore, we ruled out the explanation that parental cells had survived selection.

Testing the hypothesis that our infinite life span populations represented overgrowth of senescing populations by revertants

A third possible explanation for the infinite life spans of 38 of the 39 hybrids from putatively complementary parents is that the original hybrid cells possessed a full complement of the genetic material that confers a finite life span, but progeny cells subsequently lost some or all of the critical genetic material and, by doing so, regained an infinite life span. If a strain were to begin to senesce before such an immortal progeny cell (revertant) has arisen, this would be reflected by a temporary plateau in the growth curve. Such plateaus were reported by Pereira-Smith et al. (1990) for hybrids of HeLa cells fused with human diploid fibroblasts. We examined the growth curves of our hybrids (Fig. 1 and 2) for plateaus. Only one of our 38 immortal hybrids derived from putatively complementing parents exhibited such a temporary decrease in growth rate (Fig. 1C, closed diamonds). The decrease began at pdl 22, and the cells returned to their previous rapid rate of growth at pdl 30. However, we also noted that the rate of growth of several slow-growing hybrids (e.g., Fig. 1C, closed triangles) increased at approximately pdl 22, the time at which drug selection was discontinued. We had originally decided to discontinue drug selection at the end of the initial expansion phase (i.e., when the clonal population had filled the flask [pdl 22])

Figure 1. Growth curves of hybrids formed by fusion of HT1080-CHAT^{s}Oua^{r} cells with cells representative of immortality complementation groups A, B, and C

Drug selection was discontinued at pdl 22, as indicated by the arrows. Where growth curves of several clones fall upon one another (e.g., panel A, clones A-F), a single representative curve is depicted, and the number of clones in the group is indicated (e.g., Panel A', 6 clones).

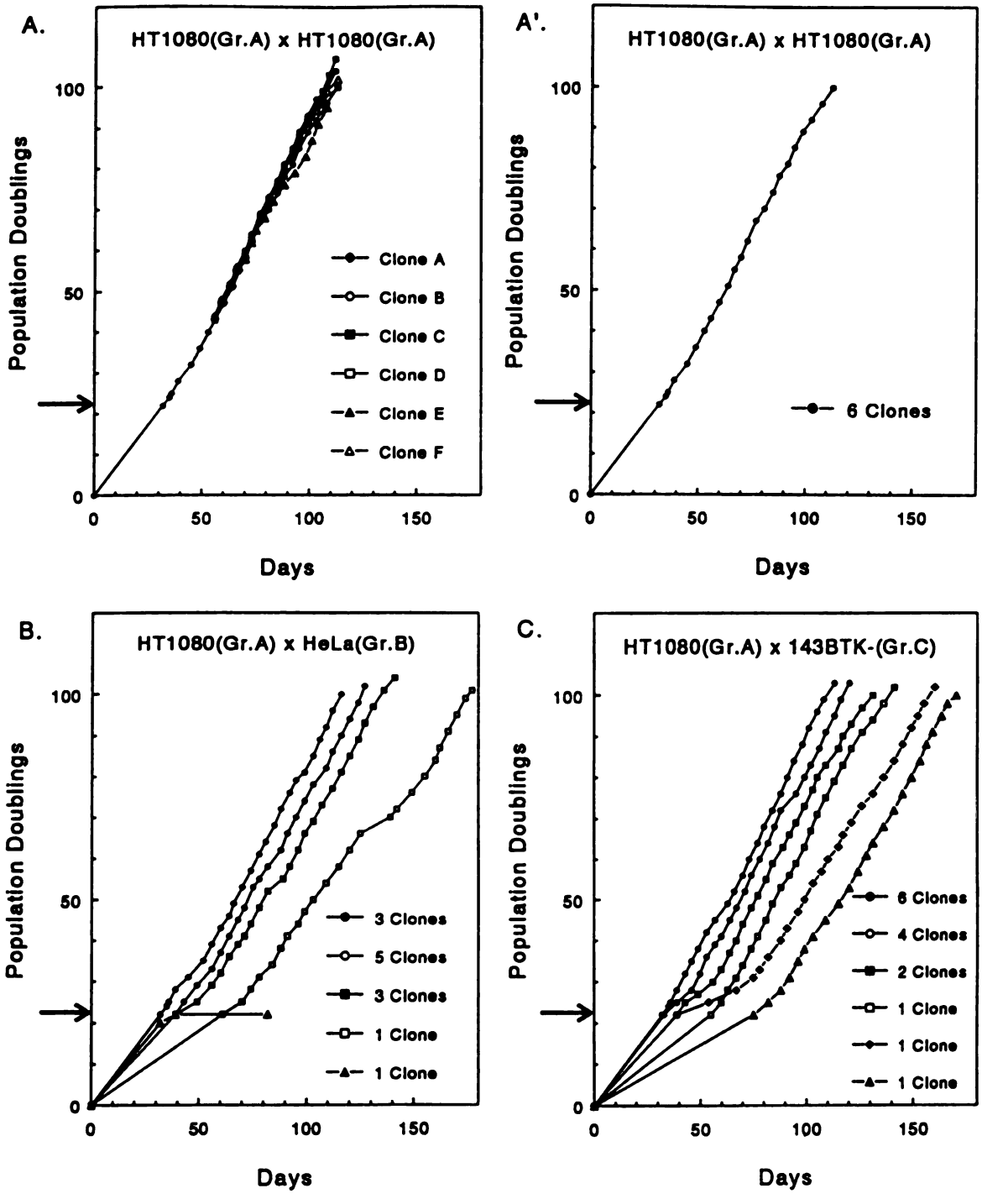

Figure 1. Growth curves of hybrids formed by fusion of HT1080-CHAT^{s}Oua^{r} cells with cells representative of immortality complementation groups A, B, and C

Figure 2. Growth curves of hybrids formed by fusion of A1698-CHAT^{s}Oua^{r} cells with cells representative of immortality complementation groups A, B, C, and D

Drug selection was discontinued at pdl 22, as indicated by the arrows.

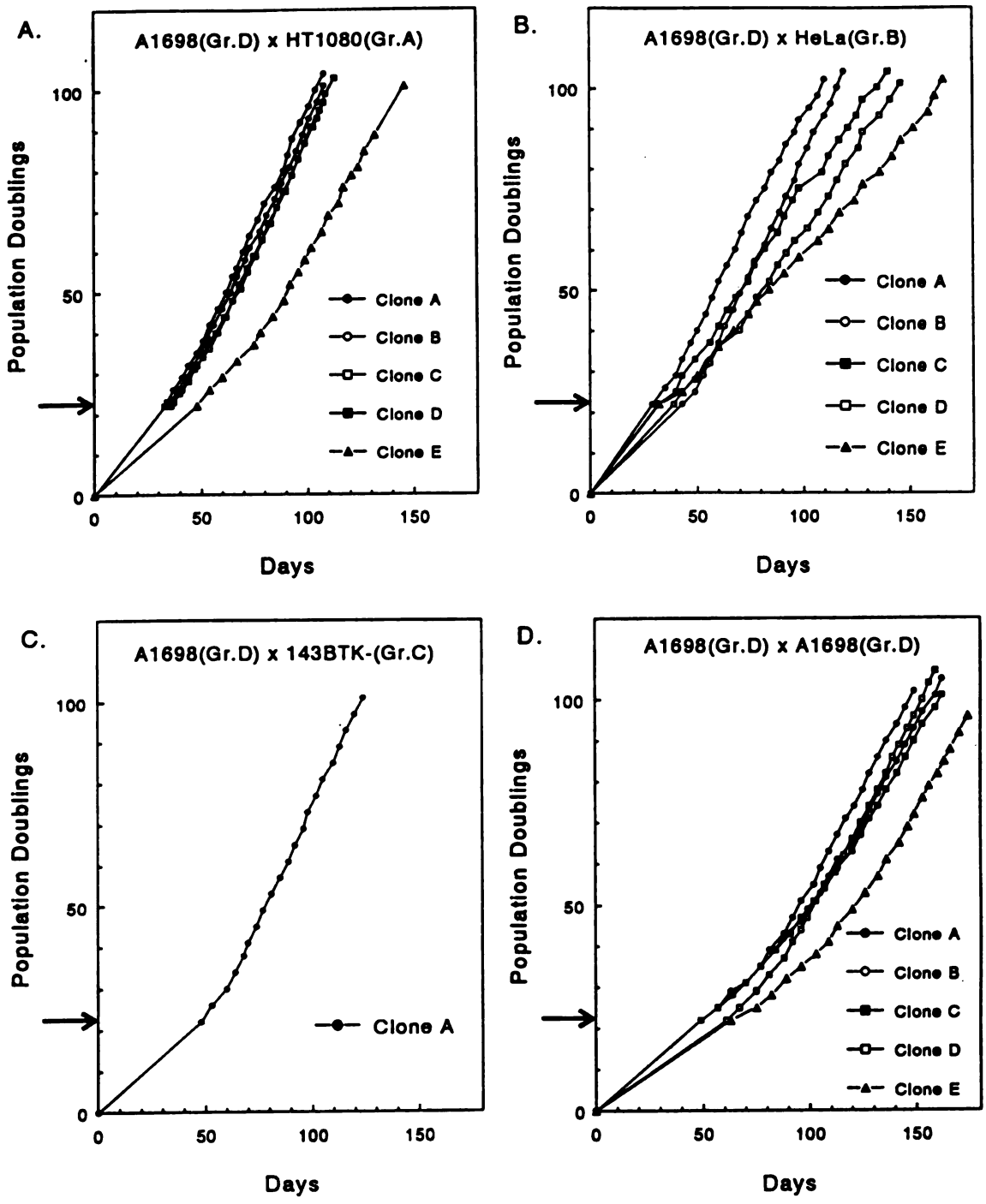

Figure 2. Growth curves of hybrids formed by fusion of A1698-CHAT^{s}Oua^r cells with cells representative of immortality complementation groups A, B, C, and D

because we noted that during expansion of the hybrids in selective medium, the cells, although resistant to both drugs, gradually became granular in appearance. Some populations doubled more slowly than the others, and the cells in these populations tended to detach from the flask. When our hybrid cells were no longer under selection, they lost their granularity. The slow-growing hybrids became more firmly attached to the flask and began to proliferate as fast as the others.

Testing the effects of long-term drug selection on senescence

Since the hybrid cells appeared much healthier after discontinuation of drug selection, we hypothesized that a longer period of drug selection can result in cell death. If so, the difference between our drug selection regimen and that of Pereira-Smith and Smith (1988) could account, at least in part, for the difference between our results and theirs. To test whether continuous propagation of hybrids in selective medium can result in cessation of growth, we thawed cells from 22 of our hybrid clones that had been frozen at pdl 22-26 and repeated the life span assay, passaging each hybrid strain in parallel flasks in the presence and absence of drug selection. Growth curves of representative hybrids are shown in Figure 3. When a cell population was grown under non-selective conditions, its growth curve closely matched that which we had obtained previously under these conditions (Fig. 3, open circles). However, when cells were propagated under continued drug selection (Fig. 3, closed circles), toxic effects were observed. Eight of the 22 hybrids continued to proliferate at a steady rate, but slightly slower than the parallel cultures grown in the absence of drug selection (e.g., Fig. 3A). The other 14 hybrids, when kept under selective conditions, entered a period of rapid cell death

Figure 3. Growth curves of hybrids formed by fusion of HT1080-CHAT^{s}Oua^{r} cells with HeLa cells

Closed symbols, continuous drug selection; open symbols, drug selection discontinued at pdl 22. Arrows indicate the beginning and end of the period of cell crisis.

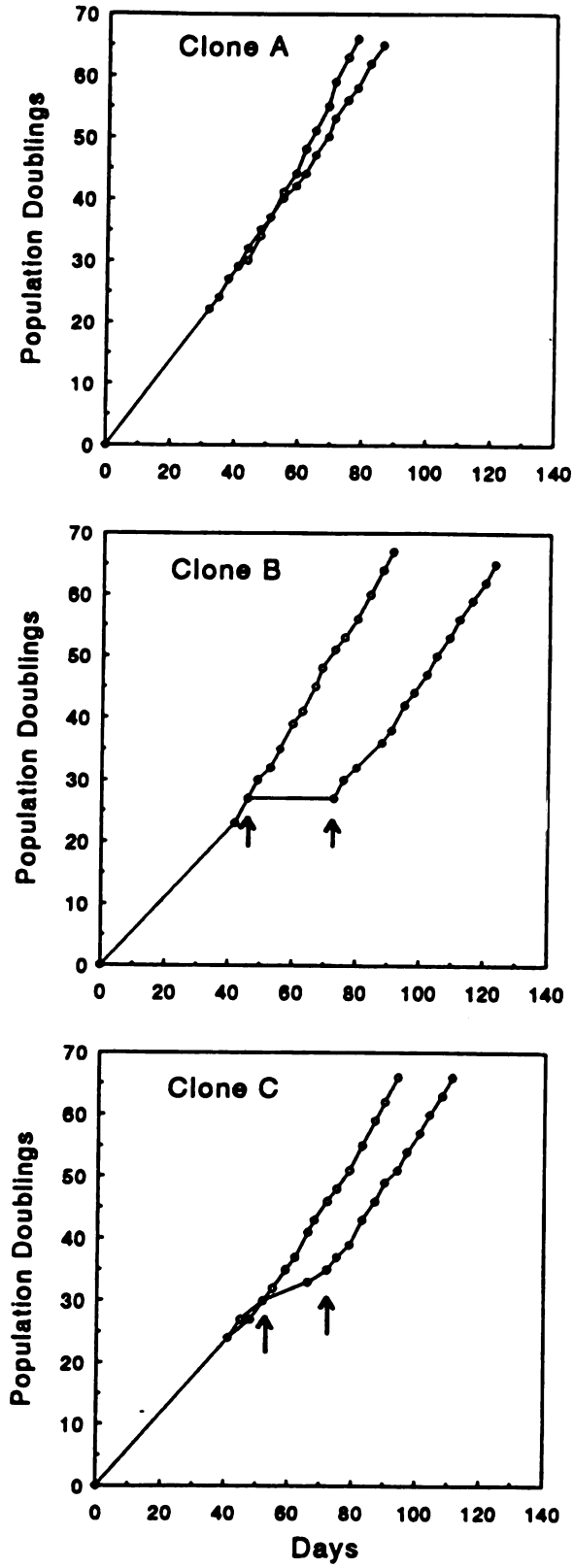

Figure 3. Growth curves of hybrids formed by fusion of HT1080-CHAT^{s}Oua^{r} cells with HeLa cells

between pdl 22 and 40; that, is the cells became increasingly more granular and then began detaching and disintegrating. Destruction of the majority of the cells occurred over a period of 1-4 weeks. In some populations this cell destruction was very rapid, and only a few cells survived. These cells formed individual clones which, when dispersed, slowly repopulated the flask over a period of 3 to 4 weeks. From the time the cells began dying until the flask was repopulated, cell division was occurring, but by definition, the cell population as a whole did not double, so the growth curve was flat (e.g., the period in Figure 3B delineated by arrows). Once the flask was repopulated, the population doubled in selective medium at the same rate as did the corresponding population grown in the absence of drug selection. In other hybrid populations cell destruction was slower. In these, a high rate of cell death competed with cell division over a period of two to four weeks, resulting in a slow rate of population doubling during the period of cell destruction (e.g., the period in Figure 3C delineated by arrows).

We interpret the growth patterns illustrated in Figure 3B,C as reflecting cell death caused by toxicity from long term exposure to CHAT and ouabain, with subsequent repopulation of the flask by surviving variant cells that have greater resistance to the drug. The eight hybrids which did not exhibit this rapid cell death apparently were more resistant to the selection than were the other 14 tested. Table 4 summarizes our results and compares them with those of Pereira-Smith and Smith (1988). The incidence of rapid cell death that we observed with continuous drug selection closely matches the incidence of "senescence" reported by Pereira-Smith and Smith (1988) for clones assayed under the same conditions (column 4 of Table 4). In contrast, when we discontinued drug

TABLE 4. Effects of short-term versus long-term drug selection on the life span of hybrid cells

Reported complementation groups of cells fused[1]	Drug selection for only 22 pdls: Hybrids that senesced per total hybrid clones (%)[2]	Drug selection throughout life span assay: Hybrids that exhibited rapid cell death per total hybrids assayed (%)[2]	Drug selection throughout life span assay: Hybrids that senesced per total hybrid clones (%)[3]
A X A	0/6 (0)	0/2 (0)	0/85 (0)
D X D	0/5 (0)	0/2 (0)	0/14 (0)
A X B	1/13 (8)	6/8 (75)	88/95 (93)
A X C	0/15 (0)	2/2 (100)	22/22 (100)
D X A	0/5 (0)	2/2 (100)	31/31 (100)
D X B	0/5 (0)	3/5 (60)	23/23 (100)
D X C	0/1 (0)	1/1 (100)	99/105 (94)

[1]For the present study, the cell strains fused are the ones shown in Table 2; for the study by Pereira-Smith and Smith (1988), the cell lines fused are designated in Tables 2-5 of that paper.

[2]Results from the present study.

[3]Results reported by Pereira-Smith and Smith (1988).

selection at pdl 22, 98% of our hybrids showed no senescence or evidence of rapid cell death (column 2 of Table 4). We interpret our results to indicate that immortal cell lines, when fused, cannot complement each other to yield hybrids with finite life spans. In addition, our results strongly suggest that the cell death observed in hybrids from these cell strains by Pereira-Smith and Smith resulted from the toxic effect of long term drug selection.

DISCUSSION

Our results showing that fusion of infinite life span MSU-1.1 cells with their own finite life span parental LG1 cells, as well as with the finite life span fibroblast cell line WI-38, yields finite life span hybrid cells confirm what has been reported by many investigators, namely that immortalization of human cells is a recessive trait (i.e., results from loss of the function of one or more of the genes responsible for senescence). However, the results of our fusions of MSU-1.1 cells with various other immortal cell lines failed to support the claim of Pereira-Smith and Smith (1983, 1988) that immortal cells can complement one another (i.e., supply the missing function(s) to yield hybrids with finite life spans). When MSU-1.1 cells were fused to representative cell lines from each of the four complementation groups established by these investigators, none of the resulting hybrids senesced. Therefore, one would have to assign MSU-1.1 cells to all four of the complementation groups, rather than just one. This implies that the MSU-1.1 cells lack

each of the sets of senescence genes represented by the four groups. Shortly after we obtained this result, another research group using the same approach was able to assign the SV40-immortalized human cell line BET-1 to a single complementation group, namely group D (Whitaker et al., 1992). However, when they later applied the method to three other SV40-immortalized human cell lines, each of these cell lines were found to belong to more than one complementation group (Duncan et al., 1993). These results, along with our own, indicate that complementation among immortal cells is a more complicated phenomenon than the studies of Pereira-Smith and Smith suggest. This conclusion is strengthened by the results of our fusions of cells reported by Pereira-Smith and Smith (1988) to be from different immortality complementation groups. Instead of obtaining hybrid cells that senescenced, 38 out of 39 of our hybrids from putatively complementary parents exhibited unlimited life spans. Flow cytometry analysis of the immortal cells from these fusions verified that they were hybrids.

Pereira-Smith and Smith proposed two two theories to explain why finite life span hybrids might appear to have infinite life spans. In one, they propose that very soon after fusion the tetraploid cell loses the gene or genes that limit proliferation (rapid chromosomal segregation)(Pereira-Smith and Smith, 1988; Pereira-Smith et al., 1990). Even if the combined genomes of two parental cells confer on a hybrid cell the full complement of the genes limiting cellular life span, immediate loss of the critical genes would render the progeny of the hybrid immortal. Such immortal hybrids comprised only 4-5% of the hybrids of complementary immortal cell lines in the 1988 study of Pereira-Smith and Smith. Even among the hybrids from HeLa D98 fused with human diploid fibroblasts, which Pereira-

Smith et al. (1990) reported have a high rate of reversion to immortality, only 13% (5/39) failed to show at least some evidence of senescence (i.e., did not exhibit any slowing of the rate of population doubling). In contrast, 95% of our hybrids (38/39) failed to show any evidence of senescence. It seems highly unlikely that such a high proportion of our hybrids should have undergone "rapid chromosomal segregation" and acquired an infinite life very soon after fusion.

The second theory proposes that the loss of critical genes occurs in a progeny cell of the expanded finite life span clonal population after the rate of population doubling has slowed rather than soon after fusion (Pereira-Smith and Smith, 1983; Pereira-Smith et al., 1990). The resulting immortal cell proliferates and overgrows the culture as the senescing population ceases cell division. One might interpret the slight increase in growth rate which occurred for several of our immortal hybrid clones when selection was discontinued (Fig. 1 and 2) as evidence that this phenomenon was occurring. However, we consider this highly unlikely, since even the immortal control hybrids we obtained from self fusions of immortal cell lines (e.g., A1698 cells X A1698 cells) exhibited such an increase in growth rate when drug selection was discontinued (see Fig. 2D). This provides evidence that the increase in growth rate represents the cells' recovery from the toxic effects of the drugs. For the hybrid population shown in Fig. 1C, we interpret the temporary reduction in growth rate (closed diamonds) following discontinuation of drug selection as delayed recovery from the toxic effects of the drugs. In addition, it could be that the clonal population that ceased dividing at population doubling 22 (Fig. 1B, closed triangles) was too damaged to recover.

In our study where hybrids were deliberately maintained on drug

selection throughout their life span assay, the incidence of rapid cell death closely matched the incidence of senescence reported by Pereira-Smith and Smith (1988)(Table 4). The cell destruction we observed was too rapid to be attributed to what is normally referred to as senescence. In populations that were rapidly dividing in selective medium, a majority of the cells suddenly detached and disintegrated. The abruptness of the cessation of growth and the rapidity of the onset of disintegration of the cells suggest that instead of senescence we were observing necrosis or apoptosis triggered by the cumulative cytotoxic effect of the drugs. According to the description by Wyllie (1981) and Wyllie et al. (1980), apoptosis involves nuclear condensation, membrane blebbing, and cytoplasmic condensation, and it ends in cell fragmentation, with death of an entire population of cells occurring over a period of several days. In the case of necrosis, the dominant morphologic event is cellular swelling from loss of control of cell volume, followed by rupture of the plasma membrane, again with rapid destruction of whole populations of cells (Wyllie, 1981; Wyllie et al., 1980). Both processes can be initiated by a variety of cytotoxic drugs.

In contrast to apoptosis and necrosis, cell senescence is not a rapid process. The population dynamics of normal finite life span cells include first, a period of rapid proliferation; secondly, a gradual decrease in proliferative rate over a period of many weeks, accompanied by deteriorative changes in the cells; and finally, complete cessation of growth (Hayflick and Moorhead, 1961; Hayflick, 1965; Bayreuther et al., 1988). In an extensive review on cellular senescence citing 575 references, Stanulis-Praeger (1987) states " 'True' senescence thus comprises the prolonged period of proliferation sterility preceding the

death of the cells." The non-dividing senescent cells enlarge and deteriorate but remain attached to the dish for weeks or months before disintegrating or detaching. We observed just such a prolonged period of proliferation sterility during our analysis of the life span of normal fibroblastic LG1 clones and of MSU-1.1 X LG1 hybrid clones. However, the cell death and detachment which occurred when the hybrids from our immortal cell X immortal cell fusions were kept under continuous drug selection was not preceded by this cessation of cell division.

It is important to know whether the limited life spans exhibited by the progeny of the hybrid clones reported by Pereira-Smith and Smith (1988) resulted from cellular senescence or from drug cytotoxicity. However, to determine this, one would need to know the appearance and behavior of their cells as division ceased and the cells died. In their 1988 publication identifying the four immortality complementation groups, Pereira-Smith and Smith do not provide the details of the cessation of cell division by the progeny of cell hybrids formed by the fusion of immortal cells with immortal cells. Instead, they refer readers to their 1981, 1982, and 1983 publications for the details of their culturing of hybrid cells and their method of determining the proliferative potential of the hybrids. Their 1981 article provides a detailed description of hybrids derived from the fusion of immortal VA13 or GM 639 cells with finite life span normal human diploid fibroblasts. They report that the clones went through two identifiable phases of growth: a period of active division, followed by a period of complete cessation of division. They stated that the period of complete cessation of cell division "...resembled the 'crisis' phenomenon observed during viral transformation of normal cells in vitro. During this period, a large number of cells

lysed. Micronucleation, nuclear lobulation, and nuclear blebbing were observed in the cells." This growth pattern is not consistent with the gradual decrease in growth rate of typical finite life span cell populations. Sudden lysis of a large number of cells is not characteristic of senescing cells. Instead, the sudden cessation of division and the cell destruction described are more characteristic of the necrosis or apoptosis process described above. Stein (1985) reports that the cell crisis exhibited by SV40-transformed cells results from increased cell death among cells that are still traversing the cell cycle because they have lost their ability to enter the "normal G-1 arrested senescent state". We have been unable to determine from the papers by Pereira-Smith and Smith whether or not an abrupt crisis phenomenon was a common feature of the division cessation of the majority of their hybrids.

Clearly, careful distinction among different types of cell death is necessary for interpreting the results of studies on cellular immortality. The ambiguities discussed above highlight the importance of finding definite and easily identifiable specific markers for cellular senescence, necrosis, and apoptosis. Several markers for senescence have already been proposed, but none of these provide a positive, quantitative marker that is characteristic only of senescing cells as distinct from non-growing quiescent cells. Necrosis can be detected by abnormal permeability to certain molecular markers (Hoffstein et al., 1975). Apoptosis is recognized by apoptotic body formation and by fragmentation of chromatin into characteristic nucleosome ladders (Wyllie et al., 1980). Resolution of the conflicting results obtained with hybrids from fusions of immortal cells with immortal cells will require further research employing careful microscopic and biochemical techniques to distinguish among these

phenomena.

If, as our data suggest, immortality is recessive in hybrids obtained from fusion of finite life span cells with immortal cells, but immortal cells cannot complement each other to yield hybrids with finite life spans, what does this say about the genetic alterations responsible for the immortal phenotype? One possible explanation is that all immortal cells share a common defect in the gene(s) that control(s) life span. This is highly unlikely since at least two different human chromosomes, i.e., 1 and 4, have been implicated in cellular senescence (Ning et al., 1991; Suguwara et al., 1990). It is more likely that several genes control life span, and, therefore, defects in any one of several different genes result in an infinite life span. In this case, a possible explanation for the failure of cells to complement each other comes from the work of Harris (1988). He proposed a theory based on genetically imprinted patterns of differentiation to explain the lack of complementation among malignant cells. If one applies his theory to the infinite life span phenotype, the essential genetic lesion determining immortality would be the loss of the ability to complete a program of terminal differentiation. According to that view, fusions between finite life span cells and immortal cells yield hybrids with finite life spans because the differentiation program of the finite life span cell can be executed in the hybrid. The lack of complementation among immortal cell strains could be attributed to the fact that a cell committed to one program of differentiation cannot simultaneously execute another, and that the genetic imprints that determine major lineage of differentiation are stably maintained in cell hybrids. Accordingly, Cell A, committed to differentiation program A, but exhibiting immortality because of its

inability to complete that program, could not be expected to complement the defect in an immortal cell B from another lineage because the genes carried in cell A for differentiation program B have been inactivated by stable imprinting. For the same reason, cell B cannot complement cell A.

This explanation could account for the inability of different types of cells, (i.e., cells that are derived from independent differentiation programs) to complement each other, e.g., HT1080, from a fibrosarcoma, and HeLa, from a cervical carcinoma. However, when cells of the same tissue type, e.g., both fibroblastic in origin, fail to complement each other, as we observed with MSU-1.1 cells and HT1080 cells, one would have to conclude that the cells share a common defect in their program of differentiation.

Even without knowledge of the underlying mechanisms involved, our data support the hypothesis that the phenotype of infinite life span in human cells results from recessive changes in a genetic program for cellular senescence, but they suggest that immortal human cells cannot complement one another to yield hybrid cells with finite life spans. Our data also indicate that long-term dual drug selection is deleterious to hybrid cells, even though the cells carry resistance markers for both drugs. Therefore, investigation of senescence using cell fusion demands careful discrimination among different types of cell death. The lack of complementation that we observed in hybrids between immortal cells is not easily explained by simple Mendelian genetics. More complex genetic mechanisms may need to be invoked to explain the phenotypes of these hybrids.

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CHAPTER III

Suppression of the Tumorigenicity of H-, N-, or v-K-*ras* Oncogene-Transformed Malignant Human Fibroblast Strains by Fusion with their Infinite Life Span, Non-Tumorigenic Parental Strain, MSU-1.1

INTRODUCTION

Activated oncogenes of the *ras* family have been detected in a variety of rodent and human tumors (reviewed in Barbacid, 1987). The results of studies in which an activated *ras* oncogene has been transfected into rodent or human cells in culture have shown that the expression of an activated *ras* oncogene can contribute to the malignant transformation of mammalian cells, but have indicated that one or more changes in addition to the expression of an activated *ras* oncogene are required for a cell to become malignant.

Malignant transformation by *ras* of rodent cells in culture requires either that the cells first be immortalized or, if transformation is to be accomplished in primary or early passage rodent cells, that a second oncogene be transfected along with *ras* (Land et al., 1983; Ruley, 1983; Newbold and Overell, 1983; Parada et al., 1984; Oshimura et al., 1985). The oncogenes, e.g., *myc*, that have been found to cooperate with *ras* in the malignant transformation of rodent cells are ones that are considered to be involved in causing cellular immortalization. Similarly, *ras*-induced malignant transformation of human fibroblasts in culture is successful when infinite life span fibroblasts are used as the recipients of the gene, but not when finite life span fibroblasts are used (Hurlin et al., 1987; Hurlin et al., 1989; Wilson et al., 1989; Wilson et al., 1990; Fry et al., 1990). One interpretation of this finding is that, in addition to expression of the *ras* oncogene, other genetic alterations must be acquired for a cell to become malignant, and that a cell must have an infinite or greatly extended life span to achieve the number of cell divisions required for these changes to occur.

Other observations made during these studies support the hypothesis that *ras*-induced malignant transformation of infinite life span human fibroblasts requires changes in addition to the expression of a *ras* oncogene. Hurlin et al. (1989) selected *ras*-expressing cells from transfected populations of infinite life span fibroblasts by isolating morphologically transformed colonies of cells (foci) that overgrew the monolayer of treated fibroblasts. They reported that, although all six of the clonal isolates expressed higher than normal levels of the activated *ras* protein, only four were tumorigenic. This suggests that the four tumorigenic isolates had acquired an additional change that is required to produce the malignant state.

Cell fusion studies suggest that the loss of the function of a tumor suppressor gene is required for *ras*-induced malignant transformation of infinite life span cells. Sager and her colleagues used the H-*ras* oncogene of the of the human bladder cancer cell line EJ, also known as T24, to malignantly transform infinite life span Chinese hamster embryo fibroblast (CHEF) cells (Smith et al., 1982). They later fused the tumorigenic *ras*-transformed CHEF cells with the infinite life span, non-tumorigenic CHEF cells from which the tumorigenic cells were derived (Craig and Sager, 1985). Although the hybrid cells expressed p21-*ras* protein at levels comparable with that of the malignant parental cells, they had a decreased tumor-forming ability in nude mice. These researchers concluded that the infinite life span non-tumorigenic CHEF cells contain a suppressor gene capable of inhibiting the tumorigenic effect of the activated EJ H-*ras* oncogene.

The results of other intraspecies rodent cell hybridization studies also support the hypothesis that normal cells contain genes that suppress

ras-induced tumorigenicity. Willecke et al. (1987) reported that the EJ H-*ras*-induced tumorigenicity of Rat-1 cells can be suppressed by fusion of these cells with rat embryo fibroblasts. Because the tumorigenic cells were fused to cells that were at least two steps removed from the tumorigenic cells (i.e., not *ras*-transformed and not infinite life span), and because three of the four hybrids expressed less p-21-*ras* protein than did the tumorigenic parental cells, this study is less convincing than that of Craig and Sager (1985).

Oshimura et al. (1988) showed that tumorigenicity of *ras/myc*-transformed Syrian hamster cells can be suppressed by fusion of these cells with Syrian hamster embryo cells. They reported that the majority of cell hybrids senesced, as would be expected since infinite life span has been shown to be a recessive characteristic (Bunn and Tarrant, 1980; Muggleton-Harris and Desimone, 1980; Pereira-Smith and Smith, 1981, 1983), but that some hybrids escaped senescence and grew indefinitely. Although these non-senescent hybrid cells produced high levels of the mutant *ras* protein, tumorigenicity was significantly suppressed.

Geiser et al. (1986) studied the suppression of the EJ H-*ras* in human cells by fusing EJ carcinoma-derived cells with normal human fibroblasts. They reported that all six of the hybrid clones that were isolated proliferated beyond 200 population doublings (see below) and were non-tumorigenic. After a period of time in culture, several tumorigenic segregants arose within the non-tumorigenic hybrid populations. The levels of expression of the mutant H-*ras* were similar in the EJ cell line, the non-tumorigenic hybrid cells, and the tumorigenic segregants. Their results suggest that *ras*-induced malignant transformation of human cells requires inactivation of a tumor suppressor gene that is present in normal

human cells.

As did the rodent cell fusions of Willecke et al. (1987) and Oshimura et al.(1988), the human cell fusion of Geiser et al. (1986) spanned at least two steps of transformation, i.e., immortalization (EJ cells have an infinite life span) and *ras*-transformation. In light of the strong evidence from the studies referenced above that infinite life span is a recessive characteristic, one would have expected most of the hybrids obtained by the fusion of EJ cells with normal fibroblasts to have a finite life span, which has been shown to be less than 100 population doublings in normal cells. Although Geiser et al. (1986) did not indicate any evidence of senescence in their report, it is likely that finite life span hybrid cells were obtained, but senesced too soon to form colonies that could be isolated.

In light of the cell fusion studies described above and the report by Hurlin et al. (1989) that some *ras* oncogene-expressing MSU-1.1 cells failed to produce tumors, I hypothesized that the malignant transformation of MSU-1.1 by an activated *ras* oncogene requires, in addition to the expression of the activated *ras* oncogene, the inactivation of a tumor suppressor gene that is present in MSU-1.1 cells. I tested this hypothesis by fusing cells from six independent malignant MSU-1.1-*ras* strains with the infinite life span , non-tumorigenic, precursor MSU-1.1 cells and assaying the hybrids for tumorigenicity. Although tumorigenicity was clearly suppressed in the majority of the hybrid cell strains, the degree of suppression varied among the hybrid strains and did not correlate with the mutant *ras* protein expression levels in the hybrid cells. The results of a comparison of the *ras* protein expression of tumorigenic cells at various passages suggested that *ras* expression is

increased by growth of the cells in athymic mice and decreased by growth of the cells in tissue culture dishes.

MATERIALS and METHODS

Cells and cell culture

The cell strains used and their sources are listed in Table 1. The cells were routinely cultured as previously described (Chapter II, Materials and Methods, pp. 66 and 69).

Cell fusion

Cell fusions and the selection and initial expansion of hybrid clones were performed as previously described (Chapter II, Materials and Methods, pp. 69-70).

Assay for tumorigenicity

BALB/c athymic mice, ages 6 to 9 weeks, were injected subcutaneously with 10^6 to 10^7 exponentially growing cells per site. Tumor growth was monitored on a weekly basis.

Immunoprecipitation of p21 *ras* protein

The amount of p21 **ras** protein was assayed by the method described by Wilson et al., 1990, with the following modifications. Cells were plated at 10^6 cells/100 mm dish. The antibody, Y13-259, which reacts with H-,K-, and N-*ras* p21s was used. The *ras* protein migrates as a doublet band (e.g., see autoradiographs depicted in Hurlin et al., 1989, and Wilson et

TABLE 1. Cell strains

Cell strain designation	Previously reported *ras* protein expression relative to MSU-1.1 cells	Reference
MSU-1.1	1.0	Morgan et al., 1991
MSU-1.1-Hg^{r}CHAT^{s}	--	Chapter II - this thesis
MSU-1.1-vK-*ras*-2T[1]	1.2-1.3	Fry et al., 1990
MSU-1.1-vK-*ras*-3T[1]	--	unpublished studies
MSU-1.1-H-*ras*-2T[1]	2.0-5.0	Hurlin et al., 1989
MSU-1.1-H-*ras*-3T[1]	2.0-5.0	Hurlin et al., 1989
MSU-1.1-N-*ras*-3T[1]	3.0-7.0	Wilson et al., 1990
MSU-1.1-N-*ras*-8T[1]	3.0-7.0	Wilson et al., 1990

[1]T: denotes that this is a strain derived from a tumor produced by the respective *ras*-transformed MSU-1.1 strain.

al., 1990). The band densities were quantified using Visage 110 BioImage System (Kodak Co., Ann Arbor, MI). For determination of H-*ras* expression levels, only the upper band of the doublet (which contains only H-*ras*) was quantified. For determination of N-*ras* expression levels, only the bottom band of the doublet (which contains both N- and K-*ras*) was quantified. The *ras* protein expression levels reported are relative to that of MSU-1.1-Hg^{r}CHAT^s which was assigned a value of 1.

RESULTS

Fusion of malignant MSU-1.1-*ras* cells with the finite life span, non-tumorigenic MSU-1.1 precursor cells.

MSU-1.1-Hg^{r}CHAT^s cells were fused with cells from six independently derived malignant *ras*-transformed MSU-1.1 strains. The later are Hg^{s}CHAT^r. From each of the cell fusions two or three independent hybrid clones were isolated, and the clonal populations were expanded. The parental and hybrid cells were injected into athymic mice to determine their tumorigenic potential (Table 2). All six of the malignant parental cell strains formed tumors at each of four injection sites within two months post injection. In contrast, MSU-1.1-Hg^{r}CHAT^s cells failed to form tumors at any of five injection sites over a period of 8.5 months. The tumorigenic potential of the hybrid cell strains varied. Although some were as tumorigenic as the malignant parental cells, most exhibited some degree of suppression of tumorigenicity.

The degree of tumor suppression in the hybrid strains, summarized in Table 3, was scored as follows. If, for a particular hybrid strain,

TABLE 2. Tumorigenicity of cell strains

Cell strain	Injection sites producing tumors	Approximate time (mos) until tumor size 1 cm^3
MSU-1.1-Hg^rCHAT^s	0/5	--
MSU-1.1-vK-*ras*-2T	4/4	2.0
MSU-1.1-vK-*ras*-3T	4/4	0.5
MSU-1.1-H-*ras*-2T	4/4	2.0
MSU-1.1-H-*ras*-3T	4/4	1.0
MSU-1.1-N-*ras*-3T	4/4	1.0
MSU-1.1-N-*ras*-8T	4/4	2.0
Hyb-vK-*ras*-2.1	0/4	--
Hyb-vK-*ras*-2.2	0/4	--
Hyb-vK-*ras*-2.3	0/4	--
Hyb-vK-*ras*-3.1	0/4	--
Hyb-vK-*ras*-3.2	0/3	--
Hyb-H-*ras*-2.1	0/4	--
Hyb-H-*ras*-2.2	2/4	5.0, 5.0
Hyb-H-*ras*-2.5	3[1]/4	2.5, 4.5, 4.5
Hyb-H-*ras*-3.1	0/2	--
Hyb-H-*ras*-3.4	2[1]/4	1.5, 4.0
Hyb-H-*ras*-3.9	0/4	--
Hyb-N-*ras*-3.1	2[1]/4	2.0, 4.5
Hyb-N-*ras*-3.3	3[1]/4	2.5, 2.5, 2.5
Hyb-N-*ras*-3.7	0/4	--
Hyb-N-*ras*-8.1	4/4	1.0, 1.0, 1.5, 1.5
Hyb-N-*ras*-8.2	4/4	1.0, 1.0, 1.0, 1.0
Hyb-N-*ras*-8.3	0/2	--

[1]Unable to adequately assess tumor formation at remaining sites because mice were sacrificed prior to 8.5 months post injection. No tumors were present at these sites at the time the mice were sacrificed.

tumors arose in all injection sites within two months, the hybrid strain was scored as exhibiting no suppression of tumorigenicity. If no tumors arose in any of the sites by 8.5 months post injection, the hybrid strain was considered to be completely suppressed for tumorigenicity. If a hybrid strain produced tumors but tumors did not arise at all sites, or if some tumors formed after two months post injection, then the hybrid strain was scored as being partially suppressed for tumorigenicity.

All five of the hybrids obtained by fusion of MSU-1.1-Hg^{r}CHAT^s cells with MSU-1.1-vK-*ras* cells were completely suppressed for tumorigenicity. Half of the six hybrids obtained from fusion of MSU-1.1-Hg^{r}CHAT^s cells with MSU-1.1-H-*ras* cells were completely suppressed for tumorigenicity; the other half were partially suppressed. The six hybrids obtained from fusion of MSU-1.1-Hg^{r}CHAT^s cells with MSU-1.1-N-*ras* cells ranged from complete, to partial, to no suppression, with two hybrids falling into each category.

TABLE 3. Suppression of tumorigenicity in hybrid strains.

Cell fusion	No. of hybrid strains		
	not suppressed	partially suppressed	completely suppressed
MSU-1.1-vK-*ras* X MSU-1.1-Hg^{r}CHAT^s	0	0	5
MSU-1.1-H-*ras* X MSU-1.1-Hg^{r}CHAT^s	0	3	3
MSU-1.1-N-*ras* X MSU-1.1-Hg^{r}CHAT^s	2	2	2

Expression levels of *ras* protein in parental cells, hybrid cells, and tumor-derived hybrid cells.

Because the tumorigenicity of the MSU-1.1-H-*ras* and MSU-1.1-N-*ras* strains was reported to be dependent upon a high expression level of the mutant *ras* protein (Hurlin et al.,1989; Wilson et al., 1990) and the suppression of the *ras*-induced tumorigenicity appeared to correlate inversely with the reported expression levels of the *ras* protein in the malignant parental cells (Table 1), I hypothesized that the suppression of the *ras*-induced tumorigenicity in the hybrids cells may be secondary to a dilution of the *ras* protein in the hybrid cells. To test this hypothesis, I determined the *ras* protein expression of the H- and N-*ras* parental cells, the hybrid cells obtained from fusion of these parents with MSU-1.1-Hg^{r}CHAT^s cells, and cells that were derived from tumors that were formed by these hybrids strains. Figures 1 and 2 depict the *ras* protein expression of these cell strains relative to that of MSU-1.1-Hg^{r}CHAT^s, which was assigned a value of 1.

The *ras* protein expression levels of the MSU-1.1-H-*ras*-2T and MSU-1.1-H-*ras*-3T cell strains were respectively 4.0 and 4.9, and that of the hybrid strains obtained from fusion of MSU-1.1-Hg^{r}CHAT^s with these two malignant strains ranged from 1.4 (Hyb-H-*ras*-3.4) to 22.1 (Hyb-H-*ras*-2.5). The *ras* protein expression levels of the MSU-1.1-N-*ras*-3T and MSU-1.1-N-*ras*-8T cell strains were respectively 45.0 and 2.7, and that of the hybrid strains obtained from fusion of MSU-1.1-Hg^{r}CHAT^s with these malignant strains ranged from 7.8 (Hyb-N-*ras*-8.3) to 32.4 (Hyb-N-*ras*-3.7). The *ras* protein expression levels in the various hybrid strains did not correlate with their tumorigenic potentials. For example, the *ras* protein expression of the non-tumorigenic hybrid strain Hyb-H-*ras*-3.2 was 10.4

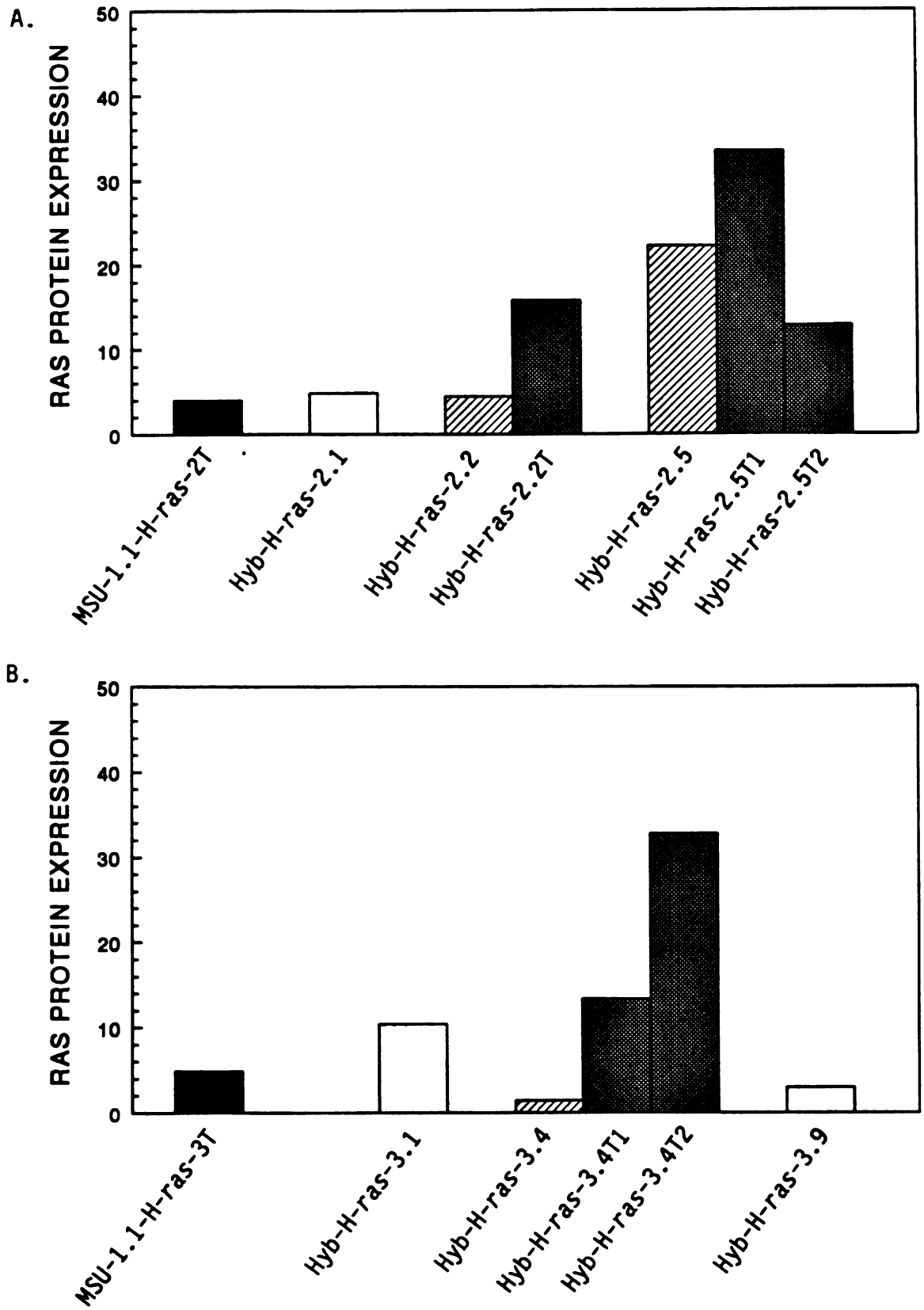

FIGURE 1. H-*ras* protein expression, relative to that of MSU-1.1-Hg^{r}CHAT^{s} cells, for H-*ras*-transformed MSU-1.1 parental and hybrid cells.

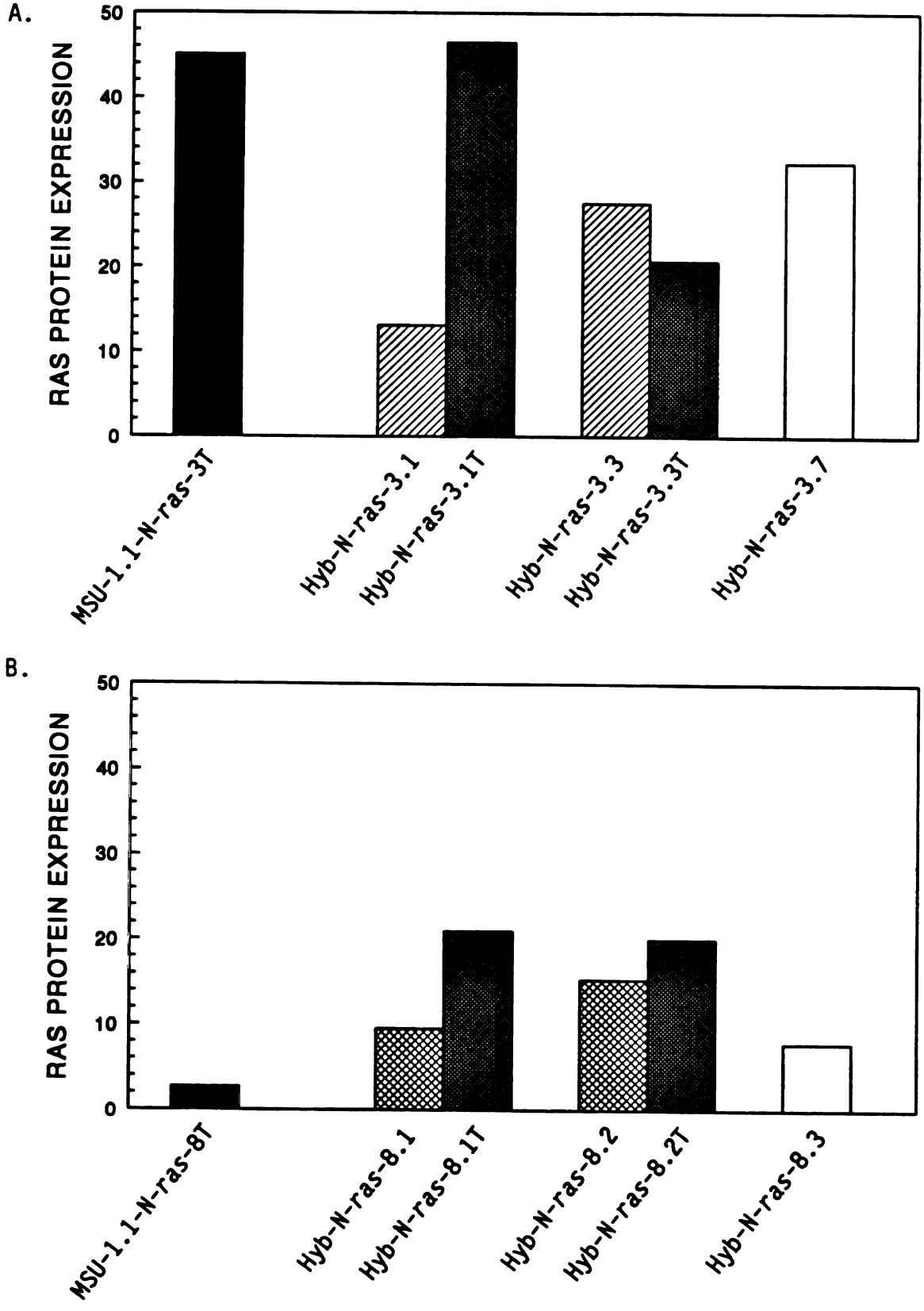

FIGURE 2. N-*ras* protein expression, relative to that of MSU-1.1-Hg^{r}CHAT^{s} cells, for N-*ras*-transformed MSU-1.1 parental and hybrid cells.

(2.1 times higher than that of the malignant MSU-1.1-H-*ras*-3 parental cells), whereas the *ras* expression of the tumorigenic hybrid strain Hyb-H-*ras*-3.4 was only 1.4 (0.28 times that of the malignant MSU-1.1-H-*ras*-3 parental cells). I found, however, that the cells that were derived from tumors that were formed by Hyb-H-*ras*-3.4 expressed much higher levels of *ras* protein, i.e., 13.3 and 32.8, than did the pre-tumor population. This appeared to be a general phenomenon, as seven of the nine tumor-derived hybrid strains expressed higher levels of *ras* protein than did their respective pre-tumor populations.

This latter finding, along with the observation that the *ras* expression that I obtained for the MSU-1.1-N-*ras*-8T cell strain was lower than that obtained previously for an earlier passage of this strain (unpublished data), led me to hypothesize that growth of cells in athymic cells increases the expression of *ras*, whereas growth of cells in tissue culture dishes decreases the expression of *ras*. As a preliminary test of this hypothesis, I compared the *ras* protein expression of late passage MSU-1.1-N-*ras*-8T cells to that of early passage cells of this strain and to that of very early passage cells that were derived from tumors formed by early and late passage MSU-1.1-N-*ras*-8T cells. The results (shown in Table 4) confirm that the late passage cells have a lower *ras* expression than do the early passage cells and show, as predicted, that the very early passage tumor-derived strains have a much higher *ras* expression than do the pre-tumor populations.

TABLE 4. The *ras* protein expression of MSU-1.1-N-*ras*-8T cells measured at various cell passages

Cell Passage	Cell strain	N-*ras* expression
late	MSU-1.1-N-*ras*-8T	0.56
early	MSU-1.1-N-*ras*-8T	2.59
very early	Tumor strain derived from late passage MSU-1.1-N-*ras*-8T	20.11
very early	Tumor strain derived from early passage MSU-1.1-N-*ras*-8T	20.33

DISCUSSION

The design of the human fibroblast cell fusion studies described here closely parallels the studies conducted by Craig and Sager (1985) with CHEF cells, and results similar to theirs were obtained. Our results suggest, as did those of Craig and Sager, that normal cells contain genes that are capable of suppressing *ras*-induced tumorigenicity, and indicate that this suppression does not result from a reduction in the expression of the *ras* protein. MSU-1.1 cells were found to suppress tumorigenicity when they were fused with malignant *ras*-transformed MSU-1.1 derivative cells, indicating that MSU-1.1 cells, although immortal, still possess a functional *ras*-transformation suppressor gene.

Although both MSU-1.1 cells and normal human fibroblasts apparently contain *ras*-transformation suppressor genes, MSU-1.1 cells can be malignantly transformed by transfection with an activated *ras* oncogene carried in a vector engineered to allow a high level of expression, whereas normal human fibroblasts cannot. A plausible explanation for this difference is that the infinite life span of MSU-1.1 cells allows them to accumulate the minimum of three genetic alterations (acquisition of an activated *ras* oncogene and inactivation of both copies of a *ras*-transformation suppressor gene) required for *ras*-induced transformation, whereas the life span of normal fibroblasts is too short to permit three sequential genetic alterations and clonal selections.

A likely explanation for the isolation by Hurlin et al. (1989) of MSU-1.1-H-*ras* strains that express high levels of mutant *ras* protein but are non-tumorigenic is that these cells still possess a functional *ras*-transformation suppressor gene. It is possible that many other non-

tumorigenic MSU-1.1-*ras* cells were present in the transfected populations in the experiments of Hurlin et al. (1989), Wilson et al. (1990), and Fry et al.,(1990), but that these were not detected because they did not form foci.

Examination of the *ras* expression of the MSU-1.1-*ras* cell strains revealed that the *ras* protein expression of a cell population is not necessarily stable. The preliminary results presented here suggest that growth of *ras*-transfected cells in athymic mice increases the *ras* protein expression of the cell population, and growth of these cells in tissue culture dishes decreases the *ras* expression of the cell population. This modulation of *ras* protein expression could be achieved by selection for cells with *ras* expression levels that are favorable to certain growth conditions or by induction of altered *ras* expression in the cells. The studies reported here were not designed to differentiate between these two possibilities. I observed, however, as have others (Wilson et al., 1990), that cells expressing a high level of mutant *ras* tend to round and detach from tissue culture dishes. This observation supports the hypothesis that the growth of cells in tissue culture dishes decreases the *ras* protein expression of the population by selecting for cells that have low *ras* expression levels. Although further studies are needed to understand these findings, the instability of the *ras* expression in these cells may explain why the measured *ras* expression levels did not correlate with tumorigenicity and indicate that caution must be exercised when interpreting measurements of the *ras* expression levels of cell populations.

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