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Senescence of immortal human fibroblasts by the introduction of normal human chromosome 6

(immortalization/growth suppressor gene/simian virus 40 transformation)

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ABSTRACT **In these studies we show that introduction of a normal human chromosome 6 or 6q can suppress the immortal phenotype of simian virus 40-transformed human fibroblasts (SV/HF). Normal human fibroblasts have a limited life span in culture. Immortal clones of SV/HF displayed nonrandom rearrangements in chromosome 6. Single human chromosomes present in mouse/human monochromosomal hybrids were introduced into SV/HF via microcell fusion and maintained by selection for a dominant selectable marker *gpt*, previously integrated into the human chromosome. Clones of SV/HF cells bearing chromosome 6 displayed limited potential for cell division and morphological characteristics of senescent cells. The loss of chromosome 6 from the suppressed clones correlated with the reappearance of immortal clones. Introduced chromosome 6 in the senescing cells was distinguished from those of parental cells by the analysis for DNA sequences specific for the donor chromosome. Our results further show that suppression of immortal phenotype in SV/HF is specific to chromosome 6. Introduction of individual human chromosomes 2, 8, or 19 did not impart cellular senescence in SV/HF. In addition, introduction of chromosome 6 into human glioblastoma cells did not lead to senescence. Based upon these results we propose that at least one of the genes (*SEN6*) for cellular senescence in human fibroblasts is present on the long arm of chromosome 6.**

Increasing attention has been directed in recent years to genes that regulate normal and tumor cell proliferation by suppressing growth. Such a phenomenon is important to our understanding of not only carcinogenesis but also the loss of cellular proliferation in aging or senescence. Human cells in culture have a limited life span, as do those of other species (1). After a number of cell generations these cells become senescent, showing characteristic morphological changes and cessation of proliferation. Most studies have exploited diploid human fibroblasts (HF) as a model system for cellular aging. Although the molecular basis for senescence has not yet been determined in full, alterations in gene expression have been demonstrated (2). In particular, these include genes for which expression and function have been closely linked to cell proliferation, such as c-*fos* (3) and the retinoblastoma-susceptibility protein (Rb-1) (4) among others. HF do not spontaneously become immortal, in contrast to rodent cells. On the other hand, many human tumors have overcome senescence and grow continuously in culture and *in vivo*. We (5–7) and others (8–10) have used the DNA virus simian virus 40 (SV40) to analyze the mechanisms involved in cellular senescence. SV40 induces DNA synthesis in senescent cells and extends the life span of HF, but SV40-transformed HF (SV/HF) are not immortal. These effects depend on the function of the virus-encoded large tumor antigen (T antigen) and are mediated, at least in part, by the ability of T antigen to inactivate the growth-suppressive properties of Rb-1 and p53 (11).

On continuous cultivation, SV/HF can give rise to rare immortal cells that are believed to originate by mutation or other loss of growth-suppressor genes. Taken together, these results with SV/HF are consistent with a multistage model involving inactivation of the effect of growth-suppressor genes. In the first stage, T antigen inactivates Rb-1 and p53. In the second stage, which is not directly dependent on T antigen function, a gene whose expression is responsible for senescence is inactivated through mutational mechanisms (10, 12). By karyotypic and molecular genetic analyses of matched preimmortal and immortal SV/HF, we have recently demonstrated that loss of sequences on the long arm of chromosome 6 is specifically associated with appearance of the immortal SV/HF (7). Nonrandom alterations in chromosome 6 have also been reported by two other laboratories (13, 14). Thus, a putative senescence gene is present on human chromosome 6q.

To test further this hypothesis, our laboratories have introduced an intact human chromosome 6 derived from HF of limited life span into SV/HF by microcell-mediated chromosome transfer (MMCT) using a monochromosomal hybrid. Such an approach has been useful in demonstrating growth-suppressor genes on a variety of human chromosomes—including chromosomes 1, 5, 6, 11, 17, 18, and X (15–20)—as assessed by their inhibition of tumor formation in nude mice. In some cases, growth inhibition in culture has been observed after the transfer of a chromosome, as with chromosomes 17 (16) and 4 (21). In the latter case, hybrids underwent characteristic morphological changes associated with senescence, suggesting that a biochemical change responsible for immortalization was being affected. Consistent with our model for immortalization of HF by SV40, we have found that introduction of chromosome 6 into SV/HF shows morphological changes characteristic of senescence and that such effects are dependent on persistence of the donor chromosome 6 and specific to sequences on 6q. We therefore conclude that the previously described genetic alterations on chromosome 6 are an essential feature of immortalization. We propose that inactivation of such senescence or growth-suppressor gene is also responsible for immortalization and carcinogenesis in human tumors that are associated with

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Abbreviations: SV40, simian virus 40; HF, diploid human fibroblasts; SV/HF, SV40-transformed HF; MMCT, microcell-mediated chromosome transfer; T antigen, large tumor antigen; TG, 6-thioguanine; Rb-1, retinoblastoma susceptibility protein.

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altered chromosome 6, including melanoma and ovarian carcinoma among others.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. HALneo and 39neo are immortal subclones derived from the human diploid fibroblast HS74 after transformation by origin-defective mutants of SV40 (5). HALneo is derived from HAL, a 6-thioguanine (TG)-resistant clone of SVtsA/HF-A (7). It contains a heat-labile T antigen, is resistant to G418 after transfection with pRSVneo (22), and is propagated at 35°C. 39neo is derived from SV/HF-5/39 (6) and is resistant to G418 after infection with the retrovirus vector MX 1112neo (23) (M. Small, personal communication). The human glioblastoma tumor cell line T98G was obtained from the American Type Culture Collection.

Members of a panel of mouse/human hybrid cell lines, each containing a single different human chromosome (monochromosomal hybrids), were used as a source of chromosomes. The human chromosome in each case is "tagged" with the dominant selectable marker Ecogpt (24, 25). Such hybrid cell lines are currently available for 19 different chromosomes in the laboratory of one of us (R.S.A.). The details of production and characterization of these hybrid cell lines will be published elsewhere (R.S.A., unpublished work). Cell lines used in the present studies included the hybrids representing normal human chromosomes 2, 6, 6q, 8, and 19, which originated from human cells.

All cell lines except HALneo were grown at 37°C in a 7.5% CO_2/92.5% air atmosphere in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% fetal bovine serum. The medium was supplemented with mycophenolic acid (25 μg/ml) and xanthine (70 μg/ml) (MX medium) for the growth of donor hybrid cell lines and for the selection and maintenance of chromosome-transfer clones.

MMCT. Micronucleation in chromosome-donor hybrid cell lines was induced by mitotic block with colcemid (0.2 μg/ml) for 36 hr. Microcells were prepared by zonal centrifugation of micronucleated cells in discontinuous Ficoll gradients and fused with recipient cells as described (24). In brief, 1×10^6 recipient cells were seeded in a 100-mm dish 24 hr before fusion. The purified microcells were then overlaid in the presence of phytohemagglutinin-P (100 μg/ml) and incubated for 15 min to facilitate adherence. Phytohemagglutinin-P medium was removed, and a 50% (wt/vol) solution of PEG 1500 (Boehringer Mannheim) was added for 2 min to induce cell fusion. After 48 hr, medium was replaced with MX selection medium containing G418 (400 μg/ml) to eliminate any unfused parental cells and intact cells in a microcell preparation. Chromosome-transfer colonies that appeared in the selection medium during the following 2–4 weeks were propagated in MX medium for further analysis.

Analysis of Chromosome-Transfer Clones. *Cell Senescence.* Chromosome-transfer clones were cultured continuously in MX medium to determine the total duration of survival. Each clone was also assessed for cellular morphology at different stages of growth in parallel with the parental human cells. Each independent colony was marked and followed individually.

Segregation of Transferred Chromosome. A chromosome-transfer clone HALneo6, isolated from fusion of HALneo and RA6, was cultured in nonselective medium to allow for segregation of the tagged human chromosome. Because the parental HALneo is $hprt^-$, the hybrid cells that lost the *gpt*-tagged human chromosome can multiply in medium containing TG (40 μg/ml, TG medium), whereas the ones containing the transferred chromosome are eliminated. Colonies that grew in TG medium were isolated individually. 39neo is $hprt^+$; therefore, segregant clones involving it were isolated on the basis of cell morphology. Immortal segregants thus generated were analyzed for growth properties and presence of the donor human chromosome.

Analysis for the Presence of Transferred Chromosomes. Transferred chromosomes were followed by PCR amplification of a segment of the *gpt* gene. Genomic DNAs purified from chromosome transfer and segregant clones were PCR amplified with oligonucleotide primers AGCCGACTGATGCCTTCTGA (GPT1) and ATAAATCCAGTTGCCGCCACA (GPT2). These primers amplify a 750-bp fragment of the *gpt* gene. Amplifications were done by using 100 ng of cellular DNA in 50 μl of standard reaction mix. The PCR conditions consisted of initial denaturation at 94°C for 4 min followed by 30 cycles of 94°C (1 min), 65°C (1 min), and 72°C (3 min). Amplified DNA products were separated by electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

Cellular sequences of the donor chromosome 6 in microcell hybrids and segregant clones were assessed by polymorphic markers. Microsatellite markers *D6S87* (at 6q22) polymorphic for (CA) repeats (26) and *ESR* (at 6q25) polymorphic for (TA) repeats (27), and a *VNTR* marker *D6S37* (at 6q26) detected with the probe JCZ30 (28) were assessed by PCR and Southern blot hybridization, respectively, as described (7).

RESULTS AND DISCUSSION

Normal human chromosomes tagged with a dominant selectable marker and present individually in mouse/human hybrid cells were transferred into two immortal derivatives of SV/HF. Four different human chromosomes (chromosomes 2, 6, 8, and 19) were introduced into HALneo and/or 39neo cells by MMCT. An outline of the experimental approach is given in Fig. 1. Results of chromosome-transfer experiments are presented in Table 1. In a total of six different experiments for the transfer of chromosome 6 or 6q, 12 independent colonies were recovered. These colonies were named as HALneo6

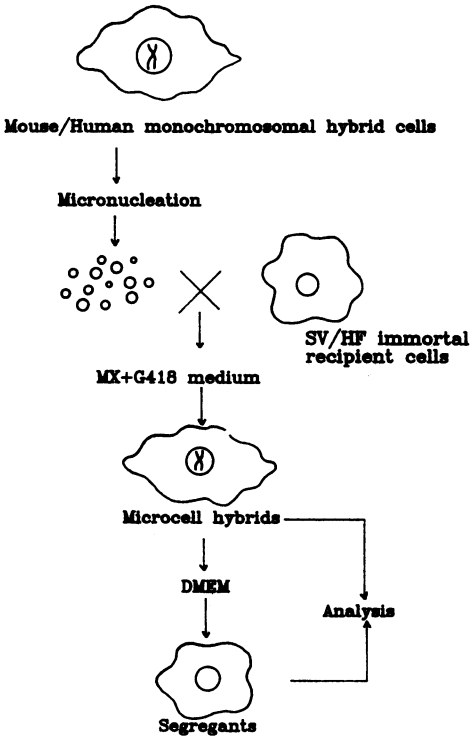

FIG. 1. Outline of experimental approach for identification of human chromosomes carrying a senescence gene(s).

Table 1. Chromosomes transferred into immortal cell lines and the phenotype of resultant clones

Exp.	Recipient cell line	MC donor cell line*	Human chromosome	Colonies, no.	Suppressed, no.	Population doublings†
3	HALneo	RAiso6q	6q	5	5	4–20
1	HALneo	RA6	6	1	1	15
1	39neo	RA6A	6	5	5	4–10
1	39neo	RA6q	6q	1	1	6
1	HALneo	RA2	2	2	0	ND
1	39neo	RA8A	8	4	0	Continuous
1	39neo	RA19A	19	5	0	Continuous
2	T98G	RA6A	6	10	0	Continuous

ND, not done. MC, microcell.
*Hybrids RA6 and RA6A contain human chromosome 6 of different origin; RAiso6q and RA6q contain isochromosome for 6q and 6q, respectively. These chromosomal arms are the same as present in RA6.
†Data are estimated from cell number observed in terminal clones or cultures.

(1-6) and C1 39neo6 (1-6), respectively. In our evaluation 39neo was relatively more efficient for cell fusion as compared with HALneo and was chosen for the transfer of other chromosomes (Table 1). A total of four and five colonies recovered for the transfer of chromosome 8 and chromosome 19, respectively, were named as C1 39neo8 (1-4) and C1 39neo19 (1-5).

Cell Morphology and Survival. Both HALneo and 39neo display characteristic features of transformed immortal human cells (Fig. 2). Introduction of normal human chromosome 6 or 6q into either of these cell lines leads to gross morphological alterations including enlarged and vacuolated cells typical of a senescent cell population (Fig. 2). Chromosome 6 originating from two different human cell lines produced a similar effect on transfer into both of the recipient cell lines (Table 1, Fig. 2). The colonies bearing chromosomes 2, 8, and 19 were morphologically similar to parental recipient cells and did not display morphological alterations observed after the introduction of chromosome 6 or 6q.

Various chromosome-transfer clones bearing chromosomes 2, 6, 6q, 8, and 19 were either isolated individually into separate dishes or followed in the original plates to determine the length of sustained growth. The results of this analysis are presented in Table 1. Cells in all colonies containing chromosome 6 or 6q senesced after a limited number of doublings (Table 1). Such colonies shared extended doubling times and multiplied to a number varying generally from 60 to 300 cells before total growth cessation, except for HALneo6-1 and C1 39neo6-1, which could be subcultured.

Identification of the Donor Chromosome 6 in Suppressed Hybrids. To verify that the poorly growing colonies were, indeed, microcell hybrids and did not originate by reinsertion of the *gpt* marker elsewhere into the recipient cell genome, we examined the suppressed hybrids for the donor chromosome 6. In the case of HALneo6-1 and C1 39neo6-1 there was sufficient cell growth to prepare DNA for PCR analysis. (In addition, cells that floated into medium due to progressive cell death were also collected for DNA of other colonies.) PCR analysis of these DNAs show the presence of *gpt* sequences (Fig. 3*A*, lanes 4 and 8) as in the donors RA6 and RA6A (lanes 3 and 7) but not in the parental HALneo (lane 2) or 39neo (lane 6). Most important, presence of the normal human chromosome 6 in senescing colonies was also demonstrated by the dinucleotide repeat sequences characteristic of the donor chromosome, as shown in Fig. 4. Immortal HALneo cells are hemizygous for the highly polymorphic microsatellite $(CA)_n$ repeat at *D6S87*, as reported (7) and shown in Fig. 4*A*. The donor chromosome 6 carries a

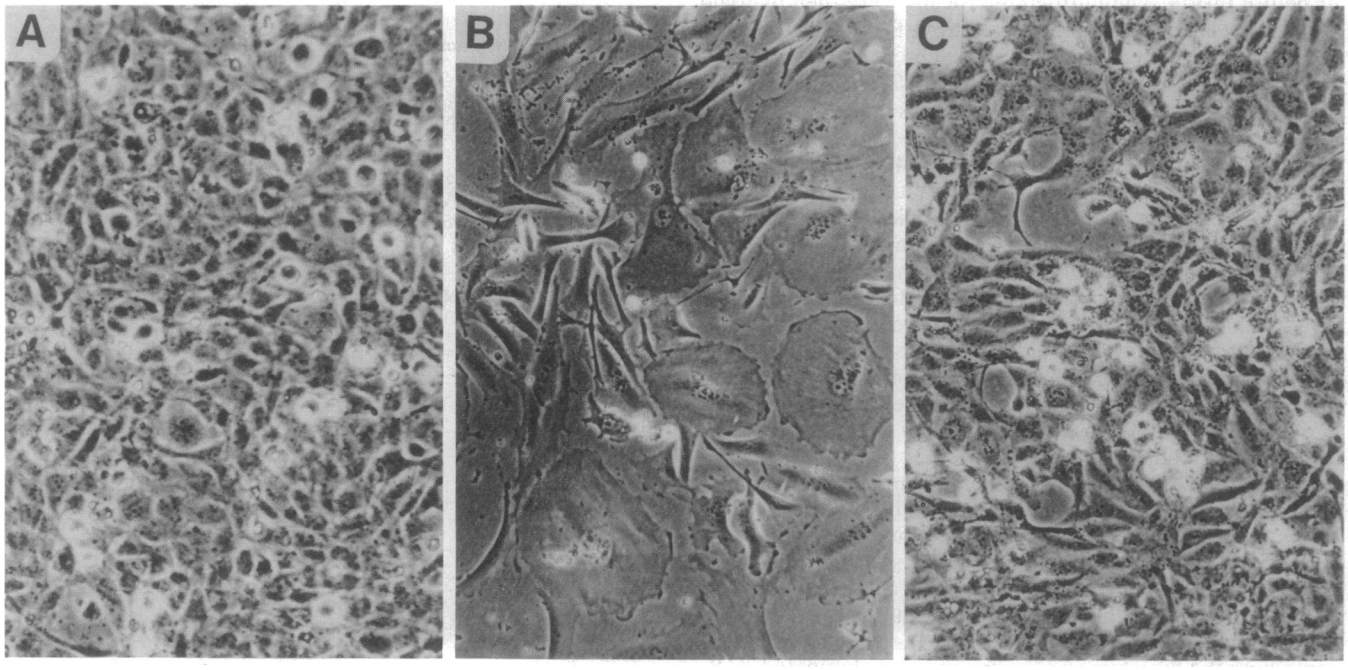

FIG. 2. Photomicrographs of cells. (*A*) Parental immortal HALneo cells. (*B*) HALneo6-1 showing senescing cells. (*C*) Segregant clone HALneo6/TG, which lost the transferred chromosome 6. (×90).

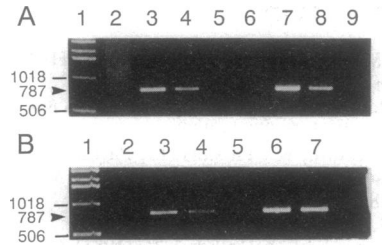

FIG. 3. Presence of *gpt* sequences in microcell hybrids determined by PCR amplification of genome DNA. Amplification occurred only in donor monochromosomal hybrids and the microcell hybrids of immortal cells bearing the transferred human chromosomes. (*A*) Lanes: 1, molecular markers; 2, HALneo; 3, RA6; 4, HALneo6-1; 5, HALneo6/TG; 6, 36neo; 7, RA6A; 8, C1 39neo6; and 9, A9 (mouse cells). (*B*) Lanes: 1, markers; 2, 39neo; 3, RA8A; 4, C1 39neo8; 5, A9 (mouse cells); 6, RA19A; and 7, C1 39neo19.

different allele than the recipient cells and therefore can be followed by analysis for *D6S87*. Senescing cells from HALneo6 (Fig. 4*A*, lane 4) are heterozygous for this locus, and the additional allele corresponds to the microcell donors RA6. Similar results were obtained by analysis for *VNTR* sequences (data not shown). 39neo contains an intact and rearranged chromosome 6 (P. Patsalis, personal communication) such that it is heterozygous for *D6S87* and *ESR*. Although the microcell donor RA6A shares one of the *D6S87* alleles (data not shown), it has a distinct microsatellite $(TA)_n$ repeat at *ESR*, which is also present in C1 39neo6 (Fig. 4*B*, lane 5). Finally, we demonstrated the presence of intact donor chromosome 6 in a suppressed colony by recovery into mouse cells as described below.

Rescue of Suppressor Chromosome 6 into Mouse Cells. Senescing cells from HALneo6-1 and C1 39neo6-1 were fused with ouabain and TG-resistant rodent RAG-OR cells, and hybrid cells were recovered by selection in MX medium/ouabain. The tagged human chromosome now present in human/RAG cell hybrids was further transferred to mouse A9 cells by MMCT. The resultant microcell hybrids obtained by selection in MX medium were analyzed for the identity and integrity of the rescued human chromosome. Such A9 hybrids contained molecular markers diagnostic of the original donor human chromosome 6 and a cytogenetically intact chromosome 6 (Fig. 5).

Suppression Is Specific to Chromosome 6. Specificity for suppression of the immortal phenotype in HALneo and 39neo by chromosome 6 was assessed by introduction of other human chromosomes into these cell lines and transfer of chromosome 6 into a glioblastoma tumor cell line T98G (see Table 1), which is not transformed by SV40 and is a member

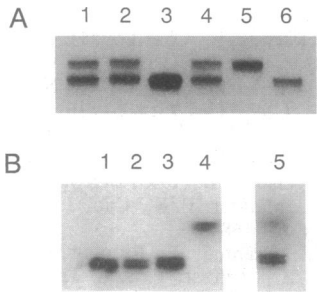

FIG. 4. Presence of transferred human chromosome 6 in suppressed microcell hybrids determined by polymorphism for dinucleotide repeat (*A*) *D6S87* locus. Lanes: 1, HF; 2, preimmortal SV/HF; 3, HALneo; 4, HALneo6; 5, RA6; 6, HALneo6/TG (segregant). (*B*) *ESR* locus. Lanes: 1, normal HF; 2, preimmortal SV/HF; 3, 39neo; 4, RA6A; 5, C1 39neo6-1.

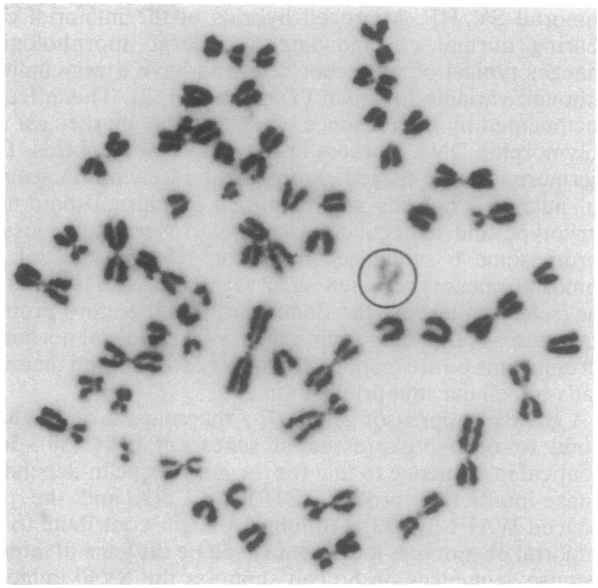

FIG. 5. Metaphase spread of a microcell hybrid showing intact chromosome 6 (circled) rescued from a senescing HALneo6-1 clone.

of a different complementation group for immortalization (29).

The clones of 39neo bearing chromosome 8 and 19 [C1 39neo8 (1-4) and C1 39neo19 (1-5)] remained morphologically similar to the parental cells, displayed an immortal phenotype, and were readily propagated. HALneo2, a clone containing transferred chromosome 2, was morphologically similar to the parental cells. However, this clone was lost due to contamination and could not be assessed for the suppression of immortal phenotype. Introduction of chromosome 6 into a glioblastoma cell line T98G had no effect on the growth characteristics of these cells. Taken together, these results show that at least one of the genes that specifically inhibits the immortal growth of SV40-transformed HALneo and 39neo cells is present on the long arm of chromosome 6.

Immortal Segregant Clones. To verify that suppression is, indeed, dependent on the normal chromosome 6, as shown by the preceding experiments, we tested whether loss of chromosome 6 results in restoration of the immortal phenotype. We therefore attempted to isolate segregant clones that have lost the introduced chromosome 6 for characterization.

Cells from clones HALneo6-1 and C1 39neo6-1 displaying the senescent phenotype were cultured in nonselective medium. Removal of selection pressure would permit random segregation of the tagged chromosome and facilitate the appearance of immortal segregant clones. This is particularly efficient for HALneo6 because HALneo is an $hprt^-$ cell line, and segregant clones were isolated by selection in the medium containing TG and on the basis of morphology as well. Such putative segregants were analyzed for loss of donor chromosome 6. (*i*) Colonies of the segregant immortal cells failed to grow in MX medium. (*ii*) DNA sequences specific for the donor chromosome 6—i.e., *gpt* and *D6S87*, determined by PCR analysis—were absent in HALneo6/TG as shown in Fig. 3*A*, lane 5, and in Fig. 4*A*, lane 6, respectively.

CONCLUSIONS

Prior studies in the laboratory of one of us (H.L.O.) (7) and others (13, 14) have shown that loss of sequences on chromosome 6q are specifically associated with the immortal phenotype of SV/HF. In this study, we demonstrate that introduction of an intact normal chromosome 6 or 6q results in the suppression of cell proliferation of two independent

immortal SV/HF. Microcell hybrids of the immortal cells bearing normal chromosome 6 undergo morphological changes typical of senescent cells and have a very limited, although variable life span (Table 1, Fig. 2). This effect is documented by the presence of a selectable marker *gpt* and polymorphic DNA markers in the suppressed hybrids. Furthermore, the *gpt*-tagged chromosome rescued into sequential microcell hybrids was identified as chromosome 6 by karyotype and molecular analysis. Most critically, loss of chromosome 6 sequences results in reappearance of the immortal phenotype when such segregants are isolated by backselection against the donor *gpt*. We therefore propose that expression of one or more genes on the distal portion of chromosome 6q are responsible for senescence, and their loss leads to cellular immortalization.

A growth-suppressor gene *SDI-1* that maps to 6p has been found to be overexpressed in senescent cells (30). It is identical in sequence to that for the human cyclin-dependent kinase-interacting protein (C1P1 or Cdi1) and the p53-induced WAF1 (31–33). Although it might contribute to the immortal phenotype, it cannot in itself be the gene of interest because sequences on 6q can suppress the SV40 immortal cell lines. Rare immortal segregants obtained in C1 39neo6-1 maintained in selective MX medium are being analyzed for loss of sequences to further localize this gene (unpublished data). A candidate locus for a tumor-suppressor gene has been mapped to 6q in human melanoma cells based on chromosomal rearrangements (34) and suppression of tumor formation in nude mice after MMCT (17). Most recently, transfection of Mn-superoxide dismutase, which is located at 6q25, into melanoma cells has been shown to inhibit tumor transplantation (35).

On the basis of cell-fusion experiments, Pereira-Smith and Smith (29) have reported that a limited number of genes may be responsible for immortalization. In that study SV/HF-5/39 or "clone 39," the parent of 39neo, was classified as a member of the complementation group A. More recently it has been shown that introduction of chromosome 4 into HeLa cells and other members of complementation group B results in growth suppression (21). We propose that a second senescence gene (*SEN6*) exists on 6q. Two considerations of the SV/HF experimental system make it unlikely that mutations in several growth-suppressor genes are involved in addition to *SEN6*. (*i*) These cell lines have undergone a limited number of cell generations in contrast to those of a human tumor, which is then repeatedly passaged in culture. (*ii*) SV40 T antigen inactivates Rb-1, p53, and possibly other growth suppressors as well, obviating the requirement for mutations in those genes. It should be noted that several human tumors, most notably melanoma, ovarian carcinoma, non-Hodgkin lymphomas, among others, have nonrandom rearrangements involving the long arm of chromosome 6 (36). We speculate that such malignancies have acquired the immortal phenotype during carcinogenesis *in vivo* by loss of function of *SEN6* and that this may be a common gene involved in a wide range of tumors.

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