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A Gene Involved in Control of Human Cellular Senescence on Human Chromosome 1q

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Normal cells in culture exhibit limited division potential and have been used as a model for cellular senescence. In contrast, tumor-derived or carcinogen- or virus-transformed cells are capable of indefinite division. Fusion of normal human diploid fibroblasts with immortal human cells yielded hybrids having limited life spans, indicating that cellular senescence was dominant. Fusions of various immortal human cell lines with each other led to the identification of four complementation groups for indefinite division. The purpose of this study was to determine whether human chromosome 1 could complement the recessive immortal defect of human cell lines assigned to one of the four complementation groups. Using microcell fusion, we introduced a single normal human chromosome 1 into immortal human cell lines representing the complementation groups and determined that it caused loss of proliferative potential of an osteosarcoma-derived cell line (TE85), a cytomegalovirus-transformed lung fibroblast cell line (CMV-Mj-HEL-1), and a Ki-*ras*$^+$-transformed derivative of TE85 (143B TK$^-$), all of which were assigned to complementation group C. This chromosome 1 caused no change in proliferative potential of cell lines representing the other complementation groups. A derivative of human chromosome 1 that had lost most of the q arm by spontaneous deletion was unable to induce senescence in any of the immortal cell lines. This finding indicates that the q arm of human chromosome 1 carries a gene or set of genes which is altered in the cell lines assigned to complementation group C and is involved in the control of cellular senescence.

It has been well documented that normal human cells in culture have a limited division potential, which has been used as a model for the aging process at the cellular level (9). Cell lines established from human tumors or normal cells transformed by carcinogens or viruses exhibit unlimited growth potential in vitro and have escaped from senescence (i.e., are immortal). Studies of cell fusions between normal and immortal human cells, as well as similar interspecies, hamster-human fusions, have demonstrated that the phenotype of cellular senescence is dominant and that the changes which lead to immortalization are recessive events (2, 3, 11, 23, 25, 30). These data also indicate that cellular senescence is under the control of programmed genetic processes rather than the result of random, stochastic events. Exploiting the recessive nature of immortality, various immortal human cell lines were fused with each other, and to date more than 30 immortal human cell lines have been assigned to four complementation groups for indefinite division (7, 24; unpublished observations). Complementation group assignment is not dependent on the embryonal layer of origin, the presence of a specific activated oncogene, or the carcinogen or viral genes used for transformation. Recently, Duncan et al. (7) have assigned three immortal simian virus 40-transformed cell lines to more than one of the previously identified complementation groups, indicating that they may have immortalized through recessive genetic events involving more than a single set of genes of the senescence program. However, the caveat that has been discussed is that it takes a single immortal segregant in a population to mask the senescent phenotype, and the possibility exists that the data reflect the same. Nonetheless, the identification of four complementation groups has narrowed the search for the set of genes or pathways responsible for escape from senescence and have further supported a genetic basis for the phenomenon of cellular senescence.

To identify the chromosomes which contain the genes necessary for this growth control process, several investigators have attempted to complement the immortal defect which allows for indefinite division in established cell lines of various species (11, 17–19, 30, 32). Ning et al. (17) identified human chromosome 4 as able to selectively reverse the immortal defect in human cell lines assigned to complementation group B, and Klein et al. (11) reported loss of cell proliferation following the introduction of a normal human chromosome X into a nickel-transformed rodent cell line. Additionally, Ogata et al. (19) recently reported that human chromosome 7 suppresses proliferation of two nontumorigenic, 4-NQO (4-nitroquinoline *N*-oxide)-immortalized fibroblast cell lines, one of which was previously assigned to complementation group D (24). Sugawara et al. (30) demonstrated that a single normal human chromosome 1 was able to reverse the immortal phenotype of a hamster cell line. To determine whether human chromosome 1 carried a gene(s) which was involved in the control of senescence in human cells corresponding to one of the four complementation groups for indefinite division defined by Pereira-Smith and Smith (24), we used the technique of microcell fusion to introduce this chromosome into immortal human cell lines representative of each of the groups. The parental cell lines and the resulting microcell hybrid clones were analyzed for their proliferative potential in culture and morphological changes characteristic of senescence. Cytogenetic and DNA polymorphism analyses confirmed the presence of the introduced chromosome.

The results demonstrate that human chromosome 1 re-

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TABLE 1. Proliferative behavior of microcell hybrids

Recipient immortal human cell line	Complementation group	No. of microcell fusions	No. of microcell hybrids	Range of PD prior to loss of division	No. with division cessation/total
EJ (bladder carcinoma)	A	2	13	—[a]	0/13
T98G (glioblastoma)	B	2	9	—	0/9
TE85 (osteosarcoma)	C	2	15	59–80	7/15
CMV-Mj-HEL-1 (cytomegalovirus-transformed lung fibroblasts)	C	8	29	1–6	20/29[b]
143B TK^- (Ki-*ras*$^+$-transformed TE85)	C	3	4	24–38	4/4
A1698 (bladder carcinoma)	D	1	8	—	0/8

[a] —, achieved >100 PD without loss of proliferative potential.
[b] In three of the clones, a focus of proliferating cells overgrew the culture and achieved >100 PD.

versed the immortal phenotype and caused loss of proliferative potential in three immortal human cell lines assigned to complementation group C but was unable to affect proliferation of cell lines assigned to the other complementation groups. Additionally, a derivative human chromosome 1, which resulted from the spontaneous deletion of most of the q arm during serial passage in the mouse A9 background, was unable to induce senescence in any of the immortal human cell lines. Therefore, a gene(s) involved in cellular senescence resides on the q arm of human chromosome 1 and is inactivated in immortal human cell lines assigned to complementation group C.

MATERIALS AND METHODS

Cell culture. The immortal human cell lines used in this study are listed in Table 1. The cell line used as the donor in the microcell fusions (A9+1) consisted of a single copy of a normal human chromosome 1, tagged with the selectable marker *neo*, in an A9 mouse fibroblast background (12). The immortal human cells, the microcell donor line, and the microcell hybrid clones were propagated in minimum essential medium supplemented with Hanks' salts or Earle's salts and 8% fetal bovine serum plus 2% supplemented calf bovine serum. G418 (0.5 mg/ml) was added to the medium of the microcell donor and hybrid clones to maintain selection for the *neo*-marked human chromosome 1. The numbers of cells and cumulative population doublings (PD) were determined at each subculture (weekly, unless otherwise indicated). A culture was considered immortal when it reached >100 PD without the loss of division potential. Detailed cell culture conditions and calculation of the in vitro life span have been described elsewhere (22).

Microcell-mediated chromosome transfer. The procedure for the preparation of microcells, microcell fusion, and isolation and subculture of the microcell hybrids has been previously detailed (10, 12, 17, 30).

Cytogenetic analysis. Analysis of the immortal human cell lines, the monochromosomal hybrid cell line, and each microcell hybrid clone was performed by using standard G banding of metaphase spreads. G-11 staining was used to determine whether mouse chromosomes were present in the hybrids (1).

Polymorphism analysis of genomic DNA. The presence of the introduced chromosome in all microcell hybrids was verified by performing either standard Southern or PCR-based polymorphism analysis. In the Southern analysis, we used 7 μg of restriction enzyme-digested genomic DNA isolated from each of the hybrid cell lines approximately 25 PD after the introduction of the donor chromosome (14). A series of random-primed [α-^{32}P]dCTP-labeled probes to known polymorphic regions of human chromosome 1 were used to detect restriction fragment length polymorphisms (4) (American Type Culture Collection). The probe designated D1S74 (see Fig. 4) identifies a single-copy DNA segment at the q terminus of human chromosome 1 and also detects a variable number of tandem repeats (VNTR) ranging from 2 to 12 kb with a heterozygosity of 96% (16).

In the case of small microcell clones (<500 cells), cell lysates were analyzed by PCR amplification since purified genomic DNA could not be obtained (29). A series of primers to DNA polymorphisms characterized by the ability to detect variable lengths of simple-sequence tandem repeats were tested. These primer sets were also used on hybrids with which the Southern analysis had proven uninformative. PCR was performed upon cell lysates in the presence of [α-^{32}P]dCTP (27), and the resulting products were purified and resolved by electrophoresis on 6% polyacrylamide sequencing gels. The primer set designated D1S245 (GenBank no. Z17011) detects amplified products in the range of 0.235 to 0.253 kb with a heterozygosity of 83% in the region of 1q41-qter. PCR was run at a denaturing temperature of 94°C for 1 min, an annealing temperature of 55°C for 1 min, and an elongation temperature of 72°C for 1 min over 35 cycles.

RESULTS

Analysis of proliferation of microcell hybrids containing an intact chromosome. Microcell hybrids were generated with the immortal human cell lines listed in Table 1. Microcell fusions were initially done with a cell line from each of the complementation groups, and several hybrid clones were obtained. Hybrids isolated from fusions with EJ, T98G and A1698, which represent complementation groups A, B, and D, could be serially subcultured with no alteration in proliferative potential through 100 PD, which is our criterion for immortality. The growth rates and morphological characteristics of the microcell hybrid clones were similar to those of the parental cell lines (Fig. 1A, B, and D), indicating that chromosome 1 had no detectable effect on the growth of these cells.

Different results were obtained with cell line TE85, assigned to group C. We obtained 15 microcell hybrids, 7 of which had lost proliferative potential and exhibited morphological characteristics indicative of cellular senescence. The hybrid clones achieved a range of PD (59 to 80) before cell division ceased (Table 1). A representative growth curve (Fig. 1C) of one microcell hybrid compared with that of the immortal parent demonstrates that the cells initially proliferated rapidly, then entered a period of slowed growth, and subsequently lost proliferative potential at PD 59. The nondividing cells remained metabolically active and were maintained for 5 weeks without a significant change in cell number. At this point, the cells also exhibited a morphology similar to that of senescent

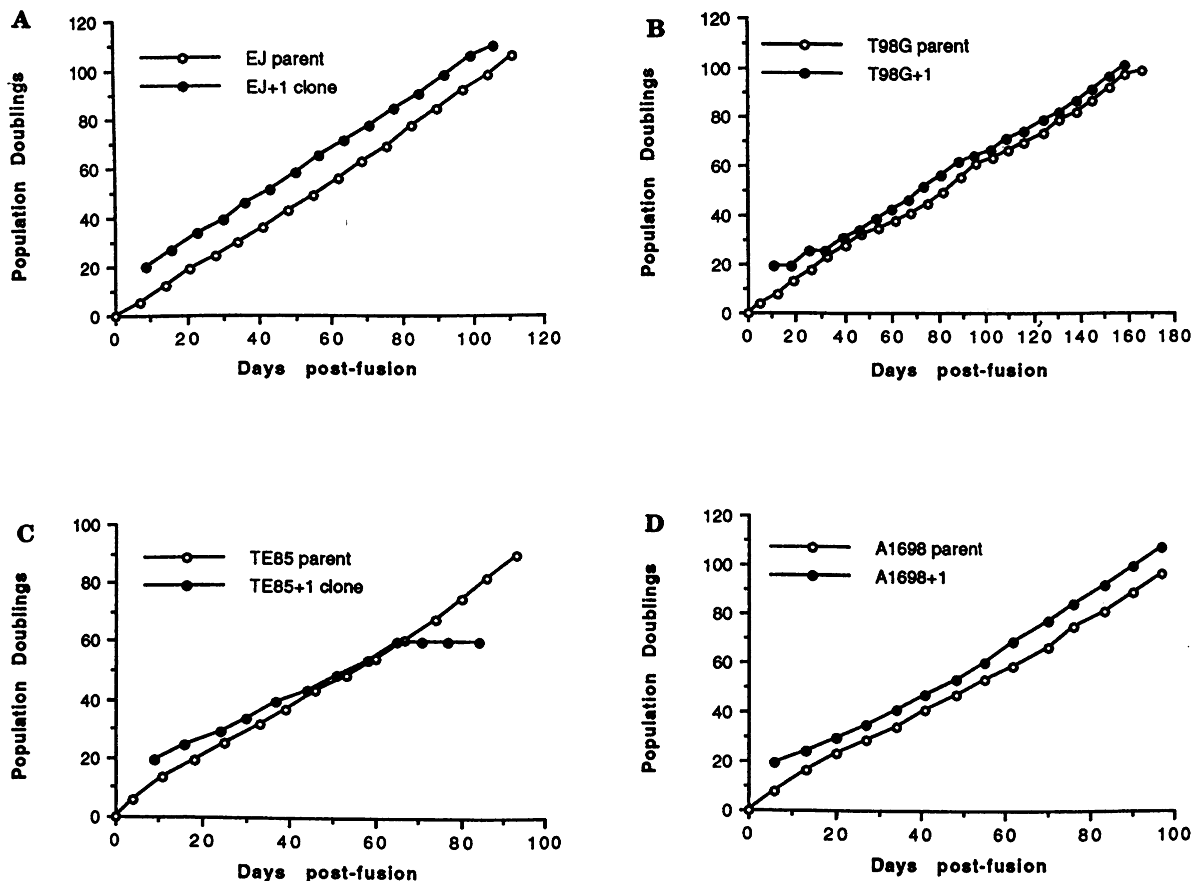

FIG. 1. Representative growth curves for each parental cell line and a microcell hybrid clone. The numbers of cells and PD were calculated each week until the population reached >100 PD. (A) EJ and EJ+1 cl 1; (B) T98G and T98G+1 cl 4; (C) TE85 and TE85+1 cl 11; (D) A1698 and A1698+1 cl 2.

cells, becoming enlarged and flattened (Fig. 2C). This morphology was distinct from that of the parental cell line (Fig. 2A) and cells of the same clone 3 weeks prior to loss of proliferation (Fig. 2B). Eight microcell hybrid clones exhibited no obvious change in growth or morphology. However, it was most likely that an immortal cell had arisen in the population and outgrew those cells that became senescent; thus, the cells appeared to exhibit an immortal phenotype. Similar observations have been made in other studies (18, 25, 30).

To rule out the possibility that through a dosage effect, the introduction of any human chromosome would cause the TE85 cells to senesce, we obtained six microcell hybrids with TE85, using a monochromosomal hybrid composed of A9 cells and a *neo*-marked human chromosome 11. None of these hybrids lost proliferation. We also included two additional cell lines that were assigned to group C in the analysis (Table 1). Microcell fusions with CMV-Mj-HEL-1 yielded a total of 29 hybrids, of which 20 exhibited morphologic (Fig. 3A and B) and growth characteristics indicative of senescence, soon after the introduction of the chromosome. The hybrid clones achieved a limited number of 1 to 6 PD (Table 1) before losing proliferative potential. These senescent clones could be maintained for more than 6 weeks without a change in cell number. The other nine hybrids exhibited an initial slow growth rate followed by rapid proliferation to achieve >100 PD. In three of these clones, a focus of rapidly proliferating cells was observed during the initial period of slow growth. The cells in the foci subsequently outgrew the more slowly growing cells in the culture and were probably an immortal variant subpopulation similar to that described above.

Four microcell hybrid clones were obtained with cell line 143B TK^-, and all lost proliferation and became senescent after undergoing 24 to 38 PD (Table 1). These clones were also maintained for 3 to 6 weeks after they became senescent (Fig. 3C and D).

Proliferative behavior of microcell hybrids containing der(1). In the course of studies with the CMV-Mj-HEL-1 cell line, a derivative human chromosome 1, der(1), was observed in the hybrid clones by cytogenetic analysis. Most of the q arm had been deleted in the derivative chromosome, which was present in all the microcell hybrid clones generated from two separate fusions (Table 2). Cytogenetic analysis of the donor cell line A9+1 revealed that 65% of the cells within the population contained der(1). The spontaneous deletion of the q arm had occurred during serial culture of the donor cell line and was not caused by the microcell fusion procedure or the CMV-Mj-HEL-1 cell line itself. der(1) was unable to induce senescence in the microcell hybrids generated with CMV-Mj-HEL-1 (Table 2).

Subcloning of the A9+1 donor cell population led to the isolation of a homogeneous population of cells containing der(1). This cell line, A9+der(1), was used as the donor in microcell fusion experiments with TE85 and 143B TK^- (Table 2). Five microcell clones obtained with TE85 and four hybrid

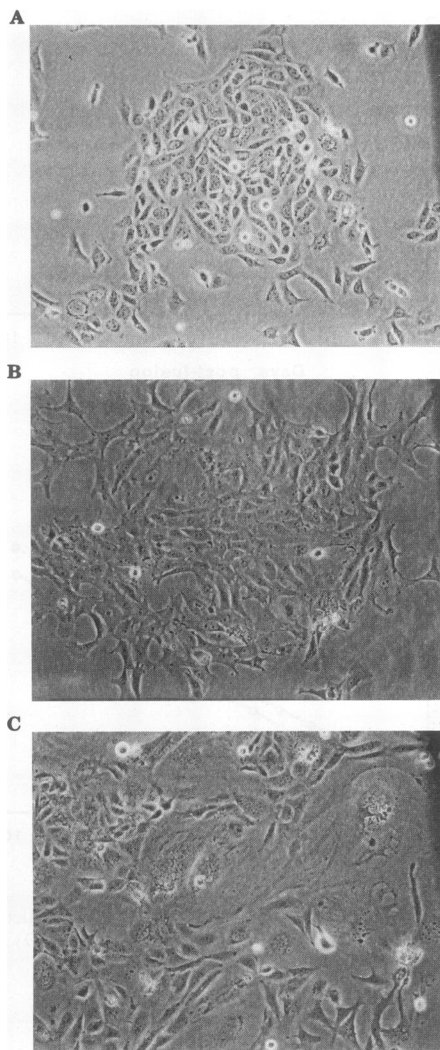

FIG. 2. Phase-contrast micrographs of TE85 cells (A) and a representative microcell hybrid clone, TE85+1 cl 13, PD 77, just prior to the onset of senescence (B) and 3 weeks after senescence (C).

clones of 143B TK$^-$ showed no alterations in morphology or proliferative potential and achieved >100 PD (Table 2).

Cytogenetic analysis. Cytogenetic analysis was performed on each parental cell line and the microcell hybrid clones that could proliferate extensively. The presence of the introduced chromosome was not always definitive because of variation in the number of chromosomes 1 in the individual cells of the culture (Table 3). However, it was informative for some of the microcell hybrids. For example, clones 1 and 7 obtained with the EJ cell line had five copies of human chromosome 1 (Table 3), and since the EJ cell line has a range of three to four copies of this chromosome, the result indicates a successful introduction of an additional chromosome. The microcell hybrid clones from the other cell lines that had cytogenetically informative results are also listed in Table 3.

In the case of hybrids in which cytogenetics was not informative, the numbers of chromosomes 1 present were usually within the range observed in the parental cell line. However, in some cases, we found a reduced number of chromosome 1. Other cell fusion studies have demonstrated that chromosomal segregation occurs and can account for the loss of the original complement of resident chromosomes from cell hybrids (21, 30). Therefore, some of the microcell clones that retained the introduced chromosome had lost one or more copies of the parental chromosome, perhaps to compensate for the additional introduced DNA. The cytogenetic analysis failed to reveal fragments or unidentifiable pieces of human or mouse DNA in any of the microcell hybrids.

DNA polymorphism analysis. To demonstrate the presence of the donor chromosome 1 in the hybrids for which cytogenetics was not informative and in hybrids which could not proliferate for at least 25 PD, DNA polymorphism analysis was performed. Genomic DNA was isolated from the microcell donor, recipient cell lines, and the resulting hybrids (14). A series of probes representing known polymorphic regions from various loci of chromosome 1 were tested for the ability to distinguish the donor and recipient alleles (5). Southern analysis with a probe to the terminus of 1q, designated D1S74, demonstrated that the donor allele was absent in each of the parental cell lines and evident in each representative microcell hybrid clone (Fig. 4).

In the case of small clones from which adequate amounts of genomic DNA could not be obtained for Southern analysis, such as the CMV-Mj-HEL-1 microcell hybrids, a PCR-based analysis of polymorphic regions of the chromosome was performed. One set of primers, D1S245, which detects a series of polymorphisms due to length variations in blocks of simple-sequence tandem repeats, yielded amplified bands that were different in the donor and recipient CMV-Mj-HEL-1 cells and demonstrated the presence of the introduced chromosome in the hybrids (Fig. 5).

DISCUSSION

It is clear that the progression to senescence and immortality involves multigenic events, and there are a relatively small number of genes which may be the cornerstone of normal growth control and the aberrant progression to neoplasia. The ability of normal cells to complement the immortal phenotype of established cell lines from humans (3, 15, 21–25) and other species (30), the assignment of many immortal human cell lines to four complementation groups for indefinite division (24), and the mapping of senescence genes to specific chromosomes support a genetic basis for human cellular senescence. The isolation of these genes has become possible as a result of studies such as those described here, in which normal human chromosomes that specifically reverse the immortal phenotype of cells in culture are identified.

The data in this report indicate that human chromosome 1 carries a gene involved in the control of human cellular senescence. This conclusion supports the previous studies in which human chromosome 1 was able to induce senescence in an immortal Syrian hamster cell line, 10W-2 (30), and in a human uterine endometrial carcinoma cell line, HHUA (32). The results suggest that this particular senescence-related gene may be functionally conserved across evolutionary boundaries. The strength of our analysis resides in the fact that chromosome 1 induces senescence in three immortal human cell lines assigned to the same complementation group, C, and has no effect on cell lines representing the other three groups. The loss of proliferation caused by this chromosome is not merely due to a dosage effect, because other chromosomes, i.e., chromosomes 11 (17; unpublished data), 6, and 9 (32), when introduced into these cell lines had no effect on proliferative potential, although in some cases there were effects on other properties such as tumorigenicity. In addition, the chromosome 1 microcell hybrid clones lost proliferative potential after

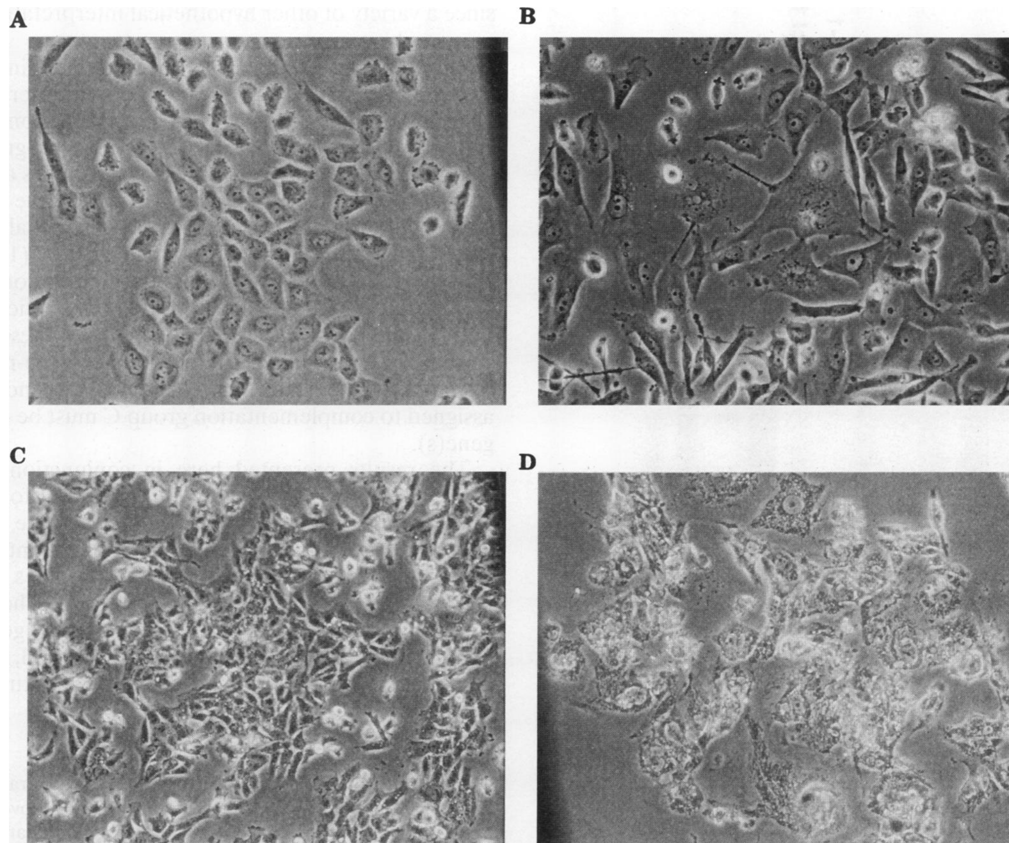

FIG. 3. Phase-contrast micrographs of CMV-Mj-HEL-1 (A), CMV-Mj-HEL-1+1 cl 5, PD 5, 5 weeks postfusion (B), 143B TK$^-$ (C), and 143B TK$^-$+1 cl 2, PD 34, 9 weeks postfusion (D).

achieving a range of PD levels, which would not be expected if gene dosage was involved.

The function of the gene on chromosome 1 in the control of cellular senescence will not be clear until it has been isolated; however, a model is suggested by the data presented here. The group C cell lines clearly become senescent in response to the incorporation of the normal human chromosome 1. The range of cumulative PD at which the hybrids ceased dividing has been observed in previous whole cell and microcell fusion studies (18, 23). It is also established that the in vitro life span of a culture is dependent on the cumulative PD and not chronological time (6, 26). Cells can be frozen for long periods of time or subjected to multiple freeze-thaw cycles with relatively little perturbation of the PD at which onset of senescence occurs. These findings indicate that there is a clock or counting mechanism which triggers events required to initiate the final stages of the senescence program once cells reach their finite proliferative potential. Therefore, a cell which is at an early time in the clock or counting mechanism would have a large proportion of its in vitro life span remaining, and a cell at a

TABLE 2. Proliferative behavior of der(1)-containing microcell hybrids[a]

Recipient immortal human cell line	No. of microcell fusions	No. of microcell hybrids	No. with division cessation/total
TE85 (osteosarcoma)	2	5	0/5
CMV-Mj-HEL-1 (cytomegalovirus-transformed lung fibroblasts)	2	8	0/8
143B TK$^-$ (Ki-*ras*$^+$-transformed TE85)	4	4	0/4

[a] All cell lines were of complementation group C; all hybrids achieved >100 PD without loss of proliferative potential.

TABLE 3. Cytogenetic analysis of recipient lines and microcell hybrid clones

Complementation group	Cell line[a]	Chromosome no./cell	No. of intact chromosome 1/cell
A	EJ	74–88	3–4
	EJ+1 cl 1		5
	EJ+1 cl 7		5
B	T98G	100–119	4–5
	T98G+1 cl 4		6
	T98G+1 cl 10		6
C	TE85	48–49	3
	TE85+1 cl 11		4
	TE85+1 cl 13		4
	TE85+1 cl 14		4
	143B TK$^-$	50–93	2–3
	CMV-Mj-HEL-1	45–47	2
D	A1698	73–76	3–4
	A1698+1 cl 1		4–5
	A1698+1 cl 2		5

[a] cl, clone.

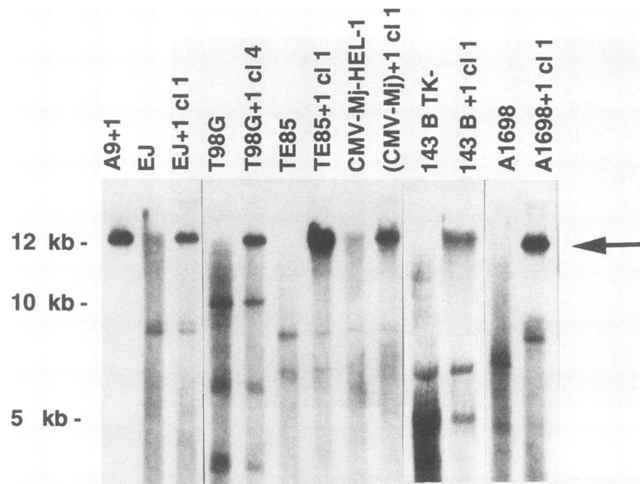

FIG. 4. Southern blot analysis of immortal human cell lines and microcell hybrid clones. Genomic DNA was digested with *Msp*I and probed with ^{32}P-labeled D1S74, which detects a polymorphic region at the terminus of 1q. The band marked with the arrow indicates the allele of the donor cell line A9+1 and its presence in each of the microcell hybrids.

later time or count would have very few divisions remaining before the onset of senescence. If the cell line TE85 developed a mutation in this clock at an early time point, introduction of the gene necessary for counting the divisions would restart the clock and the hybrids would undergo many PD before ceasing to divide. Similarly, if cell line CMV-Mj-HEL-1 developed a mutation in the clock at a late time point, resumption of the counting mechanism would allow very few population doublings to an already old cell. In this manner, the TE85 cell line could be maintained in culture for an extended period of time after the introduction of the chromosome (59 to 80 PD), and the CMV-Mj-HEL-1 cell line would enter senescence relatively soon after the introduction of the clock (1 to 6 PD). However, this is not the only model that could be proposed,

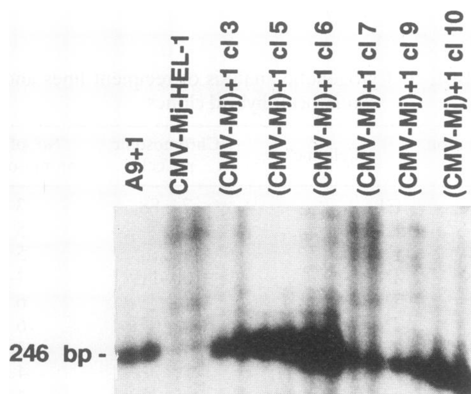

FIG. 5. DNA polymorphism analysis of small microcell hybrid clones from fusions with CMV-Mj-HEL-1. Purified DNA and DNA from cell lysates were amplified by PCR using D1S245 primers, and the resulting products were resolved on a sequencing gel. The band present at 246 bp indicates that the donor chromosome is present in each of the microcell hybrids, including 3, 7, and 9, which were amplified from cell lysates.

since a variety of other hypothetical interpretations of our data are possible.

The importance of human chromosome 1 in cellular senescence is supported by the results reported here. Studies have also indicated that this chromosome may contain a putative tumor suppressor gene(s) involved in the progression of some tumors to malignancy (33). Structural changes and deletions in chromosome 1 have been documented in the case of neuroblastomas (8), breast carcinomas (4), colorectal adenomas (20, 31), and uterine endometrial carcinomas (13). Since only human cell lines assigned to group C were complemented by chromosome 1, and the derivative chromosome which had lost most of the q arm was unable to induce senescence, the data strongly suggest that a human senescence-related gene(s) resides on the q arm of this chromosome and that cell lines assigned to complementation group C must be deficient in this gene(s).

The results presented here in conjunction with previous studies (18, 30, 32) and the identification of four complementation groups for indefinite division indicate that there are multiple chromosomes involved in the control of cellular senescence. Multiple genes and chromosomes have also been shown to control tumor growth (17, 32). The isolation and functional characterization of each of these genes will aid in the understanding of normal growth control, differentiation, and senescence and the multistep process to tumor formation.

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