Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5635–5639, July 1991
Genetics

Genetic analysis of indefinite division in human cells: Evidence for a cell senescence-related gene(s) on human chromosome 4

(microcell hybrids/cell proliferation/cell immortalization/aging)

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Communicated by Ruth Sager, March 26, 1991 (received for review November 12, 1990)

ABSTRACT **Earlier studies had demonstrated that fusion of normal with immortal human cells yielded hybrids having limited division potential. This indicated that the phenotype of limited proliferation (cellular senescence) is dominant and that immortal cells result from recessive changes in normal growth-regulatory genes. In additional studies, we exploited the fact that the immortal phenotype is recessive and, by fusing various immortal human cell lines with each other, identified four complementation groups for indefinite division. Assignment of cell lines to specific groups allowed us to take a focused approach to identify the chromosomes and genes involved in growth regulation that have been modified in immortal cells. We report here that introduction of a normal human chromosome 4 into three immortal cell lines (HeLa, J82, T98G) assigned to complementation group B resulted in loss of proliferation and reversal of the immortal phenotype. No effect on the proliferation potential of cell lines representative of the other complementation groups was observed. This result suggests that a gene(s) involved in cellular senescence and normal growth regulation resides on chromosome 4.**

Normal human cells in culture exhibit limited division potential, which is accepted as a model for cellular senescence (1, 2). Tumor-derived as well as carcinogen- or virus-transformed cells have escaped from senescence and are capable of indefinite division (immortal). In cell fusion studies involving normal and immortal human cells the hybrids had limited life-span (3–8). These results led to the conclusion that the phenotype of cellular senescence is dominant and that immortality results from recessive changes in growth-regulatory genes of the normal cell. They also indicated that senescence was the result of active genetic mechanisms rather than random events. Further evidence in favor of a genetic basis for senescence came from our studies in which fusion of various immortal human cell lines with each other led to the identification of four complementation groups for indefinite division (9). This indicated that at least four sets of genes or processes were involved in cellular senescence and could be modified to yield immortal cells. To determine which chromosomes were involved in senescence, we used microcell fusion to introduce single human chromosomes into immortal human cell lines representing the various complementation groups.

Since Sager and coworkers had demonstrated that cellular senescence was one mechanism for tumor suppression (10, 11), we began our studies with chromosomes that have been implicated in tumor suppression by others. Whole-cell fusion studies had shown that the tumorigenic phenotype of HT-1080, a human fibrosarcoma cell line, could be suppressed following fusion with normal human fibroblasts. Cytogenetic comparison of nontumorigenic hybrids with tumorigenic segregants revealed a correlation between the loss of chromosomes 1 and 4 and reexpression of tumorigenic potential (12). We therefore introduced a normal human fibroblast-derived chromosome 4 into HT-1080 (complementation group A) and additional immortal cell lines representing the other complementation groups. We observed that this chromosome was able to reverse the immortal phenotype of cell lines assigned to complementation group B (HeLa, J82, and T98G) but had no effect on the proliferation of cell lines assigned to other groups, including HT-1080. This effect was specific for chromosome 4, as in a separate study we observed no loss of cell proliferation when a normal human fibroblast-derived chromosome 11 was introduced into immortal cell lines representing the four complementation groups (13).

The presence of the introduced chromosome 4 in these intraspecies microcell fusions was demonstrated by a new class of DNA polymorphic markers (14, 15), analyzed using the polymerase chain reaction (PCR). This allowed the inclusion of microcell hybrid clones having very limited division potential in our analysis. These data demonstrate the feasibility of using intraspecies fusions to identify senescence-related genes. More important, they provide evidence that a gene(s) modified in immortal cell lines assigned to complementation group B is present on human chromosome 4 and that the normal counterpart of this gene(s) is required for expression of the senescent phenotype.

MATERIALS AND METHODS

Cell Culture. The immortal human cell lines used as recipients for microcell-mediated chromosome transfer are listed and described in Table 1. The microcell donor, HA(4)A, was generated by one of us (A.M.K.). It has a single copy of a *neo* (neomycin-resistance gene)-marked human chromosome 4 in the A9 mouse fibroblast background. The microcell donor line and microcell hybrids were propagated in medium containing G418 (500 μg/ml) to maintain selection for the *neo*-marked chromosome 4. The number of population doublings (PD) achieved was determined at each subculture. A culture was considered immortal if it achieved 100 PD without division cessation. Cell culture procedures and estimation of *in vitro* life-span have been described (5).

Microcell-Mediated Chromosome Transfer. Procedures for micronucleation, microcell fusion, and hybrid isolation have been described (13, 16).

Cytogenetic Analysis. G-banded metaphase spreads were prepared and analyzed by standard techniques. The absence of mouse chromosomes in the microcell hybrids was determined by G-11 staining (17).

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Abbreviation: PD, population doubling(s).

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Table 1. Division potential of microcell hybrids

Recipient immortal human cell line	Description	Complementation group	Number of microcell hybrids	Range of PD achieved prior to loss of division	Division cessation, no. of clones
HT-1080	Fibrosarcoma	A	5	—	—*
EJ	Bladder carcinoma	A	2	—	—
HeLa	Cervical carcinoma	B	11	4–41†	10/11‡
J82	Bladder carcinoma	B	6	6–27	4/6§
T98G	Glioblastoma	B	7	6–35	6/7
TE85	Osteosarcoma	C	3	—	—
A1698	Bladder carcinoma	D	4	—	—

*Achieved >100 PD without loss of proliferative potential.
†A normal human chromosome 11 did not affect the proliferation of this cell line, serving as a control for this study (ref. 13).
‡The clone that achieved >100 PD did not increase cell number during the initial 4 weeks postfusion.
§One of the two clones that achieved >100 PD exhibited a period of distinct slowing in growth rate before resuming rapid proliferation.

DNA Polymorphism Analysis. An abundant class of human DNA polymorphisms based on length variations in blocks of simple-sequence tandem repeats such as $(dC\text{-}dA)_n(dG\text{-}dT)_n$ was identified recently (14, 15). This type of polymorphism may represent a size difference as small as a single repeat unit [2 base pairs (bp)] and is detected by PCR amplification followed by resolution in DNA sequencing gels.

Genomic DNA from the microcell donor, recipient cell lines, and hybrids was isolated according to Miller *et al.* (18). Three chromosome 4 $(dC\text{-}dA)_n(dG\text{-}dT)_n$ polymorphic markers used as primers were *D4S171* (19), *D4S174* (20), and *D4S175*. The *D4S175* sequences, determined from clone Mfd38, were as follows: CA strand, ATCTCTGTTCCCTCCCTGTT; GT strand, CTTATTGGCCTTGAAGGTAG. Allele frequencies estimated from 148 chromosomes of unrelated CEPH (Centre d'Etude du Polymorphisme Humain) family parents were 134 bp, 0.02; 132 bp, 0.20; 130 bp, 0.08; 128 bp, 0.08; 126 bp, 0.24; 124 bp, 0.16; 118 bp, 0.07; 114 bp, 0.01; and 112 bp, 0.13. Polymorphism information content was 0.82. The *D4S175* repeat sequence was of the form $(AT)_{14}(AC)_{14}A$. Primer end-labeling with ^{32}P and PCR were performed as described (14). The resulting amplification products were resolved by electrophoresis in polyacrylamide DNA sequencing gels, followed by autoradiography.

In the case of small (<100 cells) microcell hybrid clones, slightly different conditions were used. Cell lysates rather than purified DNA were used for PCR amplification (21) through 42 cycles at an annealing temperature of 58°C. The amplified DNA was labeled by the incorporation of [α-[^{35}S]thio]dATP into the interiors of both strands and the polymorphic DNAs were resolved in nondenaturing polyacrylamide gels (22).

RESULTS

Determination of Proliferative Potential of Microcell Hybrids. Microcell hybrids were obtained with all the recipient lines used in the study (Table 1). Initially we used HT-1080, HeLa, TE85, and A1698 as recipient cell lines representative of complementation groups A–D, respectively. The microcell hybrids involving HT-1080, TE85, and A1698 could be subcultured weekly until they achieved >100 PD without loss of division potential (our criterion for the immortal phenotype). The growth rates of the clones were similar to that of the recipient cell lines. However, when a normal human fibroblast-derived chromosome 4 was introduced into HeLa cells, the microcell hybrids exhibited limited proliferative potential and became nondividing, after achieving a range of PD (Table 1) similar to that observed in the earlier whole-cell fusion studies (5, 6).

Eleven HeLa+4 microcell hybrid clones were obtained and studied. Seven of the clones had very limited growth potential and comprised only 10–30 cells 3 weeks after fusion. No change in cell number was observed when they were maintained with weekly refeeding for 2 additional weeks. However, changes in morphology occurred during this time, with the cells becoming significantly larger and resembling typical senescent cells (Fig. 1). Three microcell hybrid clones (C8–C10) could be subcultured following isolation and ceased proliferation after achieving 18, 22, and 41 PD, respectively. One clone (C11) grew very slowly during the first 4 weeks postfusion, attaining <200 cells. However, in the fifth week, the growth pattern changed and some cells within the population began to proliferate vigorously. These cells continued to grow well and achieved >100 PD. We believe that this was the result of the appearance of a variant immortal cell in the population. Similar proliferative behavior was observed in hybrids obtained from whole-cell fusions involving normal and immortal human cells (5–8). However, since the majority of the microcell hybrids lost division potential, the results indicated that a growth-regulatory gene(s) that had been modified in HeLa cells was present on chromosome 4.

To determine whether this gene was related to cellular senescence and specific for complementation group B, we performed microcell fusions using J82 and T98G, other group-B cell lines, as recipients (Table 1).

Four of six hybrids obtained with J82 had very limited life-span and comprised <50 cells 2 weeks after fusion. They behaved similarly to the HeLa+4 small microcell hybrid clones, in that they exhibited no change in cell number during the subsequent 2 weeks and morphologically came to resemble senescent cells (Fig. 2). Two of the hybrid clones (C1 and C2) could be subcultured and achieved >100 PD, but one of the clones showed a slowing in growth rate at PD 27, achieving <2 PD per week for 6 weeks. A definite increase in growth rate was then observed, with the cells achieving ≈4 PD per week, behavior indicative of an immortal variant cell taking over the population. Again, similar growth patterns were observed in whole-cell hybrids involving normal and immortal cells (5–8).

We obtained seven microcell hybrids when T98G was the recipient cell line (Table 1). Three clones had very limited doubling, comprised <50 cells 3 weeks after fusion, and exhibited a senescent morphology (Fig. 2). Three clones achieved 15, 17, and 35 PD before ceasing division. One clone (C1) achieved 100 PD without any significant slowing in growth, similar to the apparently immortal clone obtained with J82. This was most likely because an immortal variant arose in the population, early in the life-span of the clone.

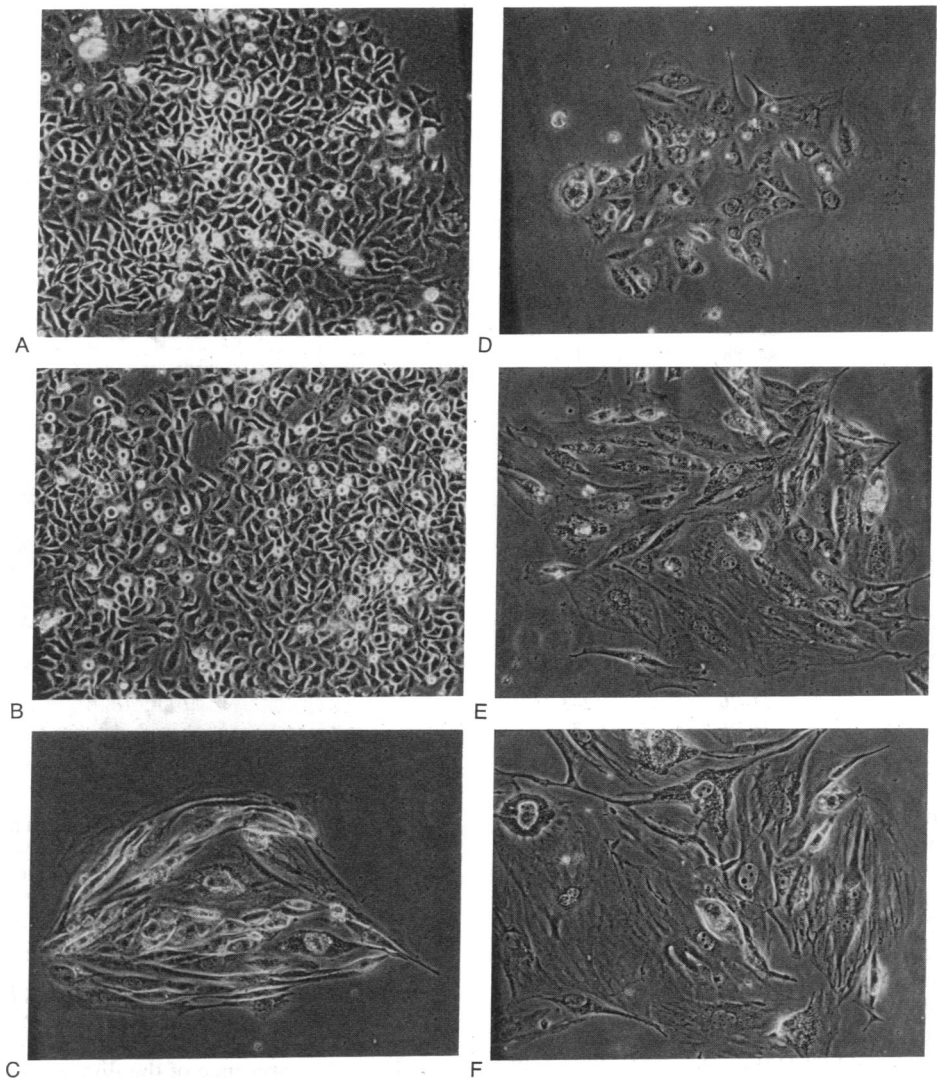

FIG. 1. Phase-contrast micrographs of HeLa cells (*A*) and representative microcell hybrid clones: HeLa+11 clone 1, 4 weeks postfusion (*B*); HeLa+4 clone 1, 4 weeks postfusion (*C*); HeLa+4 clone 2, 3 weeks (*D*), 4 weeks (*E*), and 5 weeks (*F*) postfusion.

We also used the cell line EJ (that assigned to group A with HT-1080) as a recipient in fusions because only one of the five

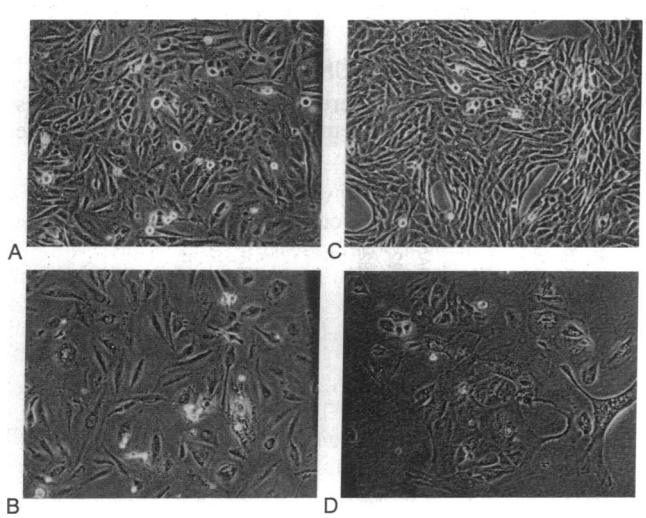

FIG. 2. Phase-contrast micrographs of J82 (*A*), T98G (*C*), and representative microcell hybrid clones: J82+4 clone 5, 4 weeks postfusion (*B*); T98G+4 clone 5, 4 weeks postfusion (*D*).

HT-1080+4 microcell hybrid clones retained an intact introduced chromosome (see cytogenetic analysis below). We felt that more clones with cell lines in group A had to be studied to provide strong experimental evidence for any conclusions. Two microcell hybrids were obtained with the EJ cell line (Table 1). These clones exhibited no loss in cell proliferation before achieving >100 PD, indicating that the result with the HT-1080+4 microcell hybrid, which retained an intact introduced chromosome 4, reflected the behavior of cell lines assigned to complementation group A when chromosome 4 was introduced into them. These data provide strong evidence that a senescence-related gene, specifically modified in cell lines assigned to complementation group B, is indeed present on human chromosome 4.

Cytogenetic Analysis. Results of cytogenetic analysis are summarized in Table 2. In the case of recipient cell lines in which the chromosome 4 copy number was consistent (HT-1080, EJ, and TE85), microcell hybrids that could grow extensively were cytogenetically analyzed after ≈25 PD. HT-1080 comprises a population in which all the cells have a pseudodiploid karyotype. Four of five HT-1080+4 microcell hybrid clones had either a pseudodiploid or tetraploid karyotype with additional cytogenetically unidentifiable fragments of DNA. One of the hybrids was tetraploid with two additional intact copies of chromosome 4. Both the EJ+4 and

Table 2. Cytogenetic analysis of recipient lines and microcell hybrid clones (C*n*)

Complementation group	Cell line	Chromosome number per cell	Copy number of intact chromosome 4 per cell
A	HT-1080	46	2
	HT-1080+4 C1	94	6
	HT-1080+4 C2–C5	46 or 92 plus fragments	2 or 4
	EJ	74–88	2
	EJ+4 C1	82–86	3
	EJ+4 C2	84–88	3
B	HeLa	75–81	2–4
	HeLa+4 C9	75–77	3
	HeLa+4 C10	79–81	5
	HeLa+4 C11	76–79	4
	J82	65–121	1–3
	J82+4 C1	102–116	3
	T98G	100–119	3–4
	T98G+4 C1	115–120	5
C	TE85	48–49	1
	TE85+4 C1	49–50	2
	TE85+4 C2	49–50	2
	TE85+4 C3	48–49	2
D	A1698	73–76	2–3
	A1698+4 C1	74–76	3
	A1698+4 C2	74–76	3

the three TE85+4 microcell hybrid clones had an additional copy of an intact chromosome 4.

Cytogenetic analysis of HeLa, T98G, J82, and A1698 was noninformative, because the chromosome 4 copy number per cell varied (Table 2, Fig. 3). However, even in the cases where a consistent chromosome number was observed, the intraspecies cytogenetic analysis was not definitive, because the donor chromosome could not be unequivocally distinguished from those in the recipient cell. In addition, some of the microcell hybrids involving HeLa, J82, and T98G comprised only a small number of cells, making cytogenetic analysis impossible. We therefore performed DNA polymorphism analysis to confirm the presence of the introduced chromosome 4 allele in the hybrid clones.

DNA Polymorphism Analysis. The chromosome 4 polymorphic marker *D4S175* confirmed the presence of the HA(4)A donor allele in microcell hybrids involving HT-1080, EJ, TE85, and A1698 (Fig. 4 *A–D*). A total of 14 microcell hybrid clones obtained from fusions with group B cell lines were analyzed: nine derived from HeLa, three from J82, and two from T98G. The HeLa+4 microcell hybrid clones, which proliferated extensively, contained the donor alleles identified by three different markers: *D4S171*, *D4S174*, and *D4S175* (Fig. 5). For J82 and T98G, polymorphism markers *D4S175* and *D4S174*, respectively, were informative and used for analysis. The presence of the donor allele in both cases was evident (Fig. 4 *E* and *F*). In the case of small hybrid clones comprising <50 cells, cell lysates rather than purified DNA were used and the presence of the HA(4)A allele in the clones was demonstrated (Fig. 6).

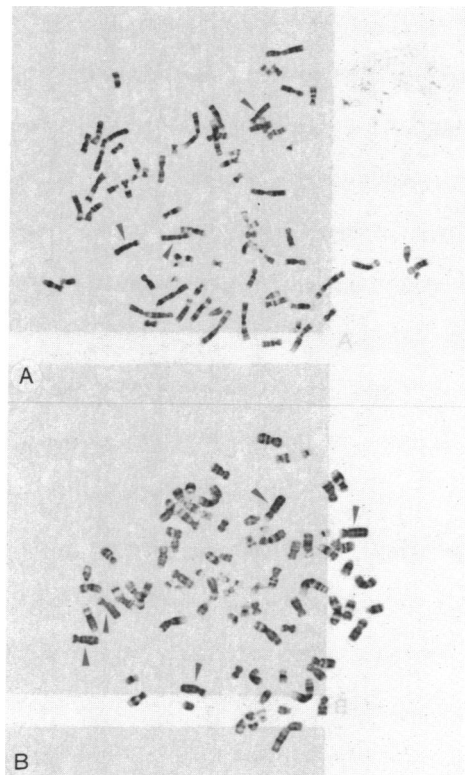

FIG. 3. Cytogenetic analysis of microcell hybrid clones involving HeLa. G-banded metaphase chromosome spreads of HeLa+4 clone 9 (*A*) and clone 10 (*B*) are shown. Arrowheads indicate the intact copies of chromosome 4. The chromosome 4 copy number varies from 2 to 4 per cell in the HeLa population. Therefore, it is only in the case of hybrid clone 10 that cytogenetics is informative.

Polymorphism analysis was repeated at PD 80–100 in clones that achieved this doubling potential (Table 1), confirming that the introduced chromosome 4 was retained throughout subculture.

DISCUSSION

The assignment of many different immortal human cell lines to a limited number of complementation groups for indefinite

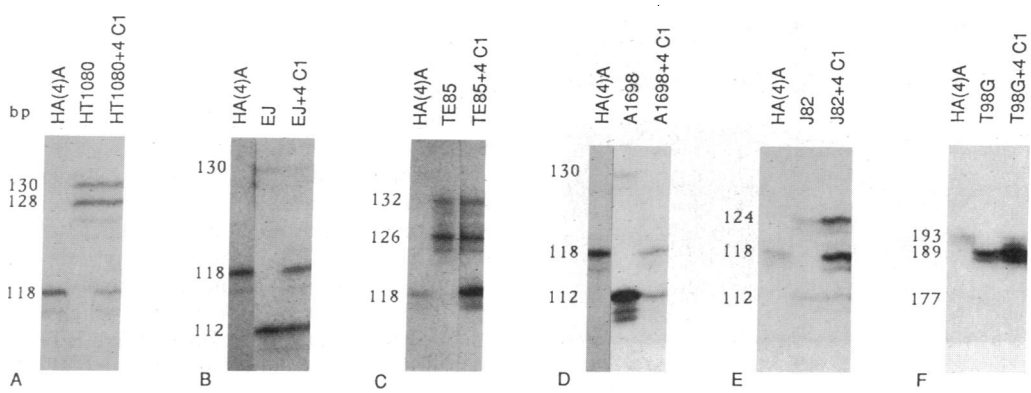

FIG. 4. DNA polymorphism analysis of microcell hybrids. ^{32}P-end-labeled CA-strand primers *D4S175* (*A–E*) and *D4S174* (*F*) were used to amplify DNA from the microcell donor [HA(4)A] and various recipient cell lines and microcell hybrid clones. C1, clone 1.

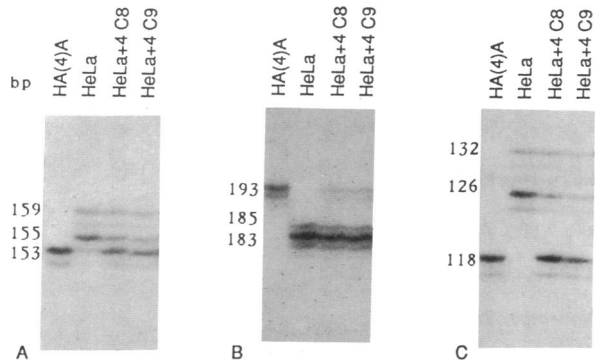

FIG. 5. DNA polymorphism analysis of microcell hybrids involving HeLa. Three sets of ^{32}P-end-labeled CA-strand primers were used to amplify DNA from HA(4)A, HeLa, and HeLa+4 clones 8 and 9. The primers were *D4S171* (*A*), *D4S174* (*B*), and *D4S175* (*C*).

division suggests that escape from senescence to the immortal phenotype involves changes in a limited number of highly specific genes. The microcell fusion data reported here show that such a gene(s) resides on human chromosome 4. This conclusion is strengthened by the following: (*i*) chromosome 4 specifically affected the proliferation of only the cell lines assigned to complementation group B; no effect on proliferation was observed when chromosome 4 was introduced into cell lines representing the other three complementation groups; (*ii*) the effect on group-B cell lines was not nonspecifically caused by the mere introduction of an extra human chromosome, because in a separate study we observed that human chromosome 11 had no effect on the proliferative potential of HeLa cells; (*iii*) the microcell hybrids achieved a range of PD before losing division potential, which would not be expected if a nonspecific gene dosage effect was occurring; (*iv*) the range of PD and appearance of immortal variant clones were similar to those observed previously in whole-cell fusion studies involving normal and immortal human cells.

In this series of experiments we introduced a single chromosome derived from normal human fibroblasts into immortal cells. The senescent phenotype could have easily been obscured by a mutation in a critical senescence gene(s) that would result in the appearance of an immortal variant cell, which would overgrow the culture. However, we did obtain microcell hybrid clones that lost proliferative potential. Although immortal variants were also observed, they occurred at a low frequency. In addition, the development of a new class of highly polymorphic DNA markers and the PCR-based detection allowed us to include the small microcell hybrid clones in our analysis, since we could unequivocally demonstrate the presence of the introduced chromosome 4 in them. The polymorphic DNA markers were also useful in analysis of microcell hybrid clones that could proliferate more extensively, since cytogenetic analysis was noninformative in most cases. Assignment of the markers to specific regions on the chromosome will aid future studies to identify the gene(s) involved in senescence.

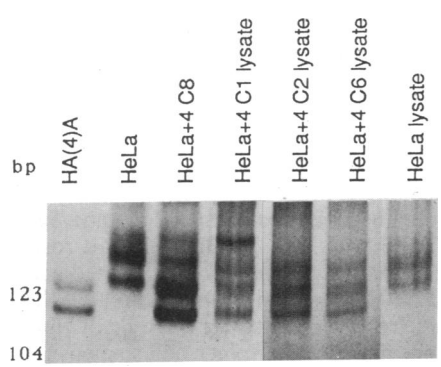

FIG. 6. DNA polymorphism analysis of small microcell hybrid clones involving HeLa. Purified DNA or DNA from cell lysates was amplified using *D4S175* as primer and the polymorphic DNAs were resolved in nondenaturing 6% polyacrylamide gels.

It could be argued that the unaffected proliferation of microcell hybrids involving cell lines assigned to complementation groups other than B was due to an immortal variant arising in the population. We do not consider this likely, because the probability of obtaining 0 out of 14 senescent hybrid clones from groups A, C, and D (Table 1) is $\approx 10^{-11}$, if one assumes an expected frequency of 0.83 (20 out of 24 for group B).

The identification of four complementation groups for indefinite division, coupled with the results presented here, indicates that more than one gene or chromosome must be involved in the pathway to cellular senescence. Indeed, Sugawara *et al.* (23) have implicated human chromosome 1 in cellular senescence in interspecies hamster–human whole-cell hybrids. The involvement of this chromosome was directly demonstrated by transfer of a human chromosome 1 via microcell fusion into an immortal hamster cell line.

We thank Mercedes Lovell in the laboratory of Dr. Linda Taylor for the cytogenetic analysis. This work was supported by National Institutes of Health Grants AG05333, PO1-AG07123, HG00248, and GM37999 and by the Pilgeram Fund for Aging Research.

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