MOLECULAR AND CELLULAR BIOLOGY, May 1992, p. 2273–2281
0270-7306/92/052273-09$02.00/0

Vol. 12, No. 5

Altered Chromosome 6 in Immortal Human Fibroblasts

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Received 2 October 1991/Accepted 20 February 1992

Human diploid fibroblasts have a limited life span in vitro, and spontaneous immortalization is an extremely rare event. We have used transformation of human diploid fibroblasts by an origin-defective simian virus 40 genome to develop series of genetically matched immortal cell lines to analyze immortalization. Comparison of a preimmortal transformant (SVtsA/HF-A) with its uncloned and cloned immortalized derivatives (AR5 and HAL) has failed to reveal any major alteration involving the simian virus 40 genome. Karyotypic analysis, however, demonstrated that all of the immortal cell lines in this series have alterations of chromosome 6 involving loss of the portion distal to 6q21. The karyotypic analysis was corroborated by DNA analyses. Southern analysis demonstrated that only one copy of three proto-oncogene loci (*ros1*, c-*myb*, and *mas1*) on 6q was retained in immortal cells. Polymerase chain reaction analysis of the microsatellite polymorphism at 6q22 (D6S87) showed loss of heterozygosity. In addition, elevated expression of c-*myb* (6q22-23) was observed. We hypothesize that the region at and/or distal to 6q21 plays a role in immortalization, consistent with the presence of a growth suppressor gene.

Spontaneous immortalization of normal diploid human fibroblasts (HDF) in vitro is an extremely rare event (25). Normal HDF grown in vitro have a limited life span, and these cells cease to divide after 30 to 50 population doublings (16). The cessation of proliferative capability inversely correlates with the age of the human donor and is termed cellular senescence. Several hypotheses have been proposed to explain cellular senescence, (discussed most recently in a review by Goldstein [12]). Senescence could be a consequence of random accumulation of DNA damage or errors in DNA, RNA, or protein synthesis. A second proposed mechanism, based on observations relating to the growth and differentiation status of cells, is that cellular senescence resembles terminal differentiation. Hybrids obtained from fusions between normal and immortal cells exhibit limited replicative potential (6, 27, 33, 34). Complementation analysis involving cell hybrids between different immortal human cells indicates that the immortalization phenotype results from a limited number of recessive genetic alterations (35), suggesting that cellular senescence is genetically determined rather than a random accumulation of DNA damage.

Study of immortalization is greatly facilitated by analysis of genetically matched preimmortal and immortal cell lines. One of the few suitable approaches uses transformation by simian virus 40 (SV40), particularly replication-defective (origin-defective) mutant genomes. Introduction of the viral SV40 A gene (17, 23, 43) encoding the large T protein (T antigen) typically increases the life span of HDF. Most important, a subpopulation of SV40-transformed fibroblasts can become immortal (28, 48), although the frequency is often quite low and the phenomenon does not occur with all transformants. We and others have proposed a two-step model for immortalization (37, 63). Cells expressing active SV40 large T antigen bypass the first stage (characterized by loss of mitogen response and growth arrest in G_1) and proliferate until the second stage is initiated. This model is supported by data that show that these cells cease to proliferate when large T antigen is removed or inactivated. Thus, large T antigen is necessary but insufficient for immortalization. The second, independent stage produces crisis; the cells attempt to divide, but fail or die in so doing. Inactivation of the second step could result in immortalization.

Detailed genetic analysis of human tumors has revealed nonrandom chromosomal rearrangements which have facilitated identification of chromosome regions containing oncogenes and tumor suppressor genes (54). Multiple independent SV40-immortalized cell lines fall within the same complementation group (35), strongly suggesting that immortalization following SV40 transformation results from loss of function of one gene product. We previously identified nonrandom alterations of parts of chromosomes 1, 6, and 11 in a series of SV40-transformed immortal cells (28). In the present studies, karyotypic analysis of an independent set of matched preimmortal and immortal SV40 transformants showed a critical region on 6q. Nonrandom deletions in chromosome 6 have been found in immortal cells which are absent in their preimmortal parental cell line. DNA and RNA analyses substantiated alteration of specific loci located on the long arm of chromosome 6.

MATERIALS AND METHODS

Cell lines and culture conditions. The human diploid fetal bone marrow fibroblast cell line HS74 was obtained from H. Smith (53) at passage 5 (P5). SV40 transformants of HS74 were isolated after introduction of an origin-defective SV40 genome encoding a heat-labile T antigen (37). HS74 was transfected with 2 μg of closed circular origin-defective pSVtsA58 DNA together with calf thymus DNA as the carrier by the calcium phosphate-DNA coprecipitation technique (13) as previously described (51). Two days after being seeded on 100-mm dishes at 35°C, 5×10^5 cells were transfected for 4 h. Two transformed foci were picked after 5 weeks. Culture conditions for HS74 and SV40 transfor-

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mants have been previously described (28, 37). The human promyelocyte leukemia cell line HL60 (50) was obtained from G. Ju and was grown in RPMI 1640 supplemented with 1% glutamine and 10% fetal calf serum. Cell number was determined with a Royco cell counter as reported previously (28).

Southern blot analysis. High-molecular-weight DNA was purified and analyzed by the Southern blot procedure, using standard conditions (44). DNA was digested with the appropriate restriction enzyme at 10 U/μg of DNA for 16 to 17 h, electrophoresed in 0.8% agarose gels, and transferred onto a nylon membrane (Nytran; Schleicher & Schuell, Inc.). Hybridization was performed by using random-primed ^{32}P-labeled probe DNA (10).

Northern (RNA) blot analysis. Total cellular RNA was isolated by a modification (52) of the procedure of Auffrey and Rougeon (1). Cells were grown to 70 to 80% confluence, washed once with phosphate-buffered saline, harvested by scraping, and then pelleted at low speed. The pellet was resuspended by vigorous vortexing in 1 ml of ice-cold 6 M urea–3 M LiCl per 100-mm dish of cells. DNA was sheared with a Brinkman Polytron (PT 3000) homogenizer for 60 s at 30,000 rpm. The suspension was incubated for at least 5 to 17 h at 4°C. RNA was pelleted by centrifugation at 10,000 rpm in a Beckman SW41 rotor at 4°C. The pellet was then digested with 100 μg of proteinase K per ml in 10 mM Tris-HCl (pH 7.5)–10 mM EDTA–0.5% sodium dodecyl sulfate at room temperature for 30 min. RNA was purified by phenol-chloroform extraction and precipitated by ethanol. Poly(A)$^+$ RNA was isolated by using standard conditions (2). The isolated cellular RNA was analyzed by the Northern blot procedure, using standard conditions (44); 10 μg of total RNA or 2 μg of poly(A)$^+$ RNA was used per sample. RNA samples were electrophoresed in a 1% agarose–0.66 M formaldehyde gel and then transferred onto a nylon membrane. Hybridization was performed by using random-primed ^{32}P-labeled probe DNA.

Karyotyping. A detailed cytogenetic analysis was performed in normal, preimmortal, and immortal cell lines. Cultures were harvested by standard methods. Slides were prepared by air drying and stained with trypsin-Giemsa for banding. Cytogenetic studies were done as described previously (42). Chromosomes were identified according to the international system for human cytogenetic nomenclature (14). At least 50 metaphase plates were examined per cell line.

DNA probes. Plasmid DNAs were propagated in *Escherichia coli* and isolated by standard methodology (44). Integrated SV40 sequences were assayed by using viral DNA sequences gel purified following *Eco*RI digestion of pSVtsA58, which was kindly provided by Y. Gluzman. The other DNA probes used for Southern and Northern analysis and their respective sources are the 900-bp *Pst*I fragment for (2′-5′) oligo(A) synthetase genomic DNA in P-900 (3; from M. Revel), the 2.6-kbp *Eco*RI fragment of pc-myb E2.6 (11; from S. R. Tronick), the 2.0-kbp *Sal*I fragment for *ros1* of pmcf 3-5.2 in pUC8 (47; from M. Wigler), the 700-bp *Bam*HI-*Hin*dIII fragment for *mas1* in pmasF (66; from M. Wigler), the 2.0-kbp *Xba*I fragment for the gamma interferon receptor in pHuIFN-γ R8 (20; from S. Pestka), and the 530-bp *Xba*I-*Hin*dIII fragment for glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) cDNA (60; from R. Wu). The microsatellite polymorphic dinucleotide repeat $(CA)_n$ sequence at the D6S87 locus (Mfd47) was assayed by polymerase chain reaction using conditions described by Weber et al. (62) with GT-strand primers end labeled with ^{32}P (2). Amplification products were resolved by electrophoresis in 6% polyacrylamide DNA sequencing gels and subjected to autoradiography.

TABLE 1. Growth properties of SVtsA/HF-A[a]

Passage no.	Efficiency of colony formation at 35°C (%)	Doubling time (h)		Saturation density (10^6)[b]	
		35°C	39°C	35°C	39°C
2	18	28	28	3.0	0.6
6	4.0	35	40	6.0	1.9
10	0.9	44	72	ND[c]	ND
13	0.8	60	>96	ND	0.1
18	1.0	80	NG[d]	ND	NG
24	ND	52	NG	ND	ND
38	1.0	48	NG	>2.5	NG
44	8.0	39	NG	ND	NG

[a] Cells were passaged and analyzed as described in Materials and Methods.
[b] Cell number per 60-mm dish.
[c] ND, not determined.
[d] NG, no growth observed after shift of cultures to 39°C.

RESULTS

Properties of SvtsA/HF-A. A morphologically transformed focus, isolated after transfection with origin-defective pSvtsA58 DNA and grown to mass culture, was designated SVtsA/HF-A, P0. The cells were passaged at 1:3 - 1:5 subcultures at 35°C to determine life span as previously reported for the normal parent HS74 and other SV40-transformed HS74 (28). SVtsA/HF-A grew well in mass culture throughout its passage history except for a transient slowing in the apparent growth rate at P14 to P16 in different experiments. It was maintained in continuous culture for over 60 passages (greater than 200 generations) and is considered to have become immortal. Examination of SVtsA/HF-A growth properties at different cell passages at 35°C, however, revealed that additional changes were occurring within the population (Table 1). There was an increase in generation time and a fall in efficiency of colony formation between P6 and P18 (stage I). At subsequent passages (stage II), these properties became reversed, with progressive improvement in cell proliferation. These results are consistent with the gradual appearance of senescence in SVtsA/HF-A during stage I and the emergence of a population of an immortalized derivative without overt crisis of the mass culture. Further passage of stage II cells resulted in improved growth, as previously noted for other SV40 transformants (28). The efficiency of colony formation remained close to or higher than 1% throughout, suggesting the presence of a minority of long-lived or immortal cells at all times. At P41, a colony (designated HAL) was isolated at 35°C (after mutagenesis with ethyl methanesulfonate and selection for resistance to thioguanine). Therefore, early-passage stage I SVtsA/HF-A cells represent predominantly a preimmortal transformed cell line, whereas late-passage cultures (i.e., after P20, including HAL cells) are predominantly or exclusively immortal SVtsA/HF-A. An additional immortal subline, AR5, derived from SVtsA/HF-A by clonal isolated at early passage, was previously reported (37).

The functions of the viral large T antigen in the preimmortal and immortalized cell lines were compared to determine whether the immortal phenotype could be attributed to altered viral gene function. The copy number and integration pattern of SV40 sequences were determined for the SV40

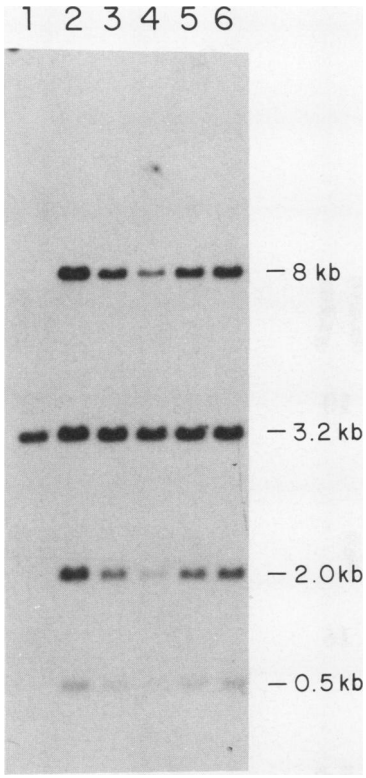

FIG. 1. Comparison of *Bst*XI-digested DNA from preimmortal and immortal SVtsA/HF-A cells. High-molecular-weight DNA prepared from untransformed HS74 cells (lane 1) and different passages of SVtsA/HF-A cells (lanes 2 to 6) was digested with *Bst*XI and analyzed by the Southern procedure. ^{32}P-labeled probes for SV40 and oligo(A) synthetase were radiolabeled separately and mixed together for simultaneous hybridization to the blot. Both probes were verified to be free of plasmid sequences. The cellular 3.2-kb band of oligo(A) synthetase provides an internal standard for quantitation. Lanes: 1, HS74; 2, immortal HAL cells; 3, immortal AR5 cells; 4, preimmortal SVtsA/HF-A cells, P7; 5, immortal SVtsA/HF-A cells, P21; 6, immortal SVtsA/HF-A cells, P44.

transformants after digestion of their respective DNAs with *Bst*XI (which cuts pSVtsA58 once within the SV40 sequence). Southern analysis revealed three bands (8, 2, 0.5 kb) with homology to SV40 DNA (Fig. 1). In addition, *Bst*XI generated a 3.2-kb band for the cellular oligo(A) synthetase gene, which was used as an internal standard for quantitation. Although the DNA patterns were similar, the immortal cell lines showed a higher level of hybridization to the SV40 probe than did the preimmortal SVtsA/HF-A. Densitometry measurements and normalization to the endogenous oligo(A) synthetase gene demonstrated that immortal SVtsA/HF-A (P21) and AR5 had approximately twofold the levels of both the 8- and 2-kb bands compared with those observed in SVtsA/HF-A (P7). Both SVtsA/HF-A cells at P44 and the subclone HAL had a still higher level of SV40 sequences. However, the level of viral large T and/or small t proteins as determined by immunoblot was minimally or not increased (data not shown). No rearrangement of the viral sequences was evident when DNA isolated from cell lines at different passages was digested with *Bst*XI (Fig. 1) or other enzymes (data not shown).

Large T protein function is temperature sensitive (*ts*) for both preimmortal and immortal cell lines. Both AR5 and HAL fail to form colonies at 39°C and cease to proliferate after one to two generations when growing cultures at 35°C are shifted to 39°C (37, 40). As shown in Table 1, uncloned SVtsA/HF-A cells are similarly temperature dependent for growth, although the preimmortal cells are able to proliferate at 39°C to a limited extent at the very earliest passage. Those cells show a reduced saturation density (0.6×10^6 to 1.9×10^6 cells) similar to that of normal HS74 and lower than that observed for transformed HS74 (28). As expected, HS74 cells transformed by a non-*ts* origin-defective genome show equivalent or enhanced growth at 39°C compared with 35°C (data not shown). T antigen binds the cellular protein p53 (22); preimmortal SVtsA/HF-A cells have temperature-dependent T-p53 complexes (data not shown), as previously reported for AR5 and HAL cells (40). Hence, large T protein is required for cell proliferation in both preimmortal and immortal cell lines, and the properties of SVtsA58 T antigen are unchanged upon immortalization.

Karyotype analysis. Since the immortalized phenotype cannot be explained simply by alteration in SV40 function, we sought changes in the cellular genome. Karyotypes of preimmortal and early immortal SVtsA/HF-A and of cloned immortal derivatives were analyzed. HS74 has a normal diploid karyotype, as previously reported (53). Early preimmortal cells show minimal rearrangements, with 65% of the cell population missing only one chromosome, chromosome 16. A typical karyotype of these cells is shown in Fig. 2. The uncloned immortalized SVtsA/HF-A had, in addition to a loss of chromosome 16, a predominantly tetraploid chromosomal number. The presence of a consistent anomaly substantiates that the immortal cell lines were derived from the parental line. This immortal cell line also showed deletion of the long arm of chromosome 6 with loss of the entire portion of 6q distal to 6q21. A representative karyotype of such an SVtsA/HF-A cell is given in Fig. 3. The other immortal cell lines (AR5 and HAL) lost one entire copy of chromosome 6. The deletion of chromosome 6 was a nonrandom event; all uncloned and cloned immortal SVtsA/HF-A had changes in chromosomes 6 and 16 only. These results are summarized in Table 2. Other chromosomal abnormalities were evident in immortal SVtsA/HF-A, AR5, and HAL. In addition, different passages of the respective sublines showed characteristic changes with persistence of prior rearrangements, suggesting a progression of karyotypic alterations. As an example, both immortal SVtsA/HF-A and its clonal derivative HAL had alterations in chromosomes 4 and 7 and a translocation involving chromosomes 10 and 12. On the other hand, the two sublines of AR5 lack these alterations but have common translocations involving (5;18) and (11;22).

Loss of copy number for loci on chromosome 6. Nonrandom loss of chromosome 6q was examined in further detail by Southern analysis. The copy number for three loci which span the distal region of 6q was measured to determine the extent of loss for chromosomal DNA observed in the karyotypes of the SV40-immortalized cells. DNAs from normal HS74 and the different SVtsA/HF-A cell lines were digested and probed for the proto-oncogene c-*myb* (6q22-23) (Fig. 4). The immortal sublines had one copy of c-*myb*, compared with two copies for normal HS74 and early preimmortal cells. Identical results were obtained when the same DNAs were digested and probed for *ros* (6q21-22) and *mas1* (6q24-27) (data not shown). Thus, the loss of the long arm of chromosome 6 observed in the immortal karyotypes represents a true loss of chromosomal DNA rather than an inapparent translocation. Rearrangement of the retained

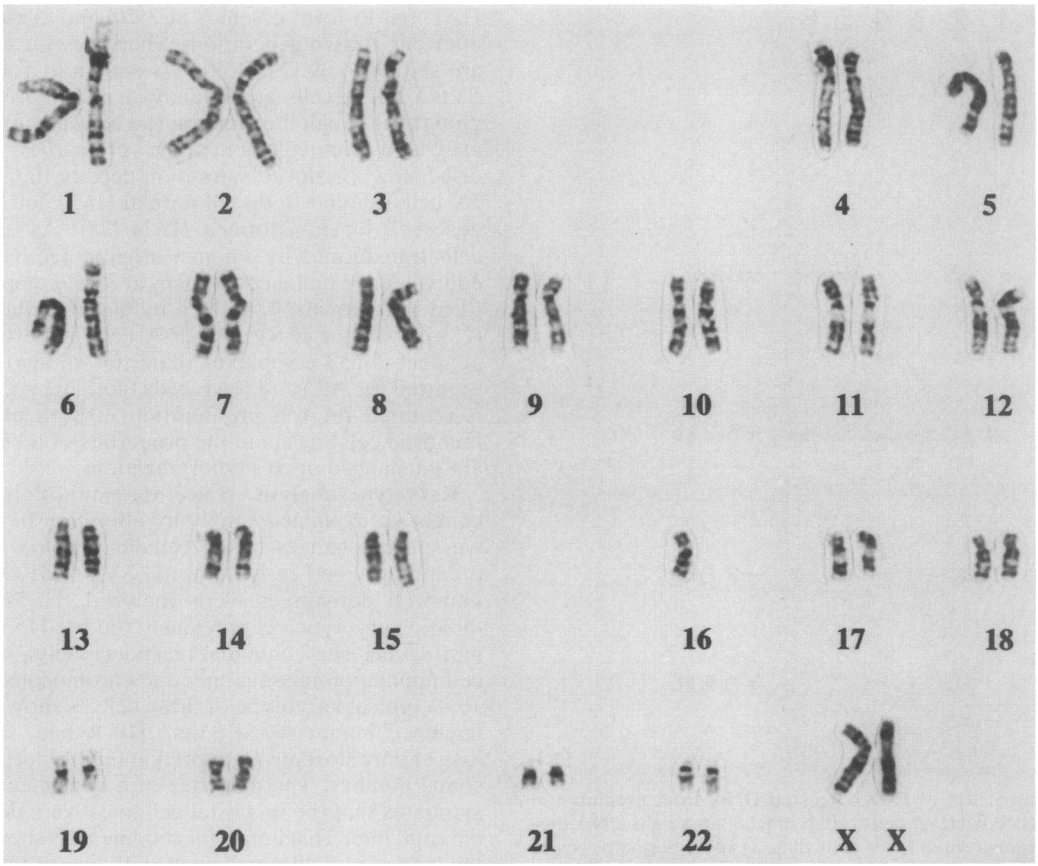

FIG. 2. G-banded karyotype of typical metaphase plates of early-passage SVtsA/HF-A cells at P5, showing clonal deletion of chromosome 16.

copy of these three loci was not detected by the enzyme used for Fig. 4 or when other enzymes were used (data not shown).

Loss of DNA was confirmed as loss of heterozygosity for the highly polymorphic microsatellite $(CA)_n$ repeat (Mfd47 at the D6S87 locus) (62) at 6q22 (61), as shown in Fig. 5. Whereas HS74 and preimmortal SVtsA/HF-A cells are heterozygous (lanes 2 and 3) with two discrete bands, the immortal cell lines have a single band in all cases. Interestingly, the two immortal cell lineages noted in the karyotypic analysis described above have retained different alleles of Mfd47, as shown in Fig. 5 for AR5 (lanes 5 to 7) and HAL (lane 8).

Expression of chromosome 6-specific loci. Consistent aberrant cytogenetic alterations involving deletions and/or translocations on a specific chromosome can direct initial studies to genetic regions which might be of potential importance. There are several genes for proto-oncogenes, growth factors, and growth factor receptors on chromosome 6. Consequently, we measured the mRNA levels of *ros* (47), c-*myb* (11), *mas* (66), and the gamma interferon receptor (20) by Northern analysis. Expression of mRNA levels for *ros* (4), c-*myb* (49), and *mas* (19) should not be detectable in HDF. Gamma interferon receptor mRNA (36) should be expressed in all cell lines. *ros* and *mas* were not expressed in normal cells or in SV40-transformed cell lines (data not shown). Figure 6A shows Northern analysis for the detection of the 2.3-kb transcript of the gamma interferon receptor encoded by a gene between 6q16 and 6q21, proximal to *ros*. There was no obvious difference between the mRNA levels of HS74 cells and the SV40 transformants. However, the data for c-*myb* were unexpected (Fig. 6B). Whereas c-*myb* was virtually undetectable in HS74 (lane 1), a band was evident at 3.5 kb in the preimmortal SVtsA/HF-A (lane 2). It was further elevated in the cloned immortalized cell lines AR5 (lane 3) and especially HAL (lane 4) to a level approaching that of the promyelocytic cell line HL60 (lane 6), in which high levels are constitutively expressed (49, 50). In Fig. 6C, the same filter was rehybridized with a probe for GAPDH, a gene which is expressed at a similar level in a wide variety of cell lines and not located on chromosome 6. All of the fibroblast cell lines show equivalent levels of this mRNA, verifying that the differences observed for c-*myb* are not due to methodological factors. Densitometry measurements indicate that the level of c-*myb* mRNA in HAL cells is approximately 60% that in HL60 cells when normalized to the level of GAPDH in the same lane. The molecular basis for the expression of c-*myb* is presently unknown.

DISCUSSION

We have exploited SV40 transformation of HS74 to analyze the basis for immortalization in human fibroblasts. One mechanism commonly involved in the growth characteristics of cancer cells is the loss of function of specific gene products. Paradigms for this type of mechanism are the retinoblastoma susceptibility gene and the p53 gene products (22, 54). SV40 T antigen, as well as viral proteins for other

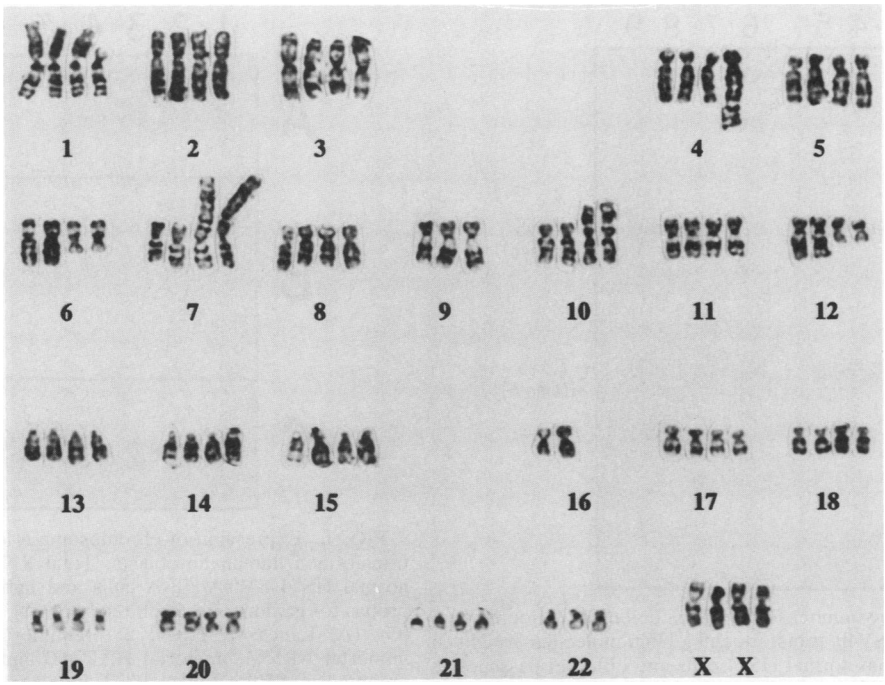

FIG. 3. G-banded karyotype of typical metaphase plates of immortal SVtsA/HF-A cells at P22.

DNA tumor viruses, has been shown to form complexes with both proteins in human and rodent transformed cells (34, 40). This interaction has been interpreted to result in the inactivation of their growth-inhibitory functions. Such complex formation might explain aspects of the transformed phenotype in cells expressing an SV40 genome, but they are insufficient to explain all aspects of the transformed phenotype in immortalized human fibroblasts. This conclusion is most simply based on the fact that SV40 transformants with a limited life span and those that are immortal do not show significant differences in viral gene function. We first observed this in a series of cell lines generated with an SV40 genome encoding a non-*ts* T antigen (28); the data reported in this paper further support this conclusion. The two- to threefold increase in copy number of SV40 sequences in the immortalized SVtsA/HF-A sublines is not associated with an increase in T-antigen levels or a change in its *ts* phenotype. Preliminary in situ hybridization analysis (32) indicates that the amplification is probably at the single site of integration. There were no free SV40 sequences, as expected for an origin-defective genome (data not shown).

The results are consistent with a model (37, 63) which proposes at least two classes of cellular functions as requirements for transformation and immortalization. One class can

TABLE 2. Karyotypic analyses

Cell line	No. of cells	Chromosome description														
		Chromosome no.[a]										Translocation[b]				
		1p+	1p−	4q+	−6	6q−	7p+	11d	−13	−16	18p+	−X	5;18	10;12	11;22	2,14,19	NC[c]
SVtsA/HF-A	150[d]																
P5	80									X							
	52																X
P22	50[d]																
	10									X							
	47[e]		X	X		X	X			X				X			
	3																X
HAL, P12	132[d,e]			X	X		X	X	X	X	X	X		X	X		X
AR5																	
P15	150[d,e]				X					X			X		X		X
P31	125[d]																
	75				X				X	X		X	X		X	X	X
	35				X				X	X		X	X		X		X

[a] Abbreviations: d, derivative; −, deletion, +; duplication.
[b] Translocations: t(5:18)(q22;q21), t(10;12)(q11;p11), t(11;22)(q13;p11), and t(2;14;19)(q21;q11;p13).
[c] NC, nonconsistent random abnormalities.
[d] Total number of cells examined. Discrepancies between this number and the subtotals reflect the presence of minor subpopulations.
[e] Mixture of 2N and 4N cells.

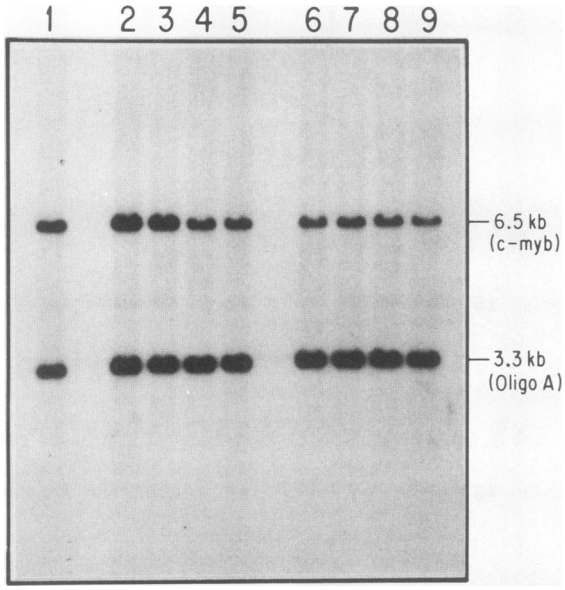

FIG. 4. Loss of copy number for c-*myb* as detected by Southern analysis in immortal SV40 transformants. High-molecular-weight DNA was prepared from normal HS74 cells and different passages of SVtsA/HF-A cells. DNA was digested with *Hin*dIII for analysis by the Southern procedure and hybridized simultaneously with ^{32}P-labeled probes for c-*myb* and oligo(A) synthetase which had been radiolabeled separately. The 3.3-kb band of oligo(A) synthetase provides an internal standard for quantitation. Lanes: 1, HS74; 2, preimmortal SVtsA/HF-A, P2; 3, preimmortal SVtsA/HF-A, P7; 4 and 5, immortal SVtsA/HF-A, P21; 6, immortal SVtsA/HF-A, P43; 7, immortal SVtsA/HF-A, P44; 8, immortal HAL; 9, immortal AR5.

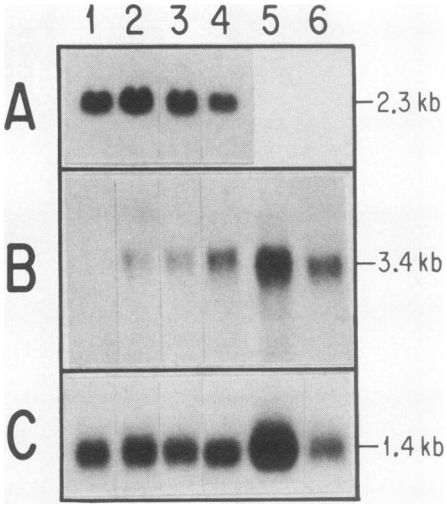

FIG. 6. Expression of chromosome 6 loci in normal and SV40-transformed human fibroblasts. Total RNAs were prepared from normal HS74 SVtsA/HF-A cells and hybridized with ^{32}P-labeled probes for gamma interferon receptor (A), c-*myb* (B), and GAPDH (C). (A) Lanes: 1, HS74; 2, preimmortal SVtsA/HF-A, P5; 3, immortal AR5; 4, immortal HAL. (B and C) Lanes: 1, HS74; 2, preimmortal SVtsA/HF-A, P5; 3, immortal AR5; 4, immortal HAL; 5, polyadenylated HAL RNA; 6, HL60. The RNAs from lanes 1 to 4 of panels B and C were from the same RNA preparations used in panel A.

be observed in typical SV40 transformants and is associated with an extension of the life span of human cells. It is dependent on continuous T function, as shown by the *ts* growth properties of preimmortal SVtsA/HF-A in this report and others (18, 29). A second class of function is required for indefinite cell growth, i.e., immortalization. Pereira-Smith and Smith used cell fusion to assign human immortal cells into four complementation groups (35), suggesting that there is a limited number of gene products which are key factors involved in senescence and immortalization processes. Our observations indicate that chromosome 6q contains such a growth suppressor or senescence-related gene.

The design of this study allows determination of differences between normal, preimmortal, and immortal cells having the same genetic background. The data on both SV40 and the karyotypes show that the immortal cell lines had the same preimmortal parent. Several chromosomal changes were noted in each of the two sets of immortal sublines, but none other than that involving chromosome 6 is unique to all of the immortal cell lines. Furthermore, since the chromosomal alterations in this set of SV40 transformants are few, the nonrandom changes found in the immortal cells with respect to chromosome 6 become even more significant. Analyses of cellular DNA sequences confirm the karyotype data that sequences distal to 6q21 are consistently deleted, resulting in a reduction in gene dosage. Interestingly, the allele of the minisatellite Mfd47 retained in AR5 clonally isolated from SVtsA/HF-A at early passage is different from that retained in the other immortal cell lines. Since immortalization is recessive to limited life span in cell hybrids, the simplest model is that small deletions or point mutations are present in the apparently intact chromosome 6, and immortalization results from a combination of mutation and loss of gene(s) on the rearranged chromosome. One of these events would have occurred early in the life span of the preimmortal SVtsA/HF-A. We would, however, have to predict independent events in the two cell lineages, since a different (retained) chromosome 6 would be expected to be mutated in AR5 versus the others. This model is somewhat inconsistent with the observation that immortalized derivatives are obtained at higher frequency in SVtsA/HF-A (i.e., the absence of overt crisis) than in other preimmortal SV40 transformants. Hence, alternate interpretations should be considered. For example, reduced dosage of a gene on 6q could be partially responsible. In view of the typical low frequency observed for immortalization, a combination of events would need to occur, for example, an additional change in a locus other than those on the long arm of chromosome 6.

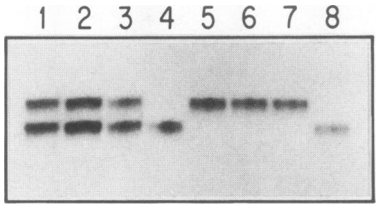

FIG. 5. Loss of heterozygosity of polymorphic dinucleotide repeat in the D6S87 locus on chromosome 6 in immortal SV40 transformants. ^{32}P-end-labeled GT-strand primers for D6S87 were used to amplify DNA in normal and SV40-transformed cell lines. Lanes: 1, HS74; 2, preimmortal SVtsA/HF-A, P6; 3, preimmortal SVtsA/HF-A, P8; 4, immortal SVtsA/HF-A, P23; 5, immortal AR5, P17; 6, immortal AR5, P32; 7, immortal AR5, P67; 8, immortal HAL.

Indeed, other changes in the cellular genome are evident. All SVtsA/HF-A cell lines have a reduced number of chromosome 16. Another notable change that occurred frequently and in all of the cloned sublines but was not evident in uncloned SVtsA/HF-A was a translocation between chromosomes 11 and 22, t(11;22) (q13:p11). Chromosomal region 11q13 is the region for Bcl-1, which has recently been shown to be a member of a new class of cyclins (24, 26, 64). The preponderance of tetraploid cells in some cases (e.g., Fig. 3) should not be considered to be a necessary precondition for immortalization. All karyotypes were performed on cell lines which had been maintained in frozen storage and thawed shortly before the karyotypes were determined. Preliminary data obtained at the time of isolation showed a lower proportion of tetraploid cells in immortal SVtsA/HF-A and AR5 (41). In a prior study involving a different series of SV40-transformed HS74, there were multiple karyotypic changes. Alterations in chromosome 6q as well as other nonrandom changes were observed (28). These changes (1p+ and 11p+) were not observed in our study, however. While this report was in preparation, we learned that Ray and Kraemer (38), using solely karyotypic analysis, have also implicated alteration of sequences at 6q21 in immortalization of SV40-transformed human fibroblasts. Taken together, studies involving two independent series of transformants in one cell system (HS74) and additional transformants in unrelated human fibroblasts serve to argue strongly for the functional significance of this consistent alteration, in any case.

Studies using a variety of tumor cell lines provide increasing evidence for one or more putative tumor suppressor genes localized on chromosome 6. Loss of heterozygosity and/or nonrandom chromosomal alterations have been noted for the long arm of chromosome 6 in neuroectodermal tumors (56), acute lymphoblastic leukemia (15), ovarian carcinomas (9, 21), non-Hodgkin's lymphoma (46), breast carcinoma (8), and malignant melanoma (59). Introduction of a normal human chromosome 6 by microcell-mediated chromosome transfer into malignant melanoma (58) and uterine endometrial carcinoma cells (65) resulted in suppression of the tumorigenicity of these cell lines. Allelic loss of chromosome 6 at high population doublings in fibroblasts of patients with Li-Fraumeni cancer syndrome, which involves a mutation in the gene for p53, also suggests that chromosome 6 may be important in view of the high frequency of spontaneous immortalization observed for these cells (5).

The strikingly increased levels of mRNA for c-*myb* in the SV40-transformed cells indicate that its genomic site may be a region of interest for further study. Whether c-*myb* is directly involved in immortalization is unknown. However, c-*myb* has been shown to regulate cell growth and differentiation in cells of hematopoietic lineage (23, 49). Introduction of a functional cDNA for c-*myb* has been recently shown to induce the expression of insulinlike growth factor 1 in 3T3 mouse fibroblasts (57), which allows cells to transverse the G_1/S boundary. This finding is particularly important because older human fibroblasts grown in culture have been reported to have an increased need for insulinlike growth factor 1 supplementation to overcome slow growth (7). The presence of c-*myb* mRNA in these SVtsA/HF-A cell lines is an unusual finding in any case. It has been shown that there is a defect in transcription of the c-*myb* locus near the 5′ end of the gene (in intron 1) due to deficiency of a sequence-specific DNA-binding protein in fibroblasts (39). This block must be bypassed in our transformants since stable, polyadenylated, putative full-length mRNA is detected by our probe directed against the 3′ end of the gene (Fig. 6B, lane 5). One might also consider the possibility that the sequences are expressed from the recently described partially homologous A-*myb* or B-*myb* gene (31), although we consider this unlikely because of the size of the mRNA (like that in HL60) and the intensity of hybridization.

The identification of senescence-related genes or genes leading to immortalization in SV40 cells may be facilitated by further study of specific regions localized on chromosome 6. There is evidence that human chromosome 4 has a senescence gene important for complementation group B (HeLa, J82, and T98G) (30) and that human chromosome 1 induces senescence in immortal Syrian hamster cells (55). These studies were done by using transfer of individual human chromosomes by microcell fusion. We are currently using this approach to determine whether suppression of growth occurs upon introduction of a wild-type copy of chromosome 6 in SV40-transformed immortal cells (45).

In conclusion, we have extended our earlier findings with a second series of genetically matched SV40-transformed preimmortal and immortal cell lines, demonstrating that a change in the cellular genome is required for immortalization. Karyotypic and DNA analyses indicate that loss of sequences in the distal portion of the long arm of chromosome 6 is involved. Altered expression of a cellular oncogene, c-*myb*, in the affected region has also been observed.

ACKNOWLEDGMENTS

This work was supported by grants awarded to H.L.O. and to A.S.H. Research conducted by H.L.O. was supported by the National Institute on Aging (NIA) of the National Institutes of Health (AG 04821). Research conducted by A.S.H. was supported by the National Institutes of Health [RO1-ES(CA)-05735], Office of Naval Research (N00014-90-J-1266), and Department of Energy (DE-FG01-89-CE34023) and by a PSC-CUNY award. K.H.-S. is a recipient of a Minority Investigators Award from the NIA. A portion of the research was performed under the auspices of the RCMI Center for Gene Structure and Function at Hunter College (Institute for Bimolecular Structure and Function).

We thank Michael Small for helpful discussions and review of the manuscript, Diane Muhammadi for expert typing, James Weber for sharing unpublished data, and Y. Gluzman, S. Petska, M. Revel, S. R. Tronick, M. Wigler, and R. Wu for providing recombinant DNAs.

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