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Analysis of molecular changes upon immortalization of SV40-transformed human fibroblasts

Pardinas, Jose Ramon, Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

ANALYSIS OF MOLECULAR CHANGES UPON IMMORTALIZATION OF SV40-TRANSFORMED HUMAN FIBROBLASTS

BY

JOSE RAMON PARDINAS

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May 23, 1994
Date

Executive Officer

Michael B. Small

Mark L. Steinberg

Ann Henderson

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

ANALYSIS OF MOLECULAR CHANGES UPON IMMORTALIZATION OF SV40-TRANSFORMED HUMAN FIBROBLASTS

by

Jose Ramon Pardinas

Adviser: Dr. Harvey Leon Ozer

Normal human diploid fibroblasts (HDF) undergo a pattern of proliferative decline in tissue culture which, by analogy to aging in the organism, has been termed cellular senescence. HDF transformed by the DNA virus SV40 have an extended lifespan, but inevitably succumb to a pattern of growth cessation designated "crisis". As a rare event, SV40-transformed fibroblast (SV/HF) populations may give rise to cell variants which are immortal. I have examined the organization and expression of viral and cellular sequences in preimmortal and immortal SV/HF in an effort to understand the molecular basis for immortalization. While rearrangements were not detected in the SV40 integrant, the immortal cells show a consistent 2-3 fold higher level of hybridization to the SV40 probe than the preimmortal progenitor. Examination of cellular DNA organization revealed several interesting changes. First, nonrandom alterations in the long arm of chromosome 6 (6q) were noted in association with the emergence of immortal cells. I have demonstrated a 2-fold reduction in the copy number of DNA sequences distal to 6q21 in the immortalized transformants consistent with the presence at this locus

of a growth suppressor whose complete loss (or dosage reduction) is critical for immortalization. Secondly, since loss of telomeric DNA has been implicated in the proliferative decline of cellular senescence, telomere lengths were determined at various times during the lifespan of normal HDF, as well as of multiple matched sets of preimmortal and immortal SV/HF. In our cell lines telomeres also display progressive shortening with cumulative population doublings of normal HDF and, futhermore, are found to continue to decrease during the extended lifespan of preimmortal SV/HF. However, telomere lengths increase and stabilize as SV/HF become immortal. These results argue against a causal role for telomere shortening in cellular senescence, and imply that immortalization induces or is partly dependent on activation of telomere maintenance mechanisms.

In order to identify mRNA species that are differentially expressed in the preimmortal and immortal SV/HF, I prepared a preimmortal cDNA library in λgt10 and analyzed it by subtractive hybridization. A number of cDNAs have been isolated representing mRNAs which are overexpressed in either the preimmortal or the immortal cells. Partial sequencing indicates that most of these (11 out of 14) correspond to novel genes.

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LIST OF ABBREVIATIONS

AMV	avian myeloblastosis virus
ARF2	ADP-Ribosylation factor 2
ATP	adenosine triphosphate
5-Aza CdR	5-Azacytidine
bp	base pair
cAMP	adenosine 3',5'-cyclic monophosphate
Cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanine triphosphate
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DTT	dithiothreitol
dTT	deoxythymidine triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EF	elongation factor
EGF	epidermal growth factor

EPC-1	early population doubling level cDNA-1
EtBr	ethidium bromide
μg	microgram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCl	hydrochloric acid
HDC	human diploid cell
HDF	human diploid fibroblast
HF	human fibroblast
Hfl	high frequency of lysogeny
HMW	high molecular weight
HPRT	hypoxanthine phosphoribosyltransferase
HSP	heat shock protein
IGF-1	insulin-like growth factor-1
IL-1	interleukin-1
Inh/Act β_A	β_A subunit of inhibin/activin
kb or kbp	kilobase or kilobase pair
KCl	potassium chloride
KOAc	potassium acetate
KH_2PO_4	potassium phosphate (monobasic)
LiCl	lithium chloride
M1	mortality stage 1

M2	mortality stage 2
5MC	5-methylcytosine
MEF	mouse embryonal fibroblast
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
ml	milliliter
MMLV	Moloney murine leukemia virus
Mn-SOD	mangano form of superoxide dismutase
MTL	mean telomere length
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
Na_2HPO_4/NaH_2PO_4	sodium phosphate (mono- and dibasic)
NaOH	sodium hydroxide
$(NH_4)_2SO_4$	ammonium sulfate
^{32}P	phosphorus-32
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PD	population doubling
PDGF	platelet-derived growth factor
PG	prostaglandin
PKC	protein kinase c

poly(A)$^+$ mRNA	polyadenylated messenger ribonucleic acid
poly(A)$^-$ mRNA	non-polyadenylated messenger ribonucleic acid
RB	retinoblastoma
RNA	ribonucleic acid
RNase I	ribonuclease I
RT	reverse transcriptase
S14	human ribosomal protein 14
SAG	senescene-associated gene
Sdi	senescent cell-derived inhibitor of DNA synthesis
SDS	sodium dodecyl sulfate
SM	bacteriophage λ storage medium
SV40	simian virus 40
TCA	trichloroacetic acid
TIMP-1	tissue inhibitor of metalloproteinase-1
TK	thymidine kinase
TRF	terminal restriction fragment
UV	ultraviolet
WS	Werner syndrome

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CHAPTER 1

INTRODUCTION

The thesis which follows represents an attempt at characterization and assessment of molecular changes that accompany the immortalization of simian virus 40 (SV40) transformed human diploid fibroblasts in tissue culture. A logical presentation of this topic requires, however, that it be considered within the larger context of human cellular senescence whose review will, therefore, be taken up first in this introduction.

As early as 1881, the biologist August Weismann postulated that the somatic cells of higher animals would be found to have limited division potential (1). However, early 20th century tissue culture investigations by Alexis Carrel and A. H. Ebeling (2,3) seemed to demonstrate the exact opposite: that cells removed from their natural milieu in the parental organism were capable of indefinite proliferation. These studies strongly suggested that senescence, the collection of deteriorative changes associated with aging, was a property of the whole animal, and not intrinsic to its cellular components. The belief became entrenched that all cells in tissue culture were inherently immortal, and that inability to propagate cells indefinitely was a result of inadequate culture conditions. Swim and Parker (4) were the first investigators to challenge this pervasive dogma after noting a finite proliferative potential in some 51 cultured human fibroblast cell strains derived from

various tissues. Hayflick and Moorhead (5) greatly extended and quantitated similar observations, inaugurating the modern study of cellular senescence or cytogerontology. These workers documented a characteristic pattern of growth for normal human diploid fibroblasts (HDF) in tissue culture which they separated into distinct phases. After outgrowth of cells from a tissue explant (phase I), there is a period of vigorous proliferation upon serial passage (phase II), followed by a time during which proliferation slows down, cells become increasingly refractory to mitogen stimulation and begin to show an accumulation of deteriorative changes (phase III), and finally an abrupt stage of proliferative arrest and decline in cell density (phase IV) from which emerges a population of long-lived postmitotic cells (6). Hayflick and Moorhead catalogued a number of seemingly inherent properties displayed by normal human diploid cells in tissue culture. Prominent among these were that cells derived from human embryos could undergo a maximum of about 50 population doublings (PD), and that even when cryogenically preserved for long periods of time and then reconstituted, these cells would "remember" the PD level at which they were preserved and undergo a number of doublings equivalent to 50 minus the number sustained prior to preservation. Furthermore, the proliferative potential of such cells in tissue culture seemed to be inversely proportional to the cell donor's age (5). These observations strongly suggested that the finite replicative capacity of cultured human fibroblasts resulted from the operation of a clock-like cellular program, and that it might be an expression of aging at the cellular level.

Attempts by other workers to reproduce Carrel's findings that normal cells in tissue culture were essentially immortal have been consistently fruitless. As a possible explanation for Carrel's results, it has been suggested that at each refeeding of his chick fibroblast cultures with an extract prepared from chick embryos, he had unwittingly introduced an inoculum of young viable cells (7). In contrast, Hayflick and Moorhead's basic observations have received widespread confirmation. It is now almost certain that most if not all cultures of human diploid cell types have a finite replicative potential (8). Furthermore, a growing number of studies have documented that a large number of cell types from diverse vertebrate species also display a finite life span in tissue culture; for example, rat lens epithelial cells (9), rat liver epithelial cells (10), mouse bone marrow cells (11), amphibian melanophores (12), chick myogenic precursor cells (13), rabbit and dog articular cartilage cells (14), rat small intestinal epithelium and squamous esophageal epithelium (12), among others.

A number of investigations following Hayflick's report have buttressed the concept that the loss of replicative potential of normal diploid cells, the so called phase III phenomenon, is a manifestation of aging at the cellular level. The initial observation by Hayflick and Moorhead that cultured human fibroblasts derived from older donors exhibited a diminished replicative potential when compared to those derived from embryos was considerably extended by Martin et al. (15). These workers isolated dermal fibroblasts from donors from fetal to 90 years of age, and found a significant inverse relationship when population doubling potential

was plotted versus the corresponding age of the donor. This inverse relationship has now been shown to hold true in a host of normal cell types originating from a large variety of tissues (16 and references therein).

Additional support for the validity of the cultured fibroblast as a model system for the study of aging has been provided by the positive correlation which has been found to obtain between the replicative potential of fibroblasts in tissue culture and the maximum life span of the donor species (17,18,19).

The initial ascription of limited proliferative potential to inadequacy of the tissue culture medium was dealt a serious blow by two lines of investigation. One was Hayflick and Moorhead's mixed culture experiment, which demonstrated that early passage (i.e. "young") cells could proliferate in the same medium in which late passage (i.e. "old") cells were deteriorating (5). Additional evidence in favor of the presence of innate, possibly genetic, cellular mechanisms responsible for the limited life span of normal cells has come from serial transplantation studies. When normal somatic tissues are transplanted to young (inbred) hosts, the transplanted cells undergo senescence even though they are in the supposedly ideal environment of a young animal's body (20,21,22,23). These studies showed that the continued survival of transplanted tissues is far more dependent on tissue donor age than on that of the recepient.

A fundamental question of importance to the premise that the limited life span of cultured diploid fibroblasts is an inherent, possibly genetic, property of

these cells is whether their decline in proliferative potential is a function of chronological time spent in culture or of the number of population doublings that have accrued. Despite some early evidence in support of the former (24), current consensus favors a decisive role for division events in the determination of in vitro life span. For example, continuously passaged clones and those maintained in stationary phase (by low serum or high density) and then subcultured attain approximately the same total number of population doublings (25). Additionally, in circular outgrowths of human fibroblasts, it has been found that cells at the circumference, which have undergone more replications, have diminished proliferative potential when compared to cells from more internal locations, which have undergone fewer doublings (26).

A number of somatic cell fusion studies have suggested the presence of a genetic program as the underlying cause of cellular senescence. These investigations have shown that the nonproliferative phenotype of in vitro senescent cells is dominant to the proliferative phenotype of younger cells as well as to that of immortal cells. These results have been interpreted to mean that senescent cells produce an inhibitor of DNA synthesis. Moreover, when senescent cells are enucleated and the resulting cytoplasts fused with young cells, DNA synthesis is inhibited with equal effectiveness. However, pre-treatment of the senescent cytoplasts with cycloheximide (to eliminate protein synthesis) is found to abolish this inhibition, and suggests the presence in old cells of a protein suppressor of DNA synthesis (27). Additional support for the existence of a DNA

synthesis inhibitor(s) comes from investigations using subcellular as well as molecular components of senescent cells. Inhibition of DNA synthesis has been brought about in young fibroblasts following their exposure to membranes prepared from senescent cells under neutral or acidic conditions. The same membranes, when prepared under basic conditions, result in stimulation rather than inhibition of DNA synthesis. The inhibitory activity in these membrane preparations is, once again, believed to be a protein on the basis of its lability to treatment with trypsin and other protein modifying agents (28,29). In a logical extension of this experimental approach, which at the same time also tested the possibility of cDNA cloning of the DNA synthesis inhibitor, Lumpkin and co-workers microinjected poly(A^+) mRNA isolated from senescent fibroblasts into young cells and found that it blocked DNA synthesis (30). The ramifications of this line of investigation will be discussed more extensively later. Overall, these experiments argue strongly in favor of the proposition that senescent cells express a gene product capable of blocking initiation of DNA synthesis in proliferation-competent young cells.

Another important question pertinent to the senescent phenotype is whether proliferative arrest in these cells occurs at a specific point in the cell cycle, and whether senescent arrest bears any relationship to the early G_1 block (termed G_0) induced in replication competent young and old cells by conditions such as low serum, high cell density, or deficiency in an essential nutrient. The results from several lines of investigation support a block to cell cycle transit in late G_1 or at the G_1/S boundary in senescent cell populations. The nuclear fluorescence intensity

of quinacrine dihydrochloride stained senescent cells resembles that of young cells after exposure to hydroxyurea, which causes arrest in late G_1, and is clearly different from the staining characteristics of serum starved (G_0 arrested) young cultures (31). Additional support for a G_1/S block comes from the equivalent induction, following serum stimulation of young and senescent fibroblasts, of various cell cycle-dependent events which are known to occur late in G_1. Notable among these is thymidine 5'-triphosphate (TTP) synthesis, which is stimulated to similar extent in both young and old cells (32). The distinct, perhaps unique, character of senescent arrest has been underscored by the isolation of a number of differentially expressed genes upon screening of G_0 subtracted cDNA libraries derived from young and old WI-38 human fibroblasts (33). One of these genes designated EPC-1 (early population doubling level cDNA-1) has a greater than 100-fold higher expression in serum-starved confluent young cultures than in similarly treated senescent cells (34). None of these observations, however, preclude a possible functional overlap between the mechanisms which are responsible for quiescent and senescent growth arrest.

The proposition that the in vitro decline in proliferative potential, the so-called phase III phenomenon, is a reflection of aging at the cellular level is still controversial. The studies and observations cited above, as well as others to be outlined below, provide support but do not prove the validity of this hypothesis. Some investigators, notably Hayflick himself, no longer see a reason, in light of the mass of accumulated evidence, to be tentative about characterizing the in vitro

behavior of fibroblasts as aging rather than as just a model for aging at the cullular level (16). Others, while acknowledging that many changes which occur during in vivo aging are also expressed in tissue culture, argue that cell culture systems may provide a valid model only for the regulation of cellular proliferation, which in its turn displays changes during aging. Even then, it has been pointed out, the in vitro fibroblast model can be expected to contribute significantly to an understanding of aging and aging-associated diseases. The latter is especially true in light of the fact that cancer and atherosclerosis, which arise from failures in the regulation of cellular proliferation, are the two most serious age-related diseases (35).

In the three decades since the publication of Hayflick and Moorhead's seminal paper (5), there has been an explosive surge of investigations and reports touching upon various aspects of the phase III phenomenon. Specifically, a rich storehouse of information has been generated documenting phenotypic and molecular changes which appear during the course of in vitro cellular senescence (for reviews see 24,36,37). It is important to note, however, that a causal role in cellular senescence has not been demonstrated for any of these alterations, and that many can be expected to turn out to be "downstream" effects of more fundamental molecular changes. Nevertheless, the identification of such differences could provide clues as to the nature of the higher-order events controlling senescence or, at the very least, help to define a distinct phenotype for senescent cells, which in turn would facilitate the design and assessment of experiments in this field. While a full consideration of this vast literature is impossible within the

bounds of this introduction, a concise overview of some of the major findings is in order.

The progressive loss of replicative potential characteristic of normal cells during phase III growth in tissue culture is accompanied by the generation of great heterogeneity in cell sizes as well as by a population shift to larger sizes with retention of the 2C or diploid DNA content (38,39). Perhaps significantly, it has been reported that larger cells have diminished replicative capacities regardless of their population doubling levels (40,41). The shift to larger cell sizes is also attended by characteristic morphological and ultrastructural alterations (42) such as an increase in the number of multinucleated cells (43), the presence of large lysosomal bodies (44), an increase in nuclear (45) and nucleolar size (46). The long-lived postmitotic population which emerges following the proliferative decline and cell death of phase III cultures can sustain only extremely low saturation cell densities, a property which has been ascribed to a hightened sensitivity to intercellular contact (24,36,37).

An array of proteins associated with the extracellular matrix (ECM) have been found to be overexpressed in cultured senescent human fibroblasts, in fibroblasts from aged donors, and in fibroblasts from donors afflicted with the accelerated aging condition known as Werner syndrome (47,48,49,50,51).

Werner syndrome (WS) is a rare inherited autosomal recessive human disorder which is characterized by growth impairment and premature aging. Individuals afflicted with WS display the early onset of a number of age-related

pathological conditions which include cataracts, graying and loss of hair, osteoporosis, insulin-resistant diabetes, atherosclerosis, neoplasia, and early death (52). Werner syndrome fibroblasts have an in vitro replicative lifespan which is markedly reduced when compared to that of age-matched normal controls (50,52). The premature replicative decline of WS fibroblasts is dominant in cell fusion studies to the proliferative phenotype of young human diploid fibroblasts (52).

Reasoning that WS might result from overexpression of senescent-specific genes, and that isolation of such genes would be relatively easier from cells exhibiting the exaggerated senescent characteristics typical of WS, a group of investigators headed by S. Goldstein prepared a cDNA library from WS fibroblasts. Screening of this library with total cDNA probes prepared from either normal or WS HDF, led to the identification of 18 distinct clones which are differentially overexpressed in the WS fibroblasts (50). These 18 clones consisted of nine known and nine (previously) unknown genes. No less than seven of the known genes code for proteins that are either intrinsic or closely associated with the extracellular matrix (ECM). Of the nine unknown genes, five contain Alu and KpnI (LINE-1) repetitive sequences. Additional validation of the in vitro fibroblast as a model system for the study of in vivo cellular senescence has come from the finding that five of the seven ECM-associated genes overexpressed in WS fibroblasts are also overexpressed in normal senescent cultured fibroblasts.

Notable among the ECM-associated proteins overexpressed in both cultured

senescent and WS fibroblasts are alpha 1 (I) procollagenase, fibronectin, collagenase, and stromelysin. Fibronectin, a prominent component of the ECM, is involved in a variety of functions which include cell spreading, substrate attachment, and in the interaction between the cell surface and other ECM components. Fibronectin is not only overexpressed in late passage fibroblasts (53), but has been reported to be qualitatively different in terms of its distribution (54), its ability to promote adhesion of both young and old cells (55), as well as in its capacity to bind to type I and II collagen (56). However, doubt has been cast on the importance of ECM involvement in the loss of proliferative capacity of senescent human fibroblasts by the observation that young fibroblasts placed in ECM derived from senescent cells experience no morphological changes or decline in replicative vigor (24).

A number of secreted proteins have also been found to be differentially expressed in early and late passage human fibroblasts, including insulin-like growth factor I (IGF-I) (57), IGF-I binding protein-3 (58), tissue inhibitor of metalloproteinase-1 (TIMP-1) (59), plasminogen activator inhibitor types 1 and 2 (60,61), and early population doubling level cDNA-1 (EPC-1) (34). Overexpression of plasminogen activator inhibitor type 2 has been reported to result from the constituitive expression in senescent cells of its transcriptional inducer interleukin 1 (IL-1) (60). Deregulated IL-1 expression in senescent human diploid fibroblasts has also been reported to lead to the overexpression of the mangano form of superoxide dismutase (Mn-SOD) (60). Overexpression of the latter is perhaps part

of a heightened response by senescent cells to the multitude of environmental stresses to which they are otherwise more susceptible (24).

Early passage human diploid fibroblasts respond to serum or growth factors by initiating DNA synthesis and undergoing mitosis. It is now well established, however, that cells senescing in vitro or those from older donors exhibit an extremely attenuated response to these same factors (62,63,64). Most studies indicate no concomitant decrease in receptor density or affinity with loss of mitogen responsiveness (65,66,67). However, the tyrosine-specific autophosphorylation of the EGF receptor from detergent solubilized and immunoprecipitated WI-38 senescent cells is lost both in the presence and absence of EGF stimulation (68). Since EGF receptor kinase function shows no alteration in intact plasma membrane preparations (69,70), it suggests the presence of a labile or modified form of this receptor in senescent cells.

Growth factor receptors mediate the action of extracellular mitogenic signals through their generation of intracellular second messenger cascades. It is, therefore, possible that the mitigated response of senescent cells to growth factor stimulation proceeds from alterations in the postreceptor components of signal transduction pathways. A number of findings are in consonance with this idea. Elevated levels of arachidonic acid and a subsequent increase in prostaglandin (PG) production have been reported in senescent cells (71,72). This could be significant given the possible role for PGs in negative growth regulation (71). Specifically, senescent cells may produce much more PG_{E2} than young cells,

and may achieve intracellular concentrations which are found to be effective in inhibiting growth of early passage cells when this compound is added exogenously. Phospholipid turnover by phospholipase $C_{\gamma}1$ in senescent IMR-90 cells has been reported to be impaired (73). Additionally, PKC translocation from the cytosol to the inner face of the plasma membrane has been reported to be defective in senescent WI-38 cells (74).

Interestingly, serum induction of c-fos transcription, which has been described as an early PKC dependent event in the G_0/S transition (75), has been found by a group of investigators to be under transcriptional repression in senescent WI-38 fibroblasts. These workers also found a lack of c-fos transcriptional induction by phorbol esters, EGF, or cyclic adenosine monophosphate (cAMP). The supression of c-fos transcription was reported to be quite specific given that serum-induced transcription of actin, a gene that is coordinately induced with c-fos in many proliferating fibroblasts, showed minimal reduction in these senescent cells (76). Members of the c-fos family complex with c-jun family members during assembly of the AP-1 family of transacting transcription factors. A deficit in fos protein has been reported to lead to a preponderance of jun-jun homodimers in senescent Hs68 human fibroblasts (77). Since the AP-1 transcription complex regulates the expression of a number of genes in response to a variety of growth signals, it is plausible that modification or reduction (77) of AP-1 activity as a consequence of c-fos repression contributes to the proliferative decline of senescent cells. Introduction of a chimeric

inducible c-fos construct under the control of a metallothionein promoter has been reported to transiently restore DNA replicative potential in senescent WI-38 fibroblasts (78). Other investigators, however, have found similar serum and phorbol ester induction of c-fos transcription in early and late passage IMR 90 human fibroblasts (79). Similarly, platelet derived growth factor (PDGF) has been reported to induce c-fos transcription in senescent AG 1523 human neonatal foreskin fibroblasts (80). Induction of c-fos in senescent IMR 90 cells by microinjection of oncogenic c-H-ras was insufficient for initiation of DNA synthesis even when an AP-1 regulated reporter gene was simultaneously shown to be efficiently expressed (81). These contradictions probably arise from cell type-specific differences, or are due to differences in experimental conditions. In any case, they underscore the complexities of the phenomenon of cellular senescence.

Molecular events downstream or beyond the G_1 locus of c-fos transcription also seem to play an important role in the block to initiation of DNA synthesis in senescent cells, and suggest the existence of multiple (convergent or parallel) pathways leading to senescent growth arrest.

Cyclins are a group of proteins which promote cell cycle transit by complexing, and thereby activating, a group of kinases known as cyclin-dependent kinases (Cdks). These kinases, in turn, phosphorylate a number of substrates, among them the retinoblastoma gene product Rb, which is one of the most important regulators of the G_1/S transition. Underphosphorylated Rb associates with, and blocks the activity of, members of the E2F family of transcription factors

(82,83). Phosphorylation of Rb by Cdks dissociates the E2F-Rb complex, freeing E2F to induce the expression of a variety of genes involved in S phase of the cell cycle (83,84,85).

Senescent cells have been reported to fail to express, or to have reduced levels of cyclins A, B, and two of the D-type cyclins, as well as of the cdc2-related p34 Cdk2 protein kinase (86,87). Reduced levels of these positive regulators of cell cycle progression may be partly responsible for the failure of serum-stimulated senescent cells to phosphorylate Rb, and to thereby inhibit its function in negative growth regulation (88). This failure may be directly responsible for the inability of senescent cells to enter S phase.

Recently, the most intensely studied of all known tumor suppressor genes, p53, has been implicated in the regulation of cell cycle progression via its indirect control of cyclin-dependent protein kinase activity. Most satisfyingly, the emergence of this highly integrative picture seems to be fully relevant to the phenomenon of G_1/S arrest in senescent cells.

The p53 tumor suppressor gene is the most frequently affected gene detected in human cancers. DNA damaging agents (radiation or radiomimetic drugs) cause p53 to accumulate and to bring about cell cycle arrest in G_1 (89). This p53-mediated growth arrest allows the cell time to carry out DNA repair before undergoing replication. Inactivation of p53 allows cells with damaged DNA to proceed through G_1 and into S phase without carrying out repair, which then leads to the accumulation of mutations and chromosomal rearrangements, and

likely to the selection of malignant cell variants. Over the last few years p53 has been found to function not only in the G_1 checkpoint for DNA damage, but also as a potent transcription factor in its own right (90).

In an attempt to identify genes whose expression leads to the inhibition of DNA synthesis which occurs in normal human cells near the end of their replicative lifespan, a group of investigators headed by J.R. Smith employed an assay involving the microinjection or transfection into young cells of full length cDNAs derived from senescent poly(A^+) mRNA and cloned in a mammalian expression vector. Three cDNA clones were isolated on the basis of their ability to block DNA synthesis in young fibroblast recipients (91). Two of these, sdi-2 and sdi-3 (for senescent cell derived inhibition of DNA synthesis), represent mRNAs whose abundance is roughly equivalent in senescent and young cells. The other, sdi-1, originates from a transcript whose level is 10-20 fold higher in senescent than in young cells (91). However, the nature of the molecular mechanism whereby sdi-1 effects its inhibition of DNA synthesis remained a mystery until very recently. Two groups of investigators have reported the simultaneous isolation of a gene which represents nothing less than the much-sought-after missing link between transcriptional activation by p53 and its role in cell cycle control (92,93). The gene, named waf1 by one group and cip1 by the other, has been found to be transcriptionally induced by wild type p53, and to suppress growth when introduced into cells carrying a mutant p53. Growth arrest by waf1/cip1 seems to be mediated by its function as a powerful inhibitor of G_1 cyclin-dependent kinases.

Strikingly, waf1/cip1 was found to be the very same gene that J.R. Smith and colleagues had isolated and named sdi-1 (91). These developments, mark the emergence of a plausible and comprehensive model for G_1 cell cycle arrest in DNA damaged and senescent cells. An outline of this model begins with the known accumulation of p53 which takes place in response to DNA damage. Accumulation of p53 or a yet-to-be identified senescence-specific signal is expected to induce transcriptional activation of waf1/cip1/sdi1. Expression of the latter would in turn result in complex formation and inactivation of cyclin-dependent protein kinases. Inactivation of Cdks would prevent the phosphorylation and consequent inhibition of pRb and its growth suppressive function. Underphosphorylated pRb would then block cell cycle progression at G_1. It is quite intriguing that neutralization of growth suppression by pRb and p53 is part of a general strategy used by DNA tumor viruses (see below) to induce infected cells to express their growth regulated genes and thus provide part of the molecular machinery that these viruses require to replicate their genomes.

Since the defining characteristic of senescent cells is their inability to replicate their DNA, a logical question is whether enzymes and replication-related processes are affected in these cells. Senescent fibroblasts are unable to express the DNA polymerase δ cofactor, proliferating cell nuclear antigen (PCNA), due to an apparent post-transcriptional block (94). Alterations in DNA polymerase α

itself have also been implicated in senescence. It has been reported that senescent fibroblasts express a form of DNA polymerase α with reduced DNA binding affinity (95). Moreover, senescent human fibroblasts fail to complement a rodent DNA polymerase α mutant (96). In short-term somatic cell fusion experiments between senescent and either young or immortal cells, DNA synthesis in the replication-competent nucleus is inhibited unless that nucleus was derived from a young or immortal cell having elevated levels of DNA polymerase α (97,98,99,100). This is very intriguing given the direct relationship that has been observed between the rate of initiation of DNA synthesis and the concentration of DNA polymerase α in cell fusion experiments (98). These studies suggest that the increase in cell volume observed in senescent fibroblasts may play an important role in their loss of proliferative potential due to the resulting dilution of some rate-limiting component of the replication apparatus.

Aging fibroblasts have been reported to exhibit a progressive reduction in their ability to respond to environmental insults. In rodents, for example, a direct proportionality appears to exist between species life span and ability to carry out repair of DNA damage (101,102). As was previously noted, a direct proportionality has also been demonstrated between species life span and in vitro replicative potential of the corresponding fibroblasts. It has been suggested, therefore, that a decline in ability to implement DNA repairs may underlie the progressive loss of viability in senescent cell populations. However, in normal as well as in Werner syndrome human fibroblasts investigators have found either no decrease or an

actual increase in DNA repair capacity (24,36), and this area remains controversial.

A number of reports have documented an attenuated induction of heat shock proteins (HSPs) in senescent cells following acute hyperthermia (103,104). Recently a group of investigators has reported the identification and cloning of a member of the hsp70 family which they have named mortalin (mot). Mortalin encodes a 66-kDa protein ($p66^{mot}$) which is expressed as two alternative forms differing by just two amino acid substitutions. One form (mot-1) is strictly cytosolic, and appears exclusively in cells of limited-proliferative potential. The second form (mot-2) has a perinuclear distribution, and is found only in immortal cells. Overexpression of mot-1 following transfection of its cDNA into immortal NIH-3T3 fibroblasts induced these cells to undergo cellular senescence. Transfection of the cDNA for mot-2 into mouse embryonal fibroblasts (MEF) did not result in immortalization of these cells, suggesting that it is the loss of mot-1 rather than mot-2 expression which is the more significant event in the overcoming of senescence by these cells. Consonant with this idea, the microinjection into senescent MEF of anti-$p66^{mot}$ antibody leads to transient stimulation of DNA synthesis (105,106).

A number of differentially expressed genes have been isolated following screening of young or senescent cDNA libraries with cDNA probes prepared from poly(A)$^+$ mRNA derived from young and senescent cells (plus/minus screening). Among these is a cDNA encoding a novel sequence which has been designated LPC-1 (late population doubling level cDNA-1). LPC-1 expression,

which is strictly cell-cycle regulated in young fibroblasts, becomes uncoupled from the cell cycle in senescent cells (34). SAG (senescence-associated gene) is another novel sequence isolated by plus/minus screening. The expression levels of SAG, while not cell cycle regulated, increase concomitantly with in vitro age. Sequencing data indicate a DNA-binding domain and makes SAG a candidate regulatory protein (107). A cDNA with homology to the multigene family of elongation factor I alpha (EF-Iα) has also been uncovered using a plus/minus screening strategy (108). Interestingly, EF-Iα has been shown to have extensive homology to statin, a protein present in both quiescent and senescent cells but undetectable in actively proliferating cells (109). Therefore, the cDNA with EF-1α homology, which was isolated on the basis of its increased hybridization to senescent-cell derived sequences, may in fact have been detecting statin (108). Statin itself was identified in the nuclei of cells no longer involved in proliferation during an immunohistochemical survey of a variety of tissues. This approach, which utilizes the generation of monoclonal antibodies directed against senescence-associated antigens, has uncovered another protein, named terminin (110). The presence of cytoplasmic terminin granules, as revealed by immunofluorescence microscopy, is reported to very accurately distinguish senescent from conditionally (serum-starved or contact-inhibited) growth arrested cells (110).

If the limited life span phenotype results from the coordinated loss and gain of specific gene functions during the differentiation-like process known as

senescence, uncovering the nature of the mechanism which is ultimately responsible for the regulation of such genes becomes of paramount importance.

Some investigators have proposed DNA methylation as a possible candidate for such a mechanism. This DNA modification, which has been implicated in the differential utilization of paternally and maternally inherited genes known as genomic imprinting, is almost certain to play an important role in the regulation of gene expression (111). An age-associated decline in the cellular levels of 5-methyl cytosine (5MC) has been reported in normal but not in (SV40 transformed) immortal human fibroblasts (112). Treatment of young fibroblasts with 5-azacytidine (5-Aza CdR), a potent inhibitor of DNA methylation, results in a sizeable decrease in the expected life span of these cells (113). An in vivo decrease with age in 5MC content has also been observed in mouse fibroblasts (114). Reactivation of a silent X-linked locus by age-related demethylation has been reported both to occur and not to occur in aging animals (115,116). In an intriguing recent paper, a group of investigators have reported the restoration of the senescent phenotype to a nickel-transformed immortal Chinese hamster cell line, carrying an Xq chromosome deletion, by transfer of a normal Chinese hamster X chromosome via microcell fusion. The frequency with which the transferred X chromosome brought about senescence was found to be inversely proportional to the passage level (i.e. age) of the cells from which the microcells were prepared. Full (100%) ability to confer senescence was restored to X chromosomes from old microcell donors when the latter were pretreated with the demethylation inducer 5-azacytidine (117). These

results suggest the existence of senescence-related genes whose activity might be under regulation by the extent or pattern of their DNA methylation. Control of cellular senescence by the random or gene-specific level of DNA methylation, while an attractive idea, still remains controversial.

The recent discovery that telomeres, the specialized repetitive structures at the end of linear eukaryotic chromosomes, decrease in length during in vitro as well as with in vivo aging has become the foundation for another theory of cellular senescence. This topic will be discussed in detail in the RESULTS section of this thesis.

On the tacit assumption that understanding of cellular senescence might be gained by the identification and analysis of conditions or agents that overcome it, there have been numerous reports dealing with various experimental protocols designed to forestall the advent of the phase III phenomenon (36). Various means have been employed to achieve life span extension in normal human fibroblasts, including exposure to intermittent fluorescent lights (118) and treatment with hydrocortisone-conditioned medium (119). However, all reported manipulations of the in vitro life span of human cells have so far merely deferred but never prevented the eventual onset of senescence.

The study of the interactions between viruses and their host cells has provided a powerful tool for uncovering the nature of many cellular processes. Virally encoded proteins often disrupt cellular regulatory mechanisms in order to generate molecular environments optimal for viral multiplication. Analysis of such

perturbations has often led to the identification of important molecular effectors of cellular control (120).

Simian virus 40 (SV40) has been particularly relevant to the study of senescence. It is a small DNA tumor virus of the papovavirus family. The SV40 genome, a closed circular double-stranded DNA molecule of 5243 base pairs, consists of two genetic regions of approximately equal size oriented in opposite directions. The early coding region (transcribed soon after infection) encodes a pre-mRNA which is differentially spliced to produce two early proteins: the small t, and large T antigens. The late coding region encodes three viral capsid proteins. SV40 large T antigen is a multifunctional protein of 708 amino acids. Among the multitude of functions attributed to large T are binding of a number of important cellular regulatory proteins such as p53, RB, and the large subunit of DNA polymerase α (121,122). It stimulates transcription from a number of cellular promoters such as those of the hsp70 gene family (123). Large T antigen induces cellular DNA synthesis, including one additional round in irreversibly arrested senescent human fibroblasts (124). Its presence overcomes the post-transcriptional block to PCNA expression observed in senescent fibroblasts (125). As discussed later large T antigen is essential to the establishment and maintenance of the transformed phenotype. Small t antigen appears to regulate protein phosphatase 2A, and has an enhancing effect on transformation by large T (126).

Human cells are semi-permissive for replication of SV40 (122). Infection of human fibroblasts by SV40 can lead to random integration of the viral genome into

that of the host. If the SV40 early coding region survives such an integration event, the resulting cells will express viral large T antigen and may exhibit a variable number of altered properties typical of transformed cells. Among these are changes in cell morphology, reduced serum requirement, increased saturation density, aneuploidy, anchorage independent growth, tumorigenicity in susceptible animals, and extended lifespan (127). The semipermissive nature of SV40 replication in human fibroblasts results in extensive killing of infected cell populations due to viral cytopathic effects. This fact has led researchers to adopt the use of replication-defective viral genomes as well as of recombinant vectors carrying only the SV40 early coding region (128).

Human fibroblasts which express large T antigen have an in vitro lifespan that extends 20-30 population doublings (PD) beyond the 50-60 PD of their normal counterparts (127). As was described earlier, part of the cellular machinery responsible for the limited lifespan of normal cells consists of the concerted antiproliferative action of two proteins: pRb and p53. SV40 large T antigen, as well as several other DNA tumor virus-encoded proteins, have been found to mediate their growth stimulating effects largely as a result of their ability to complex, and thereby inactivate, pRb and p53. The requirement for both pRb and p53 binding activities in order to confer on normal human fibroblasts an in vitro lifespan extension which is similar to that observed following SV40 T antigen expression has received support from several lines of investigation. A team of Japanese researchers depleted cellular pRb in normal human diploid fibroblasts using an

antisense-Rb oligomer and thereby achieved a lifespan extension of approximately 10 PD. Depletion of p53 with an antisense-p53 oligomer produced no effect on lifespan. However, cells simultaneously depleted for pRb and p53 attained lifespans 20-30 PD longer than those of controls (129). These results suggest a complementary role for pRb and p53 in the regulation of cellular senescence, and support the notion that lifespan extension by SV40 large T antigen probably results from the ability of this protein to bind (and thereby inactivate) both pRb and p53. Additional support for this conclusion comes from studies of human fibroblasts transfected with an SV40 large T antigen under the transcriptional control of a steroid-inducible promoter. The proliferation of such cells during their period of extended life span is dependent on the presence of large T antigen and, consequently, on the presence of steroid in the culture medium. Removal of steroid leads to de-induction of T antigen and to rapid cessation of growth. Under such conditions proliferation was found to continue only in cells that had (in addition to T antigen) been previously transfected with combinations of two other viral proteins which could together (but not separately) replace the ability of large T antigen to bind both pRB and p53 (e.g. adenovirus E1A and E1B or human papillomavirus E6 and E7) (130).

In contrast to rodent cells which are known to spontaneously generate variants exhibiting indefinite growth potential in tissue culture (i.e. which are immortal), reproducible conditions for the spontaneous immortalization of normal human diploid fibroblasts (HDF) have never been described. Similarly, the

immortalization of human cells by chemical or radiation treatment has only rarely been reported, and even then required repeated exposure to these agents. Human cells are, likewise, remarkably resistant to immortalization by transfection with viral or cellular oncogenes (127).

Although human diploid fibroblasts transformed by SV40 large T antigen exhibit an extended in vitro lifespan, they inevitably succumb to a pattern of growth cessation similar to that experienced by normal cells during phase III which has been designated "crisis". Unlike senescent cells, however, these cells are not arrested in G_1 (24). The relationship between senescent growth arrest and crisis is still not clear, but some investigators have suggested that similarities indeed exist between the two (131). Following an apparent absence of growth lasting as long as several weeks (and most often as an extremely rare event) a cluster of cells may be found to have survived the degeneration and death of crisis in the mass culture. Such cells can be subcultured for extended periods of time and are frequently immortal.

The inefficient immortalization of HF by SV40 may be attributed, at least in part, to the semi-permissive nature of the virus-cell interaction. The semi-permissiveness of human fibroblast cultures toward viral propagation is clearly indicated by the low-level production of infectious virion particles, as well as by the efficient synthesis of viral DNA in a subpopulation of infected cells (132). Such productively infected cells are likely to die due to viral cytopathic effects, thus reducing the pool of stable transformants which are potentially capable of generating immortals. It can be logically concluded that immortalization of SV40-

transformed HF involves, at least in part, a selection for alterations of the viral genome precluding viral DNA synthesis.

The extreme rarity of immortalization of human fibroblasts even after persistent expression of SV40 large T antigen (in the order of 10^{-7} in immortalization competent clones) (133), together with the recessive nature of the immortal to the limited lifespan phenotype as demonstrated by cell hybrid studies (134), has prompted investigators to postulate the inactivation of a yet-to-be identified growth suppressor gene(s) as an additional event that must take place in order for immortalization to occur (135,136). Loss of this postulated SV40-independent growth suppressor(s) is believed to occur as a result of cellular mutation, and is perhaps facilitated by the mutagenic and karyolytic properties of large T antigen (127) either directly or through its effects on p53.

The generation of human fibroblast cell lines incorporating a conditional (i.e. inducible or temperature sensitive) large T antigen has made possible the analysis of this protein's role in the pre-crisis (extended lifespan) period as well as in the immortalized cells. Such investigations have revealed that T antigen activity is absolutely required both during the extended-lifespan pre-crisis period as well as in the immortal cells that have escaped crisis (135,136). SV40 large T antigen expression can, therefore, be said to be necessary but not sufficient for the immortalization of human fibroblasts.

These ideas and findings have been formalized in a two-step (or M1/M2) model for cellular senescence and immortalization. In essence this model maintains

that immortalization requires the functional inactivation of two mortality stages, M1 and M2. Proliferative senescence in normal diploid fibroblasts arises from the operation of the M1 mechanism. Introduction of SV40 large T antigen inactivates or bypasses the M1 effectors (e.g. Rb and p53) and allows the cell to divide an additional 20-30 times more than it might otherwise have done. At this point, however, a second antiproliferative mechanism, mortality stage 2 (M2), becomes active. Only when the gene(s) responsible for the M2 mechanism become inactivated can immortalization take place. Large T antigen function is continuously required by the immortalized cells for exactly the same reason that it is required by the pre-crisis transformants: both still retain an extant M1 mechanism. Therefore, SV40 large T antigen is chiefly responsible for the neutralization of mortality stage 1 (M1), but may also have a subsidiary and indirect role in the mutational inactivation of genes controlling M2 via its generation of genomic instability (135,136).

Convincing evidence in support of a genetic basis for immortality has come from complementation analysis following cell fusion among a wide variety of human immortal cell lines. Taking advantage of the recessive nature of the immortal phenotype, a group of investigators has assigned 21 independent immortal cell lines to at least four complementation groups. The rationale behind this experiment rests on the genetic principle that cell fusion partners sharing the same molecular defect cannot complement and will produce synkaryons that would still exhibit indefinite proliferative potential. On the other hand, cell

fusion partners with defects in different molecular effectors can complement and would, therefore, be expected to generate limited life span (i.e. senescent) synkaryons. Strikingly, all SV40 immortalized cells (with the exception of xeroderma pigmentosum fibroblast line) fell into the same complementation group (137). These results suggest the exciting possibility that the number of genes involved in escape from senescence might not be very large, and that all SV40 immortalized cells share a defect in the same biochemical pathway.

Our laboratory, as part of its long-standing commitment to the elucidation of the molecular bases for the phenomena of cellular senescence and immortalization, has generated from the diploid human fetal bone marrow fibroblast cell strain HS74BM, a series of SV40-transformed human fibroblast cell lines using a replication-defective (ori-) viral genome encoding either a wild type or a temperature-sensitive large T antigen (tsA58) (128,138). From these transformants (SV/HF and SVtsA/HF), immortalized derivatives have been subsequently isolated to be used in a number of comparative studies.

The specific objectives of the present thesis were the following:

1. To assess the significance of changes in cellular or viral (SV40) genome organization to the immortal phenotype:

   I intend to compare pre-immortal and immortal members of the SVtsA/HF-A line (see Materials and Methods) in order to determine whether overt rearrangements or changes in copy number of the SV40 integrant accompany the emergence of the immortal cells.

Our laboratory has previously identified a karyotypic abnormality, involving an apparent loss of sequences present in at least one copy of the long arm of chromosome 6 (6q), that seems to be closely associated with immortalization. Using the SVtsA/HF-A line I will determine, by quantitative Southern analysis, the copy number of three oncogenes located in 6q (c-mas, c-myb, and c-ros) in the pre-immortal and the immortal cells. This will be carried out in order to ascertain that the changes observed to take place in 6q arise from a true loss of DNA sequences rather than from inapparent translocations.

The SVtsA/HF-A preimmortal and immortal cell lines represent a uniquely suitable reagent for such comparative quantitative studies in that they do not exhibit the excessive rearrangement and aneuploidy typical of most SV40 transformants.

Telomere shortening has been implicated in the phenomenology of human cellular senescence. Our laboratory has generated multiple matched sets of pre-immortal and immortal SV40-transformed human fibroblasts cell lines from the normal progenitor HS74 (see Materials and Methods). Such genetically matched cell lines at various population doubling levels can be used to assess the behavior of telomeres in the SV40 transformants during the period of extended life span leading into crisis, as well as in the immortal derivatives following crisis. Specifically, I intend to determine whether the extended life span of the pre-immortal SV40 transformants results from a selection for cells with

unusually long telomeres, and whether the telomere shortening characteristic of senescent cells continues during the period of extended life span of the pre-immortal cells. Finally, I intend to examine how the behavior of telomeres in the immortal cells compares to that observed in the preimmortal and in the normal cells.

2. To identify, using a subtractive hybridization strategy, genes which are differentially expressed in the pre-immortal SVtsA/HF-C versus either or both of the SVtsA/HF-A immortals HAL and AR5.

It is almost certain that immortalization of SV40 transformed human fibroblasts (whatever its ultimate molecular basis) must necessarily proceed via the differential expression of various genes. We envision the differential expression of such genes to result (at least in part) from an initial change involving the loss of a "primary effector" on the long arm of chromosome 6 (see Results, Chapter 3). I intend to isolate members of this set of genes in order to gain a better understanding of some of the molecular processes involved in immortalization.

CHAPTER 2

MATERIALS AND METHODS

Cell Lines and Tissue Cuture Conditions

Cells were grown in 50:50 DME:F10 (DF10) medium supplemented with 10% fetal bovine serum in the presence of the antibiotics penicillin "G" phoshate and streptomycin sulfate (from Gibco) each at 50mg/L as previously described (136,138).

Cells were subcultured (i.e. passaged) by brief treatment with a trypsin-EDTA mixture (0.5 g/L trypsin and 0.2 g/L EDTA, from M.A. Bioproducts). Cell numbers were obtained with a Royco Tissue Cell Counter after appropriate dilution of cell suspensions in phosphate-buffered saline (PBS; 150 mM NaCl, 3 mM Na_2HPO_4/NaH_2PO_4, pH 7.4). The number of generations was estimated from the number of population doublings (PD).

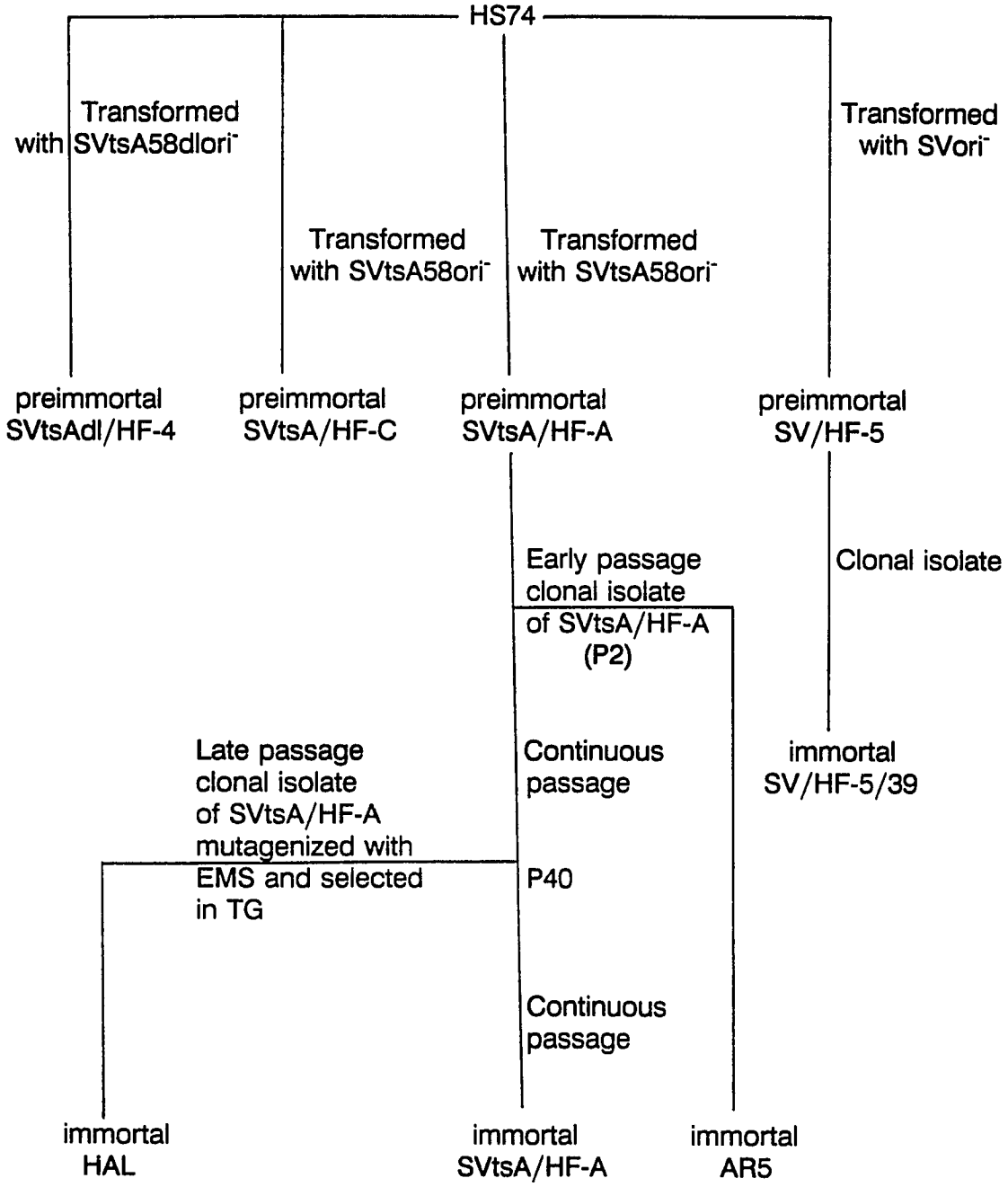
The human diploid fetal bone marrow fibroblast cell strain HS74BM was obtained from H.S. Smith (139) at passage 5 (P5) which is equivalent to 13 PD. SV40 transformants of HS74BM were isolated following introduction, by the calcium phosphate DNA co-precipitation technique (140), of an origin-defective SV40 genome encoding either a temperature sensitive (SVtsA58ori$^-$) or a wild type T antigen (SVori$^-$) (128,136,138). SVtsAdl/HF-4 is an independently derived SV40 transformant which was generated by transfection of HS74 with an ori$^-$ SV40 construct which expresses large T but not small t antigen (SVtsA58dl) (141).

Four immortalized derivatives are used in the present study: SVtsA/HF-A (at late passage), AR5, HAL, and SV/HF-5/39. AR5 was isolated as a colony from early passage (i.e. preimmortal) SVtsA/HF-A. Passage of the uncloned mass culture of SVtsA/HF-A also generated an immortal line, which is designated immortal SVtsA/HF-A. HAL was isolated as a colony from immortal SVtsA/HF-A at passage 40 in conventional medium supplemented with 5 x 10^{-5} M thioguanine following mutagenesis of the population with ethylmethane sulfonate (150 μg/ml). HAL is defective in the enzyme hypoxanthine phosphoribosyltransferase (HPRT). SV/HF-5/39 is an immortal derivative of SV/HF-5, which was generated by transformation of HS74BM with an origin-defective SV40 genome encoding a wild type T antigen (138). A concise lineage of all these cells appears in Figure 1.

Southern Blot Analysis

High molecular weight DNA was purified from confluent cultures and analyzed by the Southern blot procedure using standard conditions (142). DNA was digested with the appropriate restriction enzyme(s) at 10 U/μg DNA for 16 hrs using buffer and temperature conditions specified by the supplier. Digested DNA was electrophoresed in 0.8% agarose gel and transferred onto nylon membranes (Nytran, Schleicher and Scheull, Inc.). UV-crosslinking was carried out by placing filters (DNA side facing up) on a pre-soaked (in 5x SSC) sheet of 3MM Whatman paper and irradiating with uv (254 nm) to a final 120 mJ/cm^2 using a Stratalinker^{TM} (Stratagene). The filters were allowed to air dry and were baked

FIG. 1. Derivation of preimmortal and immortal SV40-transformants from the normal human fetal bone marrow fibroblast HS74.



EMS, Ethylmethane sulfonate
TG, Thioguanine

FIGURE 1

in a vacuum oven at 80^{0}C for 1-2 hrs. Hybridization was performed using random-primed ^{32}P-labeled probe DNA (143) in 50% formamide at 45^{0}C for 12-18 hours according to standard conditions (142). The $(TTAGGG)_5$ synthetic oligonucleotide telomere repeat probe was end-labeled with ^{32}P (144) and hybridized to filters at 37^{0}C as described by Church and Gilbert (145). For rehybridization, probes were removed from filters by soaking for 10 minutes at room temperature with slight agitation in 0.2 M NaOH, 0.1% sodium dodecylsulfate (SDS), followed by a rinse with deionized water, neutralization for three minutes in 0.5 M Tris-HCl, pH 7.0, and a final wash in 0.1% SDS. Dehybridized filters were dried at room temperature and either reused immediately or stored in heat-sealable plastic bags at 4^{0}C.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated by a modification (146) of the procedure of Auffrey and Rougeon (147). Cells were grown to 70-80% confluence (late log phase), washed once with phosphate-buffered saline (PBS), 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 seconds at 30,000 rpm. The suspension was incubated for 5 to 17 hrs at 4^{0}C. RNA was pelleted by centrifugation at 10,000 rpm in a Beckman SW41 rotor at 4^{0}C. The pellet was digested with 100 μg of proteinase K per ml in 19 mM Tris-HCl (pH 7.5)-10 mM EDTA-0.5% SDS at room temperature for 30 minutes. RNA was purified by phenol-

chloroform extraction and precipitated by ethanol. Poly(A)$^+$ mRNA was isolated by two cycles of chromatography on oligo(dT)-cellulose according to a standard protocol (144). Essentially, a 10ml capacity disposable plastic column (Stratagene) was washed with 10ml 5M NaOH, then rinsed with water. 200mg of oligo(dT) cellulose powder (Boehringer Mannheim Biochemica) was added to 5ml 0.1M NaOH. The slurry was poured into the column, allowed to drain, and then rinsed with 20ml of water. The column was equilibrated with 20ml of loading buffer (0.5M LiCl, 10mM Tris-HCl (pH 7.5), 1mM EDTA, 0.1% SDS). 2mg of total RNA were resuspended in 3ml of water and heated to 70^{0}C for 10 minutes. LiCl was added to a 0.5M final concentration from a 10M stock solution. The RNA solution was passed through the oligo(dT) column, and the column was washed with 1ml loading buffer. The eluant from this loading step was passed through the column twice more. The column was rinsed with 2ml middle wash buffer (0.15M LiCl, 10mM Tris-HCl (pH 7.5), 1mM EDTA, 0.1% SDS). The poly(A)$^+$ mRNA was eluted into a fresh tube with 2ml 2mM EDTA/0.1% SDS. The column was reequilibrated with 20ml loading buffer, and the eluted RNA carried through the entire poly(A)$^+$ selection precisely as before. Eluted poly(A)$^+$ mRNA was precipitated by adjusting the salt concentration to 0.3M sodium acetate, using a 3M sodium acetate stock solution, and adding 2.5 volumes of ethanol. The RNA was incubated overnight at -20^{0}C, and collected by centrifugation at 12,000xg for 30 min at 4^{0}C in a Beckman SW-41 rotor. The ethanol was poured off carefully, the pellet is allowed to air dry and resuspended in RNase-free TE.

The isolated RNAs were analyzed by the Northern blot procedure, using standard conditions (142); 10 μg of total RNA or 2 μg of poly(A)$^+$ RNA were used per sample. RNA was electrophoresed in 1% agarose-0.66 M formaldehyde gel, and transferred onto nylon membranes. Following transfer, filters were uv crosslinked and then baked at 80^{0}C in a vacuum oven for 1-2 hrs. Hybridization was carried out using random-primed ^{32}P-labeled probe DNA in 50% formamide at 45^{0}C for 12-18 hrs using standard conditions (142). For rehybridization, probes were removed from filters by soaking for 1 hour with gentle agitation at 90^{0}C in 0.5x Denhardt's solution (142), 25 mM Tris-HCl (pH 7.5), 0.1% SDS, followed by a 15 min wash in deionized water prewarmed to 65^{0}C. Filters were dried and either reused immediately or stored at 4^{0}C in heat-sealable plastic bags.

Data Analysis

Autoradiographs were generated at -70^{0}C with screens and developed with a Kodak X-Omat. They were scanned with a Molecular Dynamics 300B Computing Densitometer. The output was quantified by A. Marcus using Molecular Dynamics ImageQuant (v.3.15). Mean telomere length (MTL) was calculated as described by Harley et al. (148) from Msp I/Rsa I-digested DNAs probed with the $(TTAGGG)_5$ oligonucleotide. Lanes were divided into ten equal segments and MTL was determined using the formula $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is the densitometric output in each segment and L_i is the midpoint DNA fragment length for each segment. Terminal restriction fragment (TRF) length, which includes subtelomeric

and telomeric sequences, were determined using the formula $\Sigma(OD_iL_i)/\Sigma(OD_i)$. This calculation assumes the annealing of only one molecule of the subtelomeric repeat probe per chromosome end.

Construction of SVtsA/HF-C λgt10 cDNA Library

Poly(A)$^+$ mRNA was isolated from sub-confluent growing cultures of pre-immortal SVtsA/HF-C cells by two cycles of oligo(dT)-cellulose chromatography using a standard protocol (144). Double-stranded cDNA was synthesized using a Promega kit (RiboClone^{TM} cDNA Synthesis System). In brief, 2μg of poly(A)$^+$ mRNA from SVtsA/HF-C cells and 1μg of oligo(dT)$_{15}$ primer (Promega) were dissolved in 16 μl of water. The sample was heated to 70^{0}C for 5 minutes, allowed to cool to room temperature, centrifuged briefly in a microcentrifuge at 12,000 rpm to collect solvent at the bottom. The following components were then added in order at room temperature to the annealed primer/template: 5μl 10x first strand buffer [500mM Tris-HCl, pH 8.3 (at 42^{0}C), 750mM KCl, 100mM $MgCl_2$, 5mM spermidine], 5μl 100mM dithiothreitol (DTT), 5μl of 10mM each dNTP mix, 1μl (40 U/μl) RNasin ribonuclease inhibitor, 5μl 40 mM sodium pyrophosphate, and 13μl (32.5 U) of AMV reverse transcriptase. The tube was flicked gently to mix the reactants, and 10μl of the mixture was transferred to another tube containing 0.5μl (5μCi) of [α-^{32}P]dCTP (3000Ci/mmol from New England Nuclear). The latter is a tracer reaction used to calculate first strand synthesis yield by trichloroacetic

acid (TCA)-precipitable radioactivity (142). It also allows an estimation, via alkaline agarose gel electrophoresis, of the range of cDNA product sizes (142). Both reactions were incubated at 42^{0}C for 1 hour, at the end of which the tubes were placed on ice. To the tracer reaction was added 2μl of 0.2M EDTA, and water to a final volume of 100μl; it was then stored on ice for subsequent incorporation assays and gel analysis.

Second strand synthesis was initiated immediately by the sequential addition of the following components to the 40μl of unlabeled first strand reaction: 15.5μl water (for a final second-strand reaction volume of 100μl), 10μl 10x second strand buffer [500mM Tris-HCl, pH 7.2, 850mM KCl, 30mM $MgCl_2$, 1mg/ml BSA, 100mM $(NH_4)_2SO_4$], 6μl 100mM DTT, 20μl 1mM NAD, 0.5μl (5μCi) [α-^{32}P]dCTP, 4μl (10U/μl) E. coli DNA polymerase I, 2μl (1 U/μl) E. coli DNA ligase, 2μl (o.8 U/μl) E. coli RNase H. The reaction was flicked gently to mix and incubated at 14^{0}C for 2.5 hours. It was then heated to 70^{0}C for 10 minutes, centrifuged briefly to collect contents at the bottom of the tube and placed on ice. A 10μl aliquot was transferred to another tube for incorporation assays and gel analysis. To fill in the ends of the double-stranded cDNA prior to blunt-end adaptor ligation, the remaining reaction was incubated at 37^{0}C for 10 minutes with 4 units of T4 DNA polymerase, and then for an additional 10 minutes at 37^{0}C after addition of 4 units of Klenow fragment of E. coli DNA polymerase. The reaction was stopped with 20μl of 0.2M EDTA, and extracted with an equal volume of phenol:chloroform: isoamyl alcohol [25:24:1 (v/v/v)]. The aqueous phase was transferred to a fresh

tube, 0.5 volume of 7.5M ammonium acetate and 3 volumes of cold ethanol were added to precipitate the cDNA. The tube was immediately centrifuged at 12,000rpm for 15 min at room temperature, and the supernatant carefully removed. The pelleted cDNA was resuspended in 0.5ml cold ethanol, the tube was centrifuged for 1 minute and the supernatant carefully removed. The pellet was dried and redissolved in 10μl TE (10mM Tris-HCl, pH 8.0, 1mM EDTA).

The cDNA preparation was size-fractionated by electrophoresis on an ethidium bromide (EtBr) stained 0.8% agarose gel, and cDNA products larger than 500 base pairs (bp) were selectively isolated and purified on glass particles by the GeneClean (Bio101 Inc.) protocol.

Approximately 250ng of size-selected blunt-ended SVtsA/HF-C cDNA was ligated to EcoR1/Not1 adaptors (Pharmacia LKB Biotechnology). This adaptor is a duplex DNA molecule with one blunt end which ligates to the cDNA, and an exposed dephosphorylated EcoR1 cohesive end. The adaptor incorporates an internal Not1 restriction endonuclease recognition site. Ligation was carried out in a standard 30μl reaction mix containing 250ng cDNA, 250ng adaptor, 66mM Tris-HCl, pH 7.6, 10mM $MgCl_2$, 15mM DTT, 1mM spermidine, 0.2mg BSA/ml, 1mM ATP, and 3 units T4 DNA Ligase. The reaction was mixed gently, centrifuged briefly at 12,000 rpm, and incubated at 12^{0}C overnight. T4 DNA Ligase was heat inactivated at 70^{0}C for 10 minutes, and the reaction was then cooled on ice. Adaptors were phosphorylated following inactivation of the ligase by adding 4μl of 10x kinase buffer (0.7M Tris-HCl, pH 7.6, 0.1M $MgCl_2$, and 50mM DTT), 2μl

0.1mM ATP, 1μl T4 Polynucleotide Kinase, and 3μl water for a final volume of 40μl. The reaction was incubated at 37^{0}C for 30 minutes, and then extracted with an equal volume of phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)]. The aqueous phase was transferred to a fresh tube. The organic phase was re-extracted with a small volume of TE, which was combined with the aqueous phase from the preceding extraction. Excess adaptors were removed by Sephacryl S-400 column chromatography according to a protocol provided by the supplier (Pharmacia LKB Biotechnology). The "adaptored" cDNA was precipitated by adding 0.5 volume of 7.5M ammonium acetate, 2.0 volumes of ethanol, mixing, and incubating at -20^{0}C for 30 minutes. The cDNA was pelleted by centrifugation at 12,000xg for 15 minutes in a microfuge, the supernatant was carefully removed, the pellet was washed with 1ml cold 70% ethanol and dried briefly under vacuum.

The adaptored phosphorylated cDNA was ligated to EcoR1 predigested dephosphorylated λg10 arms according to a protocol provided by the supplier (Stratagene; Predigested Lambda gt10/EcoR1 cloning kit). Essentially, 250ng of cDNA were resuspended in 2.5μl TE and mixed with 1μl (1μg) of λgt10 arms. In a separate tube were mixed 0.5μl of 10x ligation buffer (500mM Tris-HCl, pH 7.5, 70mM $MgCl_2$, 10mM DTT), 0.5μl 10mM ATP (pH 7.5), and 0.5μl (2-3 units) of T4 DNA ligase. The latter solution was taken up in a pipette tip and added to the former with gentle stirring. Ligation was carried out overnight at 4^{0}C.

The recombinant λgt10 (in the 5μl ligation reaction) were packaged in lambda virions using Stratagene's packaging extracts and an accompanying

protocol (Stratagene; Gigapack II Gold Packaging Extract). Upon completion of packaging, 500μl of SM phage dilution buffer [per liter: 5.8g NaCl, 2g $MgSO_4$-$7H_2O$, 50ml 1M Tris-HCl (pH 7.5), 5ml 2% gelatin] and 20μl of chloroform were added to the reaction tube and mixed gently.

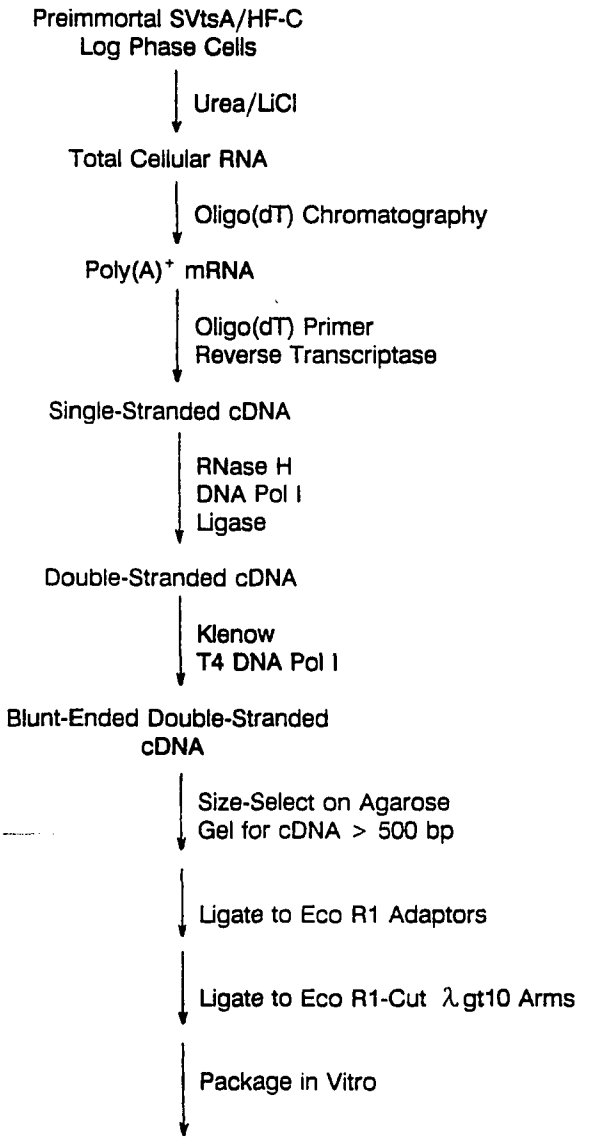
The library was titered and percentage of recombinants estimated by plating a series of dilutions of the packaging reaction on the E. coli bacterial host strains C600 and C600Hfl$^-$ according to instructions from the supplier (Stratagene). Cloning into the unique EcoR1 site of λgt10 disrupts the cI gene whose function is required to maintain lysogeny (149). The host cell function Hfl also influences the establishment of lysogeny. When λgt10 infects the E. coli strain C600Hfl$^-$ which carries a mutation predisposing to high frequency of lysogeny (Hfl$^-$), lytic growth of non-recombinant (cI$^+$) phage is repressed, while recombinant (cI$^-$) phage form plaques at normal frequency (149). C600 cells allow growth of both recombinant and non-recombinant phage, but non-recombinant phage appear as cloudy plaques due to incomplete or abortive lysogeny. Thus, these bacterial systems make it possible to estimate the percentage of recombinants in the phage lambda library. The percentage of recombinants was also estimated by picking (at random) a number of plaques grown on C600 cells, preparing phage DNA (as will be described below), digesting with EcoR1 and assaying for the presence of cDNA inserts by electrophoresis on ethidium bromide stained agarose gels. The latter approach also made possible an estimation of the distribution of cDNA insert sizes. The library was determined to be more than 95% recombinant;

cDNA insert sizes ranged from 500bp to 1.5 kb. Library yield was calculated at 5-8 x 10^5 plaque forming units (pfu) per μg of input poly(A)$^+$ mRNA. The library was verified to be representative on the basis of the number of plaques carrying sequences of known high (GAPDH, SV40 large T), as well as of known low (HPRT) abundance in the source poly(A)$^+$ mRNA population. A synoptic flow chart of the cDNA construction strategy appears in Figure 2.

Screening the SVtsA/HF-C λ gt10 cDNA Library with a Subtracted SVtsA/HF-C cDNA Probe

A [^{32}P]-labeled single-stranded (ss) cDNA probe was prepared according to a standard protocol (142). In brief, to 4μg SVtsA/HF-C poly(A)$^+$ mRNA (1μg/μl) in water, were added the following components at 4^{0}C: 10μl (1μg/μl) oligo(dT)$_{15}$ (Promega), 2.5μl 1M Tris-HCl (pH 7.6), 3.5μl 1M KCl, 2.0μl 250mM $MgCl_2$, 10μl of a mixture of all four deoxyribonucleoside triphosphates (dNTPs) each at a 5mM concentration, 5μl [α-^{32}P]dCTP (specific activity 3000 Ci/mmole or 10μCi/μl, from New England Nuclear), 2.0μl 100mM DTT, 4μl (40 U/μl) RNasin ribonuclease inhibitor, 5μl water (for a 50μl reaction volume), and 2μl (200 U/μl) Moloney murine leukemia virus (MMLV) reverse transcriptase (Pharmacia). The reaction mixture was vortexed gently, centrifuged briefly, and incubated at 37^{0}C for two hours. The incorporated [α-^{32}P]dCTP is used as a tracer to allow quantitative determination of the distribution of the cDNA during the subtractive hybridization steps which are to follow. Incorporation of [α-^{32}P]dCTP also makes possible

FIGURE 2: Scheme for Generation of SVtsA/HF-C λgt10 cDNA Library



--- Estimate the Number of Recombinant Phage from Test Plating of Serial Dilutions of the Library Using C600 and C600 Hfl⁻ Bacterial Hosts

--- Assess Degree of Representation Within the Library by Probing Plated Aliquots with Sequences of Known Abundance in the Cells

FIGURE 2

calculation of cDNA synthesis yields from trichloroacetic acid (TCA)-precipitable radioactivity, as well as a determination of product size distribution by alkaline agarose gel electrophoresis (142). The reaction was stopped by adding 2μl of 500mM EDTA (pH 8.0) and 2μl 10% SDS. Template RNA was removed by adding 10μl 0.3N NaOH and incubating at 65^{0}C for 1 hour. The solution was neutralized with 25μl of 1.0M Tris-HCl (pH 8.0) and 10μl of 2N HCl. Unincorporated radioactivity was removed by chromatography on a spin column of Sephadex G-50 (Boehringer Mannheim Biochemica) and collected in a pre-siliconized Eppenderdorf tube.

Subtractive hybridization was performed using a modified standard protocol (150). Essentially, 80μg of "driver" poly(A)$^+$ mRNA prepared from immortal HAL cells was resuspended in 100μl diethyl pyrocarbonate (DEPC)-treated water and added to the single stranded pre-immortal SVtsA/HF-C cDNA which was prepared as described in the preceding paragraph (final mixture volume 200μl). The single-stranded cDNA and poly(A)$^+$ mRNA were co-precipitated by adding 0.5 volume of 7.5M ammonium acetate and three volumes of ethanol. The pellet was washed in 1.0ml 70% ethanol, dried under vacuum, resuspended in 15μl DEPC-treated water and transferred by micropipette to a pre-siliconized 650μl Eppendorf tube. The original tube was washed with 15μl of 2x hybridization buffer [0.24M NaH_2PO_4 (pH 6.8), 1.64M NaCl, 2mM EDTA, 0.2% SDS], and this was also transferred to the 650μl tube. The solution (30μl) was covered with one drop of mineral oil and heated for 1 min at 100^{0}C to eliminate secondary RNA structures. Hybridization

was carried out at 68^{0}C for 24 hours. At the end of the incubation, the hybridization reaction was diluted with 0.5ml of 100mM KH_2PO_4 (pH 6.8) at 60^{0}C, and immediately subjected to hydroxylapatite column chromatography in order to separate the single-stranded (once-subtracted) cDNA probe from the double-stranded (cDNA-mRNA) heteroduplex fraction.

Chromatography was carried out on 0.4g of hydroxylapatite (Bio-Gel HTP from BioRad) pre-equilibrated with 100mM KH_2PO_4 (pH 6.8) in a water-jacketed column (BioRad) maintained at 60^{0}C. This system has been reported to achieve optimal separation between single and double-stranded nucleic acids when compared to several other commonly used hydroxylapatite/phosphate-buffer combinations (151). The terminated hybridization reaction (in 0.5ml KH_2PO_4) was applied to the top of a pre-equilibrated column, and 0.5ml of eluant immediately collected in order to allow the entire sample to penetrate the hydroxylapatite matrix (void volume 1.0ml). The column was eluted in 1ml steps with 100mM KH_2PO_4 pre-heated to 60^{0}C. Single-stranded material was recovered quantitatively within the first 10ml of eluant collected. The single-stranded fractions were concentrated under vacuum, desalted by chromatography on water-equilibrated Sephadex G-50 columns (which also remove small cDNA fragments), pooled, ethanol precipitated in a pre-siliconized Eppendorf tube, washed with 70% ethanol, dried briefly under vacuum, resuspended in water, co-precipitated with 80μg of HAL poly(A)$^+$ mRNA, and carried through a second round of subtractive hybridization followed by hydroxylapatite chromatography precisely as before. The percent recovery of

labeled SVtsA/HF-C cDNA after two cycles of subtraction and hydroxylapatite fractionation was approximately 2.9%.

The recovered single-stranded (twice-subtracted) SVtsA/HF-C cDNA was radiolabeled to high specific activity according to a standard protocol (142), and then used as a probe to screen 10,000 plaques of the SVtsA/HF-C λgt10 cDNA library in an attempt to isolate genes preferentially expressed in the pre-immortal cells. In brief, to the subtracted cDNA in 5μl TE, was added 5μl synthetic random hexanucleotide primers in 10x random priming buffer (Boehringer Mannheim Biochemica). The mixture was heated to 60^{0}C for 5 minutes, and after cooling on ice the following were sequentially added: 5μl 100mM DTT, 5μl of a solution containing the three deoxynucleoside triphosphates dATP, dGTP, and dTTP (each at 5mM concentration), 25μl of [α-^{32}P]dCTP (specific activity >3000 Ci/mmole), 5μl water (for a final reaction volume of 50μl), and 2.5μl (2.0 U/μl) Klenow fragment of E. coli DNA polymerase. The reaction was incubated for approximately 6 hours at room temperature, and was stopped by the addition of 1μl 500mM EDTA (pH 8.0) and 2.5μl 20% SDS. Unincorporated label was removed by Sephadex G-50 chromatography. The probe was denatured by heating to 100^{0}C for 10 minutes, placed in 20ml of Church buffer [1% bovine serum albumin (BSA) fraction V, 1mM EDTA, 500mM NaH_2PO_4 (pH 7.0), 7% SDS] (144) pre-heated to 60^{0}C, and introduced immediately into a heat-sealable plastic bag carrying 10 pre-hybridized (in Church buffer, at 60^{0}C for at least 3 hours) 150mm nylon-circles (Nytran, Schleicher and Scheull, Inc.) each representing a

1,000-plaque "lift". Filters were hybridized at 60^{0}C (with shaking) for 3 days. A replicate set of filters was hybridized to an [α-^{32}P]-labeled total cDNA probe prepared from HAL poly(A)$^+$ mRNA according to a standard protocol (142). The latter serves as a negative control to identify and rule out plaques that carry sequences which are highly expressed in the immortal HAL cells. The filters were washed using standard conditions (142), dried at room temperature, wrapped in cellophane, and subjected to autoradiography. A concise overview of the subtractive hybridization strategy appears in Figure 3.

Liquid Mini-lysate Preparation of Recombinant λgt10 DNA

Following plating and screening of the SVtsA/HF-C λgt10 cDNA library, plaques of interest were picked individually by driving the narrow end of a Pasteur pipette through the plaque and into the underlying agar. The resulting plug, which carries the plaque, was suspended in 1ml of SM (per liter: 5.8g NaCl, 2g $MgSO_4$-$7H_2O$, 50ml 1M Tris-HCl, pH 7.5, 5ml 2% gelatin) phage storage buffer, and stored (with a drop of chloroform) for 4-6 hours at 4^{0}C to allow phage to diffuse out of the top agarose. A single C600 bacterial colony was inoculated into 10ml of LB (without maltose) and allowed to grow at 37^{0}C for 4-6 hours or overnight at 30^{0}C (for an O.D.$_{600}$ < 1.0/ml). The bacteria were pelleted at 2000 rpm for 10 minutes and resuspended in half the original volume of sterile 10mM $MgSO_4$. 200μl of the bacterial suspension was combined with 100μl of the phage suspension. Phage were allowed to adsorb (without shaking) for 15-20 minutes

FIG. 3. Subtractive hybridization scheme used to identify cDNAs derived from mRNAs which are overexpressed in the preimmortal cell line SVtsA/HF-C versus the immortal AR5.

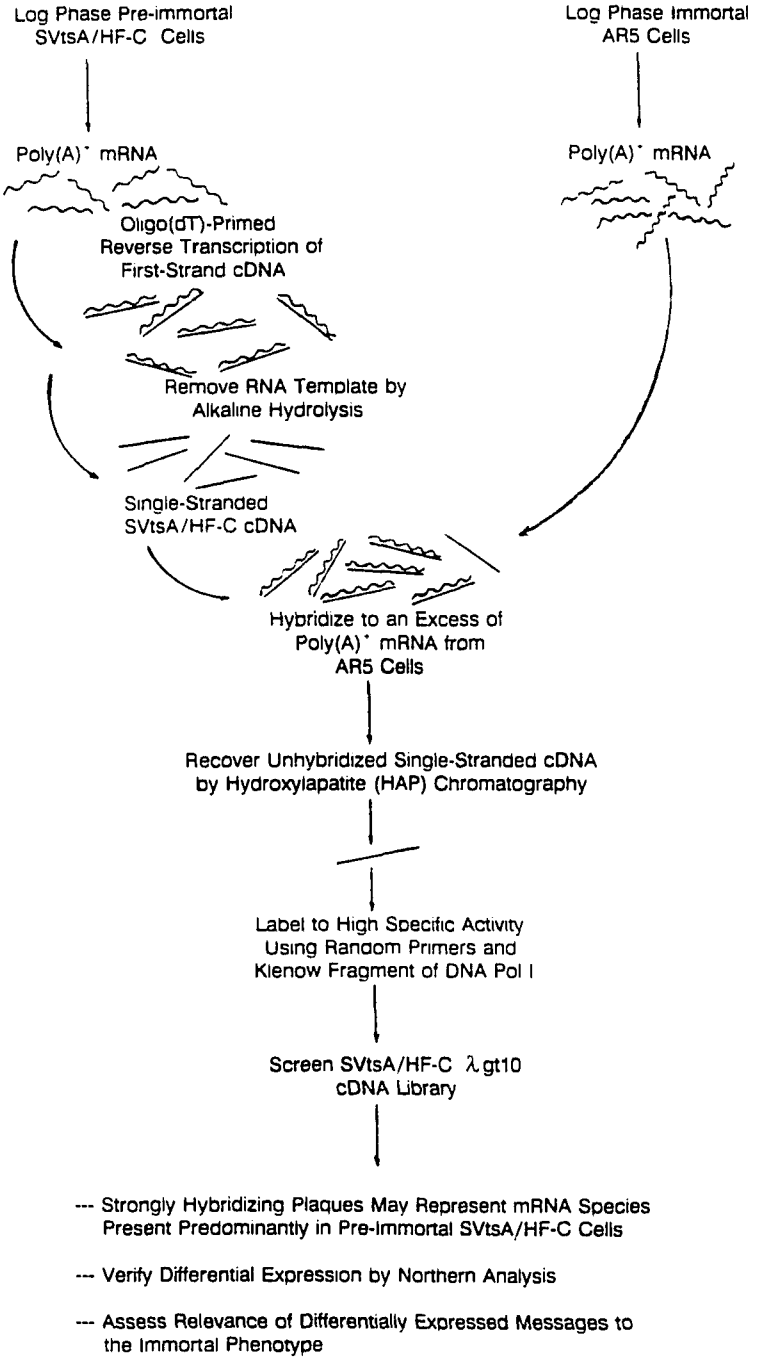

Log Phase Pre-immortal SVtsA/HF-C Cells
Log Phase Immortal AR5 Cells
Poly(A)+ mRNA
Poly(A)+ mRNA
Oligo(dT)-Primed Reverse Transcription of First-Strand cDNA
Remove RNA Template by Alkaline Hydrolysis
Single-Stranded SVtsA/HF-C cDNA
Hybridize to an Excess of Poly(A)+ mRNA from AR5 Cells
Recover Unhybridized Single-Stranded cDNA by Hydroxylapatite (HAP) Chromatography
Label to High Specific Activity Using Random Primers and Klenow Fragment of DNA Pol I
Screen SVtsA/HF-C λgt10 cDNA Library
--- Strongly Hybridizing Plaques May Represent mRNA Species Present Predominantly in Pre-Immortal SVtsA/HF-C Cells
--- Verify Differential Expression by Northern Analysis
--- Assess Relevance of Differentially Expressed Messages to the Immortal Phenotype

FIGURE 3

at 37^{0}C, after which 10ml of LB adjusted to 10mM $MgSO_4$ were added. The suspension was shaken vigorously at 37^{0}C until clearing of the culture due to bacterial lysis (approximately 3-4 hours) became evident. Chloroform was added (100μl) and shaking continued for another 15 minutes. Phage lysate (1.0ml) was transferred to an Eppendorf tube and centrifuged for 2 minutes at room temperature to remove cellular debris. The supernatant was transferred to a fresh Eppendorf tube, 1μg/ml each of RNase A and DNase I were added, and incubated at 37^{0}C for 30 minutes. The suspension was split into two fresh Eppendorf tubes, 250μl STE (1.5% SDS, 0.3M Tris-HCl, pH 9.0, 0.15M EDTA) added, mixed, and heated at 70^{0}C for 15 minutes. After cooling to room temperature, 200μl of 8M KOAc were added, mixed, and placed on ice for 15 minutes. After a 1 minute centrifugation, the supernatant was transferred to a fresh Eppendorf tube and extracted with an equal volume of phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)]. To the aqueous phase was added an equal volume of 2-propanol, mixed rapidly and centrifuged immediately for 1 minute at room temperature. The pellet was washed with 70% ethanol, dried under vacuum, and redissolved in 25μl TE. Restriction enzyme digests and polymerase chain reactions (PCR) were carried out using 10μl and 1-2μl (2-5ng) respectively of the recombinant-phage DNA solution.

Polymerase Chain Reaction (PCR) Amplification of SVtsA/HF-C cDNA Inserts Cloned in λgt10

cDNA inserts selected during screening of the SVtsA/HF-C λgt10 library

were amplified via the polymerase chain reaction (PCR) in order to obtain sufficient material for the preparation of probes to be used in Southern and Northern analysis, as well as for subcloning and sequencing. Amplification reactions were carried out using primers [5'd (AGCAAGTTCAGCCTGGTTAAGTCCAAGC) 3' and 5'd (GGTGGCTTATGAGTATTTCTTCCAGGGTA) 3'] flanking the EcoR1 cloning site of the λgt10 vector. Amplifications were carried in a standard 100μl PCR reaction mix consisting of approximately 2ng of purified recombinant λgt10 DNA, 50mM KCl, 10mM Tris-HCl, pH 8.3, 1.5mM $MgCl_2$, 0.01% gelatin, 200μM each of the four deoxynucleoside triphosphates (dATP, dGTP, dCTP, and dTTP), 1μM each of the two oligonucleotide primers, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus). Reactions were carried out in a Perkin-Elmer Model 480 thermal cycler programmed to provide an initial denaturation step of 4 min at 94^{0}C, followed by 35 cycles each consisting of 1 min at 94^{0}C, 1.5 min at 58^{0}C, and 3 min at 72^{0}C, followed by a final incubation at 72^{0}C for 5 minutes. A 10μl aliquot of each reaction was analyzed by electrophoresis on an ethidium bromide (EtBr) stained 0.8% agarose gel. PCR products were separated by agarose gel electrophoresis, and fragments of interest were purified by the GeneClean (Bio101 Inc.) protocol.

DNA Sequence Analysis

SVtsA/HF-C cDNA inserts were amplified using PCR, fractionated by electrophoresis on agarose gel, and isolated by the GeneClean protocol as already described. Purified inserts were digested with EcoR1, ethanol precipitated, washed

with 70% ethanol, dried briefly under vacuum, resuspended in TE, and ligated to EcoR1-digested and dephosphorylated pGEM-3 plasmid DNA (Promega) using standard conditions (142). The pGEM-3 multiple cloning site is flanked by oppositely oriented SP6 amd T7 promoters. The phage promoters serve as convenient priming sites for DNA sequencing reactions, and allow both strands of any DNA insert to be sequenced from opposite directions by use of the appropriate primers. Sequencing was carried out by the dideoxynucleotide chain-termination strategy (152) using the Sequenase Version 2.0 kit (United States Biochemical). SP6 and T7 promoter primers were supplied by New England BioLabs. Reactions were carried out in the presence of [α-^{32}P]dATP to allow for autoradiographic visualization of products. Products were electrophoresed on 7% polyacrylamide denaturing (7-8M urea) gels according to a standard protocol (144). Gels were wrapped in cellophane and stored at -70^{0}C for autoradiography.

CHAPTER 3

RESULTS

Changes in Organization of Viral (SV40) and Cellular Genomes

Human diploid cells (HDC) from normal tissues undergo a characteristic pattern of growth in tissue culture terminating in a non-replicative stage (Phase III) which, by analogy to aging in the organism, has been termed "senescence". Despite the very large collection of molecular changes which have been reported to take place during cellular senescence (see Introduction), there is no universally accepted explanation for this phenomenon.

One approach to understanding cellular senescence is to examine mechanisms which overcome it. While rodent cells are known to spontaneously generate variants exhibiting indefinite growth potential in tissue culture (i.e. which are immortal), normal HDC have never been found to do so. On the other hand, many human cell lines derived from various tumors have long been known to have the capacity to proliferate indefinitely in culture. They have undergone, however, multiple changes such that the predecessor of the immortal cells in not known. However, it has been observed that human diploid fibroblasts (HDF) can be transformed in culture by the oncogenic DNA virus SV40, and that a subpopulation of such transformants could become immortal, albeit at an extremely low frequency

(153,154). Hence, SV40-transformed HF could be a possible experimental system for assessing the overcoming of senescence.

Since SV40 infection of human fibroblasts is semi-permissive and it would be advantageous to study SV40-mediated transformation and immortalization using a system that is free of the complications introduced by viral DNA replication and lytic infection, our laboratory (and others) adopted the use of replication-defective viral genomes. Our laboratory generated a series of SV40-transformed HF using an origin-defective viral genome (SVori$^-$) coding for either a wild type (SV/HF) or a temperature sensitive (SVtsA/HF) large T antigen. From this series of transformants (i.e. SV/HF and SVtsA/HF), immortalized derivatives were subsequently isolated (128,138) (see Materials and Methods). These matched sets of preimmortal and immortal transformants, carrying either a temperature sensitive or wild type large T antigen, represent a unique reagent for investigations aimed at shedding light on the molecular mechanisms involved in immortalization.

Our laboratory had previously demonstrated that an enhanced transformation frequency can in fact be secured by the use of replication-defective viral constructs (128,138). Nonetheless, with one possible exception (which will be discussed below), all such transformants invariably undergo "crisis". This phenomenon, therefore, cannot be attributed to cytotoxic viral effects, which supports a model that views "crisis" as a belated manifestation of the cellular senescence associated with normal cells. Since transformation by a replication-defective SV40 genome is in itself insufficient for immortalization of human

fibroblasts, other cellular or viral events (or both) must also be involved.

I have examined the status of the resident SV40 sequences in an attempt to determine whether changes in location, arrangement, or copy number accompany the emergence of immortalized lines. Previous analysis of a preimmortal transformant generated with an ori$^-$ genome coding for a wild-type large T antigen (i.e. SV/HF-5), as well as six immortal cell lines independently derived from it, indicated that location and rearrangement of the viral genome was not responsible for immortalization (138). Quantitative changes were, however, not assessed at this time. I analyzed preimmortal and immortal SVtsA/HF-A by quantitative Southerns for possible alterations in the copy number and integration pattern of SV40. SVtsA/HF-A was isolated as a transformed focus following transfection of HS74BM with an origin-defective SV40 genome encoding a temperature sensitive large T antigen (see Materials and Methods). The SVtsA/HF-A lineage is unusual among SV40 transformed human fibroblasts in that it generated immortals with little apparent intervening crisis. The corresponding SVtsA/HF-A DNAs were digested with BstX1 (which cuts once within the SV40 sequence). Southern analysis revealed three bands (8, 2, 0.5 kb) with homology to SV40 DNA (Fig. 4). In addition BstX1 generated a 3.2-kb band for the cellular oligo(A) synthetase gene (155), which was used as an internal standard for quantitation. Although the DNA patterns were indistinguishable, 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

FIG. 4. Comparison of BstX1-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 BstX1 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.

FIGURE 4

endogenous oligo(A) synthetase gene demonstrated that immortal SVtsA/HF-A at passage 21 (i.e. P21; see Materials and Methods) and AR5 had approximately twofold levels of both the 8-kb and 2-kb bands compared with those observed in preimmortal SVtsA/HF-A (P7). Both SVtsA/HF-A cells at P44 and the subclone HAL had a still higher level of SV40 sequences. Levels of large T and/or small t antigen were found (by our colleague Bibi Zainul), to show no significant differences when assayed by immunoblot. Rearrangement of the viral sequences was similarly not detected when these DNAs were digested with a number of other enzymes (data not shown).

My data taken together with other data from this laboratory support the conclusion that immortalization of SV40-transformed HF cannot be explained simply on the basis of alterations in SV40 function. A number of studies have demonstrated that SV40 infection increases the frequency of chromosomal aberrations (e.g. aneuploidy and rearrangement) (156). We, therefore, sought to identify changes in the cellular genome which are consistently associated with immortalization. To this end, our laboratory (in collaboration with that of Dr. Ann Henderson at Hunter College) embarked on a comparison of the karyotypes of two sets of transformants (i.e. SV/HF and SVtsA/HF-A) and their immortalized derivatives (see Materials and Methods). The untransformed progenitor HS74BM had been previously shown to have a diploid karyotype (139).

Early passage preimmortal SVtsA/HF-A show minimal chromomosomal rearrangements, with approximately 65% of the cells missing only one copy of

chromomsome 16. Uncloned immortal SVtsA/HF-A has, in addition to a loss of chromosome 16, a deletion of the long arm of chromosome 6 with loss of the complete portion distal to 6q21. The immortal lines AR5 and HAL, which also derive from SVtsA/HF-A (see Materials and Methods), lost an entire copy of chromosome 6. Moreover, an earlier study involving a different transformant (i.e. SV/HF) and six of its immortalized derivatives, also noted nonrandom alterations in chromosome 6q accompanying immortalization (138). The loss of chromosome 6q was examined by me in greater detail by quantitative Southern analysis in order to determine whether the alterations observed represented a true loss or merely a rearrangement (e.g. translocation) of chromosomal material. The copy number of three loci spanning the distal region of 6q was measured to determine the degree of loss of chromosomal DNA observed in karyotypes of the SV40-immortalized cells. DNAs from the normal progenitor HS74 and different SVtsA/HF-A transformants were digested and probed for the proto-oncogenes *ros* (6q21-22) (Fig. 3), c-myb (6q22-23) (Fig. 4), and *mas* (6q24-27) (Fig. 5). Levels were normalized to oligo(A) synthetase which provides an internal standard for quantitation by densitometry. The immortal cell lines had one copy of each of the oncogenes, compared to two copies for HS74 and early preimmortal SVtsA/HF-A cells. Therefore, the loss of the long arm of chromosome 6 detected by karyotypic analysis in the immortal transformants is a true loss of DNA and not a translocation.

Deletions of specific chromosomal segments are particularly interesting in light of the recessive (i.e., loss of function) nature of the immortal phenotype as

FIG. 5. Copy number of *ros* as detected by Southern analysis of preimmortal and immortal SV40 transformants. High-molecular-weight DNA was prepared from different passages of untransformed HS74 cells (lanes 1 and 2) and of SVtsA/HF-A cells. DNA was digested with Bgl II for analysis by the Southern procedure and hybridized simultaneously with ^{32}P-labeled probes for *ros* and oligo(A) synthetase which had been radiolabeled separately. The 7.2-kb band of oligo(A) synthetase provides an internal standard for quantitation. Lanes: 1, HS74, P16; 2, HS74, P24; 3, preimmortal SVtsA/HF-A, P2; 4, preimmortal SVtsA/HF-A, P7, 5 and 6, immortal SVtsA/HF-A, P21; 7 and 8, immortal SVtsA/HF-A, P43 and P44 respectively; 9, immortal HAL cells; 10, immortal AR5.

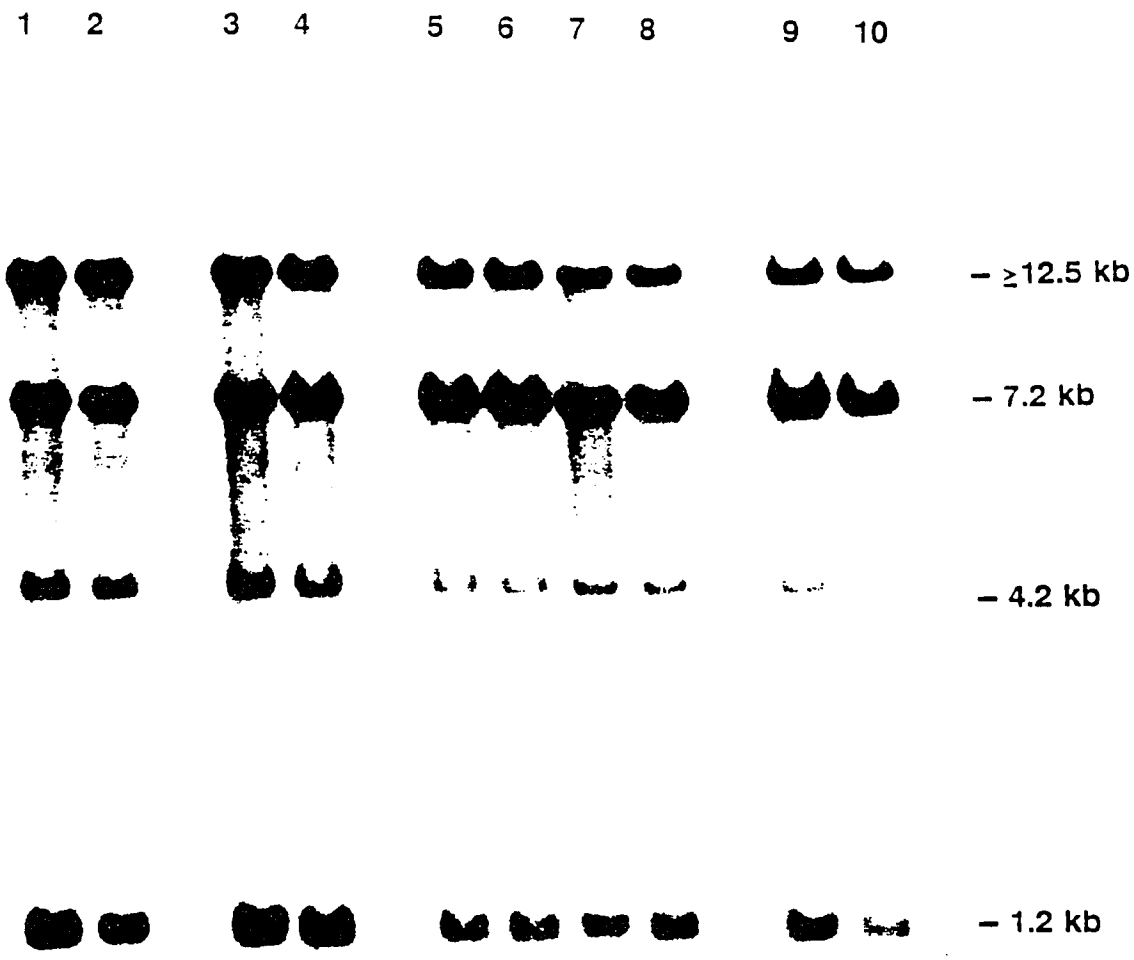


FIGURE 5

FIG. 6. Copy number of c-myb as detected by Southern analysis of preimmortal and 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 HindIII 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.

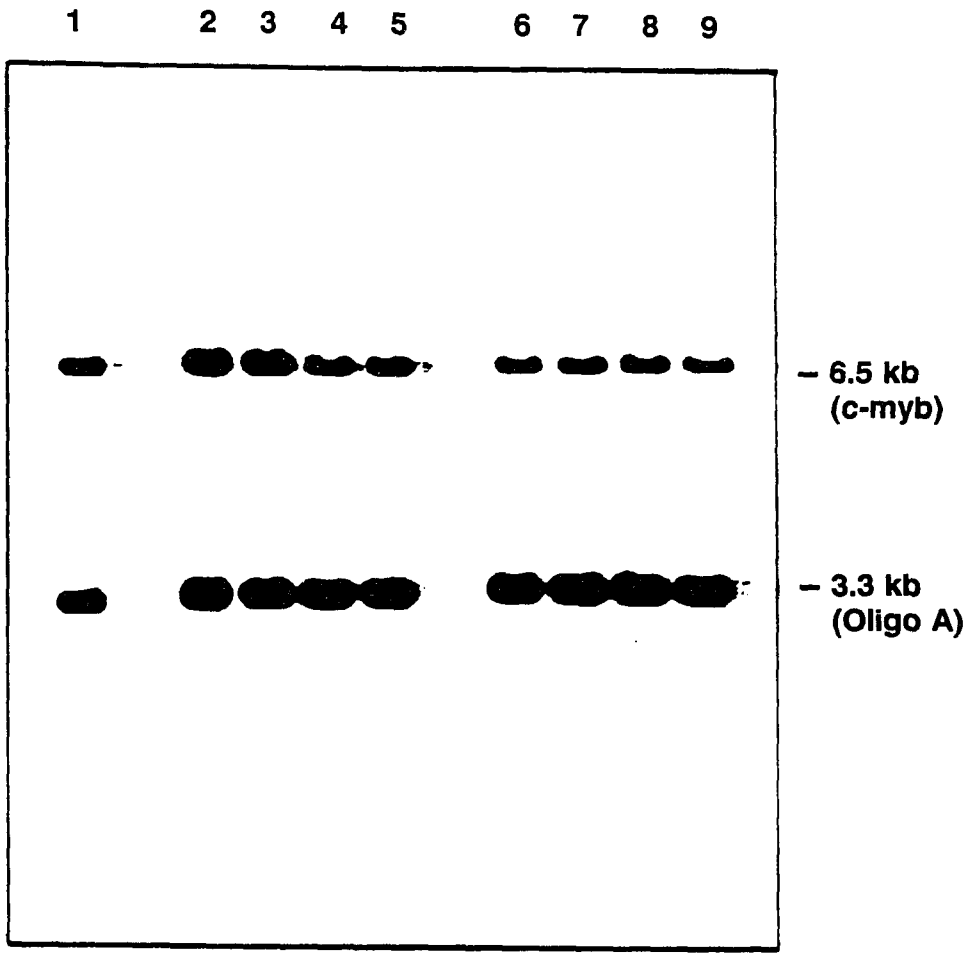


FIGURE 6

FIG. 7. Copy number of *mas* as detected by Southern analysis of preimmortal and immortal SV40 transformants. High-molecular-weight DNA was prepared from different passages of untransformed HS74 cells (lanes 1 and 2) and of SVtsA/HF-A cells. DNA was digested with Bam H1 for analysis by the Southern procedure and hybridized simultaneously with ^{32}P-labeled probes for mas and oligo(A) synthetase which had been radiolabeled separately. The 10.5-kb band of oligo(A) synthetase provides an internal standard for quantitation. Lanes: 1, HS74, P16; 2, HS74, P24; 3, preimmortal SVtsA/HF-A, P2; 4, preimmortal SVtsA/HF-A, P7; 5, immortal SVtsA/HF-A, P21; 6, immortal SVtsA/HF-A, P43; 7, immortal SVtsA/HF-A, P44; 8, immortal HAL cells; 9, immortal AR5.

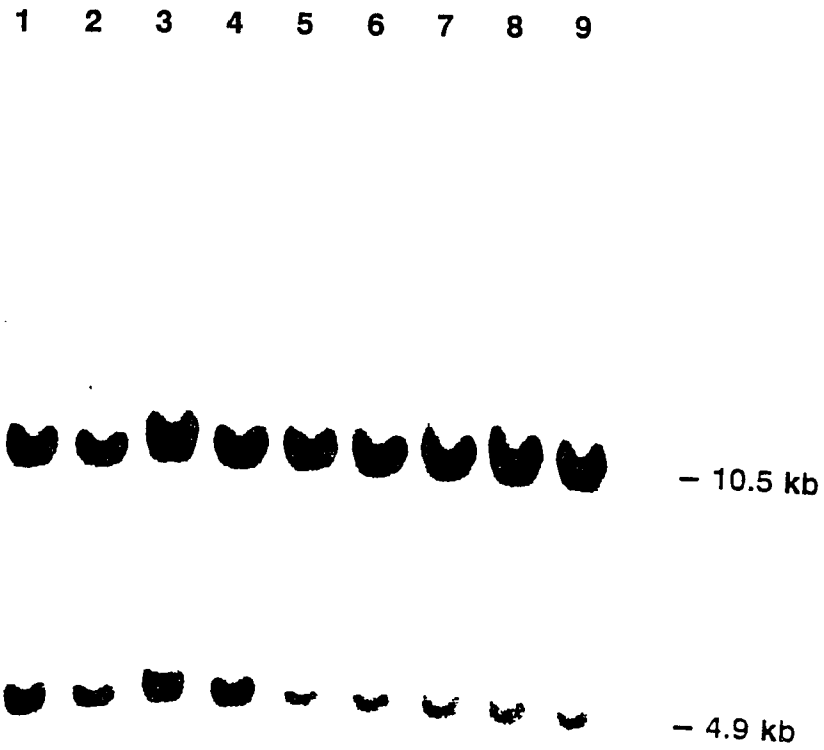


FIGURE 7

revealed by cell hybrid studies (134, and Introduction). Our data are consistent with a mechanism for immortalization requiring (beside continued large T antigen expression) the loss of both copies of a growth suppressor gene (SEN6) residing on 6q. One copy of this putative suppressor is lost together with the chromosomal fragment, while the remaining copy would be lost through inapparent mutation. Alternatively, the cell hybrid studies, and our data, are consistent with a gene dosage effect involving a two-fold reduction of some gene on 6q together with a concomitant change at an unlinked locus (see Discussion).

Telomere changes in normal, and in SV40-transformed pre-immortal and immortal human fibroblasts

The role of SEN6 does not preclude, however, other changes in the cellular genome. At the time the present work was initiated, a number of investigators had noted an apparent correlation between the loss of telomeric DNA and loss of proliferative capacity in a variety of cells. Eucaryotic linear chromosomes terminate in specialized structures called telomeres (157). Telomeres, in turn, end in a tandem series of G-rich repeats which are believed to participate in at least three vital functions. First, addition of telomeric repeats is catalyzed by a novel RNA-dependent DNA polymerase called telomerase, which provides a way around the end-replication problem inherent in all eucaryotic DNA-dependent DNA polymerases. Second, telomeres protect chromosome ends from degradation as well as from fusion and recombination with other chromosome ends. Third, the location of telomeres (proximal to the nuclear periphery) suggests their involvement in the structural organization of the nucleus (158). Vertebrate telomeres have been found to consist of arrays of TTAGGG repeats (159). Internal to these repeats chromosomes contain additional sequences called subtelomeric repeats (160,161,162), which in human cells can be as long as 5 kb (Fig. 8). It is, therefore, possible that reduction of telomeric DNA could ultimately result in the deletion of essential sequences which might, in fact, be the molecular basis for the senescent phenotype of higher eucaryotic cells (see Introduction). This

FIG. 8. Structure of terminal restriction fragments (TRFs). The physical map of chromosome ends that carry the pTH2△ subtelomeric repeat is a modification of that provided by de Lange et al. (161). The Sty I site marked * varies slightly in position at different chromosome ends.

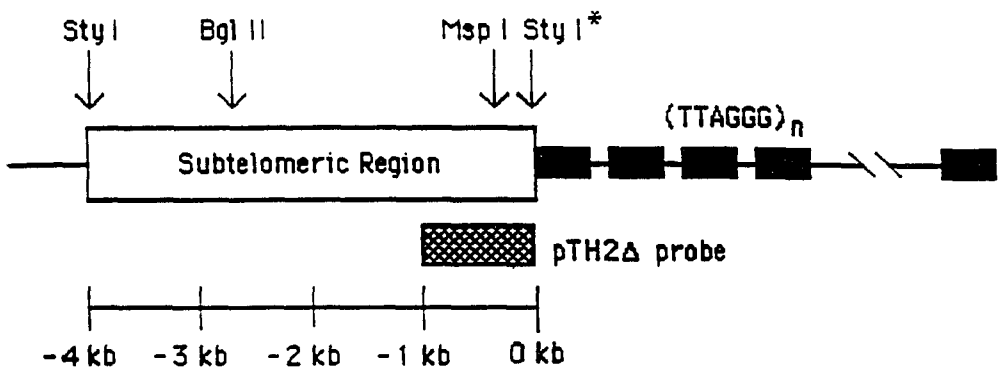

Sty I
Bgl II
Msp I
Sty I*
(TTAGGG)n
Subtelomeric Region
pTH2Δ probe
-4 kb
-3 kb
-2 kb
-1 kb
0 kb

FIGURE 8

hypothesis is based on a number of observations which will be discussed elsewhere (see Discussion).

Reduced telomere sizes have been reported in human somatic cells when compared to telomere sizes in the germ cells from the same individual (161). This observation has been interpreted to indicate an absence of telomerase activity in normal somatic cells (163). It suggests, moreover, that in cells exhibiting unlimited in vitro proliferative potential (e.g. SV40-immortalized HF) a compensatory mechanism (e.g. reactivation of telomerase function) must be in place in order to balance the progressive shortening of telomeres.

In the present study, which is the result of a collaborative effort headed by Dr. Michael B. Small of the Department of Microbiology and Molecular Genetics at UMDNJ-New Jersey Medical School, we have determined the telomere lengths at various passages in the life span of the normal human fibroblast cell line HS74BM and a number of its SV40-transformed pre-immortal and immortal derivatives.

HS74 was grown in culture and DNA isolated from cells at various population doubling (PD) levels. DNAs were digested with the restriction enzyme Bgl II, which cuts outside the telomere repeat sequences (Fig. 8). We determined the distribution of terminal restriction fragment (TRF) sizes for HS74 at passages corresponding to 28 through 64 PD, using the probe pTH2△, a repetitive DNA sequence which hybridizes to the genomic DNA immediately proximal to the telomeric repeat sequence (161). The pTH2△ probe detects sequences present on

several human chromosomes including 7, 16, 17, and 21. The use of pTH2Δ enabled us to assess the length of adjoining telomeres without concern for variation in hybridization signals due to differences in the total amount of TTAGGG repeat sequences.

As shown in Figure 9A and Table 1, the mean TRF length decreases from 11.4 kbp at 28 PD to 9.5 kbp at 64 PD, the latter is a point at which the HS74 cells are known to be senescent (164). A comparable reduction in telomere lengths was observed when another normal human fibroblast cell line, IMR 90, was similarly analyzed (data not shown). As Figure 9A shows, the pTH2Δ probe also hybridizes to discrete bands. These are believed to be repetitive sequences found internally on chromosomes (161), and serve as controls for DNA loading and integrity. To rule out the possibility that reduction in telomere lengths was due to deletions, restriction enzyme polymorphism, or some other alteration in the subtelomeric region itself, the same DNAs were digested with restriction endonuclease Sty I, which cleaves at sequences flanking the subtelomeric region (161, Figure 8). DNA digests were then analyzed for the presence of the subtelomeric region using the pTH2Δ probe. As Figure 9B shows, both early and late passage HS74 yield predominant fragments of approximately 4.7 kbp. Detection of a doublet is consistent with the presence in this region of a previously reported Sty I restriction fragment length polymorphism (161). Our data indicate that the subtelomeric sequences detected by pTH2Δ in our cell lines are invariant in length during serial passage. Consequently, the reduction in telomere length

FIG. 9. Terminal restriction fragment (TRF) length in normal human diploid fibroblasts. High molecular weight (HMW) DNA was prepared from freshly lysed untransformed HS74 at 28, 46, 58, 63, and 64 (terminal passage) population doublings (PD). Two micrograms of each DNA was analyzed by the Southern procedure. Panel A: DNAs were digested with Bgl II and hybridized with a radiolabeled probe for subtelomeric sequences (pTH2△) as described in Materials and Methods. Panel B: DNAs were digested with Sty I and hybridized with the subtelomeric repeat probe pTH2△. The 1-kbp ladder (BRL) was used for size markers. Densitometry was performed over the range of 4.7-20 kbp.

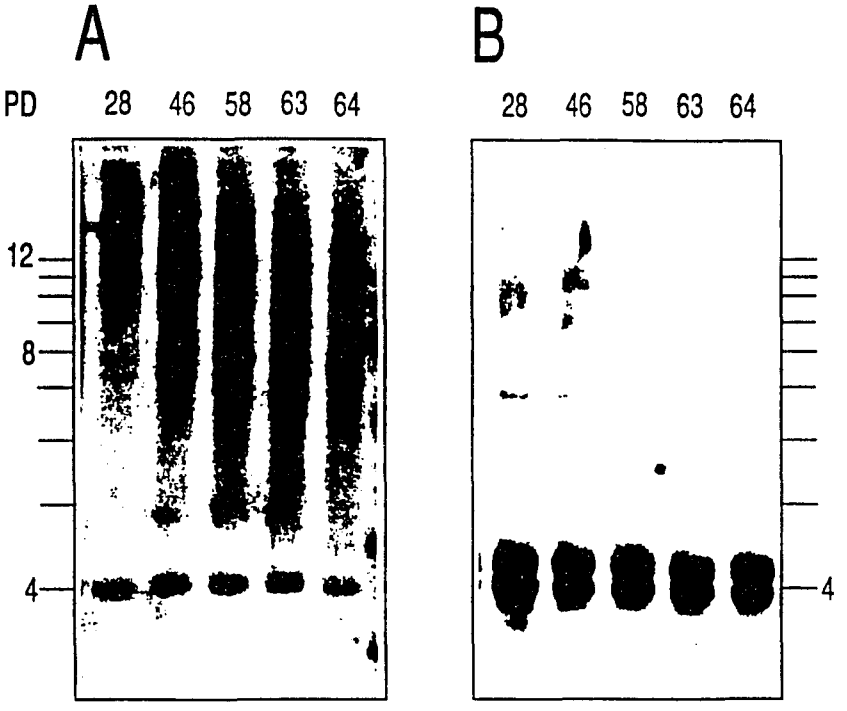


FIGURE 9

Table 1. Telomere length in normal and SV40-transformed HS74 cells

Cell Line	PD	Restriction Enzyme(s)	Probe	Mean Telomere Length or TRF (kbp)
HS74	28	Bgl II	pΔTH2	11.08,11.64
HS74	34	Bgl II	pΔTH2	10.77
HS74	46	Bgl II	pΔTH2	10.22,10.28
HS74	58	Bgl II	pΔTH2	9.50
HS74	63	Bgl II	pΔTH2	9.27,8.30
HS74	64	Bgl II	pΔTH2	9.54
HS74	28	Bgl II	TTAGGG Repeat	9.80
HS74	34	Bgl II	TTAGGG Repeat	10.52
HS74	46	Bgl II	TTAGGG Repeat	9.74
HS74	63	Bgl II	TTAGGG Repeat	8.41
HS74	28	Msp I/Rsa I	TTAGGG Repeat	3.11
HS74	46	Msp I/Rsa I	TTAGGG Repeat	2.51
HS74	58	Msp I/Rsa I	TTAGGG Repeat	2.27
HS74	63	Msp I/Rsa I	TTAGGG Repeat	1.77
SVtsA/HF-C	76	Msp I/Rsa I	TTAGGG Repeat	1.26
SVtsA/HF-C	91	Msp I/Rsa I	TTAGGG Repeat	1.10
SVtsA/HF-C	94	Msp I/Rsa I	TTAGGG Repeat	0.98
SVtsA/HF-C	103	Msp I/Rsa I	TTAGGG Repeat	0.85
SVtsA/HF-A	58	Msp I/Rsa I	TTAGGG Repeat	1.74
SVtsA/HF-A	111	Msp I/Rsa I	TTAGGG Repeat	1.40
SVtsA/HF-A	276	Msp I/Rsa I	TTAGGG Repeat	2.30
AR5	131	Msp I/Rsa I	TTAGGG Repeat	1.94
AR5	168	Msp I/Rsa I	TTAGGG Repeat	2.34
AR5	256	Msp I/Rsa I	TTAGGG Repeat	2.43
SV/HF-5A	54	Msp I/Rsa I	TTAGGG Repeat	1.16
SV/HF-5A	75	Msp I/Rsa I	TTAGGG Repeat	1.37
SV/HF-5A	120	Msp I/Rsa I	TTAGGG Repeat	1.23
SV/HF-5/39	150	Msp I/Rsa I	TTAGGG Repeat	2.00
SV/HF-5/39	213	Msp I/Rsa I	TTAGGG Repeat	2.37
SV/HF-5/39	264	Msp I/Rsa I	TTAGGG Repeat	1.94
SV/HF-5/39	273	Msp I/Rsa I	TTAGGG Repeat	2.27
SV/HF-5/39	360	Msp I/Rsa I	TTAGGG Repeat	1.86

PD, population doublings

TRF, terminal restriction fragment (Bgl II-digested DNA containing subtelomeric sequences appended to telomere TTAGGG repeats)

observed in normal cells upon serial passage represents a bona fide loss of telomeric repeat sequences.

The use of the pTH2△ probe in our Southern analysis of Bgl II-digested DNAs has corroborated previous observations of telomere shortening with in vitro serial passage of human fibroblasts. The pTH2△ probe, however, is homologous to only a subset (10% to 25%) of human chromosome ends (161), making it conceivable that reduction of TRF length applies merely to some chromosomes. Moreover, quantitation of mean TRF length based upon this analysis might underestimate the contribution of much smaller telomeres appended to subtelomeric DNA, since these would fail to be resolved by our electrophoretic conditions. Therefore, in order to determine directly the actual reduction in telomeric sequences, we repeated our analyses using as a probe the telomeric-repeat oligonucleotide $(TTAGGG)_5$, and double digests of our DNAs with the four-base pair recognition enzymes Msp I and Rsa I. This approach was based on the assumption that these enzymes, each of which is predicted to cleave at random once in every 256 (4^4) bp, would produce TRF consisting predominantly of tandem arrays of TTAGGG repeats. The subtelomeric region, in fact, has an Msp I site some 230 bp proximal to the TTAGGG telomere repeat sequences (161, Figure 8). As shown in Figure 10 and Table 1, mean telomere length (MTL) decreases from 3.1 kbp at 28 PD to 1.8 kbp at 63 PD, and is accompanied by the loss of up to 35% of total telomeric DNA over the same number of PDs. Graphical representation of the data (Figure 11A) predicts a MTL of 4.1 kbp

FIG. 10. Telomere length in normal human diploid fibroblasts. HMW DNA was prepared from untransformed HS74 at 28, 46, 58, and 63 PD. DNAs were co-digested with Msp I and Rsa I and analyzed by the Southern procedure. A radiolabeled telomere repeat probe $(TTAGGG)_5$ was used for hybridization as described in Materials and Methods. Size markers (1 kbp ladder) range from 0.5, 1.0, 1.6, and 2-12 kbp in steps of 1 kbp. Densitometry was performed over the range from 0.3-23 kbp.

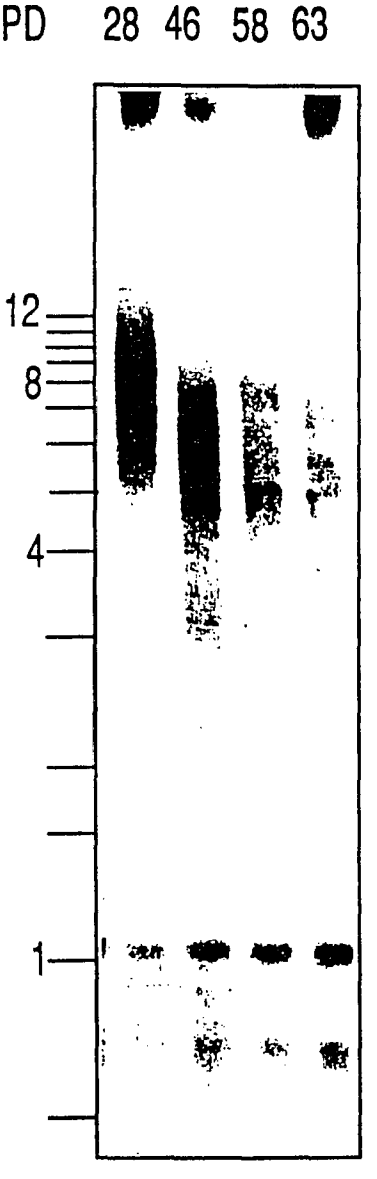
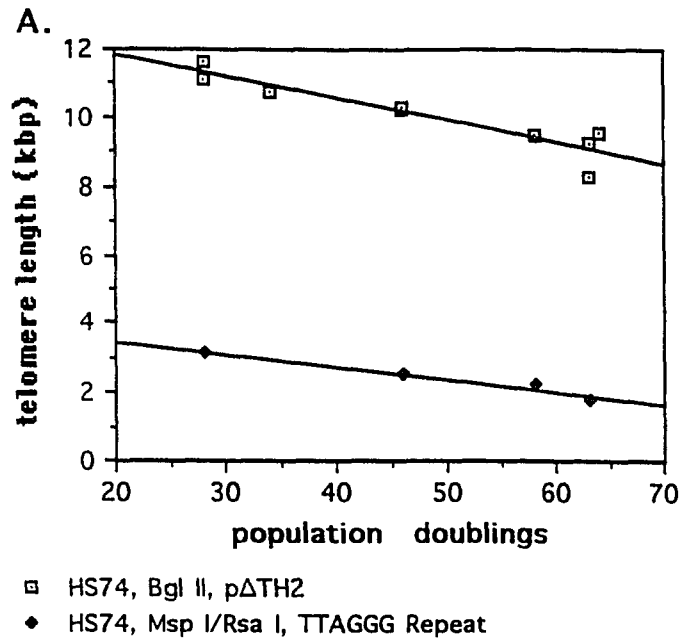


FIGURE 10

FIG. 11. Rate of telomeric sequence loss in normal HS74 human fibroblasts. Linear best fit lines were determined using Cricket Graph v.1.2. A coefficient of determination (R^2) of 1.0 indicates a perfect fit. The slope of the line reflects the rate of telomeric DNA loss per population (PD). Panel A. Rate of loss for Bgl II-digested DNAs probed with pTH2Δ (open squares) is 63 bp per PD (R^2 = 0.87) vs 35 bp per PD (R^2 = 0.94) for Msp I/Rsa I-digested DNAs probed with $(TTAGGG)_5$ (filled diamonds). Panel B. Rate of loss for Bgl II-digested DNAs probed with $(TTAGGG)_5$ (filled squares and diamonds) is 39 bp per PD (average of two sets of data; R^2 = 0.95) vs 35 bp per PD (R^2 = 0.94) for Msp I/Rsa I-digested DNAs probed with $(TTAGGG)_5$ (open squares).


A.
telomere length (kbp)
12
10
8
6
4
2
0
20
30
40
50
60
70
population doublings
HS74, Bgl II, pΔTH2
HS74, Msp I/Rsa I, TTAGGG Repeat

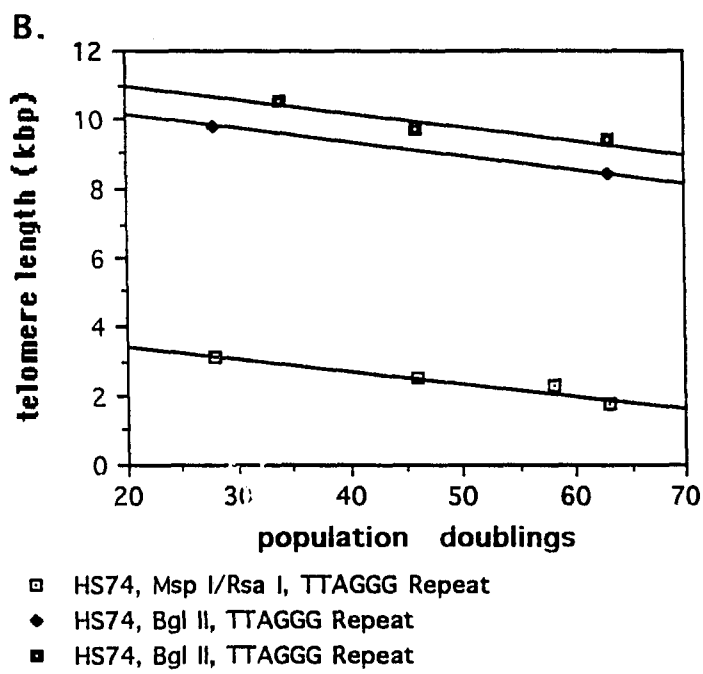

B.
telomere length (kbp)
12
10
8
6
4
2
0
20
40
50
60
70
population doublings
HS74, Msp I/Rsa I, TTAGGG Repeat
HS74, Bgl II, TTAGGG Repeat
HS74, Bgl II, TTAGGG Repeat

FIGURE 11

for HS74 at the beginning of its life span. The data also indicate that on average 35 bp are lost per PD, as has been previously reported (165). In HS74 at PD 28, telomere lengths greater than 7 kbp make a significant contribution to total optical density (approximately 25%), suggesting marked heterogeneity in either sets of chromosomes within individual cells, or among cells within the population.

Comparison of the data obtained for the same population doublings using either Bgl II or Msp I/Rsa I-digested DNAs (Figure 11A), reveals a discrepancy in the predicted rate of telomere loss per generation (63 bp for Bgl II versus 35 bp for Msp I/Rsa I). This discrepancey might reflect the loss of subtelomeric sequences. However, our initial data did not indicate this to be likely. In order to resolve this issue, we reprobed blots of Bgl II-digested HS74 DNAs with the $(TTAGGG)_5$ repeat probe. As indicated in Figure 11B and Table 1, the rate of decrease in telomere length for Bgl II-digested DNAs (39 bp per generation) is not significantly different from that determined for the Msp I/Rsa I-digested DNAs. This concordance supports our original contention that the decrease in telomere length can be accounted for by the loss of telomeric repeat sequences alone. The MTL predicted by subtraction of the subtelomeric sequences (3.1 kbp) from the densitometric values obtained for pTH2Δ -probed Bgl II-digested DNAs differed substantially from the MTL determined for TTAGGG-probed Msp I/Rsa I DNAs. This variation was detected even when Bgl II and Msp I/Rsa I digested DNAs were analyzed with the same probe. We have attributed this difference to anomalous

electrophoretic mobility caused by the appended subtelomeric sequences. This conclusion is supported by the fact that DNAs digested with Pvu II, which leaves less subtelomeric DNA (1.6 kb) attached to the telomeric repeat sequences than Bgl II, display a less pronounced variation (data not shown). The difference between predicted and observed telomere length might also be explained by hybridization of the $(TTAGGG)_5$ probe to internal telomere-related sequences (166,167), or to heterogeneity in the lengths of subtelomeric sequences other than that detected by pTH2△. In order to avoid the complications suggested by these results, we chose to use exclusively the Msp I/Rsa I-digested DNAs probed with the $(TTAGGG)_5$ telomere repeat probe in all our subsequent analyses.

We next examined the effect of both extended life span and immortalization on telomere length in several SV40-transformed derivatives of HS74. These cells were generated by transfection of HS74 with replication-defective SV40 genomes encoding either a wild-type (SV/HF-5A, SV/HF-5/39) or a temperature-sensitive T antigen (SVtsA/HF-C, SVtsA/HF-A, SVtsAdl/HF-4, AR5). All SV40 transformants were analyzed at PD levels which were either near the end or beyond the life span limit of HS74.

The SVtsA/HF-C cell line is characteristic of SV40-transformed human fibroblasts in exhibiting an extended, but finite, life span (see Introduction). MTLs were determined after Msp I/Rsa I digestion of DNAs isolated from serially passaged SVtsA/HF-C as already described for HS74. As indicated in Table 1 and Figure 12, there was a further reduction in the length of telomeric sequences

FIG. 12. Telomere length in pre-immortal SVtsA/HF-C cells. HMW DNA was prepared from SVtsA/HF-C at 76, 91, 94, and 103 (terminal passage) PD. DNAs were co-digested with Msp I and Rsa I and hybridized with the telomere repeat probe $(TTAGGG)_5$. Size markers (1 kb ladder) range from 0.3, 0.4, 0.5, 1.0, 1.6, and 2-12 kbp in steps of 1 kbp. Densitometry was performed over the range from 0.2-22 kbp.

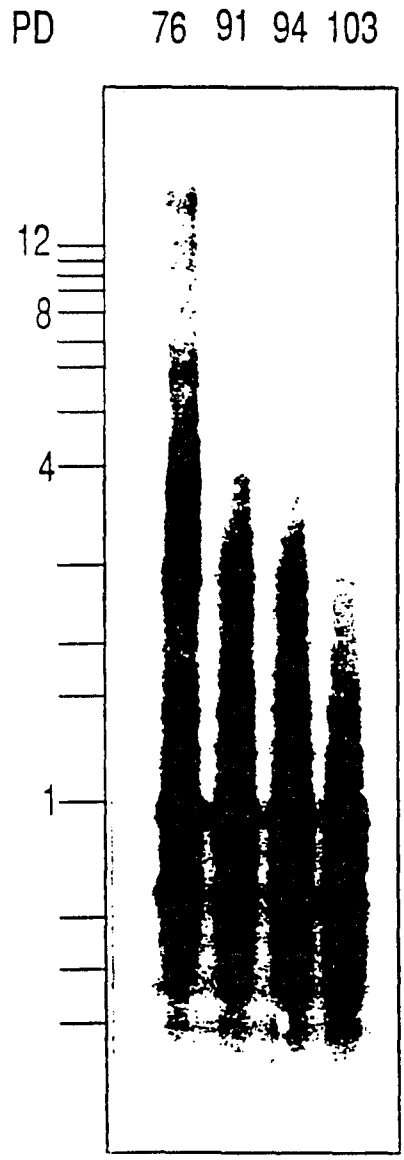


FIGURE 12

with increasing PD level. MTLs decreased from 1.3 kbp at 76 PD to 0.9 kbp at 103 PD. Interestingly, the rate of shortening in SVtsA/HF-C, as shown in Figure 13A, closely follows that in the normal parent HS74. Therefore, it appears that the mechanisms responsible for telomere reduction remain fully operant within the period of extended life span resulting from transformation by SV40. Thus senescence in HS74 is not due to the attainment of a terminal MTL. Furthermore, the extended life span of SV40 transformants does not result from the in vitro selection for a subpopulation of cells with longer telomeres.

Similar analyses were performed with an independently derived pre-immortal SV40 transformant, SVtsA/HF-A. As has already been noted, SVtsA/HF-A is an atypical transformant in that immortalization occurred with no apparent intervening "crisis". The growth characteristics of these cells have been described in detail elsewhere (136,164). Passages older than 96 PD are populated largely by immortal cells. DNAs were prepared from SVtsA/HF-A at passages ranging from 58 to 276 PD. MTLs were determined for Msp I/Rsa I-digested DNAs using the telomere repeat probe $(TTAGGG)_5$. As indicated in Table 1 and Figure 14, SVtsA/HF-A cells (58-111 PD) show a further reduction in telomere length when compared to late passage HS74 (64 PD), with a MTL decrease from 1.7 kbp at 58 PD to 1.4 kbp at 111 PD. Reduction in TRF length was also observed upon serial passage of SVtsAdl/HF-4, an independently derived SV40 pre-immortal transformant generated from another subline of HS74. SVtsAdl/HF-4 encodes large T antigen but not small t antigen, and no immortal cell

FIG. 13. Telomere alterations in normal HS74 and SVtsA-transformed pre-immortal derivatives. The linear best fit line was determined using Cricket Graph v.1.2. Panel A. Untransformed HS74 (open squares), pre-immortal SVtsA/HF-C (open circles), pre-immortal (58 and 111 PD) and immortal (276 PD) SVtsA/HF-A (filled triangles), immortal AR5 (filled circles). Panel B. Untransformed HS74 (open squares), pre-immortal SV/HF-5A (filled diamonds), immortal SV/HF-5/39 (filled squares). Dotted lines reflect hypothetical alterations in telomere length for related SV40-transformed cell lines during their transition from pre-immortal to immortal phenotypes.

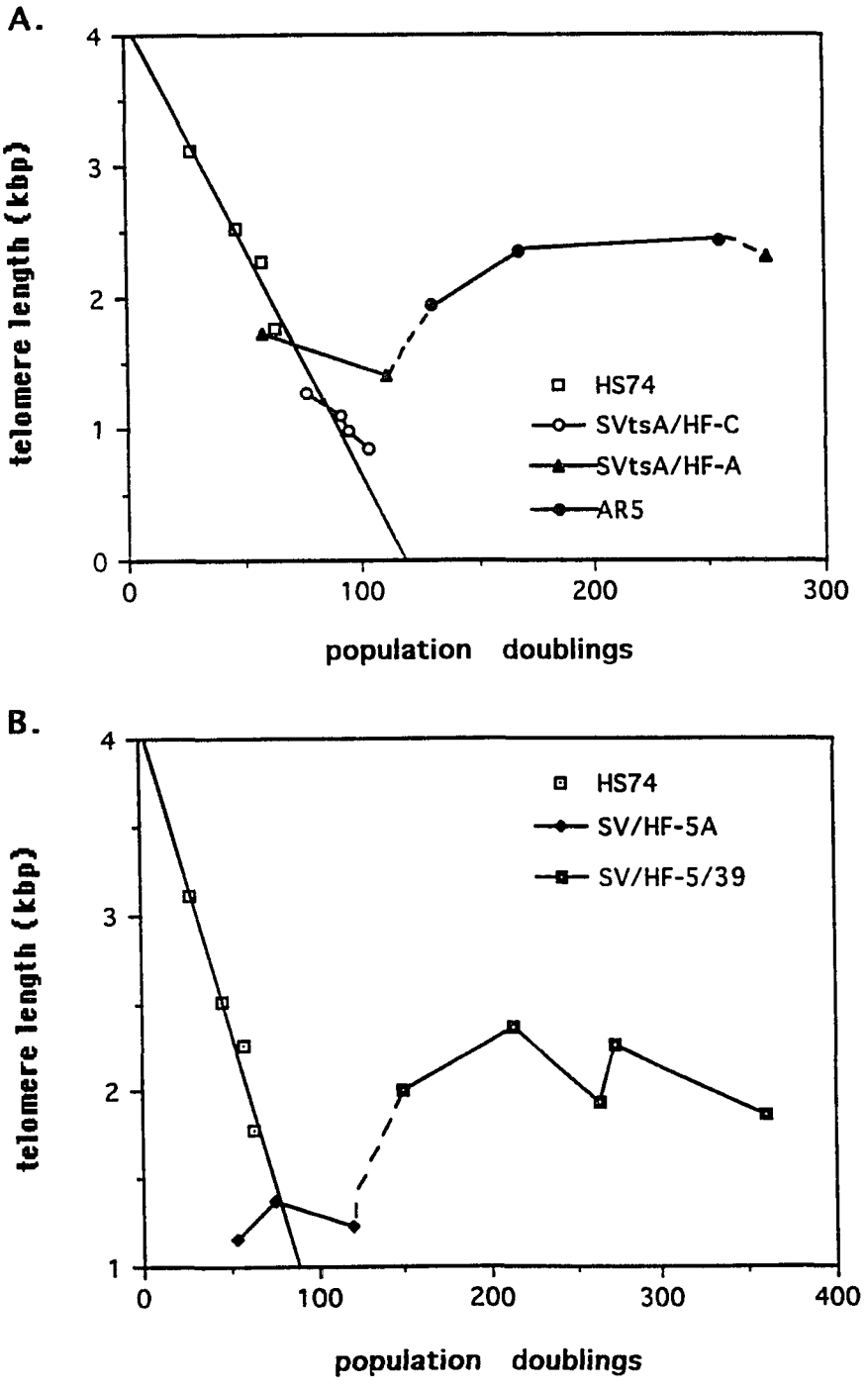

A.
telomere length (kbp)
4
3
2
1
0
0
100
200
300
population doublings
HS74
SVtsA/HF-C
SVtsA/HF-A
AR5
B.
telomere length (kbp)
4
3
2
1
0
100
200
300
400
population doublings
HS74
SV/HF-5A
SV/HF-5/39

FIGURE 13

FIG. 14. Telomere length in SVtsA-transformed pre-immortal and immortal cells. HMW DNA was prepared from SVtsA/HF-A at 58 (pre-immortal), 111 and 276 (immortal) PD, and AR5 (immortal) at 131, 168, and 256 PD. DNAs were co-digested with Msp I and Rsa I and hybridized with the telomere repeat probe $(TTAGGG)_5$. Size markers (1 kb ladder) are as described in legend to Figure 9. Densitometry was performed over the range from 0.3-24 kbp.

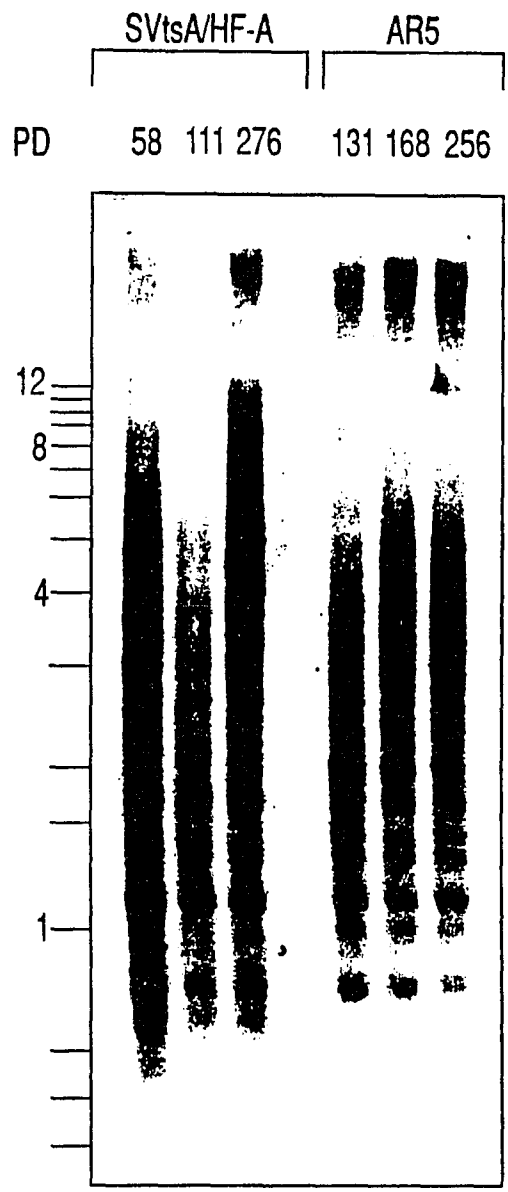


FIGURE 14

lines have yet been isolated from it (Fig. 15, and Materials and Methods). Our data, therefore, consistently show that telomere reduction is a continuing process in SV40-transformed human fibroblasts despite their temporarily extended life span. The rate of shortening of telomeres in SVtsA/HF-A is slower than that observed in SVtsA/HF-C (Figure 13A). The earliest passage of SVtsA/HF-A analyzed (58 PD) has a MTL similar to that of HS74 at a comparable age. The deviation from predicted rate of telomere reduction in SVtsA/HF-A at 111 PD may reflect an increasing preponderance of immortal cells in this population (see below).

With continued passage of SVtsA/HF-A (276 PD), MTL increases (Figure 14, and Table 1) from 1.4 kbp to 2.3 kbp (compare 111 PD to 276 PD). The fact that these cells, despite having undergone more population doublings, contain larger telomeres, suggests that a reactivation of telomere maintenance mechanisms may have occured. In order to explore further this possibility, we determined the telomere sizes in AR5, an immortal line clonally isolated from SVtsA/HF-A at early passage (see Materials and Methods). AR5 cells at 131 PD (Figures 13A, 14, and Table 1) exhibit a decreased MTL (1.9 kbp) relative to late passage HS74 (2.3 kbp at 58 PD), but have slightly longer telomeres than the pre-immortal SVtsA/HF-A cells at 111 PD. As Figure 14 and Table 1 indicate, passage of AR5 beyond 131 PD results in an increase in MTL to 2.4 kbp at 256 PD. The filter corresponding to Figure 14 was reprobed for the single-copy gene oligo(A) synthetase (155, 164) in order to rule out DNA degradation or incomplete digestion as the reason for the observed results. No degradation was observed (data not shown).

FIG. 15. Terminal restriction fragment (TRF) length in SVtsA-transformed fibroblasts. HMW DNA was prepared from untransformed HS74 at 28 and 64 PD; SVtsA/HF-A at 58, 63, 68 (pre-immortal), 111 and 276 PD (immortal); SVtsAdl/HF-4 at 62 and 85 PD; AR5 (immortal) at 131, 168, and 256 PD. DNAs were digested with Bgl II and hybridized with the subtelomeric repeat probe pTH2△. The 1kbp ladder was used for size markers.

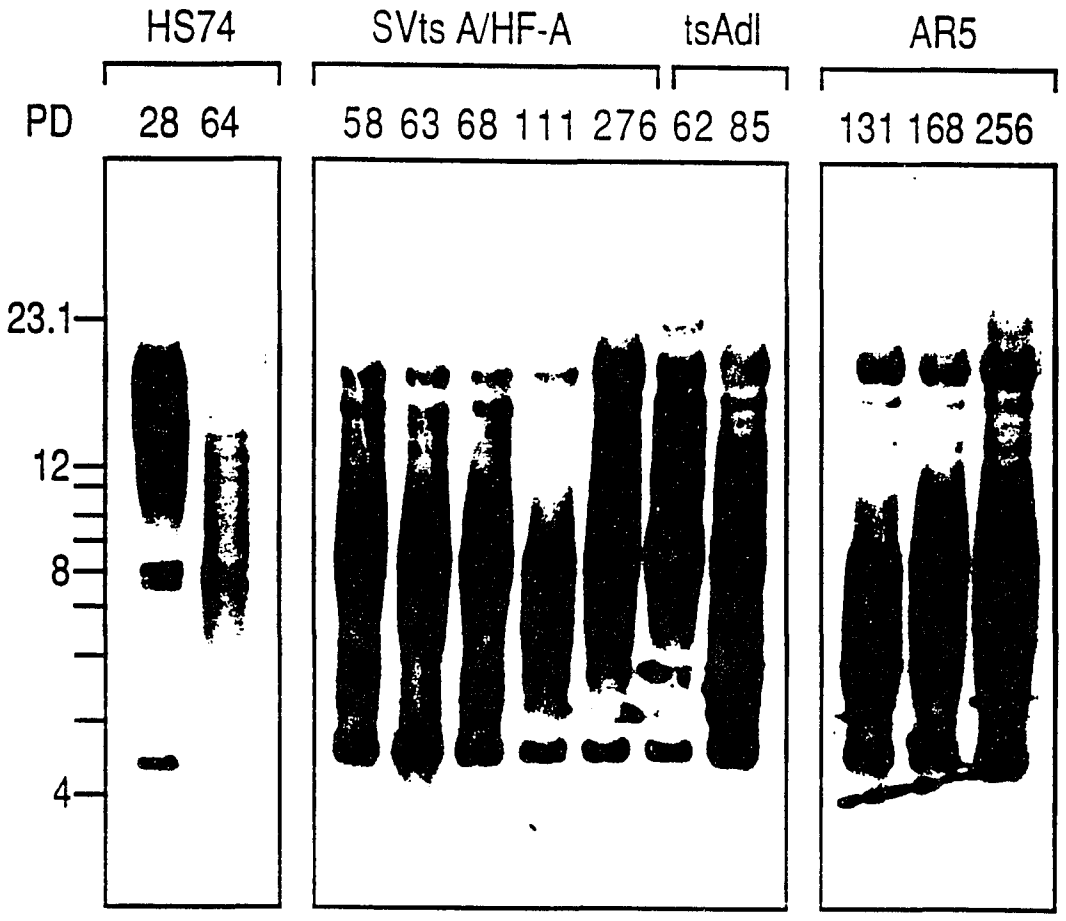

HS74
SVts A/HF-A
tsAdl
AR5
PD
28 64
58 63 68 111 276 62 85
131 168 256
23.1
12
8
4

FIGURE 15

Since the preceding experiments were performed with cells transformed by a temperature sensitive large T antigen which may be functionally diminished in some unexpected manner, we also examined an independent line of transformants which express a non-temperature sensitive large T (and small t) antigen. SV/HF-5/39 is an immortal cell line derived from the pre-immortal transformant SV/HF-5A as previously described (138). DNAs were isolated from SV/HF-5A at 54, 75, and 120 PD (terminal passage), and from SV/HF-5/39 at 150, 213, 264, 273, and 360 PD. SV/HF-5A cells show a marked decrease in MTL when compared to the parental HS74 cells at their terminal passage (Figure 16). No significant further shortening of telomeres is observed in SV/HF-5A from 54 to 120 PD (Figure 13B), indicating (as our laboratory had previously ascertained by other means) that by 54 PD this population was composed predominantly of immortal cells. Immortal SV/HF-5/39 displays a striking heterogeneity of telomere length distribution (Figure 16, 150-360 PD). Nontheless, the salient feature here (apparent even at the earliest analyzed passage) is a marked increase in telomere sizes, particularly in the range above 5 kbp. Densitometric analysis (Table 1, and Figure 13B) supports this interpretation.

In the present study we demonstrate that telomeres shorten upon serial passage of normal untransformed, as well as SV40-transformed pre-immortal fibroblasts. Our studies also document an interesting increase in telomere length in the immortalized SV40 transformants, suggesting that immortalization induces (or is partly dependent on) a reactivation of mechanisms for telomere stabilization.

FIG. 16. Telomere length in wildtype SV40-transformed fibroblasts. HMW DNA was prepared from untransformed HS74 at 33 and 63 PD; SV/HF-5A (pre-immortal) at 54, 75, and 120 (terminal passage) PD; SV/HF-5/39 (immortal) at 150, 213, 264, 273, and 360 PD. DNAs were co-digested with Msp I and Rsa I and hybridized with the telomere repeat probe $(TTAGGG)_5$. The 1 kb ladder was used as size markers. Densitometry was performed over the range of 0.35-24 kbp.

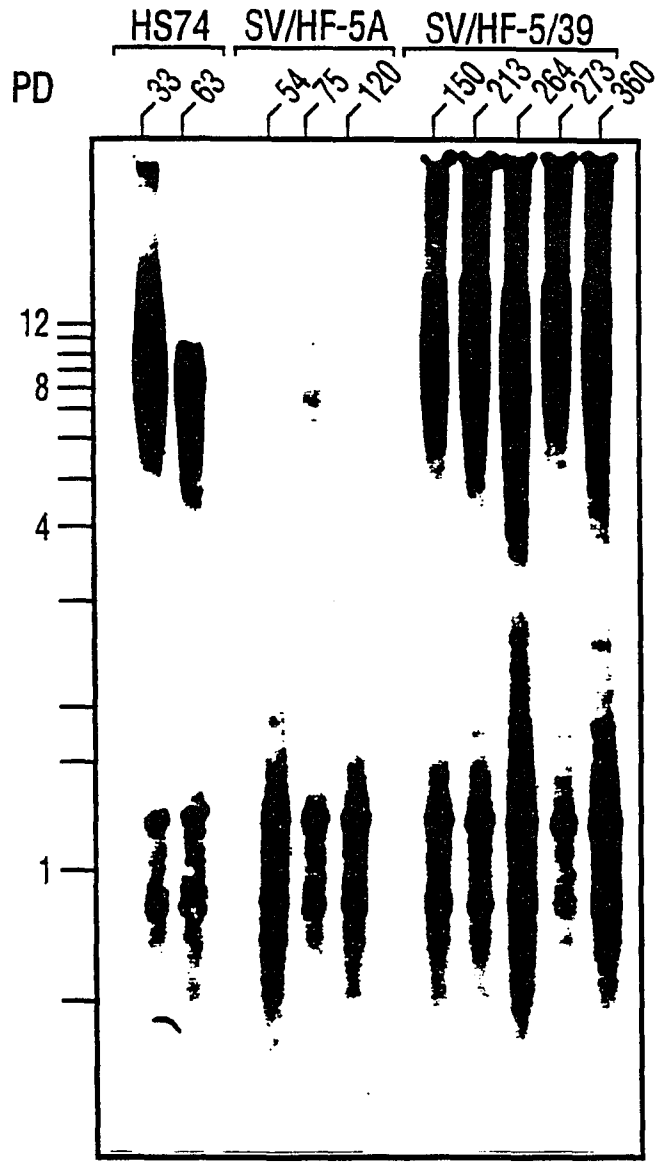

HS74
SV/HF-5A
SV/HF-5/39
PD
33
63
54
75
120
150
213
264
273
360
12
8
4
1

FIGURE 16

CHAPTER 4

RESULTS

Identification by Subtractive Hybridization of Differentially Expressed Genes

The attrition of telomeric sequences which occurs during the replicative lifespan of normal and SV40-transformed preimmortal HDF has been implicated in the loss of proliferative potential which these cells eventually undergo (161,163, and this thesis). The restoration of telomere maintenance mechanisms in immortal cells suggests, moreover, that these processes might be of importance to the emergence of the immortal phenotype.

Our laboratory (and others) has shown that SV40 large T antigen while being absolutely required for the continued viability and proliferation of immortal as well as of preimmortal HDF, does not seem in itself to be sufficient for immortalization of the SV40 transformants. The extremely low frequency of immortalization of SV40-transformed HF provides a compelling argument for the requirement for additional genetic alterations, most likely involving inactivation of both copies of a cellular growth suppressor gene such as SEN6 which was described in the previous chapter. SEN6 would represent the "primary effector" whose action must be overcome in order for the cell to bypass the theoretical mortality stage 2 (i.e. M2, see Introduction) block to attainment of unlimited

proliferative potential (135,136,164) in SV40-transformed HS74. While inactivation of both copies of the putative growth suppressor need not entail disappearance of its mRNA, it is likely, however, that functional loss of this primary effector could have qualitative as well as quantitative repercussions on the transcriptional activity of other downstream or "secondary effector" genes. Identification and analysis of such differentially expressed secondary effectors might in turn provide insights into the nature of the molecular mechanisms responsible for the immortalization of SV40-transformants.

In order to identify members of this set of genes, I have prepared a cDNA library in λgt10 from the preimmortal SVtsA/HF-C cells to use in a subtractive hybridization strategy (see Materials and Methods). Molecular subtraction has been used successfully by a number of investigators to isolate genes that are exclusively or predominantly expressed in one cell type or developmental stage versus another (168,169,170). In the usual protocol, cDNAs derived from the cell type of interest (i.e. tracer cDNA) are hybridized in solution with an excess of mRNA isolated from a closely matched cell type (i.e. driver mRNA). Sequences expressed in both cell populations will result in the formation of cDNA-mRNA heteroduplexes during the course of hybridization. On the other hand, tracer cDNAs derived from mRNAs which are exclusively (or predominantly) expressed only in the cells from which the tracer was prepared will remain single-stranded. Such single-stranded cDNAs can be isolated from the heteroduplex population by a technique such as hydroxylapatite chromatography which retains preferrentially

the double-stranded species (see Materials and Methods). A probe prepared from the isolated unhybridized tracer cDNA can then be used to screen a cDNA library in order to identify differentially expressed genes. The use of subtracted probes offers a number of advantages, among which are the elimination of abundant common sequences with its concomitant enrichment for those of the rarer classes. Such probe "simplification" makes possible longer autoradiographic exposures and thus the visualization of cDNAs originating from mRNAs of the lower abundance classes.

I have used a cDNA library derived from preimmortal SVtsA/HF-C in a subtractive hybridization experiment in order to identify genes which are differentially expressed in SVtsA/HF-C versus immortal AR5 (see Fig. 3 in Materials and Methods). Subtractive hybridization can be carried out in either of two "directions" to identify gene sequences overexpressed in either the preimmortal or the immortal cells. My efforts were initially directed at the identification of mRNAs whose levels are reduced in the immortalized cells (however, see below). This emphasis reflects our premise that loss-of-function is most consistent with the recessive nature of the immortal phenotype as demonstrated by cell hybrid studies. We are, nonetheless, not overlooking the possibility that loss of a regulatory function (a primary effector) might lead to enhanced expression of other "downstream" (or secondary effector) genes, and that mRNA species overexpressed in the immortal cells may be of importance to the immortalization process.

A tracer-labeled single-stranded cDNA was prepared from 4μg of poly(A)$^+$ mRNA derived from preimmortal SVtsA/HF-C cells, and was carried through two cycles of solution hybridization, using each time a 20-fold weight excess (i.e. 80μg) of "driver" poly(A)$^+$ mRNA derived from immortal AR5 cells. The hybridization solutions were chromatographed after each cycle on hydroxylapatite (i.e. the subtraction step) in order to enrich for sequences overexpressed in the preimmortal cells. The recovered unhybridized single-stranded material (~3% of input cDNA) was concentrated under vacuum, and applied to pre-equilibrated Sephadex G-50 columns in order to desalt and also to remove small double-stranded fragments which are not bound efficiently by hydroxylapatite. The resulting twice-subtracted SVtsA/HF-C cDNA was radiolabeled to high specific activity using random primers, and used to screen 10,000 plaques of a λgt10 cDNA library prepared from SVtsA/HF-C (see Materials and Methods). A replicate set of filter "lifts" (10, 150mm nylon circles) was hybridized with a total cDNA probe prepared from 4μg of poly(A)$^+$ mRNA derived from HAL cells in order to indentify and rule out sequences which are expressed at high levels in these immortal cells. Autoradiography of the latter set of filters could only be carried out for one day due to the intensity of the signal generated by the total cDNA probe. Comparison of both sets of autoradiograms led to the selection of 73 plaques for further analysis. Recombinant phage DNA was prepared from each of the chosen plaques, and the cDNA inserts were amplified by the polymerase reaction (PCR) as described in detail in Materials and Methods. Due to cross contamination by neighboring

plaques, about one third of the DNAs generated multiple cDNA inserts upon amplification by PCR (Fig. 17). Individual cDNA fragments were purified and used as probes in Northern analysis in order to verify the differential expression of their cognate mRNAs. The Northern blots used in these analyses contain approximately 10μg of each of three total RNA populations run in adjacent lanes and transferred onto nylon strips. These three RNAs were prepared from preimmortal SVtsA/HF-C and the two immortal cell lines HAL and AR5 (see Materials and Methods). The amounts of the RNAs used in the Northern blot experiments were equalized with reference to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a gene which is expressed at similar levels in a wide variety of cell lines and is commonly used as an internal standard for quantitation in Northern analysis (Fig. 18).

Such Northern analyses have revealed a number of cDNA clones which are differentially expressed in the preimmortal SVtsA/HF-C versus either or both of the two immortals HAL and AR5. These cDNA clones can be grouped into distinct sets on the basis of several criteria. One group, which consists of sequences overexpressed in the preimmortal cells, can be further divided into two classes on the basis of the abundance of the corresponding mRNAs in this RNA population. Low abundance mRNA often make it necessary to use poly(A)$^+$ mRNA (2μg per lane) on the Northern blots in order to generate detectable autoradiographic signals. Two representatives of the high abundance class which are overexpressed in the preimmortal SVtsA/HF-C cells appear in Figure 19. Two

FIG. 17. Sample PCR amplifications of cDNA inserts from plaques picked during subtractive hybridization experiment. The primers used are homologous to flanking λgt10 sequences. Contamination by neighbors leads sometimes to amplification of more than one cDNA insert per isolated plaque. Overall cDNA insert sizes range from 0.5kb to 2.0kb.

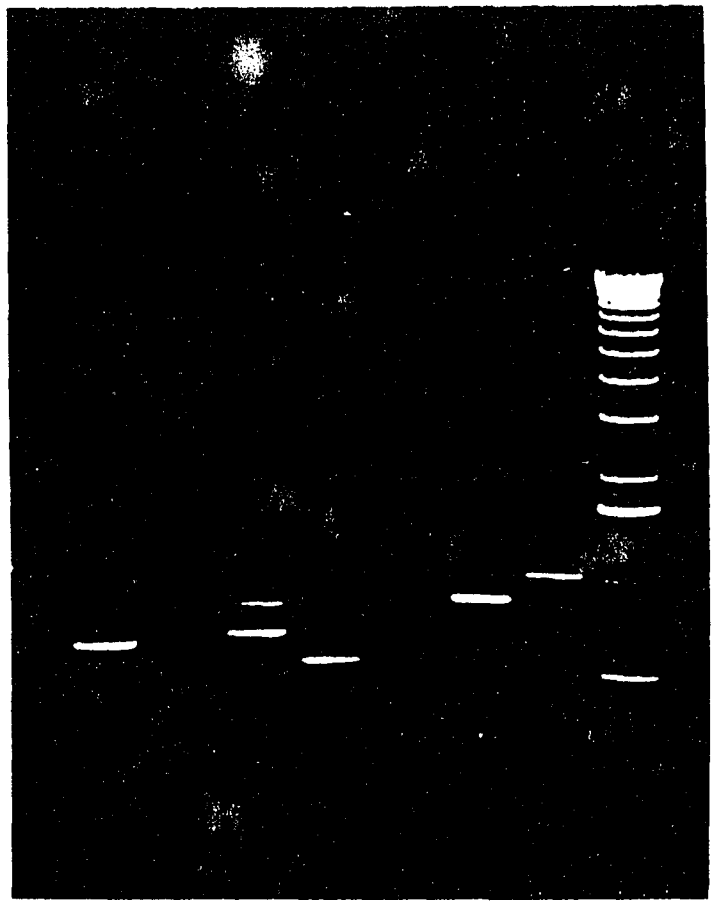


FIGURE 17

FIG. 18. Demonstration of equivalence of RNA loading when referenced to the levels of GAPDH standard in a typical Northern filter used in the verification of differential expression of genes selected during the subtractive hybridization experiment.

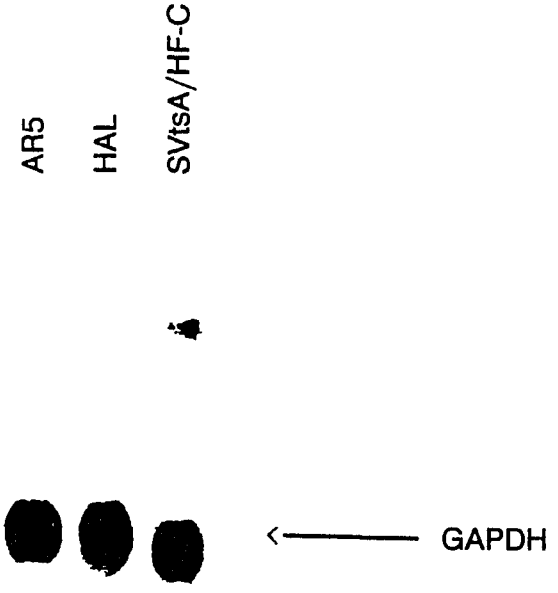


FIGURE 18

FIG. 19. Total RNA Northerns featuring two gene sequences, representative of the high abundance class, which are overexpressed in preimmortal SVtsA/HF-C relative to immortal HAL and AR5.

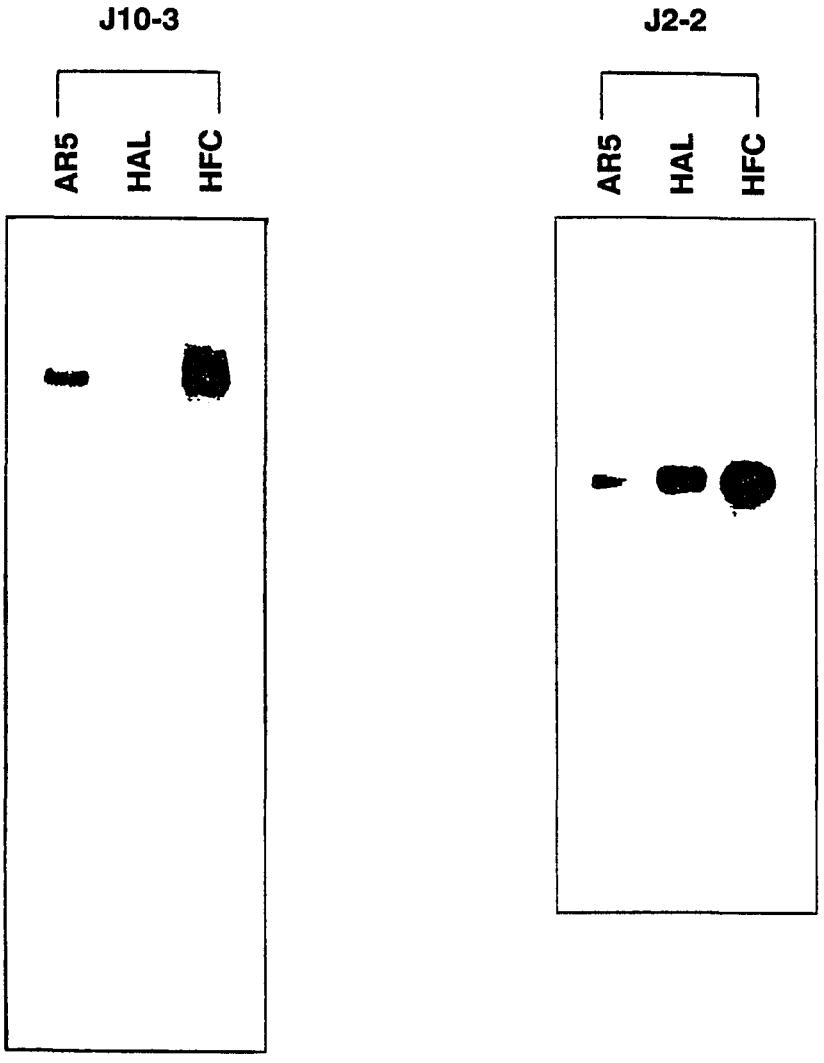


FIGURE 19

members of the low abundance class [i.e. poly(A)$^+$ mRNA blots] overexpressed in the preimmortal cells appear in Figure 20. The analysis of clones requiring poly(A)$^+$ mRNA blots has so far been confined to a comparison of expression levels between SVtsA/HF-C and AR5 (i.e. has not yet been extended to include HAL).

A second group of clones represents sequences which are overexpressed in both AR5 and HAL relative to the preimmortal SVtsA/HF-C (see Discussion). To date, no member of this group has been found to belong to the low abundance class. Representatives of this set appear in Figures 21, and 22. The clone J3-6A (Figure 22) is different in that it shows higher levels of expression in the immortal (AR5) than in preimmortal SVtsA/HF-C, but is overexpressed in SVtsA/HF-C relative to the second immortal (HAL).

A number of promising cDNA clones were subcloned into pGEM3, and partial sequencing was carried out in both directions by the dideoxy chain termination method using as primers the flanking SP6 and T7 promoter sequences (see Materials and Methods). GenBank homology searches were performed using the EuGene system at UMDNJ-New Jersey Medical School. This information was sought in order to identify which cDNAs represent novel (not yet described) gene sequences, and which show complete or partial homology to known genes or gene families. This is, of course, subject to the limitation that our cDNA clones largely represent the 3' ends of the corresponding mRNAs.

FIG. 20. Poly(A)$^+$ mRNA Northerns (2μg per lane) featuring two gene sequences, members of the low abundance class, which are overexpressed in preimmortal SVtsA/HF-C relative to immortal AR5.

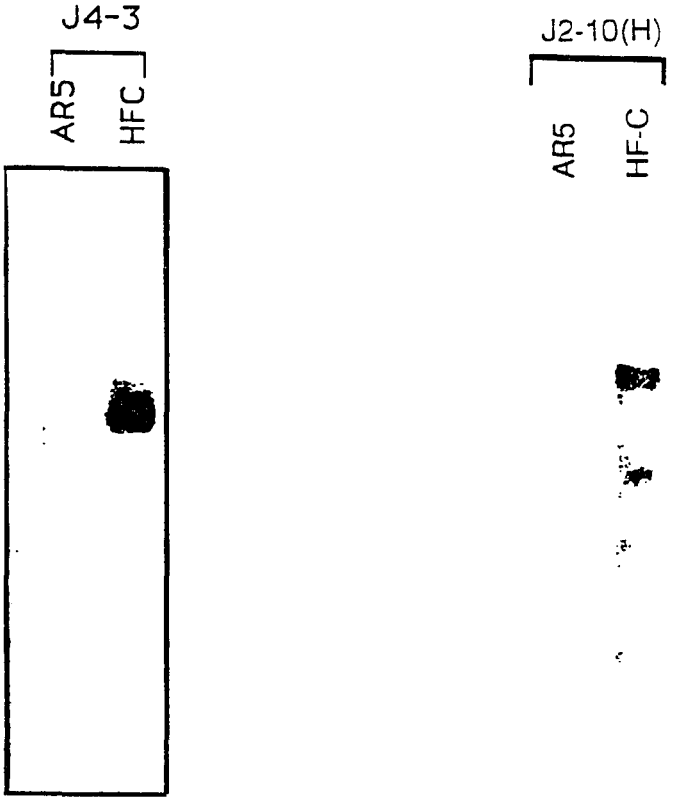


FIGURE 20

FIG. 21. Total RNA Northern blots featuring two members of a group of gene sequences which are overexpressed in the immortal cell lines HAL and AR5 relative to preimmortal SVtsA/HF-C.

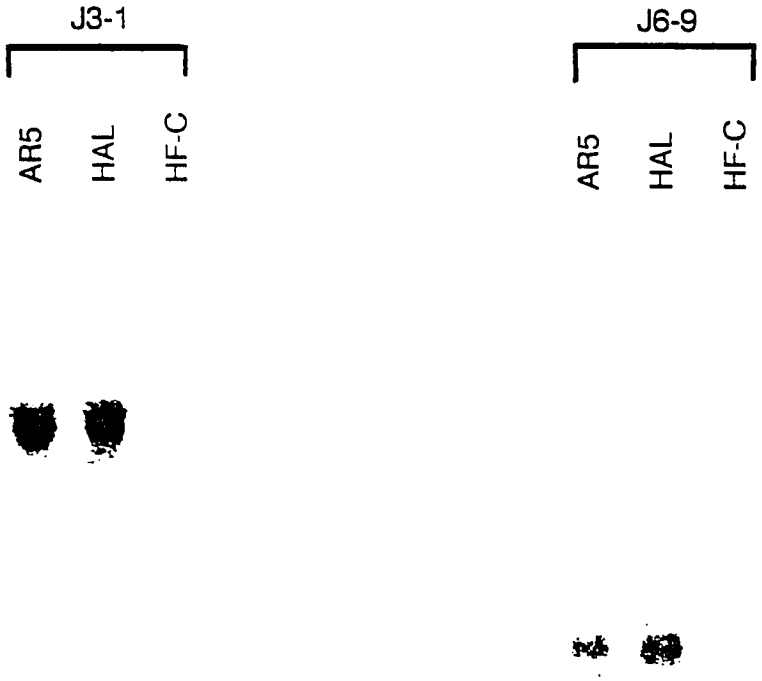


FIGURE 21

FIG. 22. Total RNA Northerns featuring two representatives of a group of gene sequences which are overexpressed in one or both of the immortal cell lines relative to preimmortal SVtsA/HF-C. The clone designated J3-6A is a "mixed" case: its level of expression, while higher in AR5 than in SVtsA/HF-C, is higher in SVtsA/HF-C than in HAL.

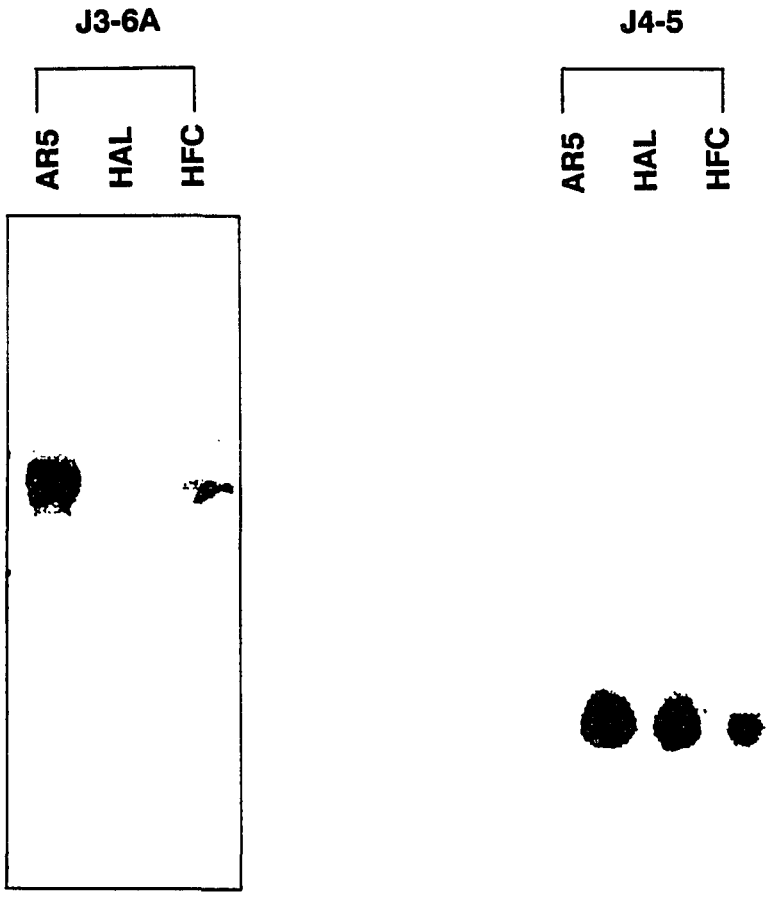


FIGURE 22

A summary of differentially expressed cDNA clones that have been partially characterized to date is given in Tables 2 and 3. Table 2 includes currently available data on sequences that are overexpressed in the preimmortal cells, while Table 3 provides data for those that are overexpressed in the immortal cells. A summary of the current state of analysis of all clones isolated during the subtractive hybridization experiment appears in Tables 4 through 10. The majority of the cDNA clones (11 out of 14) that have been partially sequenced represent genes that have not been previously described. Three cDNA clones correspond to known genes (ADP-Ribosylation factor 2, human ribosomal protein S14, and the Inhibin/Activin β_A subunit). Their possible significance in immortalization will be considered elsewhere (see Discussion).

Partial sequencing of the clone designated J3-6A (Fig. 22, Table 3), which corresponds to the β_A subunit of Inhibin/Activin, revealed the presence of a $(CA)_{17}$ repeat (171). Dinucleotide $(CA)_n$ repeats consisting of from 15 to 30 repeats per block have been found to constitute one of the most abundant human interspersed repetitive DNA families (172). Furthermore, their lengths are highly polymorphic within the human population (172), making them extremely valuable new sources of genetic markers for the study of inherited diseases and for the improvement of genetic and physical maps. The $(CA)_{17}$ repeat in the Inhibin/Activin β_A subunit was estimated (from PCR amplifications involving 122 chromosomes of unrelated individuals) to have a heterozygosity index of 0.67. The repeat was localized to chromosome 7 using a rodent-monochromosomal human somatic cell hybrid

Table 2. DIFFERENTIAL mRNA EXPRESSION:

SEQUENCES ELEVATED IN PREIMMORTAL SVtsA/HF-C

CLONE	INSERT SIZE(kb)	RNA SIZE(kb)	RNA ABUNDANCE	RNA LEVEL RELATIVE TO AR5	HAL	SEQUENCE
J2-2	0.6	7.0	+ + +	7.0	2.5	novel
J2-10(H)	1.1	9.5	low	>10	ND	novel
J2-10(L)	0.2	3.4	low	3.0	ND	novel
J3-4	2.2	4.4	+ + + +	2.0	0.9	novel
J3-6B	0.7	4.0	+ +	-	-	unknown
J4-3	0.9	1.8	low	5.0	ND	novel
J7-5	0.7	3.6	+ + + +	2.0	1.0	novel
J10-3	0.4	0.5	+ +	2.0	5.0	novel

+ to + + + + : relative intensity of signal detectable with 10μg total RNA per lane.

low : not detectable unless poly(A)$^+$ mRNA is used.

ND : not done.

unknown : sequence data not yet available.

Table 3. DIFFERENTIAL mRNA EXPRESSION:

SEQUENCES ELEVATED IN THE IMMORTAL CELLS

CLONE	INSERT SIZE(kb)	RNA SIZE(kb)	RNA ABUNDANCE	RNA LEVEL RELATIVE TO SVtsa/HF-C AR5	HAL	SEQUENCE
J2-3	2.0	2.0	+++	3.0	3.0	ARF2
J3-1	1.0	2.3	+++	6.5	6.0	novel
J3-6A*	0.7	4.0	++	3.3	0.8	INH/ACT β_A
J4-5	0.5	1.0	++++	3.9	2.6	S14
J6-7A	0.9	3.1	+	3.4	2.5	novel
J6-9	1.8	3.2	+	2.5	3.0	novel
J10-5	0.6	1.4	++	1.8	2.9	novel

INH/ACT β_A : Inhibin/Activin β_A subunit.

ARF2 : ADP-Ribosylation factor 2.

S14 : Human ribosomal protein S14 gene.

+ to ++++ : see table 2.

* : $(CA)_{17}$ repeats.

Table 4. SUMMARY OF CURRENT STATE OF ANALYSIS OF cDNA CLONES ISOLATED DURING SUBTRACTIVE HYBRIDIZATION EXPERIMENT

λ gt10 plaque designation	sizes of PCR-amplified products	differential expression
J1-1	1.0kb	---
	0.7kb	---
J1-2	0.8kb[A]	ND
J1-8	0.7kb	---
	0.5kb[B]	---
J1-10	0.5kb[B]	---
	1.2kb	---
J1-12	0.7kb[B]	---
	0.8kb	---

A : cDNA clones wihich generate no signals on Northerns carrying 10μg of total RNA per lane. They must be tested on poly(A)$^+$ mRNA Northerns

B : cDNA clones which generate, on total RNA Northerns, secondary higher molecular weight signals that are prominently overexpressed in preimmortal SVtsA/HF-C

--- : Not differentially expressed

ND : Not yet determined

Table 5. SUMMARY OF CURRENT STATE OF ANALYSIS OF cDNA CLONES ISOLATED DURING SUBTRACTIVE HYBRIDIZATION EXPERIMENT

λgt10 plaque designation	sizes of PCR-amplified products	differential expression
J2-1	0.6kb	---
J2-2	0.6kb	HF-C > HAL, AR5
J2-3	2.0kb	HF-C < HAL, AR5
J2-4	0.6kb[B]	---
J2-5	0.5kb	---
J2-6	0.2kb	ND
	0.3kb	ND
	0.5kb	ND
	0.6kb	ND
	0.7kb	ND
J2-7	ND	ND
J2-8	ND	ND
J2-9	ND	ND
J2-10	0.2kb[A]	HF-C > AR5
	1.1kb[A]	HF-C > AR5

A : cDNA clones wihich generate no signals on Northerns carrying 10μg of total RNA per lane. They must be tested on poly(A)$^+$ mRNA Northerns

B : cDNA clones which generate, on total RNA Northerns, secondary higher molecular weight signals that are prominently overexpressed in preimmortal SVtsA/HF-C

--- : Not differentially expressed

ND : Not yet determined

Table 6. SUMMARY OF CURRENT STATE OF ANALYSIS OF cDNA CLONES ISOLATED DURING SUBTRACTIVE HYBRIDIZATION EXPERIMENT

λgt10 plaque designation	sizes of PCR-amplified products	differential expression
J3-1	1.0kb	HF-C < HAL, AR5
J3-2	0.6kb	---
J3-3	0.4kb[B]	---
J3-4	2.2kb	HFC > AR5, HFC = HAL
J3-5	0.6kb	---
J3-6	0.3kb[B]	---
	0.7kb	HFC < AR5, HFC > HAL
	0.7kb	HFC > AR5, HAL
J4-1	0.7kb[A]	ND
J4-2	0.8kb[B]	---
	1.0kb[A]	ND
J4-3	0.9kb[A]	HF-C > AR5
J4-4	1.3kb[A]	ND
J4-5	0.5kb[B]	HF-C < HAL, AR5
J4-6	0.8kb	---

A : cDNA clones wihich generate no signals on Northerns carrying 10μg of total RNA per lane. They must be tested on poly(A)$^+$ mRNA Northerns

B : cDNA clones which generate, on total RNA Northerns, secondary higher molecular weight signals that are prominently overexpressed in preimmortal SVtsA/HF-C

--- : Not differentially expressed

ND : Not yet determined

Table 7. SUMMARY OF CURRENT STATE OF ANALYSIS OF cDNA CLONES ISOLATED DURING SUBTRACTIVE HYBRIDIZATION EXPERIMENT

λgt10 plaque designation	sizes of PCR-amplified products	differential expression
J5-1	0.2kb[A]	ND
J5-2	0.7kb	---
	1.2kb	---
J5-3	0.6kb	---
	0.8kb	---
	1.5kb	---
J5-4	0.4kb	---
	0.6kb	---
J5-5	0.4kb	---
	1.5kb	---
J5-6	ND	ND
J5-8	0.2kb	---
	0.7kb	---
J5-9	0.8kb[B]	---
J5-10	0.7kb[A]	ND
J5-11	0.5kb	---
J5-12	2.2kb	---

A : cDNA clones wihich generate no signals on Northerns carrying 10μg of total RNA per lane. They must be tested on poly(A)$^+$ mRNA Northerns

B : cDNA clones which generate, on total RNA Northerns, secondary higher molecular weight signals that are prominently overexpressed in preimmortal SVtsA/HF-C

--- : Not differentially expressed

ND : Not yet determined

Table 8. SUMMARY OF CURRENT STATE OF ANALYSIS OF cDNA CLONES ISOLATED DURING SUBTRACTIVE HYBRIDIZATION EXPERIMENT

λgt10 plaque designation	sizes of PCR-amplified products	differential expression
J6-1	1.9kb	---
J6-2	0.6kb	ND
	0.7kb	ND
	0.8kb	ND
	1.4kb	ND
J6-5	0.8kb[B]	---
	1.1kb[A]	ND
J6-6	0.5kb	---
J6-7	0.3kb[A]	ND
	0.9kb	HF-C < HAL, AR5
J6-9	1.8kb	HF-C < HAL, AR5
J6-10	0.8kb[B]	---
	1.2kb[B]	---
J6-11	0.5kb	ND
	0.8kb	---
J6-12	0.2kb	ND
	0.3kb	ND

A : cDNA clones wihich generate no signals on Northerns carrying 10μg of total RNA per lane. They must be tested on poly(A)$^+$ mRNA Northerns

B : cDNA clones which generate, on total RNA Northerns, secondary higher molecular weight signals that are prominently overexpressed in preimmortal SVtsA/HF-C

--- : Not differentially expressed

ND : Not yet determined

Table 9. SUMMARY OF CURRENT STATE OF ANALYSIS OF cDNA CLONES ISOLATED DURING SUBTRACTIVE HYBRIDIZATION EXPERIMENT

λgt10 plaque designation	sizes of PCR-amplified products	differential expression
J7-1	ND	ND
J7-2	0.7kb	---
	1.0kb	---
J7-3	0.6kb	Smear on Northern
J7-4	0.4kb	---
J7-5	0.7kb	HFC > AR5, HFC = HAL
J7-6	0.5kb	ND
	0.6kb	ND
	0.9kb	ND
J7-8	0.5kb	---
J7-10	1.0kb	---
	2.6kb[B]	---
J8-1	0.3kb	---
	0.5kb[B]	---

A : cDNA clones wihich generate no signals on Northerns carrying 10μg of total RNA per lane. They must be tested on poly(A)$^+$ mRNA Northerns

B : cDNA clones which generate, on total RNA Northerns, secondary higher molecular weight signals that are prominently overexpressed in preimmortal SVtsA/HF-C

--- : Not differentially expressed

ND : Not yet determined

Table 10. SUMMARY OF CURRENT STATE OF ANALYSIS OF cDNA CLONES ISOLATED DURING SUBTRACTIVE HYBRIDIZATION EXPERIMENT

λgt10 plaque designation	sizes of PCR-amplified products	differential expression
J9-1	0.4kb	---
J9-2	0.5kb	ND
J9-3	0.8kb	ND
J9-4	0.6kb	---
J9-5	0.2kb	ND
J9-6	1.6kb	ND
	2.2kb	ND
J9-7	ND	ND
J9-8	ND	ND
J9-9	0.3kb[B]	---
	1.6kb	---
J9-10	0.9kb	---
J10-1	0.8kb[A]	ND
J10-2	0.8kb[A]	ND
J10-3	1.0kb	ND
	0.4kb	HF-C > AR5, HAL
J10-4	0.5kb[A]	ND
J10-5	0.4kb	---
	0.6kb	HF-C < AR5, HAL
J10-6	0.8kb[A]	ND
J10-7	0.6kb	---

A : cDNA clones wihich generate no signals on Northerns carrying 10μg of total RNA per lane. They must be tested on poly(A)$^+$ mRNA Northerns

B : cDNA clones which generate, on total RNA Northerns, secondary higher molecular weight signals that are prominently overexpressed in preimmortal SVtsA/HF-C

--- : Not differentially expressed

ND : Not yet determined

panel. Details of this analysis have been reported elsewhere (171).

As an expected limitation of our approach to subtractive hybridization screening, a large number of cDNA clones (~80%) failed to show differential expression when tested by Northern blot analysis. However, a subset of these (all members of the high abundance class) generate unusual hybridization patterns when used as probes in Northern experiments. Autoradiograms demonstrate a principal band, which shows little or no differential expression between the preimmortal and the immortal cells. On the other hand weaker bands of higher molecular weight (HMW) are prominently overexpressed in the preimmortal RNA (Figure 23). Furthermore a number of standard well-characterized high abundance genes such as GAPDH (Fig. 18) and thymidine kinase (TK) were found to behave similarly in our Northern experiments. Others, such as the proliferating cell nuclear antigen (PCNA), did not (Fig. 24). The HMW bands contain RNA as shown in Fig. 25. Preincubation of the RNAs with RNase-free DNase I (to rule out the possibility that these differential higher molecular weight hybridizations were due to DNA contamination) failed to eradicate these signals (lanes 1 and 2). Treatment with DNase-free RNase I (lanes 3-7), however, resulted in the complete loss of all signals (Fig. 25). In order to determine whether these differential higher molecular weight signals represented polyadenylated RNA species, several positive clones (as well as the standard messages GAPDH and TK) were used as probes on poly(A)$^+$ mRNA Northerns. The results, shown for TK in Figure 26, demonstrate that while the

FIG. 23. Total RNA Northerns featuring representatives of a group of genes which generate multiple signals when used as probes in Northern experiments. The higher molecular weight hybridizations are invariably and prominently overexpressed in the preimmortal cell SVtsA/HF-C.

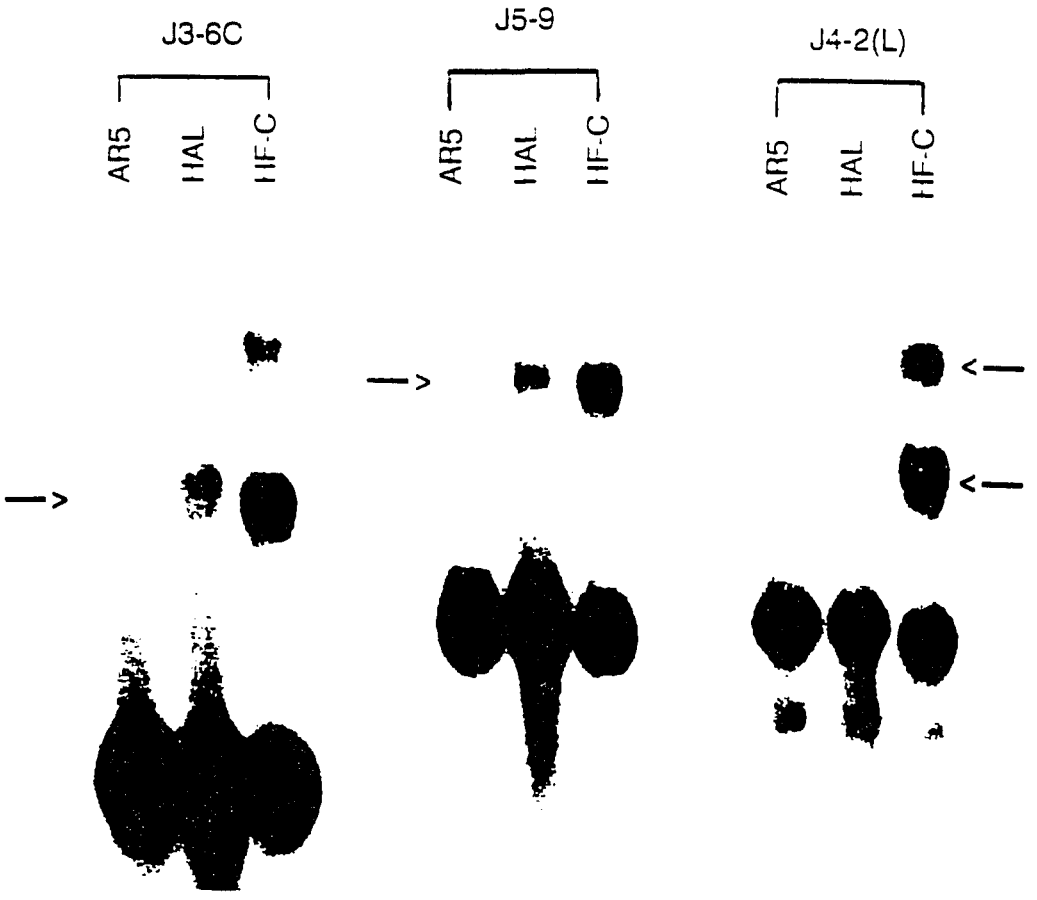


FIGURE 23

FIG. 24. Total RNA Northerns demonstrating that probes homologous to high abundance mRNAs do not always generate high molecular weight bands which are differentially overexpressed in SVtsA/HF-C. Two of the genes [J9-9(L) and J5-2(H)] were isolated by subtractive hybridization screening of a λgt10 SVtsA/HF-C cDNA library. The other is the gene for the proliferating cell nuclear antigen (PCNA).

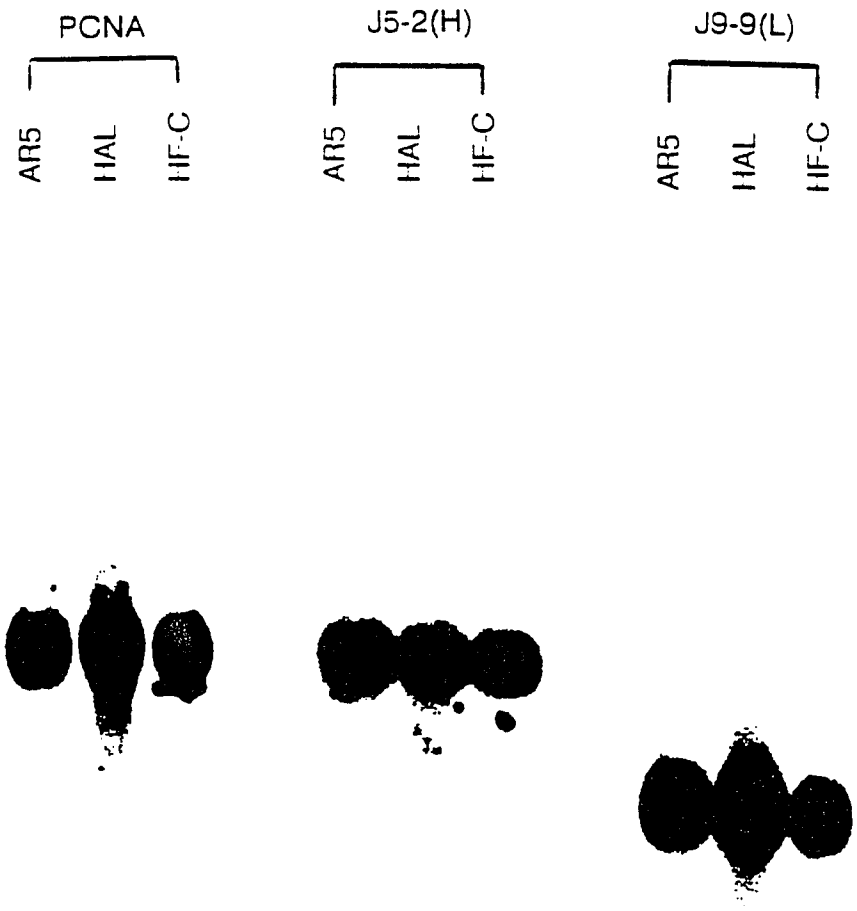


FIGURE 24

FIG. 25. Total RNA Northern demonstrating that the high molecular weight signals which are differentially overexpressed in preimmortal SVtsA/HF-C, and which are generated by a subset of the cDNA clones isolated by subtractive hybridization, are RNA-dependent and not due to DNA contamination. The cDNA clone used as probe is designated J4-2 (0.8kb). Lanes 1 through 7 carry 20μg of the corresponding total RNA, while lanes 8 and 9 carry 10μg each. Lanes: 1 and 2, SVtsA/HF-C RNA incubated for 5 minutes prior to electrophoresis with RNase-free DNase I; 3 through 7, SVtsA/HF-C RNA incubated from 1 to 5 minutes respectively with DNase-free RNase I; 8, untreated immortal AR5 RNA; 9, untreated SVtsA/HF-C RNA.

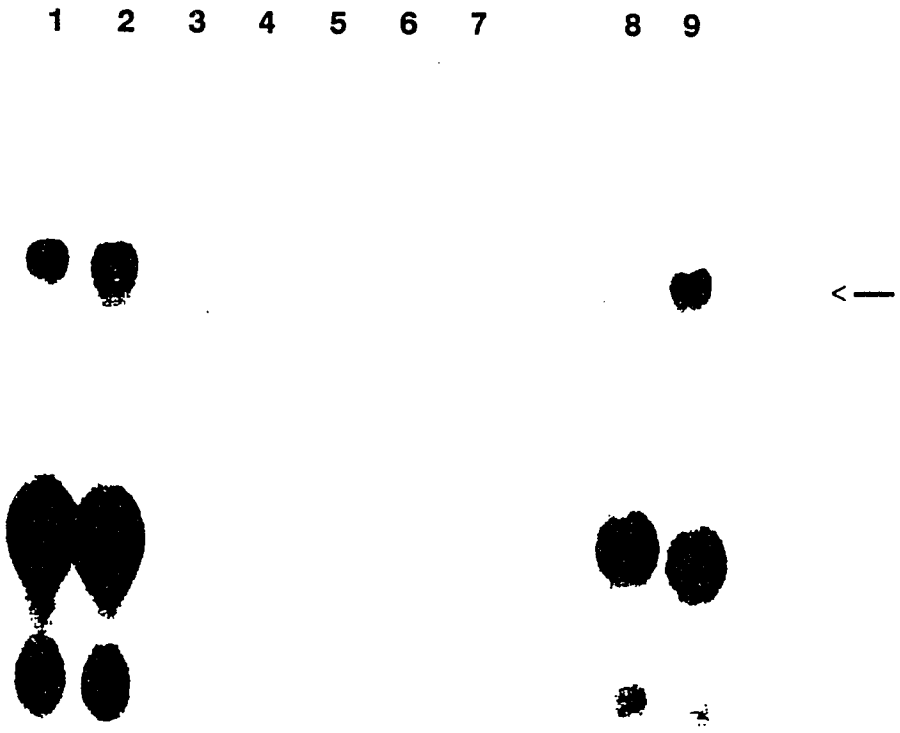


FIGURE 25

FIG. 26. Total and Poly(A)$^+$ mRNA Northerns probed with human thymidine kinase (TK) to demonstrate the disappearance, in the poly(A)$^+$ mRNA blots, of the high molecular weight hybridizations which are differentially overexpressed in preimmortal SVtsA/HF-C.

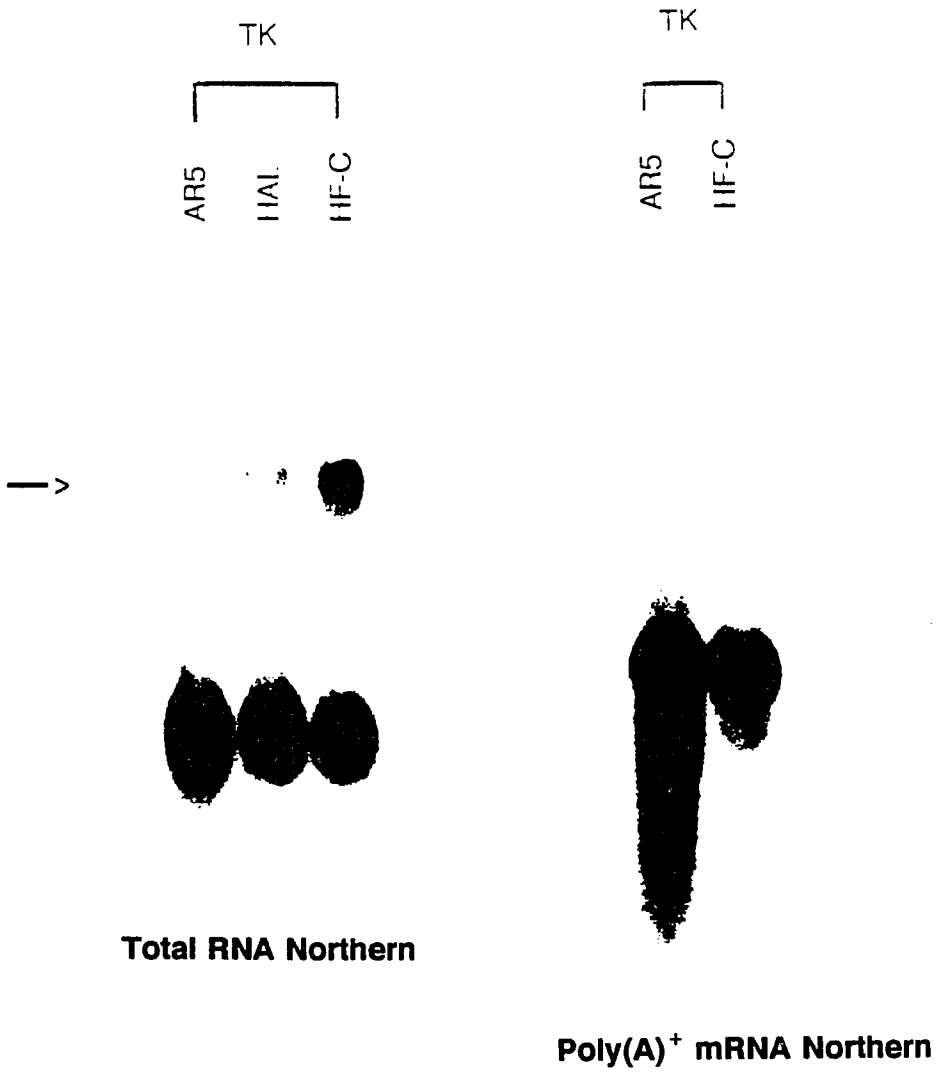

TK
AR5
IIAI
IIF-C
TK
AR5
IIF-C
Total RNA Northern
Poly(A)+ mRNA Northern

FIGURE 26

principal bands correspond to poly(A)$^+$ mRNAs, the HMW hybridizations arise from poly(A)$^-$ species. These data are consistent with the presence of a possible defect or difference in RNA processing in the preimmortal SVtsA/HF-C cells (see Discussion). The detection of this phenomenon in certain mRNAs belonging to the high abundance class does not necessarily mean that this processing difference is exclusive to transcripts expressed at relatively high levels. Since the differential high molecular weight signals are always of much weaker intensity than the accompanying main signals, it would be extremely difficult to detect them in sequences expressed at more modest levels. Moreover, since these signals correspond to poly(A)$^-$ species, poly(A)$^+$ mRNA blots cannot be used to enrich for them. Utilizing TK as a prototype for this class of gene which is differentially processed in SVtsA/HF-C, I am currently attempting by means of RT/PCR to better characterize the nature of the high molecular weight species.

CHAPTER 5

DISCUSSION

The limited in vitro proliferative potential characteristic of most (and probably all) cells derived from normal eucaryotic tissues (24) has been interpreted as a manifestation of aging at the single-cell level and has, consequently, led to its designation as cellular or replicative "senescence" (16). Since the 1961 publication of Hayflick and Moorhead's seminal paper (5), the cultured human diploid fibroblast (HDF) has become the pre-eminent experimental model system used for investigations of cellular senescence. One approach which has been used in order to gain an understanding of replicative senescence in HDF has involved the examination of mechanisms that overcome it. However, unlike rodent cells such as 3T3 mouse fibroblasts, spontaneous immortalization (i.e. escape from senescence) of HDF has never been observed to occur reproducibly in tissue culture. Moreover, HDF are extremely resistant to carcinogen, radiation, and oncogene-mediated immortalization (127). By far the most successful approach to the generation of permanent human fibroblast cell lines has involved their transformation with the DNA tumor virus SV40 (138). Expression of the SV40 large T antigen in such transformants results, among other things, in a temporary extension of their replicative lifespan. As shown previously by this laboratory and others, T antigen plays a critical role in the emergence

(albeit at a very low frequency) of immortalized derivatives (133,138). Large T antigen is the first major viral protein to accumulate in cells into which the SV40 genome has been introduced. This protein (708 amino acids) is a multifunctional effector with pleitropic consequences on cellular functions. Some of the known T antigen functions include transcriptional transactivation of a number of cellular genes, induction of cellular DNA synthesis, establishment and maintenance of the transformed phenotype, and binding of a number of important cellular regulatory proteins such as p53 and pRB. Moreover, large T antigen is absolutely required for the continued viability and proliferation of both immortal and preimmortal SV40 transformants (see Introduction). Despite intense scientific scrutiny spanning over three decades, the nature as well as the relationship between the molecular mechanisms responsible for senescence and immortalization remain largely unelucidated.

Using a set of genetically matched normal, and SV40-transformed preimmortal and immortal human fibroblasts, I have carried out a multifaceted series of comparative molecular analyses in order to identify changes in cellular gene expression, and in the organization of the viral and cellular genomes, which may be relevant to the emergence of the immortal phenotype.

However, the results of previous work in our laboratory involving the comparison of preimmortal and immortal transformants of the SV/HF line (see Materials and Methods) have suggested that immortalization is accompanied by no apparent reorganization of viral sequences nor by alterations in large T antigen

levels (138,164). I have determined, by quantitative Southern analysis, the copy number of the SV40 integrant in preimmortal and immortal members of the SVtsA/HF-A line (see Materials and Methods). The latter investigation was facilitated by the unusually stable karyotype of the SVtsA/HF-A line which, in contrast to the cell to cell variability and aneuploidy evident in SV/HF, makes possible reliable quantitative comparisons when referenced to any of a number of available internal standards (164). My analysis corroborates our laboratory's previous findings in that it reveals no overt changes in the organization of the resident SV40 sequences. However, I have detected a 2-3 fold increase in SV40 DNA in the various immortal derivatives of preimmortal SVtsA/HF-A (see Fig. 4). Duplication of chromosome 5 (the site of SV40 integration in the SVtsA/HF-A line) has been ruled out by P.C. Patsalis as the basis for SV40 amplification. Instead he has attributed the increase to the presence, in the immortal SVtsA/HF-A cells, of double minute chromosomes containing additional copies of the SV40 sequences (173). Since large T antigen expression (B. Zainul and H.L. Ozer unpublished data) seems to undergo no concomitant increase in these immortal cells, it is difficult to ascribe unambiguous significance to this amplification. Nevertheless, the generality of this trend among the immortal SVtsA/HF-A suggests the possibility that this phenomenon might constitute the basis for a selective advantage. According to current consensus, large T antigen extends the replicative lifespan of preimmortal SV40 transformants principally through its ability to bind, and thereby override, the growth suppressor functions

of p53 and pRB (129, 130, and Introduction). For reasons that are presently unknown, large T antigen cannot mediate indefinitely such lifespan extension. This fact has led to a model which postulates the existence of a secondary (i.e., to that involving p53 and pRB) cellular or other T antigen dependent mechanism acting as a barrier to the attainment of unlimited proliferative potential (135, 136, and Introduction). It is generally held that while continuous large T antigen expression is necessary for the viability of both preimmortal and immortal SV40-transformed human fibroblasts, it is by itself not sufficient to bring about immortalization. However, the possibility of quantitative effects on immortalization involving SV40 should not be dismissed too hastily, particularly (and remarkably!) since the full range of molecular effects mediated by the SV40 system have not yet been fully gleaned. Only recently, for example, (after many decades of intense investigation in countless laboratories around the world) SV40 was reported to code for a third early protein in addition to the large T and small t antigens (174). This protein, which corresponds roughly to the transforming amino-terminal domain of large T antigen, is derived from an alternatively spliced early mRNA, and was found to be present in all stably transformed as well as all lytically infected cells that were examined (174). The possibility of potentially novel effects mediated by this newly described SV40 protein becomes particularly intriguing in light of the reported mutual antagonism (vis a vis cell cycle progression) between large T antigen and Cip1 (93). The latter gene, which is also known as Waf1 or Sdi-1 (91,92), has been identified as the major p53-inducible transcript in mammalian

cells, and is a potent inhibitor of G_1 cyclin-dependent protein kinases. The levels of Sdi-1 mRNA increase progressively as cells proliferate in tissue culture, becoming 10- to 20-fold higher in senescent than in young cycling cells (175). Interestingly, Sdi-1 mRNA expression, which becomes maximal as SV40-transformed human fibroblasts enter crisis, reverts to levels comparable to those of young normal cells in the immortalized derivatives (176). In summary, while it is clear that the recessive nature of cellular immortality (134, and Introduction) can best be interpreted on the basis of loss of a cellular function, the loss of function involved may not be absolute but rather a matter of quantitative shifts in the balance between various cellular and viral effectors. For example, this requisite loss of function may be the consequence of quasi-developmental molecular "decisions" which evolve out of the complex tug-of-war interaction between viral and cellular positive and negative growth regulators.

Karyotypic analysis carried out by our laboratory in collaboration with that of Dr. Ann Henderson at Hunter College has identified a non-random alteration on the long arm of chromosome 6 (i.e. 6q) associated with the immortalization of SV40-transformed human fibroblasts (138,164). I have carried out quantitative Southern analysis of the SVtsA/HF-A line using three oncogene probes located distal to 6q21 and have demonstrated a 2-fold reduction in copy number at these loci in the immortalized cells (see Results, Chapter 3). These data are consistent with the complete loss of one copy of a putative growth suppressor located on 6q,

together with the inapparent mutational inactivation of the second copy on the seemingly normal chromosome (164) as is frequently observed during loss of function of other tumor suppressors such as pRB (177). An alternative interpretation is that immortalization results from a 2-fold dosage reduction of a critical gene on 6q, together with alterations at other loci. Additional support for an important role for a gene on 6q in the immortalization of SV40-transformed human cells has come from cytogetic data obtained by Ray and Kraemer (178). These investigators have found non-random breaks at 6q21 in SV40-transformed immortal human fibroblasts. Moreover, our laboratory in collaboration with R. Athwal (formerly of the Department of Microbiology and Molecular Genetics at UMDNJ-New Jersey Medical School), has recently demonstrated that introduction by microcell fusion of a normal chromosome 6 into SV40-transformed immortal human fibroblasts leads to restoration of the senescent phenotype (179). A large number of SV40-transformed immortal human cells fall within the same complementation group as has been established by cell hybrid studies (137). They are expected, therefore, to share the same fundamental genetic defect(s) such that suppression of the immortal phenotype via introduction of the long arm of chromosome 6 should hold for all of them if this is in fact the location of a key or defining molecular alteration leading to their immortalization.

Despite the de facto definition of an immortal cell as a cell that has escaped the normal processes of replicative senescence, it is far from clear if and how the

molecular mechanisms responsible for senescence are related to those involved in immortalization. In other words, it is conceivable that the genes which function to limit proliferative potential in normal cells may turn out not to be the same genes whose expression must be altered or eliminated in order to bring about immortalization. However, since normal senescent cells differ fundamentally from immortal SV40 transformants by virtue of the presence of large T antigen in the latter, it is probably unreasonable to expect a simple reversal of the senescent phenotype to be all that is required for immortalization. While the state of our present knowledge may not yet allow us to reach firm conclusions regarding this matter, a number of investigations do suggest that various prominent molecular alterations, which are clearly associated with senescence, are in fact reversed as cells become immortal. The behavior of Sdi-1 (described above) is a clear instance of this pattern. Another, which we have examined in some detail, is the behavior of telomeres. In a collaboration led by Dr. Michael B. Small of the Department of Microbiology and Molecular Genetics at UMDNJ-New Jersey Medical School, we have determined the telomere lengths at various passages during the replicative lifespan of normal as well as SV40-transformed preimmortal and immortal human fibroblasts (see Results, Chapter 3). At the time this study was initiated, Harley and co-workers had reported a decrease with in vitro passage in the amount and length of telomere DNA in human fibroblasts (148). We have confirmed these findings, and have extended the analysis to include an examination of the behavior of telomeres in preimmortal and immortal SV40

transformants (see Results, Chapter 3). The discovery that telomeres shorten with in vitro as well as with in vivo aging (148,180) has been advanced as the foundation for a theory of cellular senescence. It was suggested that progressive loss of telomeric sequences results in the eventual loss of genetic information at or near chromosome ends, which is in turn responsible for the replicative decline of senescent cells. A theory based on the loss of genetic material could not, however, be easily reconciled with the rapid reversible onset or the rapid escape from senescence which can be brought about when large T antigen is deinduced and reinduced by various means in SV40 transformants (135,136). In order to correct this inconsistency Wright and Shay (181) have proposed the existence of "telomere positional effects". According to this paradigm, senescence is controlled by the activity of genes located at or near the telomeric regions. These genes are in turn responsive to the status (i.e. length) of telomeres via conformational mechanisms which become operative long before chromosome ends suffer catastrophic instability due to the complete loss of telomere sequences. We have documented that the attrition of telomeres characteristic of normal senescent human fibroblasts continues (at very nearly the same rate) in the preimmortal SV40 transformants which are capable of proliferating well beyond the maximal lifespan of their normal untransformed progenitors (182, and this thesis). Our findings, together with reports by others, of no change in telomere length during aging in Saccharomyces cerevisiae (183), cast doubts on the proposition that telomere loss plays a causal role in cellular senescence. However, it is probably premature

probably premature to dismiss the significance to proliferative senescence of telomere shortening in human fibroblasts purely on the basis of what has been observed in yeast cells. Even our documentation of continued telomere shortening during the extended lifespan of the preimmortal SV40 transformants should not be overstressed given that even here we are in fact dealing with two distinctly different cellular systems (i.e. normal versus SV40-transformed human cells). The presence of large T antigen could conceivably antagonize the putative senescence-inducing signal generated by telomere shortening. This would in fact be consistent with current evidence which characterizes "crisis" in preimmortal SV40 transformants as a manifestion of delayed senescence (131,175). Moreover, we have made the interesting observation (182, and this thesis) that telomere lengths stabilize and may increase in the immortal SV40-transformants. This finding suggests that immortalization induces (and is at least partly dependent on) a reactivation of mechanisms for telomere maintenance (i.e., telomerase). Telomerase reactivation, however, is unlikely to be the only molecular event required for immortalization given that when senescent cells are hybridized to immortal cells (which express telomerase) the resulting synkaryons exhibit limited proliferative potential (134,184). The dominance of the senescent cells in such studies suggests that they express a growth suppressor whose loss is critically important in immortalization. In any case, actual proof of an important connection between the behavior of telomeres in somatic human tissues and human cellular senescence (and immortalization) may have to await the

experimental manipulation of telomerase expression.

In an attempt to better define the molecular genetic basis of immortalization in SV40-transformed human fibroblasts, I have prepared a cDNA library in λgt10 from preimmortal SVtsA/HF-C cells and have used it in a subtractive hybridization approach in order to identify mRNA species which are differentially expressed in preimmortal SVtsA/HF-C versus either or both of the immortal lines HAL and AR5 (see Materials and Methods). As has already been pointed out (see Results, Chapter 4) our subtractive hybridization scheme was formally designed to identify sequences underrepresented in the immortal cells. However, as we have documented (Chapter 4), a number of cDNAs corresponding to species overexpressed in the immortals were also isolated, which requires an explanation. As already detailed, our experiment involved a screening of 10,000 plaques of the SVtsA/HF-C λgt10 cDNA library with an SVtsA/HF-C cDNA probe which had been depleted, by molecular subtraction, of sequences also present in mRNA populations derived from immortal AR5 (see Fig. 3 in Materials and Methods). The decision as to which plaques should be picked was made on the basis of a comparison between the intensities of the signals generated by the lifts hybridized to the subtracted probe, and those generated by a replica set of lifts hybridized to a total cDNA probe prepared from poly(A)$^+$ mRNA from immortal HAL cells (see Materials and Methods). Essentially, strong hybridizations in the former corresponding to weak hybridizations in the latter were interpreted as the signatures of mRNA species which are more abundant in the preimmortal than in

the immortal cells (see Table 2). On a number of occasions, however, I detected the combination of a weak signal on the filters hybridized to the subtracted probe together with an exceptionally robust signal on the filters hybridized to the immortal total cDNA probe. Plaques generating such signals were picked, since I suspected that they might represent sequences overexpressed in the immortal cells. These isolates are no doubt responsible for our identification of a number of such sequences (see Table 3). The remaining clones of this type, which cannot be attributed to such deliberate isolation, may have arisen from the inevitable "noise" inherent in all subtractive hybridization schemes (see Materials and Methods).

Our initial intention to emphasize the identification of mRNAs whose levels are reduced in the immortal cells reflected our belief that the known recessive nature of the immortal phenotype is most consistent with a loss of function. However, while the loss of function of a "primary effector" is logically required by the recessive nature of the immortal phenotype, its loss may result in the enhanced expression of multiple dependent (secondary effector) genes, and these are certainly of interest in their own right. Moreover, identification of an mRNA whose expression decreases dramatically in the immortal cells is not unequivocal proof that it is in fact the putative primary effector. That is, transcriptional inactivation of a secondary effector through whose "downstream" function the primary effector operates would be phenotypically equivalent to loss of the primary effector itself. Furthermore, inactivation of the postulated primary effector need

not involve disappearance of its mRNA (i.e. inactivation may reside at other than the transcriptional level). In fact, if the latter circumstances were the case, subtractive hybridization could never reveal the identity of the primary effector.

A number of the cDNA clones which have been shown by Northern analysis to exhibit differential expression were subcloned in the pGEM3 vector and partially sequenced in both directions using as primers the flanking SP6 and T7 promoters (see Materials and Methods). GenBank homology searches were carried out in order to identify which cDNAs represented novel sequences, and which were completely or partially homologous to previously described genes. As summarized in Tables 2 and 3, most of the sequences correspond to genes that have not been previously described. Only three of the cDNA clones represent known genes (see Table 3). The three are ADP-Ribosylation factor 2, human ribosomal protein S14, and the Inhibin/Activin β_A subunit, all are members of the group which is overexpressed in the immortal cells (Table 3). The overexpression in the immortal cells of the gene encoding the β_A subunit of Inhibin/Activin is very interesting. Inhibins A and B are heterodimers made up of an α-subunit combined respectively with a β_A- or a β_B-subunit. Activins A and B are homodimers of the β_A- and β_B-subunits respectively (183). The β subunits share extensive sequence homology with transforming growth factor β (TGF-β), and activins are classified as members of the TGF-β superfamily (185). Activins and inhibins are known to exert opposite biological effects on a large number of systems. This represents a particularly striking example of how dimerizing with different partners can lead to

dramatically altered activities (185). Activins and inhibins regulate a vast range of processes including cell proliferation, differentiation, and cell cycle (186). For example: erythroid differentiation factor (EDF) has been found to be encoded by the same mRNA as that of the inhibin β_A subunit (187). Inhibin A (a heterodimer of an α- and the β_A-subunit) functions as a gonadal tumour-suppressor gene (188), and activins have also been shown to promote the survival of neurons in culture (189). Taken together these observations strongly suggest that a differential shift in the expression level of one or another of the inhibin/activin subunits could have important consequences for cellular survival and proliferation. Similarly, overexpression in the immortal cells of the gene encoding human ribosomal protein S14 is intriguing in light of the large number of reports documenting an association between increased levels of the mRNAs of specific ribosomal proteins and various cancers (190,191,192,193). These studies have demonstrated that the overexpression of distinct ribosomal mRNAs in different tumors is not accompanied by increased synthesis of ribosomes, nor by increased translational activity. It must, therefore, be postulated that at least some of the ribosomal proteins serve as more than merely structural elements. In fact certain ribosomal proteins have already been implicated in regulatory processes (190). The remaining cDNA clones with homology to a previously reported gene corresponds to ADP-ribosylation factor 2. ADP-ribosylation factor 2 was originally identified as a GTP-binding protein necessary for the action of cholera toxin in mammalian cells (194). It has subsequently been shown to be a protein that is highly concentrated in non-

clathrin-coated vesicles, where it apparently participates in the modulation of a novel pathway of vesicle budding and intracellular transport via controlled GTP hydrolysis (195).

Among the differentially expressed genes that I have isolated, a number exhibit elevated expression in preimmortal SVtsA/HF-C cells versus only one or the other of the two immortal cells used in our study (i.e. HAL or AR5). While these results may represent instances of inconsequential clonal variation, it should be borne in mind that functional inactivation of a gene need not entail the disappearance of its mRNA.

A subset of cDNA clones generate unusual hybridizations on total RNA Northerns (see Fig. 23, Chapter 4). When any of this group is used as a probe, autoradiograms typically display a principal band which is not differentially expressed, plus one band at higher molecular weight which is prominently overrepresented in the preimmortal RNA. I have shown that the higher molecular weight hybridizations do not arise from DNA contamination, and that they correspond to poly(A)$^+$ mRNA species (Chapter 4). The nature of these secondary differential hybridizations is currently being investigated and the analysis extended to include other preimmortal cells. This phenomenon may represent an interesting defect or difference in RNA splicing and polyadenylation between the preimmortal and the immortal cells, and could conceivably have important consequences for cellular proliferation.

In summary, I have generated a λgt10 cDNA library from SVtsA/HF-C

poly(A)$^+$ mRNA in order to identify sequences which are differentially expressed in these preimmortal cells versus the SVtsA/HF-A immortals HAL and AR5. We elected to use the preimmortal SVtsA/HF-C rather than the more closely matched preimmortal SVtsA/HF-A because of our concern (in light of its unusually high frequency of immortalization) of the possibility that SVtsA/HF-A might have already undergone some of the critical genetic changes required for immortalization. A total of 10,000 plaques were screened with a subtracted cDNA probe (see Results, Chapter 4), and 73 plaques selected for further analysis. Our analysis has thus far involved the following steps: PCR amplification and isolation of the cDNA inserts, use of the cDNA inserts for Northern analysis to verify differential expression between SVtsA/HF-C and either or both of the two immortal lines HAL and AR5, and partial DNA sequencing of promising clones. Of 14 clones that have so far been partially sequenced, 11 represent novel genes and 3 correspond to known sequences (Tables 2 and 3). Although my initial efforts were aimed at the identification of mRNA populations whose levels are reduced in the immortal cells (most consistent with the recessive nature of the immortal phenotype), I have also identified a number of sequences whose expression is elevated in the immortal cells (Table 3). These are not necessarily less valuable, since although immortalization may ultimately require the loss of a putative primary effector, the latter may turn out to be a negative regulator whose loss results in the overexpression of a number of "downstream" secondary effectors. Characterization of these cDNA clones continues. Specifically, Northern analysis of selected clones

must be extended to encompass a larger panel of our preimmortal and immortal cell lines in order to discount such differences as may arise from immortalization-irrelevant clonal variation. Additionally, more complete (preferably full-sized) copies of selected cDNA clones must be isolated from an appropriate library in order to better assess their functional significance.

In conclusion, I should point out that a previous effort involving plus/minus screening of a much larger number of plaques of the SVtsA/HF-C λgt10 library resulted in the identification of no differentially expressed sequences. It is, therefore, gratifying that despite the recognized technical pitfalls of molecular subtraction, I have been able to isolate a number of genes that are differentially expressed between such closely matched cells lines as are SVtsA/HF-C and AR5 (or HAL). It remains to be determined what (if any) role these genes play in the immortalization of SV40-transformed human fibroblasts.

BIBLIOGRAPHY

1. Weismann, A. Essays upon Heredity and Kindred Biological Problems, 2nd. edn., Claredon Press, Oxford, 1891.
2. Carrel, A., On the permanent life of tissue outside of the organism. *J. Exp. Med.* **15**: 516-528, 1912.
3. Ebeling, A.H., The permanent life of connective tissue outside of the organism. *J. Exp. Med.* **17:** 273-285, 1913.
4. Swim, H.E., and R.F. Parker. Culture characteristics of human fibroblasts propagated serially. *Am. J. Hyg.* **66**: 235-243, 1957.
5. Hayflick, L., and P.S. Moorhead. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**: 585-621, 1961.
6. Matsumura, T., Z. Zerrudo, and L. Hayflick. Senescent human diploid cells in culture: survival, DNA synthesis and morphology. *J. Gerontol.* **34**: 328-334, 1979.
7. Hayflick, L. The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* **37**: 614-636, 1965.
8. Martin, G.M. The interaction of aging and environmental agents: the gerontological perspective. *Prog. Clin. Biol. Res.* **228**: 25-80, 1987.
9. Tassin, J., L. Simmoneau and Y. Courtois, Epithelial lens cells. A model for studying in vitro aging and differentiation. In Y. Courtois and F. Regnault (eds.), Biology of the Epithelial Lens Cell in Relation to Development, Aging and Cataract, INSERM, Paris, 1976, pp. 145-163.

10. Matsumura, T., Sequence of cell life phases in a finitely proliferative population of cultured rat cells: A gerontological study. *J. Cell Physiol.,* **119**: 145-154, 1984.

11. Lipchutz, D.A., and K.B. Udupa. Effect of donor age on long-term culture of bone marrow in vitro. *Mech. Ageing Dev.* **24**: 119-127, 1984.

12. Kondo, H., and H. Ide. Long-term cultivation of amphibian melanophores. In vitro ageing and spontaneous transformation to a continuous cell line. *Exp. Cell Res.* **149**: 247-256, 1983.

13. Quinn, L.S., M. Nameroff, and H. Holtzer. Age-dependent changes in myogenic precursor cell compartment sizes. *Exp. Cell Res.* **154**: 65-82, 1984.

14. Evans, G.H., and H.I. Georgescu. Observations on the secescence of cells derived from articular cartilage. *Mech. Ageing Dev.* **22**: 179-191, 1983.

15. Martin, G.M., C.A. Sprague, and C.J. Epstein. Replicative lifespan of cultivated human cells-effects of donor age, tissue, and genotype. *Lab. Invest.* **23**: 86-92, 1970.

16. Hayflick, L., Aging under glass. *Exp. Gerontol.* **5**: 291-303, 1970.

17. Rohme, D., Evidence for a relationship between longevity of mammalian species and life-span of normal fibroblasts in vitro and erythrocytes in vivo. *Proc. Natl. Acad. Sci. USA* **78**: 5009-5013, 1981.

18. Hayflick, L., The cell biology of aging, in: *Clinics in Geriatric Medicine, The Aging Process,* **Vol. 1**, No. **1**, pp. 15-27.

19. Macieira-Coelho, A., Metabolism of aging cells in culture. *Gerontology* **22**: 3-8, 1976.

20. Albright, J.W., and T. Makinodan. Decline in the growth potential of spleen-colonizing bone marrow stem cells of long-lived aging mice. *J. Exp. Med.* **144**: 1204-1213, 1976.

21. Daniel, C.W., K.B. Deohme, J.T. Young, P.B. Blair, and L.J. Faulkin. The in vivo lifespan of normal and preneoplastic mouse mammary glands: a serial transplantation study. *Proc. Natl. Acad. Sci. USA* **61**: 52-60, 1968.

22. Krohn, P.L., Heterochronic transplantation in the study of ageing. In: *Proceedings of the Royal Society. Review Lectures on Senescence. Series B.* London: The Royal Society, 1962, **vol. 157**, p. 128-147.

23. Siminovitch, L., J.E. Till, and E.A. McCulloch. Decline in colony-forming ability of marrow cells subjected to serial transplantation into irradiated mice. *J. Cell Physiol.* **64**: 23-31, 1964.

24. Cristofalo, V.J., and R.J. Pignolo. Replicative Senescence of Human Fibroblast-Like Cells in Culture. *Physiological Reviews.* **Vol. 73, No. 3**, pp. 617-638, 1993.

25. Goldstein, S., and D.P. Singal. Senescence of cultured human fibroblasts: mitotic versus metabolic time. *Exp. Cell Res.* **88**: 359-364, 1974.

26. Harley, C.B., and S. Goldstein. Cultured human fibroblasts: distribution of cell generations and a critical time limit. *J. Cell Physiol.* **97**: 509-516, 1978.

27. Drescher-Lincoln, C.K., and J.R. Smith. Inhibition of DNA synthesis in senescent-proliferating human cybrids is mediated by endogenous proteins. *Exp. Cell Res.* **153**: 208-217, 1984.

28. Pereira-Smith, O.M., S.F. Fisher, and J.R. Smith. Senescent and quiescent cell inhibitors of DNA synthesis. Membrane-associated proteins. *Exp. Cell Res.* **160**: 297-306, 1985.

29. Stein, G.H., and L. Atkins. Membrane-associated inhibitor of DNA synthesis in senescent human diploid fibroblasts: characterization and comparison to quiescent cell inhibitor. *Proc. Natl. Acad. Sci. USA* **83**: 9030-9034, 1986.

30. Lumpkin, C.K.J., J.K. McClung, O.M. Pereira-Smith, and J.R. Smith. Existence of high abundance antiproliferative mRNAs in senescent human diploid fibroblasts. *Science Wash. DC* **232**: 393-395, 1986.

31. Gorman, S.D., and V.J. Cristofalo. Analysis of the G_1 arrest position of senescent WI-38 cells by quinacrine dihydrochloride nuclear fluorescence - evidence for a late G_1 arrest. *Exp. Cell Res.* **167**: 87-94, 1986.

32. Olashaw, N.E., E.D. Kress, and V.J. Cristofalo. Thymidine triphosphate synthesis in senescent WI-38 cells. Relationship to loss of replicative capacity. *Exp. Cell. Res.* **149**: 547-554, 1983.

33. Dogget, D.L., M.O. Rotenberg, R.J. Pignolo, P.D. Phillips, and V.J. Cristofalo. Differential gene expression between young and senescent quiescent WI-38 cell. *Mech. Ageing Dev.* **65**: 239-255, 1992.

34. Pignolo, R.J., M.O. Rotenberg, and V.J. Cristofalo. Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G_0 state. *J. Biol. Chem.* **268**: 8949-8957, 1993.

35. Martin, G.M., and C.A. Sprague. Symposium on in vitro studies related to atherogenesis. Life histories of hyperplastoid cell lines from aorta and skin. *Exp. Mol. Pathol.* **18**: 125-141, 1973.

36. Stanulis-Praeger, B.M., Cellular senescence revisited: a review. *Mech. Ageing Dev.* **38**: 1-48, 1987.

37. Norwood, T.H., J.R. Smith, and G.H. Stein. Aging at the cellular level: the human fibroblastlike cell model. In: *Handbook of the Biology of Aging,* edited by E.L. Schneider and J.W. Rowe. San Diego, CA: Academic, 1990, p. 131-154.

38. Cristofalo, V.J., and D. Kritchevsky. Cell size and nucleic acid content in the human diploid cell line WI-38 during aging. *Med. Exp.* **19**: 313-320, 1969.

39. Greenberg, S.B., G.L. Grove, and V.J. Cristofalo. Cell size in aging monolayer cultures. *In Vitro Cell Dev. Biol.* **13**: 297-300, 1977.

40. Angello, J.C., W.R. Pendergrass, T.H. Norwood, and J. Prothero. Proliferative potential of human fibroblasts: an inverse dependence on cell size. *J. Cell Physiol.* **132**: 125-130, 1987.

41. Pendergrass, W.R., J.C. Angello, M.D. Kirschner, and T.H. Norwood. The relationship between the rate of entry into S phase, concentration of DNA polymerase alpha, and cell volume in human diploid fibroblast-like monokaryon cells. *Exp. Cell Res.* **192**: 418-425, 1991.

42. Robbins, E., E.M. Levine, and H. Eagle. Morphological changes accompanying senescence of cultured human diploid cells. *J. Exp. Med.* **131**: 1211-1222, 1970.

43. Matsumura, T., Multinucleation and polyploidization of aging human cells in culture. *Adv. Exp. Med. Biol.* **129**: 31-38, 1980.

44. Brandes, D., D.G. Murphy, E.B. Anton, and S. Barnard. Ultrastructural and cytochemical changes in cultured human lung cells. *J. Ultrastruct. Res.* **39**: 465-483, 1972.

45. Mitsui, Y., and E.L. Schneider. Increased nuclear sizes in senescent human diploid fibroblast cultures. *Exp. Cell Res. **100**: 147-152, 1976.*

46. Bemiller, P.M., and L.H. Lee. Nucleolar changes in senescing WI-38 cells. Mech. Ageing Dev. **8**: 417-427, 1976.

47. Kleinsek, D.A., Selection of mRNAs expressed during cellular senescence in vitro. *Age* **12**: 55-60, 1989.

48. Mann, D.M., P.J. McKeown-Longo, and A.J. Millis. Binding of soluble fibronectin and its subsequent incorporation into the extracellular matrix by early and late passage human skin fibroblasts. *J. Biol. Chem.* **263**: 2756-2760, 1988.

49. Millis, A.J., J. Sottile, M. Hoyle, D.M. Mann, and V. Diemer. Collagenase production by early and late passage cultures of human fibroblasts. *Exp. Gerontol.* **24**: 559-575, 1989.

50. Murano, S., R. Thweatt, R.J. Shmookler-Reis, R.A. Jones, E.J. Moerman, and S. Goldstein. Diverse gene sequences are overexpressed in Werner syndrome fibroblasts undergoing premature replicative senescence. *Mol. Cell Biol.* **11**: 3905-3914, 1991.

51. Sottile, J., D.M. Mann, V. Diemer, and A.J. Millis. Regulation of collagenase and collagenase mRNA production in early and late-passage human diploid fibroblasts. *J. Cell Physiol.* **133**: 281-290, 1989.

52. Thweatt, R., S. Murano, R.D. Fleischmann, and S. Goldstein. Isolation and characterization of gene sequences overexpressed in Werner syndrome fibroblasts during premature replicative senescence. *Exp. Gerontol.* **27**: 433-440, 1992.

53. Kumazaki, T., R.S. Robetorye, S.C. Robertorye, and J.R. Smith. Fibronectin expression increases during in vitro cellular senescence: correlation with increased cell area. *Exp. Cell Res.* **195**: 13-19, 1991.

54. Edick, G.F., and A.J.T. Millis. Fibronectin distribution on the surfaces of young and old human fibroblasts. *Mech. Ageing Dev.* **27**: 249-256, 1984.

55. Chandrasekhar, S., and A.J.T. Millis. Fibronectin from aged fibroblasts is defective in promoting adhesion. *J. Cell Physiol.* **103**: 47-54, 1980.

56. Chandrasekhar, S., J.A. Sorrentino, and A.J.T. Millis. Interaction of fibronectin with collagen: Age specific defect in the biological activity of human fibroblast fibronectin. *Proc. Natl. Acad. Sci. USA* **80**: 4747-4751, 1983.

57. Ferber, A., C. Chang, C. Sell, A. Ptasznik, V.J. Cristofalo, K. Hubbard, H.L. Ozer, M. Adamo, C.T. Roberts,Jr., D. LeRoith, G.Dumenil, and R. Baserga. Failure of Senescent human fibroblasts to express the insulin-like growth factor-1 gene. *J. Biol. Chem.* **268**: 17883-17888, 1993.

58. Goldstein, S., E.J. Moerman, R.A. Jones, and R.C. Baxter. Insulin-like growth factor binding protein 3 accumulates to high levels in culture medium of senescent and quiescent human fibroblasts. *Proc. Natl. Acad. Sci. USA* **88**: 9680-9684, 1991.

59. Millis, A.J., M. Hoyle, H.M. McCue, and H. Martini. Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase genes in aged human fibroblasts. *Exp. Cell Res.* **201**: 373-379, 1992.

60. Kumar, S., A.J. Millis, and C. Baglioni. Expression of interleukin 1-inducible genes and production of interleukin 1 by aging human fibroblasts. *Proc. Natl. Acad. Sci. USA* **89**: 4683-4687, 1992.

61. West, M.D., W.E. Wright, and J.W. Shay. Transcriptional mechanisms regulating the over-expression of plasminogen activator inhibitor-1 in senescent fibroblasts (Abstract). *Proc. Ann. Meet. Am. Gerontol. Soc. 44th San Francisco* CA 1991, p. 314.

62. Cristofalo, V.J., D.L. Doggett, K.M. Brooks-Frederich, and P.D. Phillips. Growth factors as probes of cell aging. *Exp. Gerontol.* **24**: 367-374, 1989.

63. Phillips, P.D., K. Kaji, and V.J. Cristofalo. Progressive loss of the response of senescing WI-38 cells to platelet-derived growth factor, epidermal growth factor, insulin, transferrin, and dexamethasone. *J. Gerontol.* **39**: 11-17, 1984.

64. Plisko, A., and B.A. Gilchrest. Growth factor responsiveness of cultured human fibroblasts declines with age. *J. Gerontol.* **38**: 513-518, 1983.

65. Gerhard, G.S., P.D. Phillips, and V.J. Cristofalo. EGF- and PDGF-stimulated phosphorylation in young and senescent WI-38 cells. *Exp. Cell Res.* **193**: 87-92, 1991.

66. Phillips, P.D., E. Kuhnle, and V.J. Cristofalo. [^{125}I]EGF binding ability is stable throughout the replicative life span of WI-38 cells. *J. Cell Physiol.* **114**: 311-316, 1983.

67. Phillips, P.D., R.J. Pignolo, and V.J. Cristofalo. Insulin-like growth factor-1: specific binding to high and low affinity sites and mitogenic action throughout the life span of WI-38 cells. *J. Cell Physiol.* **133**: 135-143, 1987.

68. Carlin, C.R., P.D. Phillips, B.B. Knowles, and V.J. Cristofalo. Diminished in vitro tyrosine kinase activity of the EGF receptor of senescent human fibroblasts. *Nature Lond.* **306**: 617-620, 1983.

69. Brooks, K.M., P.D. Phillips, C.R. Carlin, B. Knowles, and V.J. Cristofalo. EGF-dependent phosphorylation of the EGF receptor in plasma membranes isolated from young and senescent WI-38 cells. *J. Cell Physiol.* **133**: 523-531, 1987.

70. Chua, C.C., D.E. Geiman, and R.L. Ladda. Receptor for epidermal growth factor retains normal structure and function in aging cells. *Mech. Ageing Dev.* **34**: 35-55, 1986.

71. Cristofalo, V.J., P.D. Phillips, T. Sorger, and G. Gerhard. Alterations in the responsiveness of senescent cells to growth factors. *J. Gerontol.* **44**: 52-62, 1989.

72. Murato, S.I., Y. Mitsui, and M. Kawamura. Effect of in vitro aging on 6-ketoprostaglandin F_{1a}-producing activity in cultured human diploid lung fibroblasts. *Biochim. Biophys. Acta* **574**: 351-355, 1979.

73. Choudhury, G.G., V.L. Sylvia, and A.Y. Sakaguchi. Decline of signal transduction by phospholipase C_y in IMR-90 human diploid fibroblasts at high population doubling levels. *FEBS Lett.* **293**: 211-214, 1991.

74. Detata, V., A. Ptasznik, and V.J. Cristofalo. Effects of the tumor promoting agent phorbol 12-myristate 13-acetate (PMA) on the proliferation of young and senescent WI-38 human diploid fibroblasts. *Exp. Cell Res.* **205**: 261-269, 1993.

75. Greenberg, M.E., and E.B. Ziff. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature* **311**: 433-439, 1984.

76. Seshadri, T., and J. Campisi. Repression of c-fos transcription and an altered genetic program in senescent human fibroblasts. *Science Wash. DC* **247**: 205-209, 1991.

77. Riabowol, K., J. Schiff, and M.Z. Gilman. Transcription factor AP-1 activity is required for initiation of DNA synthesis and is lost during cellular aging. *Proc. Natl. Acad. Sci. USA* **89**: 157-161, 1992.

78. Phillips, P.D., R.J. Pignolo, K. Nishikura, and V.J. Cristofalo. Renewed DNA synthesis in senescent WI-38 cells by expression of an inducible chimeric c-fos construct. *J. Cell Physiol.* **151**: 206-212, 1992.

79. Shigeoka, H., and Henry C. Yang. Early kinase C dependent events in aging human diploid fibroblasts. *Mech. Ageing Dev.* **55**: 49-59, 1990.

80. Paulsson, Y., M. Bywater, S. Pfeifer-Ohlsson, R. Ohlsson, S. Nilsson, C.H. Heldin, B. Westermark, and C. Betsholtz. Growth factors induce early pre-replicative changes in senescent human fibroblasts. *EMBO* **5**: 2157-2162, 1986.

81. Rose, D.W., G. McCabe, J.R. Feramisco, and M. Adler. Expression of c-fos and AP-1 activity in senescent human fibroblasts is not sufficient for DNA synthesis. *J. Cell Biol.* **119**: 1405-1411, 1992.

82. Chellappan, S.P., S. Hiebert, M. Mudryl, J.M. Horowitz, and J. Nevins. The E2F transcription factor is a cellular target for the Rb protein. *Cell* **65**: 1053-1061, 1991.

83. Lees, J.A., M. Saito, M. Vidal, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin. The retinoblastoma protein binds to a family of E2F transcription factors. *Mol. Cell Biol. 13: 7813-7825, 1993.*

84. Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of cyclin-dependent kinases. *Cell* **75**: 805-816, 1993.

85. Nevins, J.R., E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**: 424-429, 1992.

86. Stein, G.H., L.F. Drullinger, R.S. Robetorye, O.M. Pereira-Smith, and J.R. Smith. Senescent cells fail to express cdc2, cycA, and cycB in response to mitogen stimulation. *Proc. Natl. Acad. Sci. USA* **88**: 11012-11016, 1991.

87. Won, K-A., Y. Xiong, D. Beach, and M.Z. Gilman. Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. *Proc. Natl. Acad. Sci. USA* **89**: 9910-9914, 1992.

88. Stein, G.H., M. Beeson, and L. Gordon. Failure to phosphorylate the retinoblastoma gene product in senescent human fibroblasts. *Science* **249**: 666-669, 1990.

89. Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and R.W. Craig. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* **51**: 6304-6311, 1991.

90. Raycroft, L., Wu, H., and G. Lozano. Transcriptional activation by wild-type p53 but not transforming mutants of the anti-oncogene. *Science* **249**: 1049-1051, 1990.

91. Smith, J.R., Inhibitors of DNA synthesis derived from senescent human diploid fibroblasts. *Exp. Gerontol.* **27**: 409-412, 1992.

92. Wafik, S.E-D., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. Waf1, a potent mediator of p53 tumor suppression. *Cell* **75**: 817-825, 1993.

93. Haper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**: 805-816, 1993.

94. Chang, C-D., P. Phillips, K.E. Lipson, V.J. Cristofalo, and R. Baserga. Senescent human fibroblasts have a post-transcriptional block in the expression of the proliferating cell nuclear antigen gene. *J. Biol. Chem.* **266**: 8663-8666, 1991.

95. Busbee, D., V. Sylvia, G. Curtin, S. Peng, V. Srivastava, and R. Tilley. Age-related changes in DNA polymerase alpha expression. *Exp. Gerontol.* **24**: 395-413, 1989.

96. Norwood, T.H., A. Saulewicz, F. Hanaoka, and W.R. Pendergrass. Failure of senescent fibroblasts to complement a DNA polymerase alpha mutant (Abstract). *Proc. Ann. Meet. Am. Gerontol. Soc. 44th San Francisco CA 1991,* p. 354.

97. Norwood, T.H., W.R. Pendergrass, C.A. Sprague, and G.M. Martin. Dominance of the senescent phenotype in heterokaryons between replicative and post-replicative human fibroblast-like cells. *Proc. Natl. Acad. Sci. USA* **71**: 2231-2235, 1974.

98. Pendergrass, W.R., J.C. Angello, A.C. Saulewicz, and T.H. Norwood. DNA polymerase alpha and the regulation of intry into S phase in heterokaryons. *Exp Cell Res.* **192**: 426-432, 1991.

99. Pereira-Smith, O.M., and J.R. Smith. Phenotype of low proliferative potential is dominant in hybrids of normal human fibroblasts. *Somatic Cell Genet.* **8**: 731-742, 1982.

100. Stein, G.H., R.M. Yanishevsky, L. Gordon, and M. Beeson. Carcinogen-transformed human cells are inhibited from entry into S phase by fusion to senescent cells, but cells transformed by DNA tumor viruses overcome the inhibition. *Proc. Natl. Acad. Sci. USA* **79**: 5287-5291, 1982.

101. Hart, R.W., G.A. Sacher, and T.L. Hoskins. DNA repair in a short- and a long-lived rodent species. *J. Gerontol.* **34**: 808-817, 1979.

102. Hart, R.W., and R.B. Setlow. Correlation between deoxyribonucleic acid excision-repair and life-span in a number of mammalian species. *Proc. Natl. Acad. Sci. USA* **71**: 2169-2173, 1974.

103. Liu, A.Y-C., Z. Lin, H-S. Choi, F. Sorhage, and B. Li. Attenuated induction of heat shock gene expression in aging diploid fibroblasts. *J. Biol. Chem.* **264**: 12037-12045, 1989.

104. Luce, M.C., and V.J. Cristofalo. Reduction in heat shock gene expression correlates with increased thermosensitivity in senescent human fibroblasts. *Exp. Cell Res.* **202**: 9-16, 1992.

105. Wadhwa, R., S.C. Kaul, Y. Ikawa, and Y. Sugimoto. Identification of a novel member of the mouse hsp70 family. *J. Biol. Chem.* **268**: 6615-6621, 1993.

106. Wadhwa, R., S.C. Kaul, Y. Sugimoto, and Y. Mitsui. Induction of cellular senescence by transfection of cytosolic mortalin cDNA in NIH 3T3 cells. *J. Biol. Chem.* **268**: 22239-22242, 1993.

107. Wistrom, C., and B. Villeponteau. Cloning and expression of SAG: a novel marker of cellular senescence. *Exp. Cell Res.* **199**: 355-362, 1992.

108. Giordano, T., and D.N. Foster. Identification of a highly abundant cDNA isolated from senescent WI-38 cells. *Exp. Cell Res.* **185**: 399-406, 1989.

109. Wang, E., Rapid disappearance of statin, a nonproliferating and senescent-cell specific protein, upon reentering the process of cell cycling. *J. Cell Biol.* **101**: 1695-1701, 1985.

110. Wang, E., and G. Tomaszewski. Granular presence of terminin is the marker to distinguish between the senescent and quiescent states. *J. Cell Physiol.* **147**: 514-522, 1991.

111. Cedar, H., DNA methylation and gene activity. *Cell* **53**: 3-4, 1988.

112. Wilson, V.L., and P.A. Jones. DNA methylation decreases in aging but not in immortal cells. *Science* **220**: 1055-1057, 1983.

113. Fairweather, D.E., M. Fox, and G.P. Margison. The in vitro lifespan of MRL-5 cells is shortened by 5-azacytidine-induced demethylation. *Exp. Cell Res.* **168**: 153-159, 1987.

114. Wilson, V.L., R.A. Smith, S. Ma, and R.G. Cutler. Genomic 5-methyldeoxycytidine decreases with age. *J. Biol. Chem.* **262**: 9948-9951, 1987.

115. Migeon, B.R., J. Axelman, and A.H. Beggs. Effect of ageing on reactivation of the human X-linked HPRT locus. *Nature* **335**: 93-96, 1988.

116. Wareham, K.A., M.F. Lyon, P.H. Glenister, and D.E. Williams. Age-related reaction of an X-linked gene. *Nature: 327: 725-727, 1987.*

117. Klein, C.B., K. Conway, X.W. Wang, R.K. Bhamra, X. Lin, M.D. Cohen, L. Annab, J.C. Barrett, and M. Costa. Senescence of nickel-transformed cells by an X chromosome: possible epigenetic control. *Science* **251**: 796-799, 1990.

118. Parshad, R., and K.K. Sanford. Intermittent exposure to fluorescent light extends the lifespan of human diploid fibroblasts in culture. *Nature* **268**: 736-737, 1977.

119. Phillips, P.D., K. Woolwich, and V.J. Cristofalo. Hydorcortisone stimulation of DNA synthesis in bromodeoxyuridine-selected non-dividing WI-38 cells. *Mech. Ageing Dev.* **20**: 271-277, 1982.

120. Fanning, E., and R. Knippers. Structure and function of simian virus 40 large tumor antigen. *Annu. Rev. Biochem.* **61**: 55-85, 1992.

121. Shay, J.W., O.M. Pereira-Smith, and W.E. Wright. A role for both RB and p53 in the regulation of human cellular senescence. *Exp. Cell Res.* **196**: 33-39, 1991.

122. Dornreiter,I., A. Hoss, A.K. Arthur, and Ellen Fanning. SV40 T antigen binds directly to the large subunit of purified polymerase alpha. *The EMBO Journal* **9**: 3329-3336, 1990.

123. Taylor, I.C.A., W. Solomon, B.M. Weiner, E. Paucha, M. Bradley, and R.E. Kingston. Stimulation of the human heat shock protein 70 promoter in vitro by simian virus 40 large T antigen. *J. Biol. Chem.* **264**: 16160-16164, 1989.

124. Gorman, S.D., and V.J. Cristofalo. Reinitiation of cellular DNA synthesis in BrdU-selected nondividing senescent WI-38 cells by simian virus 40 infection. *J. Cell. Physiol.* **125**: 122-126, 1985.

125. Koniecki, J., P. Nugent, J. Kordowska, and R. Baserga. Effect of the SV40 T antigen on the posttranscriptional regulation of the proliferating cell nuclear antigen and DNA polymerase-alpha genes. *Cancer Res.* **51**: 1465-1471, 1991.

126. Sack, G.H., Jr., Human cell transformation by simian virus 40 - a review. *In Vitro* **17**: 1-19, 1981.

127. Shay, J.W., W.E. Wright, and H. Werbin. Defining the molecular mechanisms of human cell immortalization. *Biochim. Biophys. Acta* **1072**: 1-7, 1991.

128. Small, M.B., Y. Gluzman, and H.L. Ozer. Enhanced transformation of human fibroblasts by origin-defective simian virus 40. *Nature* **296**: 671-672, 1982.

129. Hara, E., H. Tsurui, A. Shinozaki, S. Nakada, and K. Oda. Cooperative effect of antisense-Rb and antisense-p53 oligomers on the extension of life span in human diploid fibroblasts, TIG-1. *Biochem. Biophys. Res. Comm.* **179**: 528-534, 1991.

130. Shay, J.W., O.M. Pereira-Smith, and W.E. Werbin. A role for both RB and p53 in the regulation of human cellular senescence. *Exp. Cell. Res.* **196**: 33-39, 1991.

131. Stein, G.H., SV40-transformed human fibroblasts: evidence for cellular aging in precrisis cells. *J. Cell Physiol.* **125**: 36-44, 1985.

132. Ozer, H.L., M.L. Slater, J.J. Dermody, and M.J. Mandel. Replication of SV40 DNA in normal human fibroblasts and those from xeroderma pigmentosum. *Virology* **39**: 481-489, 1981

133. Shay, J.W., and W. Wright. Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen. *Exp. Cell Res.* **184**: 109-118, 1989.

134. Pereira-Smith, O.M., and J.R. Smith. Evidence for the recessive nature of cellular immortality. *Science* **221**: 964-966, 1983.

135. Wright, W.E., O.M. Pereira-Smith, and J.W. Shay. Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. *Mol. Cell. Biol.* **9**: 3088-3092, 1989.

136. Radna, R.L., Y. Caton, K.K. Jha, P. Kaplan, G. Li, F. Traganos, and H.L. Ozer. Growth of immortal simian virus 40 tsA-transformed human fibroblasts is temperature dependent. *Mol. Cell. Biol.* **9**: 3093-3096, 1989.

137. Pereira-Smith, O.M., and J.R. Smith. Genetic analysis of indefinite division in human cells: identification of four complementation groups. *Proc. Natl. Acad. Sci. USA* **85**: 6042-6046, 1988.

138. Neufeld, D.S., S. Ripley, A. Henderson, and H.L. Ozer. Immortalizaion of human fibroblasts transformed by origin-defective simian virus 40. *Mol. Cell. Biol.* **7**: 2794-2802, 1987.

139. Smith, H.S., B. Owens, A.J. Hiller, W.A. Nelson-Rees, and J.O. Johnston. The bilology of human cells in tissue culture. I. Characterization of cells derived from osteogenic sarcomas. *Int. J. Cancer* **17**: 219-234, 1976.

140. Graham, F.L., and A.J. Van der Eb. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**: 456-467, 1973.

141. Li, G., and H.L. Ozer. Unpublished data.

142. Sambrook, J., E.F. Fritsch, and T. Maniatis. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1989.

143. Feinberg, A.P., and B. Vogelstein. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**: 6-13, 1983.

144. Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (ed.). Current protocols in molecular biology. Greene Plublishing Associates and Wiley-Interscience, New York, 1987.

145. Church, G.M., and W. Gilbert. Genomic sequencing . *Proc. Natl. Acad. Sci. USA* **81**: 1991-1995, 1984.

146. Small, M.B., N. Hay, M. Schwab, and J.M. Bishop. Neoplastic transformation by the human gene N-myc. *Mol. Cell. Biol.* **7**: 1638-1645, 1987.

147. Aufrey, C., and F. Rougeon. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107**: 303-314, 1980.

148. Harley, C.B., A.B. Futcher, and C.W. Greider. Telomeres shorten during aging of human fibroblasts. *Nature* **345**: 458-460, 1990.

149. Lewin, B.M., Gene Expression - 3, John Wiley & Sons, Inc., New York, 1977.

150. Berger, S.L., and A.R. Kimmel (eds). Guide to molecular cloning techniques. *Methods in Enzymology,* volume **152.** Academic Press Inc., 1987.

151. Martinson, H.G., and E.B. Wagenaar. Thermal elution chromatography and the resolution of nucleic acids on hydroxylapatite. *Anal. Biochem.* **61**: 144-154, 1974.

152. Sanger, F., S. Nicklen, and A.R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463, 1977.

153. Girardi, A.J., Jensen, F.C., and H. Koprowski. SV40-induced transformation of human diploid cells: crisis and recovery. *J. Cell. and Comp. Physiol.* **65**: 69-84, 1965.

154. Gish, W.R., and M.R. Botchan. Simian virus 40-transformed human cells that express large T antigens defective for viral DNA replication. *J. Virol.* **62**: 2864-2876, 1987.

155. Benech, P., Y. Mory, M. Revel, and J. Chebath. Structure of two forms of the interferon-induced (2'-5') oligoA synthetase of human cells based on cDNAs and gene sequence. *EMBO* **4**: 2249-2256, 1985.

156. Nichols, W.W., A.J. Girardi, C.I. Bradt, R. Hill, and C. Cody. Cytogenetic changes induced in human diploid fibroblasts by tsA58 SV40 at permissive and restricitive temperatures. *Mutat. Res.* **150**: 327-332, 1985.

157. Blackburn, E.H. Telomeres: Structure and synthesis. *J. Biol. Chem.* **265**: 5919-5921, 1990.

158. de Lange, T. Human telomeres are attached to the nuclear matrix. *EMBO* **11**: 717-724, 1992.

159. Moyzis, R.K., J.M. Buckingham, L.S. Cram, M. Dani, L.L. Deaen, M.D. Jones, J. Meyne, R.L. Ratliff and J.R. Wu. A highly conserved repetitive DNA sequence, $(TTAGGG)_n$, present at the telomeres of human chromosomes. *Proc. Natl. Acad. Sci. (U.S.A.)* **85**: 6622-6626, 1988.

160. Brown, W.R.A., P.J. MacKinnon, A. Villasante, N. Spurr, V.J. Buckle, and M.J. Dobson. Structure and polymorphism of human telomere-associated DNA. *Cell* **63**: 119-132, 1990.

161. de Lang, T., L. Shiue, R.M. Myers, D.R. Cox, S.L. Naylor, A.M. Killery, and H.E. Varmus. Structure and variability of human chromosome ends. *Mol. Cell. Biol.* **10**: 518-527, 1990.

162. Weber, B., C. Collins, C. Robbins, R.E. Magenis, A.D. Delaney, J.W. Gray, and M.R. Hayden. Characterization and organization of DNA sequences adjacent to the human telomere associated repeat $(TTAGGG)_n$. *Nuc. Acids Res.* **18**: 3353-3361, 1990.

163. Allsopp, R.C., H. Vaziri, C. Patterson, S. Goldstein, E.V. Younglai, A.B. Futcher, C.W. Greider, and C.B. Harley. Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. (U.S.A.)* **89**: 10114-10118, 1992.

164. Hubbard-Smith, K., P. Patsalis, J.R. Pardinas, K.K. Jha, A.S. Henderson, and H.L. Ozer. Altered chromosome 6 in immortal human fibroblasts. *Mol. Cell. Biol.* **12**: 2273-2281, 1992.

165. Levy, M.Z., R.C. Allsopp, A.B. Futcher, C.W. Greider, and C.B. Harley. Telomere end-replication problem and cell aging. *J. Mol. Biol.* **223**: 951-960, 1992.

166. Meyne, J., R.J. Baker, H.H. Hobart, T.C. Hsu, O.A. Ryder, O.G. Ward, J.E. Wiley, D.H. Wurster-Hill, T.L. Yates, and R.K. Moyzis. Distribution of non-telomeric sites of the $(TTAGGG)_n$ telomeric sequence in vertebrate chromosomes. *Chromosoma* **99**: 3-10, 1990.

167. Pardue, M.L., Dynamic instability of chromosomes and genomes. *Cell* **66**: 427-231, 1991

168. Sargent, T.D., and I.B. Dawid. Differential Gene Expression in the Gastrula of Xenopus laevis. *Science* **222**: 135-139, 1983.

169. Timberlake, W.E., Developmental Gene Regulation in Aspergillus nidulans. *Dev. Biol.* **78**: 497-510, 1980.

170. Hedrick, S.M., D.I. Cohen, E.A. Nielsen, and M.M. Davis. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* **308**: 149-153, 1984.

171. Pang, Z., J.R. Pardinas, J. Dermody, and H.L. Ozer. Dinucleotide repeat polymorphism in the INHBA gene. *Hum. Mol. Genet.* Vol. **2**, No. 11, p.1982, 1993.

172. Weber, J.L., and P.E. May. An abundant new class of human DNA polymorphisms. *Am. J. Hum. Genet. (suppl.)* **43**: A161, 1988.

173. Patsalis, P.C., Molecular and cytogenetic analysis of human diploid fibroblast cells transformed by simian virus 40; what causes immortalization? *Doctoral dissertation,* 1993.

174. Zerrahn, J., U. Knippschild, T. Winkler, and W. Deppert. Independent expression of the transforming amino-terminal domain of SV40 large T antigen from an alternatively spliced third SV40 early mRNA. *EMBO* **12**: 4739-4746, 1993.

175. Noda, A., Y. Ning, S.F. Venable, O.M. Pereira-Smith, and J.R. Smith. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* **211**: 90-98, 1994.

176. Rubelj, I., and O.M. Pereira-Smith. SV40-transformed human cells in crisis exhibit changes that occur in normal cellular senescence. *Exp. Cell Res.* **211**: 82-89, 1994.

177. Marshall, C.J. Tumor Suppressor Genes. *Cell* **64**: 313-326, 1991.

178. Ray, F.A., and P.M. Kraemer. Frequent deletions at chromosome 6q21 and other recurrent changes in nine newly immortalized human fibroblasts cell lines. *Cancer Genet. Cytogenet.,* in press.

179. Sandhu, A.K., K. Hubbard, G.P. Kaur, K.K. Jha, H.L. Ozer, and R.S. Athwal. Senescence of immortal human fibroblasts by the introduction of normal human chromosome 6. *PNAS,* in press.

180. Hastie, N.D., M. Dempster, M.G. Dunlop, A.M. Thompson, D.K. Green, and R.C. Allshire. Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **346**: 866-868, 1990.

181. Wright, W.E., and J.W. Shay. Telomere positional effects and the regulation of cellular senescence. *Trends Genet.* **8**: 193-197, 1992.

182. Small, M.B., K. Hubbard, J.R. Pardinas, K.A. Sethi, and Alexander M. Marcus. Maintenance of telomeres in SV40-transformed and immortal human fibroblasts. *Mol. Cell. Biol.,* submitted.

183. D'Mello, N.P., and S.M. Jazwinski. Telomere length constancy during aging of Saccharomyces cerevisiae. *J. Bacteriol.* **173**: 6709-6713, 1991.

184. Counter, C.M., A.A. Avilion, C.E. LeFeuvre, N.G. Stewart, C.W. Greider, C.B. Harley, and S. Bacchetti. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *The EMBO Journal* **11**: 1921-1929, 1992.

185. Kingsley, D.M., The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes & Dev.* **8**: 133-146, 1994.

186. Attisano, L., J.L. Wrana, S. Cheifetz, and J. Massague. Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. *Cell* **68**: 97-108, 1992.

187. Murata, M., Y. Eto, H. Shibai, M. Sakai, and M. Muramatsu. Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin β_A chain. *Proc. Natl. Acad. Sci. USA* **85**: 2434-2438, 1988.

188. Matzuk, M.M., M.J. Finegold, J.J. Su, A.J.W. Hsueh, and A. Bradley. α-Inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature* **360**: 313-319, 1992.

189. Schubert, D., H. Kimura, M. LaCorbiere, J. Vaughan, D. Karr, and W.H. Fischer. Activin is a nerve cell survival molecule. *Nature* **344**: 868-870, 1990.

190. Henry, J.L., D.L. Coggin, and C.R. King. High-level expression of the ribosomal protein L19 in human breast tumors that overexpress erbB-2. *Cancer Res.* **53**: 1403-1408, 1993.

191. Sharp, M.G.F., S.M. Adams, P. Elvin, R.A. Walker, W.J. Brammar & J.M. Varley. A sequence previously identified as metastasis-related encodes an acidic ribosomal phosphoprotein, P2. *Br. J. Cancer* **61**: 83-88, 1990.

192. Pogue-Geile, K., J.R. Geiser, M. Shu, C. Miller, I.G. Wool, A.I. Meisler, and J.M. Pipas. Ribosomal protein genes are overexpressed in colorectal cancer: isolation of a cDNA clone encoding the human S3 ribosomal protein. *Mol. Cell. Biol.* **11**: 3842-3849, 1991.

193. Grabowski, D.T., R.O. Pieper, B.W. Futscher, W.A. Deutsch, L.C. Erickson, and M.R. Kelley. Expression of ribosomal phosphoprotein PO is induced by antitumor agents and increased in Mer$^-$ human tumor cell lines. *Carcinogenesis* **13**: 259-263, 1992.

194. Cassel, D., and T. Pfeuffer. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. USA* **75**: 2669-2673, 1978.

195. Serafini, T., L. Orci, M. Amherdt, M. Brunner, R.A. Kahn, and J.E. Rothman. ADP-Ribosylation factor is a subunit of the coat of golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell* **67**: 239-253, 1991.