# ANALYSIS OF LOSS OF TELOMERIC DNA DURING REPLICATIVE AGING OF HUMAN CELLS AND EXAMINATION OF THE ROLE OF THIS PROCESS IN THE INDUCTION OF CELL SENESCENCE

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# ANALYSIS OF TELOMERE SHORTENING DURING AGING OF HUMAN CELLS

## ABSTRACT

Telomeres are nucleoprotein complexes located at the ends of all eukaryotic chromosomes, and are essential for genetic stability and viability. Telomere length decreases during replicative aging of normal human diploid fibroblasts (HDF) *in vitro*. These observations imply that telomere length provides a mitotic clock for the replicative age of normal somatic cells, and that telomere shortening may cause cell senescence. To further examine these predictions, telomere length was analyzed (i) *in vitro*, in young HDFs as well as senescent and near-senescent HDFs and (ii) *in vivo*, in HDFs and candidate hematopoietic stem cells. Mean terminal restriction fragment (TRF) length decreased significantly at a rate of ~ 15 bp/year in HDFs. Also, mean TRF length decreased with increasing donor age in $CD34^{+}CD38^{lo}$ hematopoietic cells obtained from fetal donors and adult donors of ages 19 and 59 years. These observations confirm that telomere length decreases not just during aging *in vitro* but also during aging *in vivo*. They also, imply that stem cells have a finite lifespan.

The observations that telomere length is proportional to replicative capacity in cultured HDFs from normal and progeroid donors and that telomere length is shorter in HDFs at the periphery of radial outgrowths relative to HDFs at the center suggest that telomere length is a valuable marker for replicative potential.

Interclonal variability in telomere length was significantly less at senescence than at early passage for HDF clones established from the same strain, in support of the existence of a critical telomere length in senescent

cells. Calculation of mean telomere length at senescence for these clones and one other HDF strain yielded an upper estimate of 2-3 kb for the critical telomere length. However, the detection frequency of individual telomeres by fluorescent *in situ* hybridization (FISH) varied from telomere to telomere in both young and senescent cells. Furthermore, in pre-crisis SW26 fibroblasts, the long arm of one copy of chromosome 9, which has a relatively short telomere, is frequently engaged in an end-to-end dicentric chromosome. Also, a significant distribution of TRF lengths exists at all PDLs. These observations indicate that the shortest telomere length at senescence, which best represents the critical telomere length, is probably considerably less than 2-3 kb.

These observations support a causal role of telomere shortening in cell senescence. This has important implications for future treatment of cancer and therapeutic prevention of cell senescence during aging.

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## Preface

In section 2.1, the mean TRF length analysis of HDFs from various aged normal donors and progeroid donors was done by myself. The data from this analysis is presented in Figures 1-3 of this section.

In section 2.2, the mean TRF length and telomeric signal intensity analysis presented in Figure 5 was done by myself. The analysis of the standard deviation of the TRF length distribution and calculation of the mean telomeric and non-telomeric lengths at senescence was also done by me. The model for the end replication problem (Figures 1-4 and 6) was done by M. Levy and C. Harley.

In section 2.3, the establishment and culturing of the radial outgrowths of HDFs as well as the analysis of mean TRF length and telomeric signal intensity of the outgrowths was done by myself (data presented in Table 1). Analysis of mean TRF length in serially passaged, quiescent and quiescent-trypsinized HDFs was done by M. Kashefi-Aazam (Figure 2). Analysis of mean TRF length of brain tissue from various aged donors was done by E. Chang (Figure 3). Telomerase assays on brain tissue was done by J. Shay and M. Piatyszek (Figure 4).

In section 2.4, all experimental analyses were done by myself.

In section 2.5, ~50% of the mean TRF length analysis of the hematopoietic cell long term cultures (Figure 1 and 2) and all of the mean TRF length analysis of the hematopoietic cells obtained directly from fetal and adult donors (Figure 3) was done by myself. The remainder of the mean TRF

length analysis of the hematopoietic cell long term cultures was done by H. Vaziri. All hematopoietic cell samples were obtained from and cultured and sorted by P. Lansdorp.

In section 2.6, all FISH analysis of metaphase chromosomes (Figures 5 and 7) and analysis of detection frequency of telomeric signal on metaphase chromosomes (Tables I and II) were done by myself. In addition, measurement of the rate of telomere shortening by Southern analysis of TRF length for BJ cells was done by myself. All FISH analysis of interphase cells was done by S. Henderson (Figures 2, 3 and 6). Results of Southern analysis of TRF length shown in figures 4 and 8 was done by S.-S. Wang.

The major part of each paper in sections 2.1, 2.3, 2.4 and 2.6 was also written by myself.

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| | |
|---|---|
| A | deoxyadenosine-5'-phosphate |
| bp | base pair |
| BSA | bovine serum albumin |
| C | deoxycytidine-5'-phosphate |
| CD4, CD8, etc | Sets of cell surface molecules on T lymphocytes which define a certain differentiated population |
| CDK | cyclin-dependent kinase |
| cDNA | DNA complementary to mRNA |
| CLSM | confocal laser scanning microscope |
| DA | distamycin |
| DAPI | 4',6-diamidino-2-phenylindole |
| DIG | digoxigenin |
| DNA | deoxyribonucleic acid |
| dUTP | deoxyuridine-5'-triphosphate |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| FISH | fluorescent *in situ* hybridization |
| FITC | fluorescein isothiocyanate |
| G | deoxyguanosine-5'-phosphate |
| G4-DNA | tetraplex formed from G-rich DNA |
| HDF | human diploid fibroblast |

| | |
|---|---|
| HeT-A | retrotransposon-like element in *Drosophila* |
| kbp | kilobase pair |
| kDa | kilodaltons |
| M | molar |
| min | minute |
| ml | milliliter |
| mM | millimolar |
| mRNA | messenger ribonucleic acid |
| NA | numerical aperture |
| ng | nanogram |
| nm | nanometer |
| oxo$^8$dG | 8-oxo-2'-deoxyguanosine |
| p53 | tumor suppressor of molecular mass 53 kDa. |
| PBL | peripheral blood leucocyte |
| PBS | Dulbecco's phosphate buffered saline |
| PCR | polymerase chain reaction |
| pd | population doubling |
| PDL | population doubling level |
| PI | propidium iodide |
| PN | phosphate buffer containing Nonidet P-40 |
| PNM | PN buffer containing dried milk |
| pRB | retinoblastoma gene product |
| RITC | rhodamine isothiocyanate |
| RNA | ribonucleic acid |
| RT | room temperature |
| SDI1 | senescent cell-derived inhibitor, also referred to as p21 |
| SSC | sodium chloride/sodium citrate buffer |

| | |
|---|---|
| T | deoxythymidine-5'-phosphate |
| TE | Tris/EDTA buffer |
| TIMP | tissue inhibitor of metalloproteinases |
| TRF | terminal restriction fragment |
| µg | microgram |
| µl | microliter |
| µm | micrometer |

# 1. Introduction

## 1.1 Cell senescence and organismal aging.

The eventual acquisition of a senescent phenotype is universal for nearly all mitotically active somatic cells (reviewed in Finch, 1990; Stanulis-Praeger, 1987). A key feature of senescence of mitotic cells is the irreversible loss of proliferative capacity (reviewed in Goldstein, 1990). Hence the senescence of mitotic cells is often referred to as replicative senescence. The model of cell senescence was established over 30 years ago in classic experiments by Hayflick and Moorhead who established that the limited proliferative capacity (the Hayflick limit) of cultured HDFs was attributable to senescence (1961). Although senescent HDFs have lost the ability to divide, they can be maintained viable in culture for periods of a year or more (Matsumura *et al*, 1979). Senescent HDFs are not compromised in fidelity of translation and do not have increased levels of mutated or faulty proteins in general (reviewed in Stanulis-Praeger, 1987; Finch, 1990; Goldstein, 1990). Other characteristics of the senescent phenotype include increased cytoplasmic volume, reduced levels of cytosine methylation, reduced rates of protein degradation and increased levels of chromosomal aberrations (reviewed in Stanulis-Praeger, 1987; Finch, 1990; Goldstein, 1990; Dice, 1993).

The onset of cell senescence is accompanied by changes in gene expression. Many of these changes also occur in cells that are induced, either by serum starvation or contact inhibition, to enter a state of proliferative arrest called $G_0$ (quiescence). For example, both senescent and quiescent HDFs

have increased levels of statin (Wang, 1985) and decreased levels of c-*fos* (Riabowol *et al*, 1988; Seshadri and Campisi, 1990). Changes in gene expression specific to senescence include increased levels of procollagenase (West *et al*, 1989) and terminin (Wang and Tomaszewski, 1991) and decreased levels of TIMP-1 (West *et al*, 1989). It is for these reasons, loss of ability to proliferate and changes in gene expression, that cell senescence has been suggested as being akin to terminal differentiation (also see Section 1.2.2)(reviewed in Goldstein, 1990).

There is considerable correlative evidence suggesting that cell senescence does have an impact on organismal aging. The *in vitro* proliferative capacity of fibroblasts established from different species is proportional to species lifespan (Rohme, 1981), and cultured fibroblasts established from Hutchinson-Gilford progeria donors and Werner's progeria donors have significantly reduced proliferative capacities in comparison to fibroblast cultures established from age-matched normal donors (Goldstein and Harley, 1979). The primary cloning efficiency, a measure of proliferative capacity, of arterial smooth muscle cells decreases with donor age for mice and humans (Martin *et al*, 1983; Bierman, 1978). In middle-aged individuals the replicative capacity for human endothelial cell cultures established from the iliac artery, a site of relatively high hemodynamic stress and concentration of atherosclerotic plaques, appears to be reduced by 25-30% compared to endothelial cell cultures established from the iliac vein (Chang and Harley, 1995). These latter observations support the hypothesis that exhaustion of replicative capacity at focal points of high hemodynamic stress may play a causative role in atherosclerosis. Results from recent studies suggest that senescent PBLs may accumulate with donor age. Human PBLs undergo a decline in their proliferative response to mitogens in culture with

increasing donor age (Hefton *et al*, 1980). Expression of the costimulatory molecule CD28 decreases during proliferation of cultured T lymphocytes to the extent that nearly 100% of the cells at replicative senescence are $CD28^{lo}$ (Effros *et al*, 1994). Moreover, the fraction of $CD28^{lo}$ T lymphocytes in peripheral blood increases significantly as a function of donor age (Effros *et al*, 1994). Thus it is possible that clonal attenuation, which with respect to lymphocytes refers to a decreased proliferative response of individual clones to antigenic stimuli, is at least partially responsible for the decline in immune function in elderly individuals. Evidence also exists in support of a contribution of replicative senescence to skin aging, in particular aging of the dermis (reviewed in West, 1994). The dermis is largely composed of an extracellular connective matrix which consists mostly of collagen (West, 1994). In elderly individuals, there is pronounced degeneration of the extracellular matrix which is at least partially due to a decrease in collagen content (West, 1994). Fibroblasts, the primary source of collagen in the dermis, also decrease in number during aging (West, 1994). Senescent fibroblasts produce less procollagen, more collagenase and less TIMP-1 than their younger counterparts (West, 1994; Linskins *et al*, 1995). In addition, a recent study has provided direct evidence, as indicated by *in situ* detection of ß-galactosidase, for an increase in the number of senescent fibroblasts and keratinocytes in skin tissue during aging (Dimri *et al*, 1995). Thus an increase in the number of senescent fibroblasts in the dermis may account for some of the deleterious changes in this tissue with age. However, it is still controversial whether some cell types, for example stem cells, have a limited replicative capacity (Ross *et al*, 1982), or are capable of indefinite cell division (Harrison *et al*, 1984). Overall, these data support the notion that cell senescence has a non-adaptive, deleterious role in individuals of advanced

age.

It is also possible that cell senescence may have an adaptive role during the initial stages of life. It has been proposed that clonal attenuation of certain subsets of cells might be a mechanism to allow tight control of tissue growth during early development (Martin, 1993), although this has yet to be shown. Additionally, Sager has suggested that cell senescence may have a role in tumor suppression in reproductively active individuals (1991). The accumulation of damage and alterations to DNA during aging of mitotic cells (Bender *et al*, 1989; reviewed in Shigenaga *et al*, 1993 and Ames *et al*, 1993; Liu *et al*, 1994) is fundamental to this theory. Any cells that acquire DNA alterations that predispose to or directly cause loss of growth regulation, and therefore predispose to neoplasia, would not be able to grow beyond the Hayflick limit due to activation of tumor suppressors. Once the cause of cell senescence is understood, it may be possible to test these theories in model organisms or cell systems in which cell senescence is delayed or prevented.

## 1.2 Stochastic Error Theories Versus Genetic Theories of Cell Senescence

The many theories that have been proposed to explain why cells senesce can be classified into 2 broad categories: stochastic error theories and genetic theories. Stochastic error theories assume cell senescence occurs as a result of the accumulation of damaged macromolecules, whereas genetic theories are based upon the assumption that cell senescence occurs as a result of the induction of expression of certain genes. Currently, genetic theories seem to give the best explanation of cell senescence (reviewed in Goldstein, 1990; Dice, 1993). It is difficult to reconcile the observation that quiescent or

cryo-preserved cells from the same HDF strain all reach senescence, within narrow limits, at the same PDL (Hayflick, 1965; Dello'Orco *et al*, 1973). Also, there are many specific alterations in gene expression as cells become senescent which shows that cell senescence is at least partially genetically determined (reviewed in Goldstein, 1990; Dice, 1993). These 2 categories are not mutually exclusive; thus it remains possible that, under certain circumstances, accumulation of damage to macromolecules, which is a stochastic process, could induce senescence by triggering the expression of genes involved in causing cell senescence.

### 1.2.1 Stochastic Error Theories

Orgel proposed that cell senescence is caused by a low level of synthesis of aberrant proteins in young cells, leading to the further accumulation of malfunctional proteins (1963). However, fidelity of protein synthesis has repeatedly been shown to be similar for senescent and young HDFs and increased levels of mutant proteins in senescent HDFs has not been detected (reviewed in Stanulis-Praeger, 1987; Finch, 1990; Dice, 1990), suggesting that accumulation of mutated proteins does not play a role in senescence of HDFs. Aberrant forms of some proteins do accumulate in senescent HDFs, but these alterations are primarily if not entirely caused by post-translational modifications (reviewed in Dice, 1993).

The somatic mutation theory predicts that cell senescence is caused by the accumulation of alterations to the genome. As mentioned above, mutant proteins do not accumulate in senescent HDFs, which strongly argues that point mutations do not accumulate during replicative aging. However, chromosomal aberrations, particularily dicentric chromosomes, do

accumulate during aging of HDFs *in vitro* (Benn, 1976; Sherwood *et al*, 1988) and human lymphocytes *in vivo* (Bender *et al*, 1989). In addition, a chromosomal translocation resulting in loss of regulation of expression of the bcl2 gene increases in frequency in human B cells with age (Liu *et al*, 1994).

The free radical theory of cell senescence predicts that accumulation of damage incurred upon macromolecules by free radicals causes cells to senesce (reviewed in Harman, 1981; Ames *et al*, 1993). Mitochondria appear to be the greatest source of free radicals in the cell (Shigenaga *et al*, 1993). Oxidative free radicals, such as the hydroxyl radical and superoxide, are produced in the mitochondria as a by-product during normal aerobic metabolism (Shigenaga *et al*, 1993). A reduction of mitochondrial protonmotive force in lymphocytes from aged rats has been observed (Leprat *et al.*, 1990). This deficiency in mitochondrial function may be due to inactivation of mitochondrial proteins by reaction with oxidative free radicals. In addition, DNA damage, in the form of oxo$^8$dG, has been shown to be higher in senescent HDFs than in young HDFs (Chen *et al*, 1995). Moreover, reduction in oxygen tension have been shown to extend replicative lifespan (Chen *et al*, 1995), and addition of hydrogen peroxide to the culture medium of young HDFs has been shown to cause irreversible cell cycle arrest (Chen and Ames, 1994). However, it is unknown whether the effect of oxidative damage on replicative lifespan is direct or indirect (also see Section 3.4). Furthermore, the role of oxidative damage in cell senescence *in vivo* remains to be established.

Dice has suggested that the reduced rate of protein degradation observed in senescent HDFs may also be causal in cell senescence (1993). The nature of the alterations in protein catabolism in senescent HDFs that accounts for the decreased protein turnover rate is unknown (Dice, 1993).

### 1.2.2 Genetic Theories

Early genetic theories to explain cell senescence have now been largely discounted. For example, the codon restriction theory proposes different codons are used to code for the same amino acid at different stages of development (Strehler, 1986). Thus the expression of genes would change during development, ultimately leading to cell senescence. However, no change in codon usage during development has yet been reported, despite the large number cDNAs that have now been sequenced.

Some of the first evidence that cell senescence is a genetically determined process came from cell fusion experiments in the mid seventies. In one study Norwood *et al.* fused senescent cells and young cells together to form heterokaryons and subsequently assayed for DNA synthesis (1974). No DNA synthesis was observed in either nuclei. A similar observation was found when heterokaryons were formed by the fusion of senescent cells with immortal cells, that is the synthesis of DNA did not occur (Norwood *et al*, 1975). These results suggested that an inhibitor of DNA synthesis is expressed in senescent cells, resulting in the dominance of senescence when either young cells or immortal cells are fused with senescent cells. Subsequently, an activity that inhibits DNA synthesis in young cells was found to be expressed on the cell membrane surface of senescent fibroblasts (Pereira-Smith *et al*, 1985; Stein and Atkins, 1986) as well as in conditioned media from these cells (Smith, 1992), although the factor(s) responsible for this inhibition of DNA synthesis have not yet been cloned. An exception to the dominance of senescence with respect to the initiation of DNA synthesis occurs when senescent cells are fused with cells immortalized by DNA tumor viruses; in these heterokaryons, DNA synthesis is observed to occur in both nuclei (Stein

*et al*. 1982). Also, the expression of SV40 T antigen in senescent cells results in the induction of DNA synthesis (Gorman and Cristafalo, 1985). However, neither heterokaryons formed from fusing senescent cells with cells immortalized with DNA tumor viruses nor with senescent cells expressing T antigen are capable of dividing, in support of the irreversibility of cell senescence.

Further work involving the fusion of different types of immortal cells provided additional evidence for a genetic process for cell senescence. Results from these experiments suggested that all immortal cells fall into four complementation groups (Pereira-Smith and Smith, 1988), wherein the hybrids formed by fusion of two cell lines from the same complementation group undergo senescence. Thus there may be four different genes involved in the induction of senescence. In fact studies using microcell fusion to introduce single chromosomes into immortal cells indicate that genes that are causally involved in cell senescence are located on a number of different chromosomes including 1 (Sugawara *et al*, 1986), 4 (Ning *et al*, 1994) and 6 (Sandu *et al*, 1994). The identity of these genes remains largely unknown, however evidence suggests that they may include the tumor suppressors p53 and the retinoblastoma gene product (pRB) (Wright *et al*, 1989). The transformation of normal cells by DNA tumor viruses or their genes, which inactivate both p53 and pRB, results in the acquisition of an extended lifespan (Wright *et al*, 1989). The inactivation of p53 and pRB is a direct effect of the DNA tumor virus gene products, as opposed to being a result of induced genomic rearrangements, since in fibroblasts transformed with an inducible SV40 T antigen, repression of T antigen expression causes the cells to immediately senesce (Wright *et al*, 1989). Furthermore, mutation of the DNA tumor viruses to eliminate the ability to inactivate either p53 or pRB results

in the loss of the ability of the cells to divide beyond the Hayflick limit (Shay *et al*, 1991a), suggesting that inactivation of both of these proteins is required to prevent cell senescence. In addition, expression of antisense p53 and pRb in normal HDFs leads to an extension of lifespan beyond the Hayflick limit (Hara *et al*, 1991). Also, epithelial cells from individuals with Li Fraumini syndrome, in which only one functional copy of the p53 gene is present, can undergo spontaneous immortalization (Shay *et al*, 1995). These observations provide strong support for the involvement of p53 and pRB in cell senescence.

DNA tumor virus transformed cells are mortal since they can only undergo a finite number of doublings beyond the Hayflick limit before they cease dividing (Wright *et al*, 1989). At this point, referred to as crisis, most of the cells die. Only the very rare cell (1 in 2-3 x $10^7$) which survives crisis will become immortal. These observations led to the suggestion that there are 2 checkpoints, mortality stage 1 (M1) and mortality stage 2 (M2), that cells must escape before they become immortalized (Shay *et al*, 1991b). This model further suggests that cell senescence is a genetic process and that cell mortality is dependent on a number of different gene products.

It has also been proposed that cell senescence is in fact a special form of terminal differentiation, which is a genetically programmed event wherein the continual change in gene expression with increasing cell doublings culminates in irreversible cell cycle arrest (reviewed in Goldstein, 1990). However, it is debatable whether cell senescence is a programmed event that is subjected to the forces of evolution. Also, there are a number of deleterious changes in senescent cells and possibly in tissues that occur as a result of an increase in fraction of senescent cells (see Sections 1.1 and 1.2.1) which are unlikely to be present in terminally differentiated cells, at least in young

donors. Senescent cells and terminally differentiated cells are similar in that they both are in a state of irreversible cell cycle arrest. Some of the genes involved in causing cell cycle arrest appear to be common in these cells. Attention has focused on changes in levels of p53 and pRB, as well as other proteins involved in regulation of the cell cycle, particularly at the G1/S transition (Seshadri and Campisi, 1990; Stein *et al*, 1991). The steady state levels of pRB do not change at senescence, although pRB is hypophosphorylated at senescence in comparison to cycling young cells (Stein *et al*, 1990). p53 mRNA and protein levels also do not appear to change at senescence (Afshari *et al*, 1993; Atadja *et al*, 1995), although p53 DNA binding activity has recently been observed to be higher in late passage HDFs relative to early passage cells (Atadja *et al*, 1995). Recent evidence indicates that the inability of senescent cells to phosphorylate pRB is in part due to the increased expression of p21/SDI1 at senescence (Harper *et al*, 1993; Xiong *et al*, 1993; Noda *et al*, 1994). p21/SDI1 inhibits the activity of a number of cyclin dependent kinases including CDK2 and CDK4, which appear to be responsible for the phosphorylation of pRB (Harper *et al*, 1993; Xiong *et al*, 1993; Noda *et al*, 1994). In addition p21/SDI, pRB and p53 all appear to play an essential role in the development of certain types of terminally differentiated cells (Pan and Griep, 1994; Halevy *et al*, 1995; Sah *et al*, 1995). The reason for the increase in p21/SDI1 as well as alterations in expression of other gene products at senescence is still largely unknown. It is possible that induction of expression of p21/SDI1 is due to activation of p53 (Atadja *et al*, 1995). Activation of p53 is required for induction of p21/SDI1 and G1/S arrest of gamma-irradiated human cells (Dulic *et al*, 1993; Namba *et al*, 1995).

## 1.3 Structure and function of telomeres

### 1.3.1 Telomere function

The word telomere was coined by Muller from the Greek words telos meaning 'ends' and meros meaning 'part' to describe the chromosomal termini (1938). Telomeres are nucleoprotein complexes that are essential for genetic stability and cell viability (reviewed in Blackburn, 1991; Kipling, 1995). Mutant yeast and ciliate strains that are incapable of maintaining telomere length during cell division eventually become sickly, stop dividing and die (Lundblad and Szostak, 1989; Yu *et al*, 1990). Also, it has recently been shown that removal of a telomere from one end of an individual chromosome in *S. cerevisiae* causes a loss in mitotic stability of that chromosome (Sandell and Zakian, 1993). Cells which had not lost the terminally deleted chromosome either underwent cell cycle arrest or synthesized a new telomere in place of the one that was deleted. The specific functions of telomeres that make them an essential genetic element are now becoming apparent.

Telomeres appear to protect chromosomal ends from aberrant recombination and nuclease degradation. This first manifested itself from studies by McClintock who showed that the termini of broken chromosomes in *Zea mays* are recombinogenic and fusogenic, whereas the natural chromosomal termini are not (1941). More recently, it has been shown that linear DNA molecules injected into *Paramecium* undergo end-to-end fusions, however if telomeric DNA is present at the termini of the injected molecules then fusion is prevented (Bourgain and Katinka, 1991). Also, the

heterodimeric protein which binds to the ends of telomeres in *Oxytricha nova* protects the single-stranded telomeric G-strand terminus from exonuclease degradation and chemical modification *in vitro* (Gottschling and Zakian, 1986).

Telomeres may also play a role in the subnuclear organization of chromatin. That chromatin is spatially organized in the nucleus has been shown in a number of light and electron microscopy studies involving visualization of individual chromosomes in interphase nuclei (Comings, 1980; Heslop-Harrison and Bennett, 1990; Haaf and Schmidt, 1991). These studies show that chromosomes occupy discrete spatial domains in mitotic nuclei in a wide variety of species. Furthermore, in some species the telomeres appear to be clustered near to the nuclear envelope with the centromeres clustered at the opposite end of the nucleus (Comings, 1980; Agard and Sedat, 1983). This is known as the Rabl orientation (Rabl, 1885). Telomeres also appear to be attached to the nuclear envelope in some species (Byers and Goetsch, 1975; Agard and Sedat, 1983; Hilliker and Appels, 1989). This association between telomere and nuclear envelope appears to correlate with the presence of heterochromatin at chromosomal termini and the clustering of telomeres (Agard and Sedat, 1983; Young *et al*, 1983; Barnes *et al*, 1985; Gilson *et al*, 1993a). However, it is unclear whether the Rabl orientation and apparent attachment of telomeres to the nuclear envelope is biologically relevant since telomeres and centromeres become polarized during cell division as a result of forces involved in chromosome segregation. Thus the Rabl orientation may reflect absent or incomplete redispersion of chromatin in interphase nuclei following cell division. Furthermore, disruption of the telomere-nuclear envelope interaction and disassembly of telomeric heterochromatin in *S. cerevisiae* has little effect on chromosome stability

(Palladino *et al*, 1993). In mammals, telomeres appear to be neither arranged in the Rabl orientation nor attached to the nuclear envelope (Billia and De Boni, 1991; Ferguson and Ward, 1992). However, mammalian telomeres do appear to be attached to the nuclear matrix (De Lange, 1992).

Telomeres may also facilitate the pairing of chromosomes during meiosis. Unlike mitotic cells, the association of telomeres with the nuclear envelope during meiosis seems to be more of a universal phenomenon (Loidl, 1990). The association of telomeres to the nuclear envelope effectively concentrates the chromosomes into a smaller area, which may make interaction between homologous chromosomes more probable. Additionally, the pairing of homologous chromosomes and formation of the synaptonemal complex appears to initiate in subterminal regions of chromosomes in some species, although initiation at interstitial sites is also sometimes observed (Loidl, 1990). The direct involvement of telomeres in initiation of chromosome pairing is unlikely since the telomeric DNA sequence is identical for all chromosomal ends. However, G-rich oligonucleotides can form stable tetraplexes (G4-DNA) *in vitro*, in which all oligos are apparently aligned in parallel (Sen and Gilbert, 1988). Although it has yet to be shown, it is possible that the telomeric termini of the 4 sister chromatids of 2 homologous chromosomes could interact to form G4-DNA with all strands parallel *in vivo*, which could theoretically help stabilize and aid in the formation of the synaptonemal complex.

### 1.3.2 Telomere Structure

Telomeres are composed of both DNA and proteins. In general, the telomeric DNA is composed of tandem repeats of a short G/C-rich sequence,

for example the *S. cerevisiae* telomeric sequence is composed of imperfect repeats which conforms to a general consensus of $(TG)_{1-6}TG_{2-3}$ (Szostak and Blackburn, 1982). However, there are numerous exceptions to this rule (see Table 1). For instance, the telomeric repeat of *Chlamydomonas*, TTTTAGGG, is T/A rich (Petracek *et al.*, 1990). The telomeric DNA sequence of all vertebrates including humans is $(TTAGGG)_n$ (Meyne *et al*, 1989). *Drosophila* appears to be the one exception thus far for which the telomeric DNA sequence is not composed of simple repeats. Instead, *Drosophila* telomeres are composed of arrays of a retrotransposon-like repetitive element called HeT-A (reviewed in Biessmann and Mason, 1992). The HeT-A element is approximately 6 kbp in length (Valgeirdottir *et al*, 1990).

A more stringent characteristic of telomeric DNA is that the strand which extends 5' to 3' is G-rich (reviewed in Blackburn, 1991; Kipling, 1995). The reason for the evolutionary conservation of this feature of telomeric DNA is unknown but perhaps may relate to the remarkable ability of G-rich single-stranded DNA, including oligonucleotides corresponding to actual telomeric DNA sequence, to form *in vitro* intra- and intermolecular complexes stabilized by non-Watson/Crick base pairing of G residues (Fig. 1.3.1A) (reviewed in Blackburn, 1991; Kipling, 1995). These complexes include 2 stranded structures formed by the folding back of a single oligonucleotide into a hairpin, and 4 stranded structures or G4-DNA composed of 1, 2 or 4 oligonucleotides (Fig. 1.3.1A). In the latter complexes, the G residues base pair so as to form a cyclic, planar array (G-quartet) (Fig. 1.3.1B). The G-quartets are stacked together with a mono- or divalent cation located approximately at the centre of the stack of G quartets. These cations are essential for complex formation, with $K^+$ providing the greatest source of stability (Oka and Thomas, 1987; Hardin *et al*, 1991).

Table 1.3.1. Telomeric DNA sequences in eukaryotes.

**Table 3.1 Terminal repeat sequences**

| Sequence | Species |
|---|---|
| TTTTGGGG | *Euplotes, Oxytricha, Stylonychia* |
| TTGGGG | *Glaucoma, Tetrahymena* |
| TTGGGG and TTTGGG | *Paramecium* |
| TTTAGGG and TTCAGGG | *Plasmodium* |
| TTAGGG | *Trypanosoma brucei* |
| TAGGG | *Giardia lamblia* |
| $(TG)_{1-6}TG_{2-3}$ | *Saccharomyces cerevisiae* |
| $TTACAG_{1-8}$ | *Schizosaccharomyces pombe* |
| TTAGGGGG | *Cryptococcus neoformans* |
| TTAGGG | *Podospora anserina* |
| TTAGGG | *Fusarium oxysporum* |
| TTAGGG | *Neurospora crassa* |
| ACGGATGTCTAACTTCTTGGTGT | *Candida albicans* |
| TTAGGG | *Didymium iridis* |
| TTAGGG | *Physarum polycephalum* |
| $AG_{1-8}$ | *Dictyostelium discoideum* |
| TTAGGC | *Ascaris lumbricoides* |
| TTGCA | *Parascaris univalens* |
| TTAGG | *Bombyx mori* |
| HeT-A, TART (retroposons) | *Drosophila melanogaster* |
| TTAGGG | Humans, mice |
| TTTAGGG | *Arabidopsis thaliana* |
| TT(T/A)AGGG | Tomato |
| TTTTAGGG | *Chlamydomonas reinhardtii* |

Modified from Kipling (1995).

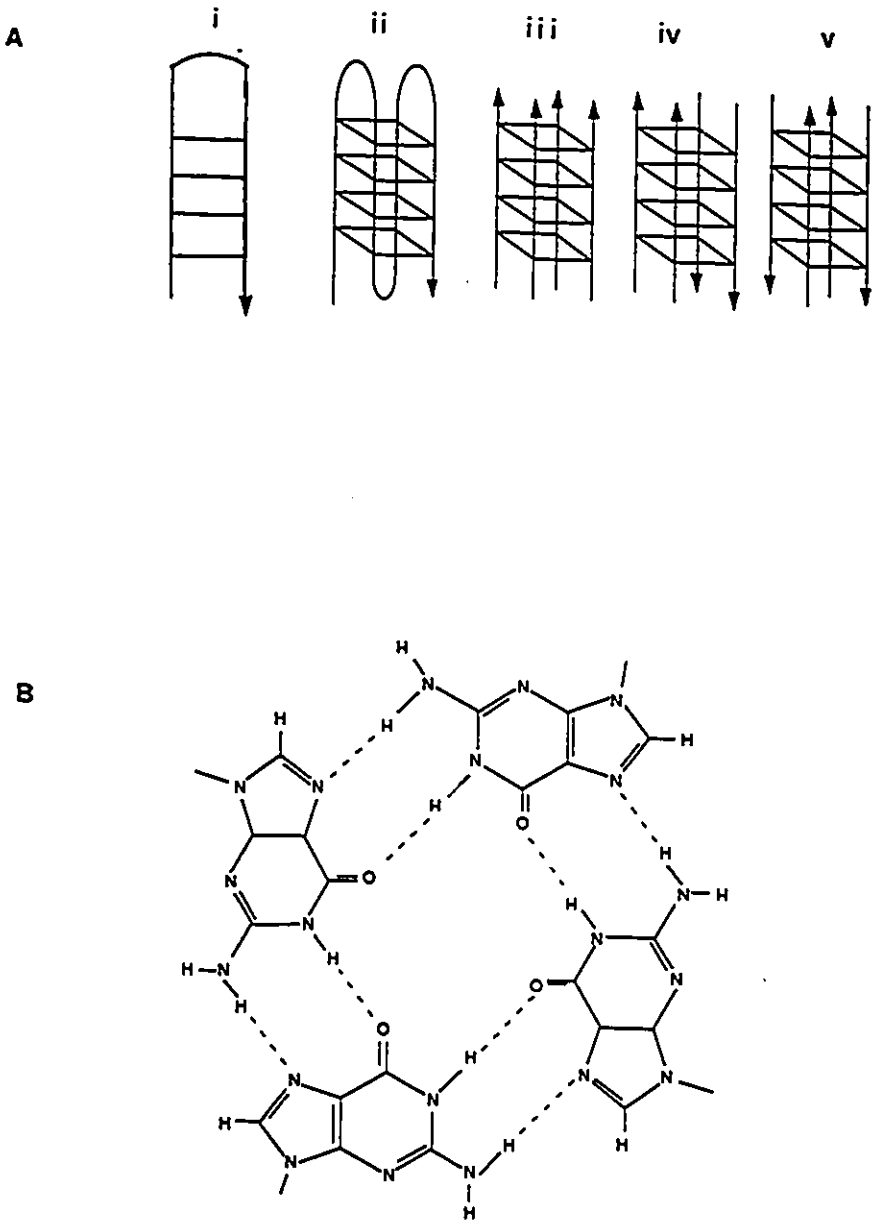


Fig. 1.3.1. Structures formed by G-rich DNA sequences based on there ability to form G·G base pairs. (A) Possible intra- and interstrand complexes involving G·G base pairing are shown for the G-rich strand of *Tetrahymena* telomeric DNA. (i) Foldback hairpin loop. (ii) Foldback G-quartet. (iii) Parallel quadruplex. (iv) and (v), Antiparallel quadruplexes. Modified from Aboul-ela *et al.* (1992).(B) The G-quartet. The G·G base pairing which confers stability to the G-quartet is shown. Taken from Kipling (1995).

Also, telomeres in various ciliate species and in the slime mold *Dydimium* have been shown to end in a 3' overhang of 12-16 bp depending on the species (Klobutcher *et al*, 1981; Henderson and Blackburn, 1989). The transient presence of 3' overhangs that are no less than 30 bp in length has also been detected in *S. cerevisiae* in late S phase (Wellinger *et al*, 1993). In this organism, experiments involving disruption of the yeast gene *KEM1*, which encodes a G4-DNA dependent nuclease, provide evidence that the 3' overhangs form foldback structures and/or intertelomeric complexes stabilized by G-G base pairing *in vivo* (Liu *et al*, 1995; Liu and Gilbert, 1994). The *KEM1* gene product binds specifically to G4-DNA and cleaves single-stranded DNA in a region 5' to the G4 structure (Liu and Gilbert, 1994). Also, homozygous deletions in *KEM1* result in telomere shortening and cell senescence (Liu *et al*, 1995). This latter observation is consistent with the presence of G4-DNA structures involving the 3' overhangs at the ends of telomeres since telomeric G4-DNA structures do not serve as substrates for telomerase (see Section 1.4)(Zahler *et al*, 1991). Thus, the *KEM1* gene product may be required to remove any G4-DNA complexes formed by the 3' overhangs in S phase to allow accessibility of the 3' termini to telomerase.

Telomeric DNA sequences also occur at interstitial sites in many species (Fanning, 1987; Forney *et al*, 1987; Ganal et al, 1991), including humans (Brown *et al*, 1990; Wells *et al*, 1990; Ijdo *et al*, 1991; Weber *et al*, 1991). In humans, < 1 % of the telomeric DNA in the genome is estimated to occur at sites greater than 16 kbp from chromosomal ends (Moyzis *et al*, 1988). Human interstitial telomeric DNA appears to consist of both telomere-like sequences as well as short stretches of $(TTAGGG)_n$ (Wells *et al*, 1990; Weber *et al*, 1991; Ijdo *et al*, 1991) (Fig. 1.3.2). One site of interstitial telomeric DNA occurs near the centromere of chromosome 2 (Ijdo *et al*, 1991). The occurrence of

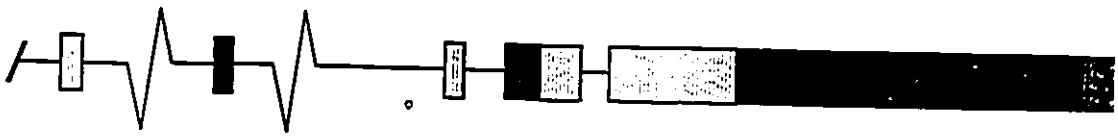

Fig. 1.3.2. Organization of telomeric and telomere-like sequences in the human genome. Solid box: telomeric sequences. Shaded box: telomere-like sequences. Solid line: non-telomeric sequences. The relative proportions of these sequences and the organization of these sequences at subterminal and interstitial sites are based upon observations by Allshire *et al.* (1990), de Lange *et al.* (1990), Wells *et al,* (1990), Brown *et al.* (1990) and Weber *et al.,* (1990), and are not representative of any telomere in particular.

interstitial telomeric DNA at this site provides additional evidence in support of the hypothesis that chromosome 2 has recently evolved from the end-to-end fusion of two smaller chromosomes (Yunis and Prakash, 1982). Results from experiments involving Southern blot analysis using degenerate telomeric probes (Allshire *et al*, 1989), analysis of BAL-31 nuclease sensitivity of telomere-like DNA clones (Wells *et al*, 1990), and direct sequencing of DNA clones from subterminal loci (Brown *et al*, 1990) show that sequences of a telomeric nature are also located directly proximal to the terminal $(TTAGGG)_n$ tract on some, possibly all, telomeres (Fig. 1.3.2). The biological relevance, if any, of telomeric DNA at interstitial sites and subterminal sites remains to be determined.

Subterminal DNA sequences have been cloned and characterized from various species. In the yeast *S. cerevisiae*, there are predominantly 2 subterminal repeat families, the X and Y' elements (Szostak and Blackburn, 1982; Chan and Tye, 1983). The number of copies of either element varies from telomere to telomere, although the Y' element is present at 0-4 copies per telomere (Szostak and Blackburn, 1982; Chan and Tye, 1983). Some yeast telomeres lack both Y' and X elements (Zakian and Blanton, 1988). A number of non-$(TTAGGG)_n$, short (10-100 bp) repeats are present in human subterminal DNA, and like in yeast, each of these human subterminal repeats appears to be present on some but not all telomeres (Brown *et al*, 1990; de Lange *et al*, 1990). The particular sub-set of telomeres associated with each repeat appears to be polymorphic (Brown *et al*, 1990). These observations suggest that subterminal DNA may not be essential for telomere function. Further evidence indicating that subterminal DNA is not required for telomere function is that in humans (Wilkie *et al*, 1990) and ciliates (Klobutcher *et al*, 1981; Spangler *et al*, 1988), broken chromosomes can be

'healed' by the *de novo* synthesis of new telomeric DNA onto the broken end. In these instances, a new telomere is formed in the absence of subterminal DNA. However, it is possible that subterminal DNA could aid in the pairing of homologous chromosomes during meiosis, as well as in chromosome healing.

Less is known about the protein component of telomeres relative to the DNA component. Telomere binding proteins have been purified and cloned from various lower eukaryote species. Information from these studies suggest that telomere binding proteins fall into 2 categories: those which bind to the single stranded 3' overhang at the telomere terminus and those which bind at intervals along the duplex telomeric DNA tract (Fig. 1.3.3).

A telomeric binding protein has been cloned from *Oxytricha nova* which consists of 2 components, a 41 kDa subunit (alpha subunit) and a 56 kDa subunit (ß subunit) (Gray *et al*, 1991). This heterodimeric protein appears to bind to the very terminus of the telomere, since in extracts from the macronucleus, this protein is found to be stably and specifically associated with a single stranded DNA fragment corresponding to the 3' tail, $(TTTTGGGG)_2$, of *Oxytricha* macronuclear DNA (Price and Cech, 1989). Furthermore, the heterodimer protects the associated DNA from degradation by nucleases and chemical modification *in vitro* (Gottschling and Zakian, 1986), suggesting a telomere stabilizing role for this protein *in vivo*. This protein does not bind to single stranded telomeric DNA which has folded back into a G-quartet structure (Raghuraman and Cech, 1990). These structures may form after DNA replication which probably requires dissociation of the telomere end binding protein from the telomeric DNA terminus. However, the ß-subunit can accelerate the change in conformation between the foldback G-quartet structure and the unfolded structure without

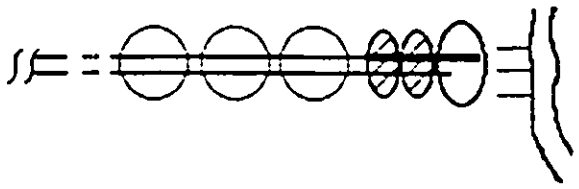

Fig. 1.3.3. The eukaryotic telomere. Proteins which bind along the length of the telomere are represented by hatched ovals and proteins which only bind at the telomeric termini are represented by the open oval. Bulk nucleosomes which bind non-telomeric DNA are represented by open circles. The G-rich strand of telomeric DNA is represented by a thickened black line. In this schematic, the terminal telomere binding protein(s) are shown to be associated with a component of the nuclear matrix, although this may not be the case in some eukaryotes. Taken from Blackburn (1991).

shifting the equilibrium (Fang and Cech, 1993). This might then allow recruitment of the alpha-subunit to the telomere terminus to form a complete heterodimer. In addition, telomerase from *O. nova* can not act on telomeric DNA substrates that are folded into a G4-DNA structure (Zahler *et al*, 1991), although it can extend telomeric DNA complexed with the heterodimer (Shippen *et al*, 1994). Together, these latter observations suggest another role for the *O. nova* telomere cap protein, that is to prevent the 3' overhangs from forming intra- or intertelomeric complexes in order to allow telomerase to maintain telomere length.

Another telomere binding protein which has been cloned and characterized in some detail is the multifunctional protein RAP1 in *S. cerevisiae* (reviewed in Gilson *et al*, 1993a). In addition to being a component of telomeric chromatin in yeast (Conrad *et al*, 1990; Wright *et al*, 1992), RAP1 also plays a role as a transcriptional regulator, and facilitates meiotic recombination at the *HIS4* locus (White *et al*, 1991). RAP1 binds to double stranded yeast telomeric DNA at roughly 18 bp intervals but appears not to bind to telomere termini (Buchman *et al*, 1988; Gilson *et al*, 1993b). RAP1 is a component of the nuclear scaffold and thus may be directly involved in the spatial organization of telomeric chromatin *in vivo* (Cardenas *et al*, 1990). Also, RAP1 may help protect telomeric chromatin from damage *in vivo* as evidenced by its ability to protect telomeric DNA from nuclease degradation *in vitro* (Gilson *et al*, 1993b). RAP1 is essential for viability (Shore and Nasmyth, 1987) and for regulation of telomere length since RAP1 mutants can cause either an increase or decrease in telomere length (Conrad *et al*, 1990; Kyrion *et al*, 1992). The telomeric position effect, which refers to the repression of genes located adjacent to telomeres, also appears to require RAP1 (Kyrion *et al*, 1993), presumably because RAP1 is essential for the

formation of telomeric heterochromatin (Gilson *et al*, 1993b; Blackburn, 1994). RAP1 appears to recruit other telomere binding or telomere associated proteins to the telomere as determined by 2-hybrid screens (Hardy *et al*, 1992; Moretti *et al*, 1994). One such protein that appears to interact with RAP1 and is also essential for proper regulation of telomere length is RIF1 (Hardy *et al*, 1992). The proteins SIR3 and SIR4 also appear to interact with RAP1 and are essential for telomere position effect (Palladino *et al*, 1993; Moretti *et al*, 1994). Unlike RAP1, none of these RAP1-interacting proteins are essential for viability or chromosome stability, indicating that stability of telomeric DNA *per se* in *S. cerevisiae* may be primarily conferred by RAP1.

Telomeric binding proteins have yet to be cloned from vertebrates. Using a band-shift assay, a potential telomeric binding protein having a molecular mass of roughly 50 kDa has been identified in human, monkey and mouse cells (Zhong *et al*, 1992). Like RAP1, this factor only binds to double stranded telomeric DNA. The sequence requirements for binding are stringent and correlate with the ability of the sequence to seed new telomeres (Hanish *et al*, 1994).

Telomeric chromatin is non-canonical. In yeast and in the ciliate macronucleus, the telomere is packaged into a large, non-nucleosomal complex (Wright *et al*, 1992; Gottschling and Cech, 1984). In humans and rodents however, nucleosomes are present in telomeres (Makarov *et al*, 1993; Tommerup *et al*, 1994). These telomeric nucleosomes co-sediment with nucleosomes from bulk chromatin in sucrose gradients. Telomeric nucleosomes differ from those found in bulk chromatin in that they are more closely spaced and contain less DNA. Furthermore, telomeric mononucleosomes are hypersensitive to nuclease digestion relative to bulk nucleosomes (Tommerup *et al*, 1994), implying that there are unique DNA-

histone interactions and/or proteins or protein modifications in mammalian telomeric nucleosomes. In human cell lines containing short (2 to 7 kbp) telomeres, the nucleosomal ladder is very diffuse above the nucleosome trimer, indicating that there are regions of telomeres in these cells which do not contain nucleosomes (Tommerup *et al*, 1994). These barren telomeric regions may also occur in cells with longer telomeres, and the inability to detect the characteristic short nucleosomal ladder in these cells is presumably because most of the telomeric chromatin is composed of regularly spaced nucleosomes (Tommerup *et al*, 1994).

## 1.4 Telomerase

In almost all eukaryotic cells, the *de novo* synthesis of telomeric DNA and maintenance of telomere length is accomplished by a ribonucleoprotein complex called telomerase. This occurs by the addition of nucleotides, one at a time, onto the 3' chromosomal termini (reviewed in Blackburn, 1991) (Fig.1.4). Telomerase was originally identified in *Tetrahymena* by the discovery of an activity in cell free extracts which adds the *Tetrahymena* telomeric DNA sequence $(TTGGGG)_n$ onto G-rich oligomers, but not onto C-rich oligomers or to blunt-ended duplex DNA (Greider and Blackburn, 1985)(Fig.1.4). Subsequently, telomerase has been detected in various other ciliate species, humans, mice, frogs and the yeast *Saccharomyces cerevisiae* (Morin, 1989; Zahler and Prescott, 1988; Prowse *et al*, 1993; Lingner *et al*, 1994; Mantell and Greider, 1994; Singer and Gottschling, 1994; Lin and Zakian, 1995; Cohn and Blackburn, 1995). Ciliate telomerase has an apparent molecular mass of 200-500 kDa as determined by column chromatography (Greider and

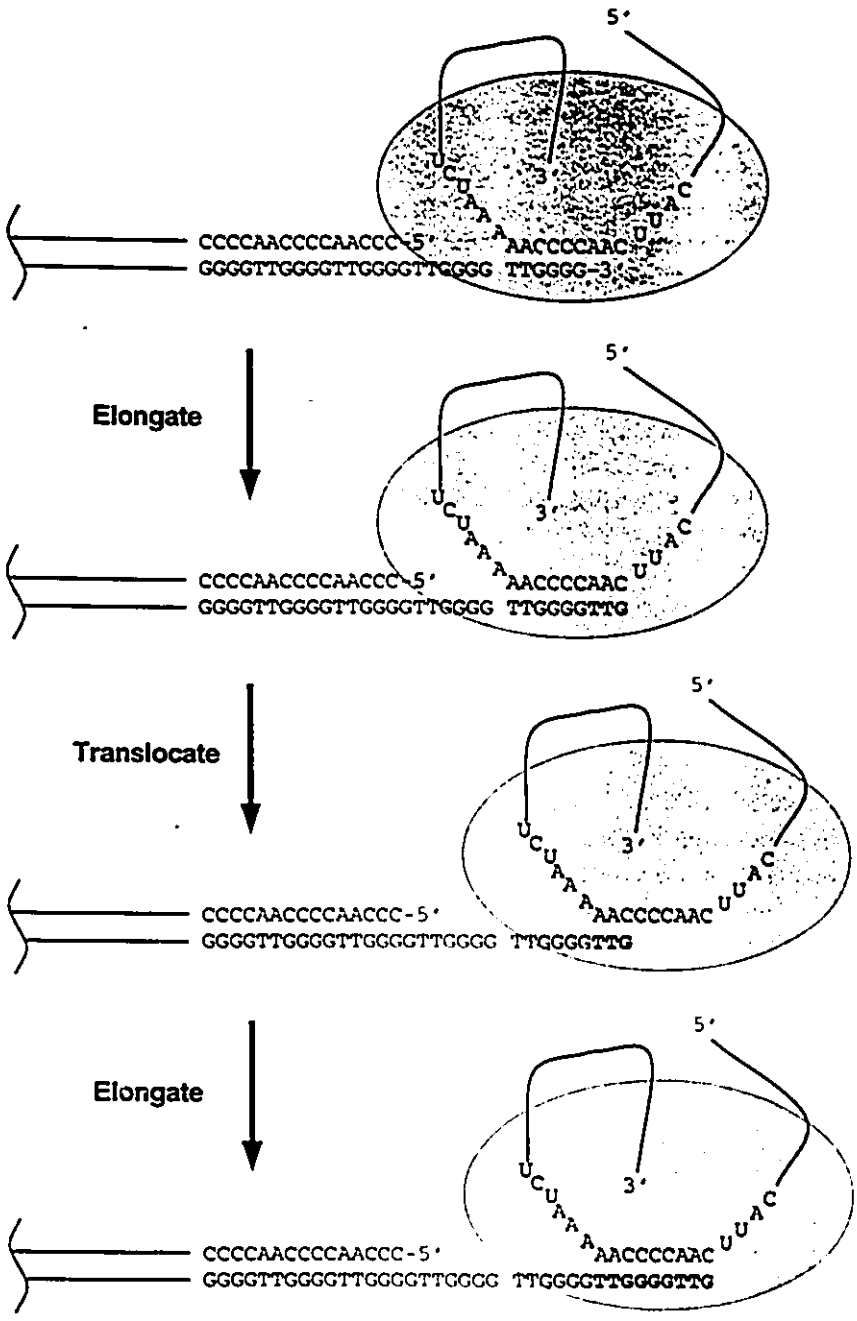


Fig. 1.4. Model for the synthesis of telomeric DNA by telomerase. This model specifically shows how telomerase might add telomeric sequences onto the ends of telomeres in *Tetrahymena*. The template region of the RNA component of telomerase, 5'-AACCCCAAC-3', plus some flanking sequence are shown. See text for further explanation. Modified from Greider and Blackburn (1989).

Blackburn, 1987). Telomerase is composed of both protein and RNA components since telomerase activity is sensitive to both ribonuclease and protease digestion (Greider and Blackburn, 1985). *Tetrahymena thermophila* telomerase consists of 2 protein components of molecular masses 80 and 95 kDa (Collins *et al*, 1995). The 80 kDa component appears to associate with the RNA component while the 95 kDa component appears to associate with the telomeric substrate (also see below). The RNA component of telomerase has been cloned from ciliates, yeast, mice and humans (Greider and Blackburn, 1989; Romero and Blackburn, 1991; Lingner *et al*, 1994; Blasco *et al*, 1995; Feng *et al*, 1995). The size of the RNA component is 160-194 nucleotides in ciliates, depending on the species (Romero and Blackburn, 1991; Lingner *et al*, 1994), 1300 nucleotides in *S. cerevisiae* (Singer and Gottschling, 1994), 430 nucleotides in mice (Blasco *et al*, 1995) and 450 nucleotides in humans (Feng *et al*, 1995). Although the primary sequence is highly divergent, even among different strains of *Tetrahymena* (Romero and Blackburn, 1991), the secondary structure, as determined by folding algorithms and evolutionary arguments based on compensatory mutations in predicted duplex regions, appears to be conserved amongst ciliate species (Romero and Blackburn, 1991; Lingner *et al*, 1994). However, a complete description of how the RNA and protein components contribute to telomerase structure and function remains to be determined.

In all of the cloned RNA components of telomerase, there is a short motif complementary to the G-rich strand of the telomeric DNA sequence of the species from which the telomerase was obtained (Greider and Blackburn, 1989; Lingner *et al*, 1994; Singer and Gottschling, 1994; Feng *et al*, 1995)(see Fig.1.4). This motif serves as a template for the synthesis of telomeric DNA by telomerase, since telomerase activity is sensitive to RNase H-directed

cleavage only when oligonucleotides that are complementary to the region of the RNA component that contains this motif are used (Greider and Blackburn, 1989). Also, oligonucleotides that are complementary to the RNA component and for which the 3' end terminates just before the predicted template region can provide substrates for telomerase *in vitro*, even though these oligonucleotides are not G-rich (Greider and Blackburn, 1989). Proof that the 'telomeric' motif of the RNA component is the template region for synthesis of telomeric DNA was provided in experiments involving single base mutations of the RNA component of *Tetrahymena* telomerase (Yu *et al*, 1990; Yu and Blackburn, 1991) and human telomerase (Feng *et al*, 1995). These mutations were placed at various sites within the predicted template region. When these mutated RNA components are over-expressed *in vivo* (Yu *et al*, 1990; Yu and Blackburn, 1991; Feng *et al*, 1995) or used to reconstitute telomerase *in vitro* subsequent to nuclease digestion of the endogenous RNA component (Autexier and Greider, 1994), the sequence of the newly synthesized telomeric DNA is found to correctly incorporate the nucleotide dictated by the mutation.

The extension of most telomeric oligonucleotides *in vitro* by telomerase from humans and ciliates is processive: hundreds of base pairs of telomeric DNA are added onto a single oligomer by telomerase before telomerase disengages and reassociates with another oligomer (Greider and Blackburn, 1985; Greider, 1991). This implies that there must be a translocation step to re-align the 3' end of the growing oligomer once it is flush with the 5' end of the template region (Greider and Blackburn, 1989)(Fig. 1.4). Translocation seems to occur once for every telomeric repeat motif added during oligomer extension as evidenced by a corresponding pause in oligomer extension (Greider and Blackburn, 1985; Greider and

Blackburn, 1989). For example, the template region of *Tetrahymena* telomerase is 5'-CAACCCCAA-3'(Greider and Blackburn, 1989), and there is a pause during oligomer extension after the addition of the 5' guanine residue in the *Tetrahymena* telomeric repeat $(TTGGGG)_n$ (Fig. 1.4). Since the *Tetrahymena* template region is composed of 1.5 telomeric repeats, this latter observation implies that the three most 3' residues of the template region do not have a template function, but are involved in binding of the oligomer. This prediction has been shown to be correct in reconstitution experiments: mutations in any of these residues in the RNA used to reconstitute telomerase does not change the sequence added (Autexier and Blackburn, 1994). The occurrence of a translocation step also implies that, in addition to the RNA:DNA interaction between the template region of telomerase RNA and the oligomer substrate, there must be a protein:DNA interaction to prevent telomerase from completely dissociating from the oligomer during re-alignment. In *Tetrahymena* telomerase, this anchoring function appears to be accomplished by the 95 kDa protein component (Collins *et al*, 1995). This is very likely, since telomerase can use numerous oligonucleotides that are not rich in guanine at the 3' end as substrates (Harrington and Greider, 1991), and also add telomeric DNA *de novo* to the ends of broken chromosomes (Yu and Blackburn, 1991).

In some cases, telomerase functions in a distributive manner, only adding one or two telomeric repeats before dissociating. This occurs *in vitro* when very short (10 bp or less) oligomers are used as substrates (Collins and Greider, 1993) and may reflect the inability of a short oligomer substrate to interact simultaneously with both the template region and with anchor site. Also, mouse telomerase appears to function in a distributive manner in certain *in vitro* assays (Prowse *et al*, 1993; Prowse *et al*, 1995), although this

may be due to loss of a loosely bound processivity factor during extract preparation. The extent to which telomerase functions processively or distributively *in vivo* remains to be determined.

Telomerase is capable of adding telomeric DNA onto pre-existing telomeres *in vivo*, as well as *de novo* synthesis of telomeric DNA since over-expression of telomerase RNA containing mutations in the template region results in the synthesis of telomeres containing the mutated sequence (Yu *et al*, 1990; Yu and Blackburn, 1991). However, little is known regarding how telomerase interacts with chromatin or how the amount of telomeric DNA synthesized by telomerase is regulated. In certain cases, telomerase can add telomeric DNA onto oligomer substrates that are not G-rich at the 3' end as long as there is a stretch of telomeric DNA somewhere in the substrate (Harrington and Blackburn, 1991). Telomerase may initially bind to the telomeric sequence of the oligomer and then slide along to the 3' end, and as this occurs the DNA between the telomeric sequence and the 3' end may loop out to bring the 3' end into the active site. However, *in vivo*, telomerase can add telomeric DNA onto broken or fragmented chromosomal ends that are devoid of any previously existing telomeric DNA and which may even lack 3' overhangs (Yu and Blackburn, 1991). Thus *de novo* synthesis of telomeric DNA may require additional factors to initiate interactions between telomerase and newly produced chromosomal ends. Also, in immortal cells, telomeres can be maintained at the same length for many cell generations (D'Mello and Jaswinski, 1991; Counter *et al*, 1992; Counter *et al*, 1994; Gilley and Blackburn, 1994). In ciliates and possibly other organisms, telomere-specific nucleases may be involved in regulation of telomere length since during macronuclear development, telomere length is initially much longer in the vegetatively growing cells than in the macronucleus (Vermeesch and

Price, 1994). The G4-DNA specific nuclease KEM1 in yeast may be an example of such a protein (Liu and Gilbert, 1994; Liu *et al*, 1995).

## 1.5 Telomere length and telomerase expression in mortal and immortal cells.

Considerable inter-species variation in telomere length exists. Telomeres in *Oxytricha* macronucleus are relatively short, each telomere being exactly 36 bp long, including the 3' overhang (Klobutcher *et al*, 1981). In many other species, telomere length is more heterogeneous. For example, telomere length in *S. cerevisiae* ranges from about 200-400 bp (Shampey and Blackburn, 1988). Telomeres in inbred mice may be very long, possibly reaching lengths up to 150 kbp (Kipling and Cooke, 1990). In humans, telomere length, the length of the terminal (TTAGG)n tract, depends on cell type. Telomeres are longest in the germ line (~10-15 kbp) (Cooke and Smith, 1986; de Lange *et al*, 1990; Hastie *et al*, 1990) and shortest in terminally differentiated cells, such as PBLs, particularily from old donors (2-3 kbp) (Hastie *et al*, 1990; Vaziri *et al*, 1993).

Perhaps the first suggestion that telomere loss occurs during *in vivo* aging was the observation that telomeres are shorter in white blood cells than in the germ line from the same donor (Cooke and Smith, 1986; de Lange *et al*, 1990). Harley *et al* were the first to directly demonstrate, by Southern analysis of terminal restriction fragment length, a gradual loss of telomeric DNA during replicative aging of normal human cells *in vitro*, as well as provide preliminary evidence for the loss of telomeric DNA during aging *in vivo* (1990). The rate of telomere shortening during division of cultured HDFs is 30-80 bp/pd (Harley *et al*, 1990), and 40 bp/year and 21 bp/year during *in vivo*

aging of PBLs and skin tissue respectively (Hastie *et al*, 1990; Lindsey *et al*, 1991; Vaziri *et al*, 1993). Loss of telomeric DNA has also been shown to occur in cultured endothelial cells and T lymphocytes at rates of 190 bp/pd (Vaziri *et al*, 1993) and 120 bp/pd (Chang and Harley, 1995) respectively. Telomere shortening also occurs during division of transformed HEK cells that have bypassed the cell senescence checkpoint. The rate of shortening, 65 bp/pd, is comparable to that observed for normal somatic cells (Harley *et al*, 1990; Counter *et al*, 1992).

In immortal cells, such as unicellular eukaryotes and cell lines established from higher eukaryotes including humans, telomere length is stably maintained (D'Mello and Jaswinski, 1991; Counter *et al*, 1992; Counter *et al*, 1994b; Klingelhutz *et al*, 1994). Telomerase has been shown to be essential for maintenance of telomere length in *Tetrahymena thermophila* (Yu *et al*, 1990) and *Sacchromycies cerevisiae* (Singer and Gottschling, 1994). In *Tetrahymena, in vivo* expression of the cloned RNA component containing a single mutated base in the template region results in the gradual loss of telomeric DNA during division of these cells (Yu *et al*, 1990). Eventually, most of the cells became enlarged, ceased to divide, and died. Also, deletion of the template region from the gene encoding the RNA component in yeast has been shown to cause gradual telomere shortening with increasing generations (Singer and Gottschling, 1994). These cells also eventually entered a state in which the doubling time increased and cell viability decreased. However, these sickly cells were subsequently taken over by faster growing, healthier cells. A similar phenomenon, that is the gradual loss of telomeric DNA leading to the cell cycle arrest and cell death followed by an overgrowth of rapidly dividing cells, has been observed in the *S. cerevisiae* strains harboring mutations in the est1 (ever shortening telomere)

gene (Lundblad and Szostak, 1989; Lundblad and Blackburn, 1993). In these surviving cells, and presumably in the surviving cells from the *S. cerevisiae* strain with the functionally inactive RNA component (Singer and Gottschling, 1994), telomere length is maintained by the amplification of the subtelomeric Y' elements (Lundblad and Blackburn, 1993). Y' element amplification provides a means to maintain telomere length since these elements are flanked by telomeric sequences (Louis and Haber, 1990). The mechanism of amplification of the Y' element is unknown but is *RAD52*-dependent and therefore most likely involves recombination (Lundblad and Blackburn, 1993). That telomeres shorten in strains with mutations in est1 suggests that EST1 may be a component of telomerase. Recent studies in which assays have been developed to detect yeast telomerase activity give conflicting results as to whether EST1 is a component of the yeast telomerase (Lin and Zakian, 1995; Cohn and Blackburn, 1995). In the study which did detect telomerase activity in a mutant est1 strain, only 20% of the est1 coding sequence remained in the mutant strain and therefore it is unlikely that the mutant EST1 protein in this strain was functional (Cohn and Blackburn, 1995). Thus EST1 is probably not essential for telomerase activity *in vitro*, although it may be essential for proper function of telomerase *in vivo*.

Telomerase also appears to be required for telomere length maintenance in immortal human cell lines and germ line cells. For virally transformed cells to become immortal, they must survive crisis (see Section 1.2.2 and Fig. 2.1.5). The activation of telomerase and stabilization of telomere length have both been shown to occur concomitant with these cells passing through crisis (Counter *et al*, 1992; Counter *et al*, 1994b). Also, telomerase activity has been detected in most human cell lines and tumors (Morin, 1990; Counter *et al*, 1992; Counter *et al*, 1994a,b; Kim *et al*, 1994), but appears to be

scarce or absent in normal somatic cells and tissue (Counter *et al*, 1992; Kim *et al*, 1994; Counter *et al*, 1995). Telomerase activity has also been detected in human germ line cells and tissue (Kim *et al*, 1994) as well as in *Xenopus* oocytes (Mantell and Greider, 1994), and therefore is probably responsible for maintenance of telomere length during species propagation.

In telomerase positive cell lines, the average telomere length remains constant (Counter *et al*, 1992; Counter *et al*, 1994b), or in some cases increases (Klingelhutz *et al*, 1994), during division of immortal human cell lines. However, the dynamics of individual telomeres during propagation of telomerase positive cell lines has not been analyzed. The lengths of individual telomeres have been monitored over a number of population doublings in a telomerase negative cell line (Murnane *et al*, 1994). Individual telomeres were observed to fluctuate up and down in length (Murnane *et al*, 1994). Specifically, analysis of the length of a single telomere in several clones from an SV40 transformed fibroblast cell line showed that the length was quite variable between clones, ranging from ~ 0 kbp to > 23 kbp. Additionally, when the length of a single telomere was monitored over a number of population doublings, it was found to shorten at a rate of 52 bp/pd, similar to the rate of shortening for normal fibroblasts in culture (Harley *et al*, 1990); once the length of the single telomere reached ~ 0 kbp, the length suddenly increased and became very heterogeneous. This particular cell line was reported not to express telomerase, and therefore it is unknown whether any immortal cell lines that express telomerase also experience very dynamic changes in telomere length.

## 1.6 The telomere hypothesis of cell aging and immortalization

The loss of DNA from the ends of chromosomes during replicative aging of normal human cells was predicted to occur long before telomeres were even defined at a molecular level (Olovnikov, 1971; Olovnikov, 1973). This prediction was based upon the inability of the normal DNA replication machinery to completely replicate the 5' ends of duplex DNA, referred to as the end replication problem (Olovnikov, 1971; Watson, 1972) (see Figures 2.2.1 and 2.3.1, as well as Section 2.2 for further discussion). Furthermore, it was also predicted at this time that the end replication problem would eventually lead to the deletion of an essential gene in actively dividing cells and this would then cause cell senescence (Olovnikov, 1973). Based upon more recent findings regarding loss of telomeric DNA and the expression of telomerase (see Section 1.5), this initial model of cell senescence has been refined (Harley, 1991). The new model is referred to as the telomere hypothesis for human cell aging and immortalization (see Figures 2.1.5 and 2.6.1). This model is based on several predictions. First, telomere length is maintained in the germ line from generation to generation by telomerase. Second, during development telomerase is repressed in most, if not all, somatic cells. Third, due to the end replication problem and/or possibly other mechanisms, telomere length will get progressively shorter as somatic cells divide. Once a critical length is reached on one or more telomeres a signal will be sent to initiate the M1 checkpoint (the Hayflick limit) and cells will cease dividing. Fourth, telomere length will continue to shorten in cells which have acquired the ability to bypass cell senescence, for example in cells transformed with DNA tumor viruses, until most of the terminal $(TTAGGG)_n$ tract has been deleted from many telomeres. At this point, crisis

or the M2 checkpoint will be signalled. Fifth, in the rare immortal cells which survive crisis, telomere length is maintained by telomerase. Strong evidence has been obtained in support of predictions 1, 2, 4 and 5 (see Section 1.5). However, the exact cause of telomere shortening is unknown. Also, the extent of telomere shortening occurring in different human cell types has not been thoroughly examined, nor has it been definitively demonstrated that telomere shortening has a causal role in human cell senescence. The examination of these issues is the focus of the work presented here.

## 2. Results

### 2.1 Analysis of the correlation between replicative capacity and telomere length in cultured HDFs.

The demonstration that telomere length decreases during replicative aging of cultured HDFs led to the speculation that telomere shortening is a cause of cell senescence (Harley *et al*, 1990). One goal of the work presented in this Section was to determine if in fact the loss of telomeric DNA during aging of HDFs *in vitro* also holds true *in vivo*. Towards this end, telomere length was measured at early passage for 43 different HDF strains established from donors ranging in age from 0 (fetal) to 90 years. The correlation between telomere length and donor age was then examined. Additionally, the rate of telomere shortening per population doubling *in vivo* was calculated and compared to the rate of telomere shortening *in vitro*.

If telomere shortening is a cause of cell senescence, then replicative capacity of individual HDF strains would be expected to be dependent on initial telomere length. Thus another goal of this Section was to test this prediction by examining the correlation between *in vitro* replicative capacity and telomere length at early passage. Also, the replicative capacity of HDF strains established from progeroid donors is short relative to the replicative capacity of HDF strains established from normal donors (Goldstein and Harley, 1978). Analysis was done to determine if telomere length is also reduced in HDF strains from progeroid donors relative to HDF strains from normal age-matched controls.

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# Telomere length predicts replicative capacity of human fibroblasts

(aging/sperm/progeria/cellular senescence)

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ABSTRACT **When human fibroblasts from different donors are grown *in vitro*, only a small fraction of the variation in their finite replicative capacity is explained by the chronological age of the donor. Because we had previously shown that telomeres, the terminal guanine-rich sequences of chromosomes, shorten throughout the life-span of cultured cells, we wished to determine whether variation in initial telomere length would account for the unexplained variation in replicative capacity. Analysis of cells from 31 donors (aged 0–93 yr) indicated relatively weak correlations between proliferative ability and donor age ($m = -0.2$ doubling per yr; $r = -0.42$; $P = 0.02$) and between telomeric DNA and donor age ($m = -15$ base pairs per yr; $r = -0.43$; $P = 0.02$). However, there was a striking correlation, valid over the entire age range of the donors, between replicative capacity and initial telomere length ($m = 10$ doublings per kilobase pair; $r = 0.76$; $P = 0.004$), indicating that cell strains with shorter telomeres underwent significantly fewer doublings than those with longer telomeres. These observations suggest that telomere length is a biomarker of somatic cell aging in humans and are consistent with a causal role for telomere loss in this process. We also found that fibroblasts from Hutchinson–Gilford progeria donors had short telomeres, consistent with their reduced division potential *in vitro*. In contrast, telomeres from sperm DNA did not decrease with age of the donor, suggesting that a mechanism for maintaining telomere length, such as telomerase expression, may be active in germ-line tissue.**

The cellular senescence model of aging was founded by landmark experiments of Hayflick and Moorhead (1), who firmly established that normal human fibroblasts have a finite life-span *in vitro*. Although much evidence supports this model (2–8), the mechanism accounting for the finite division capacity of normal somatic cells remains a mystery. Olovnikov (9, 10) suggested that the cause of cellular senescence is the gradual loss of telomeres due to the end-replication problem—i.e., the inability of DNA polymerase to completely replicate the 3′ end of linear duplex DNA (11) (for review, see refs. 12 and 13).

Telomeres play a critical role in chromosome structure and function. They prevent aberrant recombination (14–16) and apparently function in the attachment of chromosome ends to the nuclear envelope (17). Telomeres are composed of simple repetitive DNA (for review, see ref. 12), and in mammals this sequence is $(TTAGGG)_n$ (18). These telomeric repeats are elongated by telomerase, a ribonucleoprotein enzyme that extends the 3′ end of telomeres (ref. 19; for review, see ref. 20). Thus, in immortal eukaryotic cells, telomerase apparently balances telomere loss with *de novo* synthesis of telomeric DNA.

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Although it is clear that aging in cells, tissues, and organs occurs at many levels and is polygenic (13, 21), several observations implicate a role for telomere shortening in replicative senescence of cells. (*i*) Telomeres shorten during aging of cultured fibroblasts (22) and other somatic cells *in vivo*, including skin epidermal cells (23) and peripheral blood leukocytes and colon mucosa epithelia (24). In somatic cells, telomere loss may be due to incomplete DNA replication in the absence of telomerase (25, 26). (*ii*) There is an increased frequency of chromosomal abnormalities, especially telomeric associations or dicentrics, in senescing fibroblasts (27–29). Such chromosomal abnormalities are a hallmark of terminal deletions (14). (*iii*) The presence of telomerase in immortal cell lines (26, 30) correlates with stabilized telomere lengths (26). (*iv*) The length of sperm telomeres is greater than that of somatic cells (31–33). These observations have led to the telomere hypothesis of cellular aging (13), in which loss of telomeres due to incomplete DNA replication and absence of telomerase provides a mitotic clock that ultimately signals cell cycle exit, limiting the replicative capacity of somatic cells. To further explore this hypothesis, we have examined the relationship between telomere length, *in vivo* age, and replicative capacity of fibroblasts from normal donors and subjects with the Hutchinson–Gilford syndrome of premature aging (34, 35). We have also determined the relationship between telomere length in sperm DNA and donor age.

## MATERIALS AND METHODS

**Cell Culture.** Skin samples from surgical specimens or biopsy of various aged donors were cut into pieces 1 $mm^3$ or less in size and used to establish primary cultures (36). When the first 100-mm dish reached confluence ($\sim 3 \times 10^6$ cells), the cell population was assigned 16 population doublings. Except as noted, DNA was analyzed from cultures at 19 population doublings. All samples were coded at the time of tissue collection, and the data on mean terminal restriction fragment (TRF) length and culture life-span were collected without knowledge of the donor age. Of the 43 independent strains established and analyzed for TRF measurements (see Fig. 1C), 31 were selected at random for measurement of total replicative capacity (see Fig. 2). Cells were considered senescent when confluence (continuous monolayer) was not reached after 4 weeks with weekly refeeding.

**Isolation of Sperm DNA.** Sperm pellets from 200 μl of semen were washed in phosphate-buffered saline twice and lysed in 200 μl of 4 M guanidium isothiocyanate/0.1 M mercaptoethanol/25 mM sodium citrate for 1 hr at 37°C. After extensive dialysis against proteinase K digestion buffer

Abbreviations: TRF, terminal restriction fragment; MPD, mean population doubling; $H_o$, null hypothesis.

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(100 mM NaCl/10 mM Tris, pH 8/5 mM EDTA/0.5% SDS), proteinase K was added to the dialysate (final concentration, 0.2 mg/ml) and incubated at 48°C overnight. Each sample was extracted twice with 25:24:1 phenol/chloroform/isoamyl alcohol, once with chloroform, and then ethanol precipitated. Most semen samples were voluntary donations from normal individuals after informed consent. Some samples were from the infertility clinic at McMaster University. Only those samples having normal sperm count and morphology were used in this study.

**Analysis of DNA.** DNA was digested with restriction enzymes *Rsa* I and *Hin*fI and quantified by fluorometry. One microgram was resolved by electrophoresis in 0.5% agarose gels for 600–700 V-hr. Hybridization of oligonucleotide probes to dried gels was based on a modification of Mather (37). In brief, gels were dried under vacuum at 60°C for 45–60 min, soaked in 0.5 M NaOH/1.5 M NaCl for 10 min, then soaked in 0.5 M Tris, pH 8/1.5 M NaCl for 10 min, incubated in 5× standard saline/citrate (SSC) at 37°C with $^{32}$P-end-labeled $(CCCTAA)_3$ for 8–12 hr, and finally washed three times in 3× SSC at 48°C (10 min each) before exposure to preflashed Kodak XAR film for 1–2 days. Mean TRF length was determined from densitometric analysis of autoradiograms as described (22). In some experiments, dried gels were directly analyzed by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with similar results. In these experiments, the calculation of mean TRF length assumes that the amount of telomeric DNA (TTAGGG repeats) in a given TRF is proportional to the length of that TRF (22). However, recent data suggest that a large and variable fraction of the TRF is the non-TTAGGG component and that it may be better not to normalize signal strength to TRF length. Both methods, however, yield similar qualitative conclusions with relatively minor changes in calculated mean TRF length (data not shown).

## RESULTS AND DISCUSSION

**Telomere Length Decreases with Age *in Vivo*.** Because there is no reliable method of directly measuring telomere length in human cells, we and others have used mean TRF length to detect changes in the length of the terminal TTAGGG arrays (22–26, 30–33, 38, 39) (Fig. 1*A*). We have successfully used this method of telomere-length analysis to show that telomeres shorten during replicative aging of human fibroblasts *in vitro* (22, 25) and possibly *in vivo* (22). To firmly establish the effect of chronological aging *in vivo* on telomere length in fibroblasts, mean TRF length was determined for fibroblast strains at equivalent passage established from 43 donors ranging in age from 0 (fetal or newborn) to 93 yr (Fig. 1). A small, but significant decrease in TRF length with age was observed [15 ± 6 base pairs (bp) per yr; $P = 0.01$]. This loss was accompanied by a decrease in hybridization signal intensity (data not shown), indicating that the shortening of fibroblast TRF length *in vivo* was from loss of TTAGGG repeats, as was seen during aging of fibroblasts *in vitro* (22). Similar results were reported by Lindsey *et al.* (23) for telomere loss during aging of skin epidermal cells *in vivo* (20 ± 7 bp per yr).

Mean TRF length of fibroblasts capable of proliferation *in vitro* decreases ≈1.5 kbp during the life-span of humans (Fig. 1*C*). Because a substantial portion (≈4–5 kbp, on average) of the human fibroblast TRF is composed of subtelomeric (non-TTAGGG) repeats (25, 26, 32), the loss of 1.5 kbp of TTAGGG represents a significant fraction (>30%) of the initial telomeric DNA at birth. Overall, the mean TRF length of cells from old donors (≈7 kbp) is larger than that of senescent cells *in vitro* (≈6 kbp) (22, 25), consistent with the significant replicative capacity of the cell population established from these donors. Heterogeneity in the length of the non-TTAGGG component in different telomeres (K. R. Prowse, B. S. Abella, A.B.F., C.B.H., and C.W.G., unpublished data), and the presence of some TTAGGG repeats in the proximal region of the TRF can account for hybridization of the telomeric probe to some TRFs shorter than 4–5 kbp (Fig. 1*B*).

**Telomere Length Predicts Replicative Capacity of Cells.** *In vitro* life-span of cells from unrelated donors (Fig. 2*A*) decreases with age of the donor (2–8), but the correlation is relatively weak ($r = -0.42$). Both interdonor genetic varia-

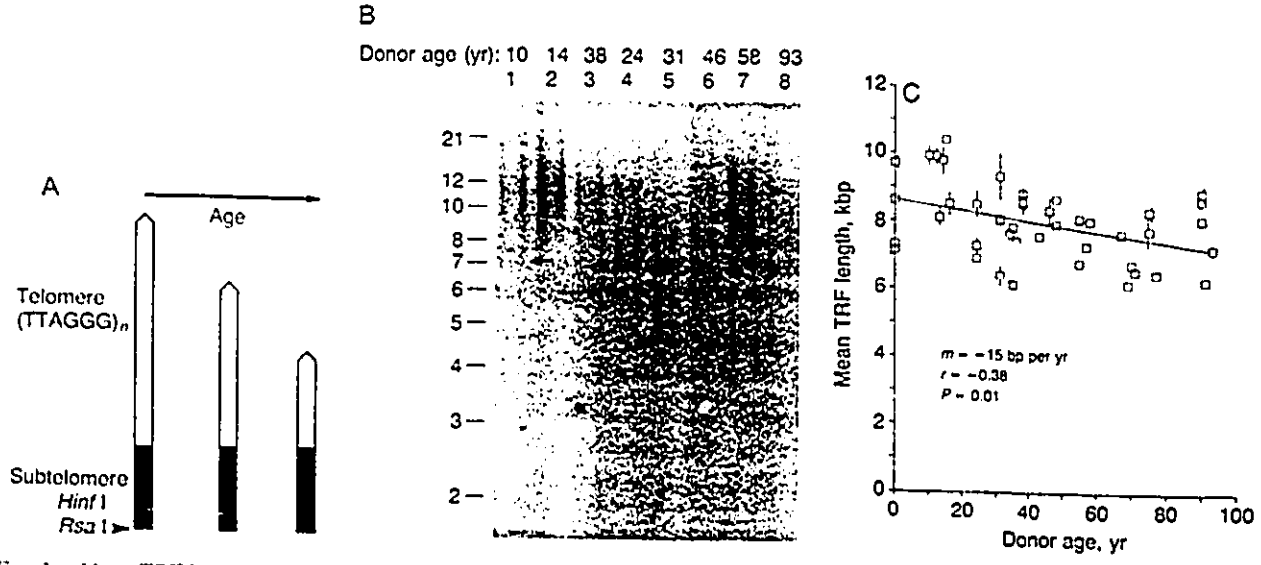


FIG. 1. Mean TRF length decreases in skin fibroblasts with increased age of human donors. (*A*) The TRF is composed of distal telomeric (TTAGGG) repeats (open bars), which hybridize to the telomeric probe, and other proximal sequences (solid bars), which do not. Arrowhead indicates most distal *Hin*fI or *Rsa* I site. (*B*) Genomic DNA from fibroblast strains established from different donors of indicated ages was prepared as described and resolved in agarose gels by electrophoresis. TRFs were detected with a $^{32}$P-labeled telomeric oligonucleotide. Size [in kilobase pairs (kbp)] and position of markers are indicated. (*C*) Mean TRF length was calculated at the earliest possible passage for fibroblast strains established from 43 normal donors ranging in age from 0 (fetal) to 93 yr. SDs for points representing the mean of two or more determinations are indicated. The slope of the regression line is significantly different than 0. Values for the slope ($m$), regression coefficient ($r$), and probability [$P$; null hypothesis ($H_0$): slope = 0] of the linear regression line are shown.

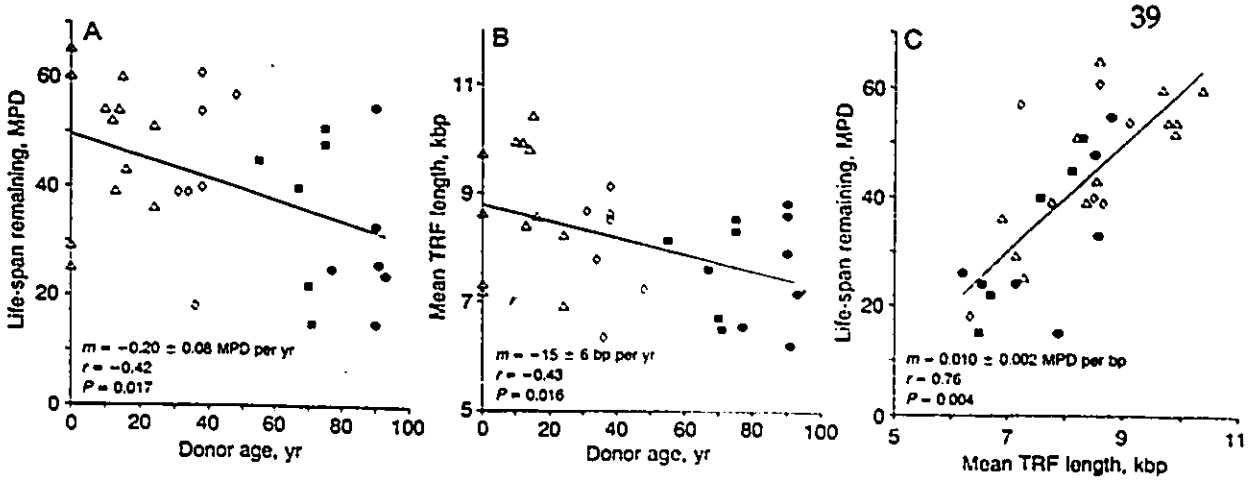


FIG. 2. Replicative capacity is proportional to TRF length. TRF length (*B*) and remaining replicative capacity (mean population doublings, MPD) after the initial 19 population doublings in culture (*A*) are shown as a function of donor age for a random subset of the strains tested in Fig. 1*C*. From these data, replicative capacity was plotted as a function of TRF length (*C*). Values for the slope (*m*), regression coefficient (*r*), and probability (*P*; $H_0$: slope = 0) of the linear regression lines are shown.

tion and clonal heterogeneity resulting from the variable *in vivo* history of cells in a single donor probably contribute to this weak correlation. Similarly, the correlation between TRF length and donor age is also weak (Figs. 1*C* and 2*B*; $r = -0.38$ and $-0.43$, respectively). If telomere length were causally linked to cellular life-span, then the initial telomere length of the culture should correlate well with the life-span of that culture, regardless of donor age. Of the 43 strains analyzed for initial TRF length (Fig. 1), 31 strains were selected at random (see *Materials and Methods*) for determination of *in vitro* life-span (Fig. 2*A*). When replicative capacity was plotted as a function of the initial TRF length for these strains, a much more significant correlation was found (Fig. 2*C*; $r = 0.76$; $P < 0.004$) than that between replicative life-span and donor age (Fig. 2*A*; $r = -0.42$; $P < 0.02$). To show that initial TRF length explains a significant portion of the variation in replicative capacity seen throughout the age range of donors, different symbols were used to record the data in Fig. 2 for donors in the age ranges 0–25 yr, 26–50 yr, 51–75 yr, and >75 yr. In each case, a clear correlation is seen between replicative capacity and initial TRF length. This key observation is important because it shows that TRF length is a much better predictor of replicative capacity than is donor age. Moreover, by providing a biomarker of cellular aging, telomere length may prove useful in assessing whether replicative senescence plays a role in age-related diseases and loss of tissue function *in vivo*.

**Telomere Length Is Reduced in Progeria Fibroblasts.** Because replicative capacity is generally reduced in accelerated aging syndromes (34, 35), we wanted to determine whether telomeres are also shortened in these disorders. To test this, TRF lengths in fibroblast cultures from Hutchinson–Gilford progeria patients were compared with those from age-matched normal individuals. We found that the pooled mean TRF length in five cell strains established from progeria donors, all of which had reduced proliferative capacity *in vitro* (ref. 34 and unpublished data), was significantly shorter than the corresponding value for five young normal donors (Fig. 3; $P < 0.001$). The reduction in both telomere length and replicative capacity in fibroblasts from the progeria donors is further evidence that telomere length is a biomarker of cellular aging. The mean TRF length in fibroblasts of parents of progeria patients, who reveal no signs of premature aging, was not different than that of age-matched normal donors (Fig. 3*B*). This result is consistent with the suggestion that Hutchinson–Gilford progeria arises from a *de novo* autosomal dominant mutation (for review, see ref. 35). The short telomeres characteristic of progeric fibroblasts could be caused by abnormal regulation of telomere length in a parental germ-line clone, a high rate of cell turnover during development of progeric individuals, or an aberrantly high rate of telomere loss with each cell division. To test the later possibility, we compared telomere loss during aging of fibroblasts from three progeria patients *in vitro* to that of age-matched normal donors. There was a small and statistically insignificant increase in the rate of telomere loss in the fibroblasts from progeria patients (data not shown). Thus, other factors are likely to contribute to the shortened telomeres seen in these cells.

**Rates of Telomere Loss *in Vivo* and *in Vitro* Are Comparable.** Based on data presented here, it is possible to estimate the rate of telomere shortening per cell doubling *in vivo*. The rate of decline of proliferative capacity of fibroblasts *in vitro* as a function of donor age is ≈0.2 population doublings per yr (refs. 2 and 3, and Fig. 2*A*). In the same donors, mean TRF length decreased ≈15 bp per yr (Figs. 1 and 2*B*). If we assume that the loss of culture life-span with donor age reflects the average cell doubling rate *in vivo* (i.e., that the total number of doublings *in vivo* and *in vitro* is constant for a given population of cells), then we obtain ≈75 bp per population doubling for the *in vivo* rate of telomere shortening in human fibroblasts. This rate is similar to that observed for telomere shortening *in vitro* (48 ± 21 bp per population doubling) (22, 25) but significantly greater than that for terminal DNA loss in yeast defective for telomere maintenance (40) or in *Drosophila* mutants with broken chromosomes (41–43). Analysis of DNA loss in these organisms suggested that as few as 2–4 bp were lost from the end of the chromosome per cell doubling. However, many factors could account for the difference between rates of telomere loss in normal human somatic cells and these eukaryotes, such as differences in the extent of incomplete replication, telomerase activity, or exonuclease activity.

**Maintenance of Telomere Length in Sperm.** Because the germ-line lineage is immortal, a mechanism to maintain telomere length must be active at some stage of germ-line development. To test this, we measured mean TRF length for sperm from 63 donors ranging in age from 19 to 68 yr and found that it increased slightly with age (Fig. 4). Analysis of the integrated signal from the telomeric probe hybridized to the TRFs showed greater experimental variation but also indicated that the TTAGGG abundance did not decrease with age (data not shown). These observations are consistent with the idea that telomerase may be active in germ-line tissue,

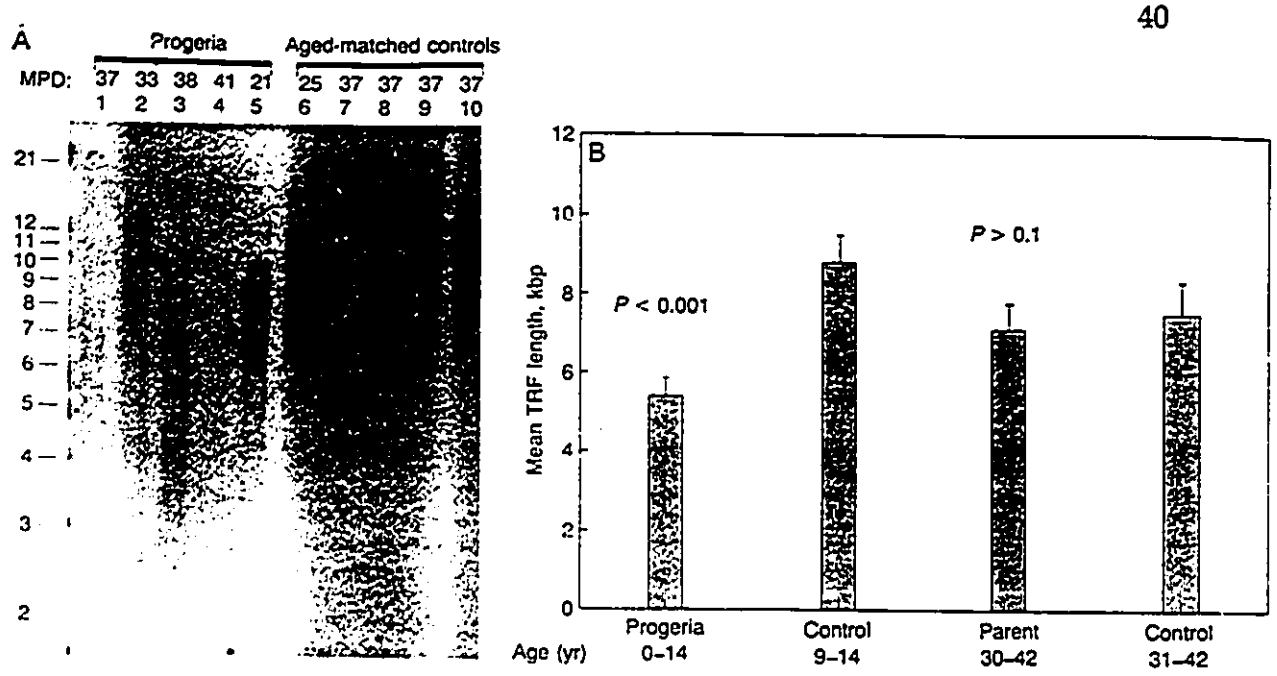


FIG. 3. Comparison of mean TRF length for fibroblast strains established from progeria donors and age-matched controls. (*A*) Genomic DNA prepared from skin fibroblast cultures established from Hutchinson–Gilford (progeria) and normal donors at the indicated MPDs was analyzed as described in Fig. 1*B*. Size and position of markers are indicated. The donor age and maximum replicative capacity of the strains in lanes 1–10 are 9, 3, 3, 4, 14, 9, 10, 14, 12, and 15 yr and 37, 33, 41, 54, 26, 54, 73, 73, 73, 79 population doublings, respectively. (*B*) The pooled mean TRF length for the progeria donors is significantly less than that for controls ($P < 0.001$). Comparison of pooled mean TRF length for the parents of progeria patients and age-matched controls showed no significant difference ($P > 0.1$). Because DNA could not be obtained at equivalent passage or donor age for all comparisons, mean TRF length was determined for normal donor strains at equal or greater population doublings or donor age.

thus maintaining telomere length between generations of the organism.

**The Telomere Hypothesis.** Telomerase can be detected in a variety of immortal tumor cell lines and transformed cells in culture (26, 30) but not in normal fibroblasts or embryonic kidney cells (ref. 26, and A. Avilion and C.W.G., unpublished data). The loss of telomeric DNA during aging of human fibroblasts (22), keratinocytes (23), leukocytes (24), epithelial cells (24, 26), and endothelial cells (E. Chang and C.B.H., unpublished data) is consistent with a general lack of telomerase in untransformed somatic cells. These observations, together with maintenance of telomere length in germ-line cells (Fig. 4), suggest a role for telomeres and telomerase in cell aging and immortalization (Fig. 5). We suggest that telomerase is (*i*) active in germ-line cells, (*ii*) repressed early in embryonic development in most somatic cells, and (*iii*) reactivated in somatic cells immortalized during tumorigenesis.

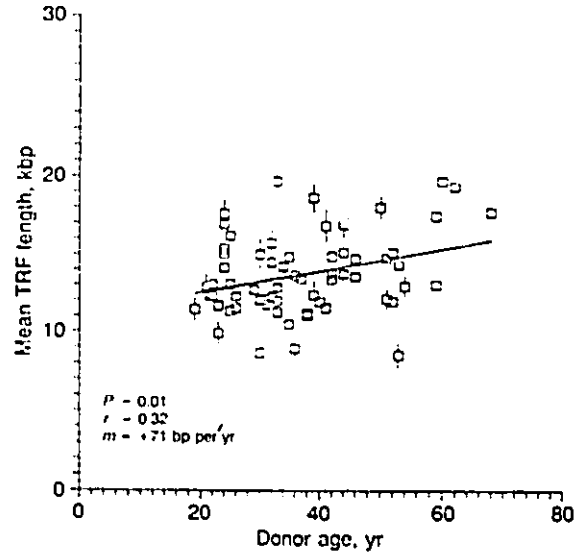


FIG. 4. Mean TRF length in sperm DNA increases as a function of donor age *in vivo*. Mean TRF length was determined for semen samples from 63 donors who ranged in age from 19 to 68 yr. SDs for points representing the mean of two or more determinations are indicated. The slope of the regression line is 71 bp per yr and is significantly different from 0 ($P = 0.01$). Values for the slope ($m$), regression coefficient ($r$), and probability ($P$; $H_o$: slope = 0) of the linear regression line are shown.

Counter *et al.* (26) showed that simian virus 40 large T antigen and adenovirus 5 oncogenes can extend the life-span of human embryonic kidney cells without directly activating telomerase. Hence, during the extended life-span phase of these cultures, telomeres continued to shorten until a "crisis" point, when most cells die. Telomerase was detected only in the relatively rare immortal clones that survived crisis, and in these cells telomere length was stable. The two breakpoints in Fig. 5 representing replicative senescence and crisis correspond to mortality phase 1 (M1) and mortality phase 2 (M2), as defined by Wright *et al.* (44). The mean telomere length at these points are designated T1 and T2. We assume that M1 is a checkpoint in cell growth when one or more telomeres reach a critical minimum length. Viral oncogenes and other agents may be able to bypass this checkpoint without activating telomerase, thus compromising genetic stability while telomeres continue to shorten. At M2, telomere length may be severely shortened on many chromosomes, leading to a dramatic increase in chromosomal instability and cell death (26). Cells that activate telomerase and stabilize telomere length may be able to survive M2.

Some studies with mice, a short-lived species, have shown that TRFs are long and that their length does not decrease noticeably with age (38, 39). However, because TRF lengths in the strains of mice examined were very heterogeneous,

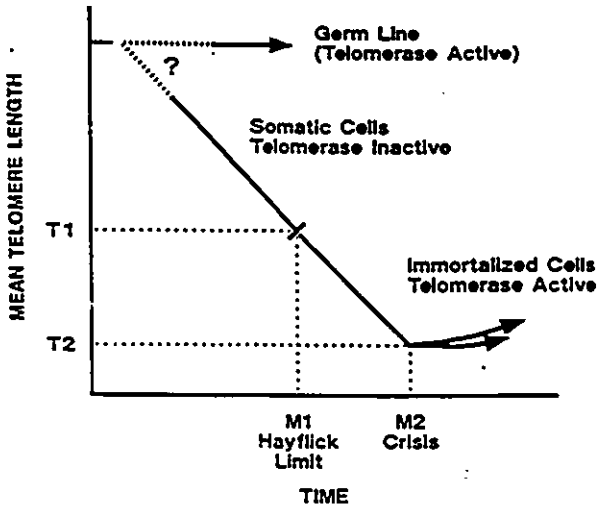


FIG. 5. Schematic of the telomere hypothesis of cell aging and immortalization. Changes in telomere length with passage of time (in cell divisions) are represented for germ line, normal somatic, and transformed cells. Events during early embryonic development at the beginning of the time axis are uncertain (dotted lines). Telomerase is presumably activated at some point in gametogenesis because telomere length is maintained in germ-line cells, but it is not known whether telomerase is active in the early embryo before germ-line development. In contrast, the observed decrease in telomere length and lack of telomerase activity in normal somatic cells suggest repression of telomerase in these cells. At the Hayflick limit (M1), we assume that one or more telomeres have lost a threshold amount of TTAGGG, signaling a checkpoint in cell growth (see text). Mean telomere length at this point is designated T1. Partially transformed cells that bypass this checkpoint without activation of telomerase continue to lose telomeres until "crisis" (M2), when most cells have critically short telomeres on many chromosomes (mean telomere length ≈ T2). Cells that activate telomerase, presumably by mutation, may survive crisis. Telomeres can then be stably maintained at any length. (Graph was modified from ref. 13.)

both in total length and number of TTAGGG repeats, TRFs with few telomeric repeats could have been obscured by the strong signal from other TRFs with long telomeric arrays. In this case, loss of a few hundred base pairs from short telomeric arrays could be important to cellular aging but go undetected. Thus, the role of telomere loss in replicative senescence of murine cells remains uncertain.

The dominance of senescence over immortality in hybrids between mortal and immortal cells (for review, see ref. 45) could be explained by a trans-acting repressor of telomerase in the mortal cell parent. The existence of complementation groups in transformed cells defined by the presence of a mortal phenotype in hybrids between different immortal cells (46, 47) is more difficult, but not impossible, to explain with the telomere hypothesis. However, cells senesce and organisms age for many different reasons, and it is certain that telomeres and telomerase are not involved in all aspects of this multifactorial process (13).

In summary, our observations suggest that the gradual shortening of telomeres in human somatic cells that lack telomerase provides a good marker of replicative capacity *in vitro* and *in vivo*. Moreover, the tight correlation of telomere length to replicative capacity suggests that telomere loss may initiate cell cycle exit once a critical or "threshold" number of telomeric TTAGGG repeats is reached. It should be possible to test whether telomere loss and telomerase expression are coincidental or causal in cell senescence and immortalization once methods become available to alter these activities in multicellular eukaryotes.

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## 2.2 Analysis of telomere length in senescent HDFs.

The telomere hypothesis predicts that the shortening of telomeres below a critical length will induce cell senescence. The critical telomere length can be estimated by measuring the mean telomere length at senescence. In this Section, a method for calculating the mean telomere length is described, and mean telomere length at senescence is determined for one HDF strain.

Telomere shortening has been predicted to occur due to the end replication problem. A model showing the effect of the end replication problem on the loss of telomeric DNA from a single chromosomal end during replicative aging is presented. This model predicts an increase in the heterogeneity of telomere lengths with each successive cell division. To examine this prediction, the size of the TRF distribution was measured for a HDF strain at several different PDLs.

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# Telomere End-replication Problem and Cell Aging

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Since DNA polymerase requires a labile primer to initiate unidirectional 5′-3′ synthesis, some bases at the 3′ end of each template strand are not copied unless special mechanisms bypass this "end-replication" problem. Immortal eukaryotic cells, including transformed human cells, apparently use telomerase, an enzyme that elongates telomeres, to overcome incomplete end-replication. However, telomerase has not been detected in normal somatic cells, and these cells lose telomeres with age. Therefore, to better understand the consequences of incomplete replication, we modeled this process for a population of dividing cells. The analysis suggests four things. First, if single-stranded overhangs generated by incomplete replication are not degraded, then mean telomere length decreases by 0·25 of a deletion event per generation. If overhangs are degraded, the rate doubles. Data showing a decrease of about 50 base-pairs per generation in fibroblasts suggest that a full deletion event is 100 to 200 base-pairs. Second, if cells senesce after 80 doublings *in vitro*, mean telomere length decreases about 4000 base-pairs, but one or more telomeres in each cell will lose significantly more telomeric DNA. A checkpoint for regulation of cell growth may be signalled at that point. Third, variation in telomere length predicted by the model is consistent with the abrupt decline in dividing cells at senescence. Finally, variation in length of terminal restriction fragments is not fully explained by incomplete replication, suggesting significant interchromosomal variation in the length of telomeric or subtelomeric repeats. This analysis, together with assumptions allowing dominance of telomerase inactivation, suggests that telomere loss could explain cell cycle exit in human fibroblasts.

*Keywords:* senescence; chromosomes; DNA replication, fibroblasts; cell kinetics

## 1. Introduction

Biochemical characteristics of DNA polymerase preclude it from fully replicating the linear ends of DNA by the normal reaction process (Olovnikov, 1971, 1973; Watson, 1972). The properties of DNA polymerase that create the "end-replication problem" are the unidirectional growth of the new chain of nucleotides and the requirement for a primer to initiate synthesis. Since the DNA duplex is antiparallel, each daughter molecule would be shortened on the 5′ end of the new DNA strand after replication (Fig. 1).

Immortal, unicellular organisms and viral genomes have evolved special mechanisms to overcome the end-replication problem. For example, the circular chromosomes of *Escherichia coli* and simian virus 40 (SV40), and the circular replicative intermediate of a number of bacteriophage simply eliminate ends. Alternatively, end-replication can be achieved by priming initiation with repeated sequences at the ends of the chromosome, as proposed for bacteriophage T7 replication, or with a "terminal protein", which is transiently linked to a nucleotide primer, as in adenovirus replication. All of these mechanisms in prokaryotes and viruses prevent incomplete replication of the ends of the chromosome. Eukaryotic cells, in contrast, have evolved a novel solution involving specialized structures called telomeres at the ends of their linear chromosomes (Blackburn & Szostak, 1984), which act as substrates for telomerase, an enzyme that apparently counteracts incomplete replication by

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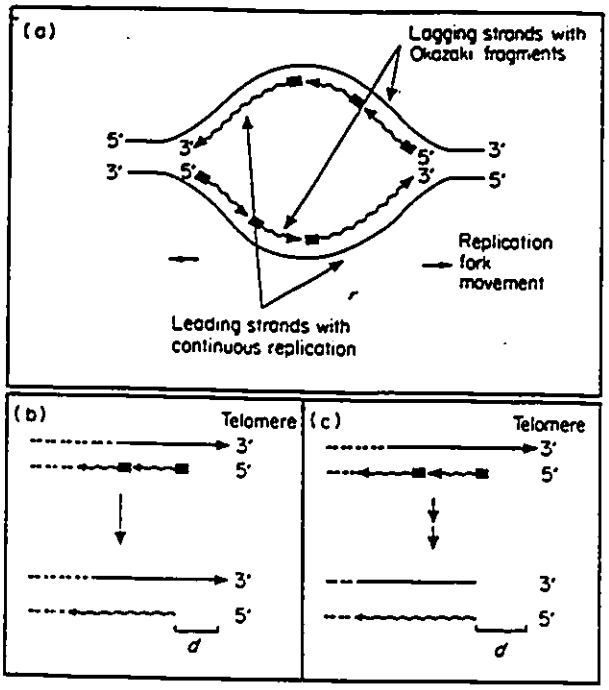


**Figure 1.** The end-replication problem. (a) Schematic of the DNA replication bubble at an origin of replication. The RNA primer (filled block) and newly replicated DNA (wavy lines) are shown. (b) and (c) Schematic of possible events on the lagging strand at either telomere. (b) After removal of the RNA primer that initiates the terminal Okazaki fragment, a single strand deletion *d* remains at the 5' end of the newly replicated strand. (c) Degradation of the overhang generated by incomplete replication results in a double-stranded deletion.

lengthening telomeres (Greider & Blackburn, 1985, 1989). Direct evidence that telomerase maintains telomere length *in vivo* comes from studies of mutations in the template region of the RNA component of *Tetrahymena* telomerase, which caused both an altered telomere sequence and altered telomere length (Yu *et al.*, 1990). In some mutants, telomeres became shorter and the cells died. Similarly, telomere shortening leads to cell death in the yeast mutant *EST1* (Lundblad & Szostak, 1989).

During evolution of some multicellular eukaryotes, notably land animals, there may have been a strong selective advantage for programmed senescence of essentially all non-germline cells. Cellular mortality confers a strict level of growth control and reduces the probability of deleterious hyperplasia or cancer (Harley, 1988). In animals, cells of many somatic tissues in fact have a finite replicative lifespan which likely contributes to senescence of the organism (Hayflick & Moorhead, 1961; Hayflick, 1965; Stanulis-Praeger, 1987). Olovnikov (1971, 1973) proposed that somatic cells may not overcome the end-replication problem and thus telomeric deletions would accumulate at each generation until a critical deletion is made that causes cell death. This hypothesis was supported by recent data showing that telomeres become shorter during aging of human cells *in vitro* and *in vivo* (Harley *et al.*, 1990; Hastie *et al.*, 1990; for a review, see Harley, 1991) and by our preliminary results indicating that telomerase activity is absent from untransformed fibroblasts and epithelial cells in which telomere shortening is observed (A. Avilion & C. W. Greider, unpublished results; Counter *et al.*, 1992). Since the mechanism of telomere loss is not known, it is important to consider the experimental evidence for this process and its possible consequences in light of different models, such as incomplete replication, simple end degradation or unequal recombination coupled with selection for cells with shorter telomeres. In this paper we describe the characteristics of the passive, incomplete replication model for telomere loss assuming no elongation by telomerase and no recombination.

## 2. Methods

A human skin fibroblast culture was derived from a 28 year old normal donor from a 4 mm punch biopsy. The skin was cut into 16 pieces (about 1 $mm^3$ each) and placed under coverslips in four 60 mm dishes (4/dish) (Harley & Goldstein, 1978; Harley, 1990). After about 2 weeks, cells from all dishes were harvested, pooled, and reseeded into four 100 mm dishes. When these dishes became confluent, cells were assigned a mean population doubling level (MPD) of 15. At intervals thereafter high molecular weight DNA was isolated, digested with *Hin*fI and *Rsa*I, and 1·0 $\mu$g electrophoresed in 0·5% (w/v) agarose. To avoid variation due to incomplete transfer of DNA to a membrane support, the gel was dried (40 to 60 min at 60°C) on Whatman 3MM paper and then carefully removed from the paper for direct hybridization with a human telomere probe ([$^{32}$P]d(CCCTAA)$_3$). The dried gel was hybridized, washed, exposed to pre-flashed X-ray film, and analyzed as described for membrane filters (Harley *et al.*, 1990). In some experiments, a PhosphorImager (Molecular Dynamics) was used to quantify the signal from the dried gel. Similar results were obtained by both methods. Replicate or triplicate gels were run for statistical analysis of the telomeric distribution. Where possible, the telomeric signal was normalized to an internal band (≈2 kb† in size, see Fig. 5(a)) to control for variation in the amount of DNA loaded. To control for the quality of the DNA in older cells, undigested DNA was analyzed in a similar manner. These experiments indicated that there was no significant signal in the 2 to 20 kb range for either young or old cells (not shown), which suggests that non-specific degradation of DNA does not contribute to the analysis of the terminal restriction fragments.

## 3. Results

We wished to theoretically model the loss of telomeres by incomplete replication in order to better understand this process. In comparison of theory and data, we assume that telomeres are the com-

†Abbreviations used: kb, $10^3$ base-pairs; TRF, terminal restriction fragment; bp, base-pair(s).

**Table 1**
*Assumptions and definitions*

A. *Assumptions*

| | |
|---|---|
| (1) | Telomeres behave identically with respect to incomplete replications, but inter- and intrachromosomal variation exists in initial telomere length |
| (2) | Telomere length affects neither interdivision time nor chromosome segregation |
| (3) | Overhangs created by single strand deletions are not degraded |
| (4) | A deletion on either or both strands of a telomere constitutes a "deletion event" |
| (5) | A checkpoint is signalled and the cell ceases to divide when a telomere shortens to a certain critical length |

B. *Definitions*

| | |
|---|---|
| TRF, | terminal restriction fragment |
| T, | telomere (terminal TTAGGG or TTAGGG-like DNA repeats) |
| X, | subtelomeric, non-TTAGGG-like DNA repeats in the TRF |
| $n$, | generation, or number of doublings a cell has undergone |
| $d$, | number of deletions on a telomere |
| $d_c$, | critical number of deletions before a checkpoint in cell growth is signalled |
| $p(d,n)$, | probability that a telomere has $d$ deletions at generation $n$ |
| $P(d_c,n)$, | probability that for a particular telomere there has been less than $d_c$ deletions at generation $n$ |
| $F(d_c,n)$, | fraction of cells in which all telomeres have fewer than $d_c$ deletions at generation $n$ |

ponent of the terminal restriction fragments (TRFs) detected by a d(CCCTAA)$_3$ oligonucleotide probe. Thus, the TRF consists of telomeres (T) and subtelomeric DNA (X), which does not hybridize to the probe (Table 1). If TRF, T and X have the same units (e.g. kb), then TRF = T+X.

If telomere loss in fact occurs by incomplete replication, we can use the model to predict the variation in telomere length generated by incomplete replication, and the relationship between the observed loss in telomeric DNA and deletion events. Moreover, if telomere loss is causally involved in signalling cell cycle exit (Harley *et al.*, 1990; Goldstein, 1990; Greider, 1990; Harley, 1991), then the model helps predict the number of deletions a telomere undergoes before replicative senescence, and the rate at which the population of cells senesce.

### (a) *The simple model*

Assuming that overhangs created by incomplete replication are not degraded (Fig. 1(b) and Table 1), a single strand deletion is inherited in the subsequent generation as a double strand deletion on one of the two daughter chromosomes, and recreated as a single strand deletion on the other (Fig. 2). We further assume that a single strand

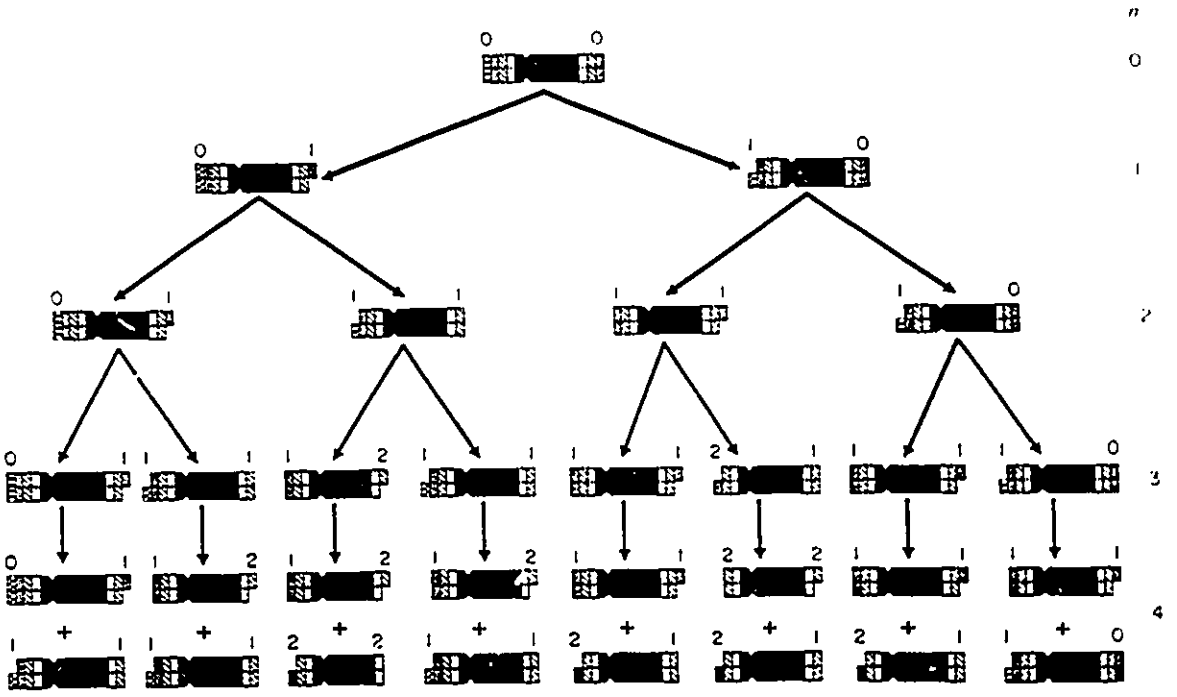


**Figure 2.** Lineage for a single chromosome showing telomeric deletions. Top strand of each chromosome is 5' to 3', left to right. Numbers above each telomere represent the number of deletions at that telomere. The initial telomere length on the long arm of this chromosome is arbitrarily shown shorter than that on the short arm. From assumption 4 (Table 1), loss of DNA on either or both strands of a telomere constitutes a "deletion".

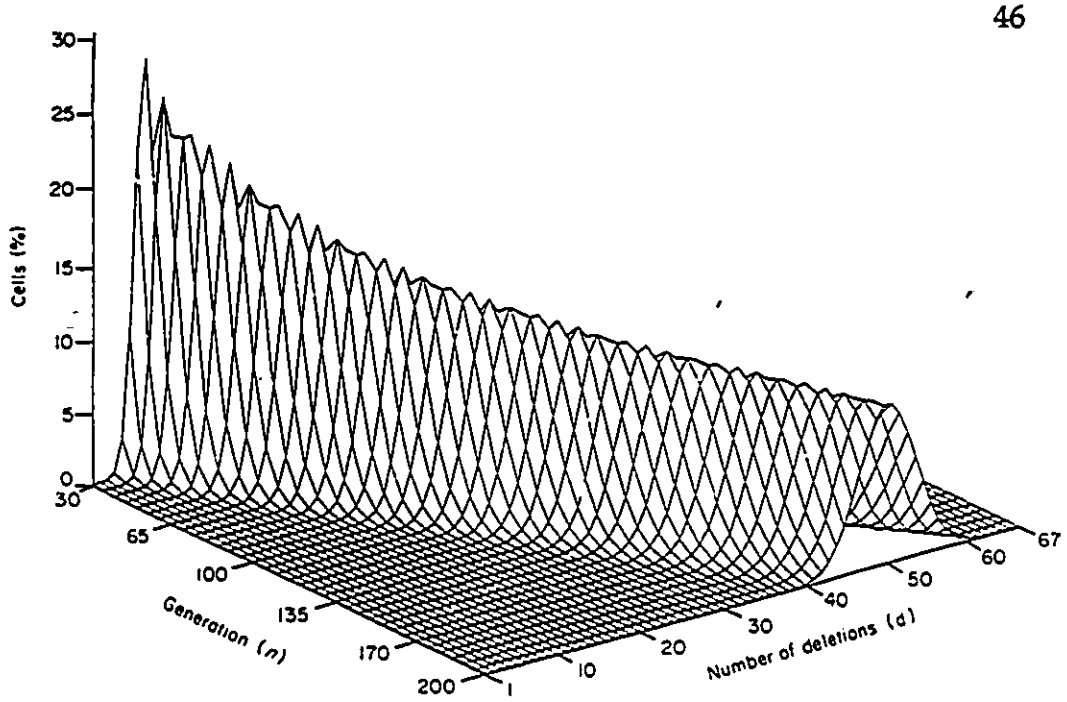


**Figure 3.** Theoretical distribution for the percentage of cells with $d$ deletions on a given telomere at generation $n$. The area under each curve is 100%. Curves are derived from eqn (1) for $n = 30$ to 200.

deletion has the same effect as a double strand deletion. Thus, numbers above each telomere shown in Figure 2 represent the greatest number of deletion events that have taken place on either strand at that telomere. The distribution of deletions $d$ on a given telomere in the $2^n$ cells at generation $n$ arises from alternate binomial coefficients (Table 2). For example, after five generations there is one cell with no deletions at that telomere, 15 cells with one deletion, 15 cells with two deletions, and one cell with three deletions. No cell would have more than three deletions at generation five. The general formula for the probability $p(d,n)$ that a particular telomere has $d$ deletions at generation $n$ is:

$$p(d,n) = \begin{cases} \binom{n+1}{2d} \Big/ 2^n & \text{if } d \leq \dfrac{n+1}{2} \\ 0 & \text{if } d > \dfrac{n+1}{2} \end{cases} \qquad (1)$$

This distribution is shown in Figure 3. It can be used to determine the fraction of cells that have accumulated more than a threshold, or critical number of deletions at a particular telomere (discussed below).

Taking into account all 92 telomeres of a diploid cell, we can predict the rate of loss of telomeric DNA and the variance in telomere length as a function of cell doublings (Fig. 4). The model predicts that mean telomere length, and hence the mean length of TRFs, decreases by one full deletion every four generations. The reason for this is that for a given telomere only half of the cells in each generation undergo one new single strand deletion at that telomere. Thus, a particular telomere undergoes, on average, a single strand deletion every other generation and a double strand deletion every fourth generation.

The mean length of TRFs decreases about 40bp/ generation for the human fibroblast cell strain

**Table 2**
*Distribution of deletions*

| Generations ($n$) | Deletions ($d$) 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| 1 | $\binom{2}{0}$ 1 | $\binom{2}{2}$ 1 | | |
| 2 | $\binom{3}{0}$ 1 | $\binom{3}{2}$ 3 | | |
| 3 | $\binom{4}{0}$ 1 | $\binom{4}{2}$ 6 | $\binom{4}{4}$ 1 | |
| 4 | $\binom{5}{0}$ 1 | $\binom{5}{2}$ 10 | $\binom{5}{4}$ 5 | |
| 5 | $\binom{6}{0}$ 1 | $\binom{6}{2}$ 15 | $\binom{6}{4}$ 15 | $\binom{6}{6}$ 1 |

Distribution of deletions ($d$) for a single chromosome is shown for generations $n = 1$ to 5 in a clonal population of cells for the model shown in Fig. 2. Numbers are derived from alternate binomial coefficients,

$$\binom{n+1}{2d} = (n+1)!/(n+1-2d)!(2d)!,$$

and indicate the number of cells having $d$ deletions on a particular telomere after $n$ generations.

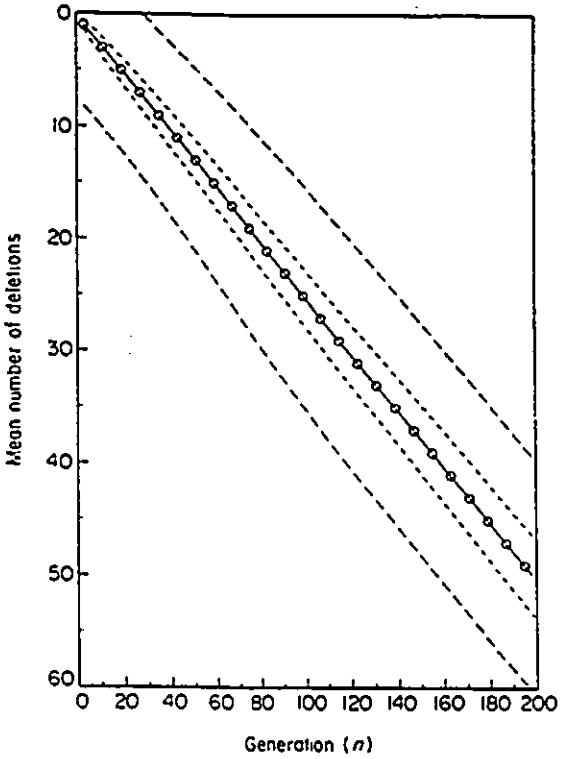


**Figure 4.** Mean number of telomeric deletions as a function of generation in a clonal population. Data were derived from the distribution shown in Fig. 3. Inner broken lines indicate ±1 s.d. The outer broken lines represent the standard deviation of the distribution assuming variability in the initial telomere length (as described in the text). The slope of the continuous line is −0·25.

reported in Figure 5. In five other fibroblast strains the rate of telomere loss ranged from 31 to 85 bp/generation (mean 48±21) (Harley *et al.*, 1990). Using about 50 bp/generation as an average rate of loss, and assuming that single stranded DNA is not degraded, the model predicts that the length of unreplicated DNA at each telomere is four times this value, or about 200 nucleotides.

In some cell strains the TRF distribution is multimodal (e.g. see Fig. 5(a) and Fig. 1(g) of Harley *et al.*, 1990), which could reflect heterogeneity in the cell population or distinguishable variation in the number of TTAGGG or subtelomeric repeats between different chromosomes. The rate of telomere loss for the distinct, high molecular weight smears seen here (about 60 bp/generation) and for other cell strains (K. Prowse *et al.*, unpublished results), are similar to those for the total TRF distribution, suggesting that qualitatively different mechanisms for telomere loss are not at work in different subpopulations of cells or telomeres.

The model also predicts that if all telomeres are initially of the same length, that the standard deviation of telomere length should increase gradually (Fig. 4, inner broken lines). At 80 generations, the standard deviation is about 2.5 full deletions (about 500 bp). We observe, however, that the standard deviation of the distribution of TRF lengths in cultured cells is relatively constant at about 3 to 4 kb (Fig. 5(b), broken lines). Variation in generation level between individual cells is unlikely to account for this discrepancy. We suspect that the large variation in TRF lengths reflects varying amounts of TTAGGG and/or other subtelomeric repeats in the TRFs of different chromosomes. If we assume a normal distribution in initial TRF lengths equivalent to about ten deletion events (about 2 kb), the new standard deviation of the TRF distribution is roughly equivalent to that observed experimentally (Fig. 4, outer broken lines).

### (b) *The model applied to replicative senescence*

Olovnikov (1971, 1973) hypothesized that incomplete DNA replication might lead to a critical deletion causing irreversible cell senescence. However, replicative senescence observed *in vitro* probably reflects a "checkpoint" or signal in growth regulation since it can be bypassed by cellular transforming agents such as SV40 T-antigen (Wright *et al.*, 1989). Thus, we assume that a cell cycle checkpoint is signalled when a specific critical length of telomeric DNA is reached on any one telomere. Since the structure of the telomere at this point is not known, it is possible that TTAGGG repeats are still present, and that further deletions would not necessarily be lethal to the cell.

It is likely that different telomeres vary to some extent in their initial number of TTAGGG repeats. Since only the chromosomes with the shortest telomeres are relevant to replicative senescence, only the deletions that occur on the shorter of these chromosomes' two telomeres need to be considered. For these telomeres, we define a critical number of deletions $d_c$ that signals a checkpoint in cell growth. Thus, the probability $P(d_c,n)$ that such a telomere has not signalled the checkpoint at generation $n$ is simply the sum of probabilities $p(d,n)$ (eqn (1); see also Fig. 3) for all values of $d$ less than $d_c$:

$$P(d_c,n) = \sum_{i=0}^{d_c-1} p(d_i,n). \qquad (2)$$

The probability that none of the chromosomes with short telomeres has signalled a checkpoint in cell growth is the product of their individual probabilities for this event. Since we are considering only the shortest telomeres, we can assume that the value of $d_c$ for each of these telomeres is identical. Thus, if only one critically short telomere is sufficient to signal cell cycle exit, then the fraction of dividing cells $F(d_c,n)$ at generation $n$ in a clonal population is:

$$F(d_c,n) = P(d_c,n)^k, \qquad (3)$$

where $k$ is the number of relevant chromosomes (i.e. ones with initially short telomeres).

Analysis of this distribution shows that $F(d_c,n)$ is relatively insensitive to $k$ for $k = 1$ to 46 (not

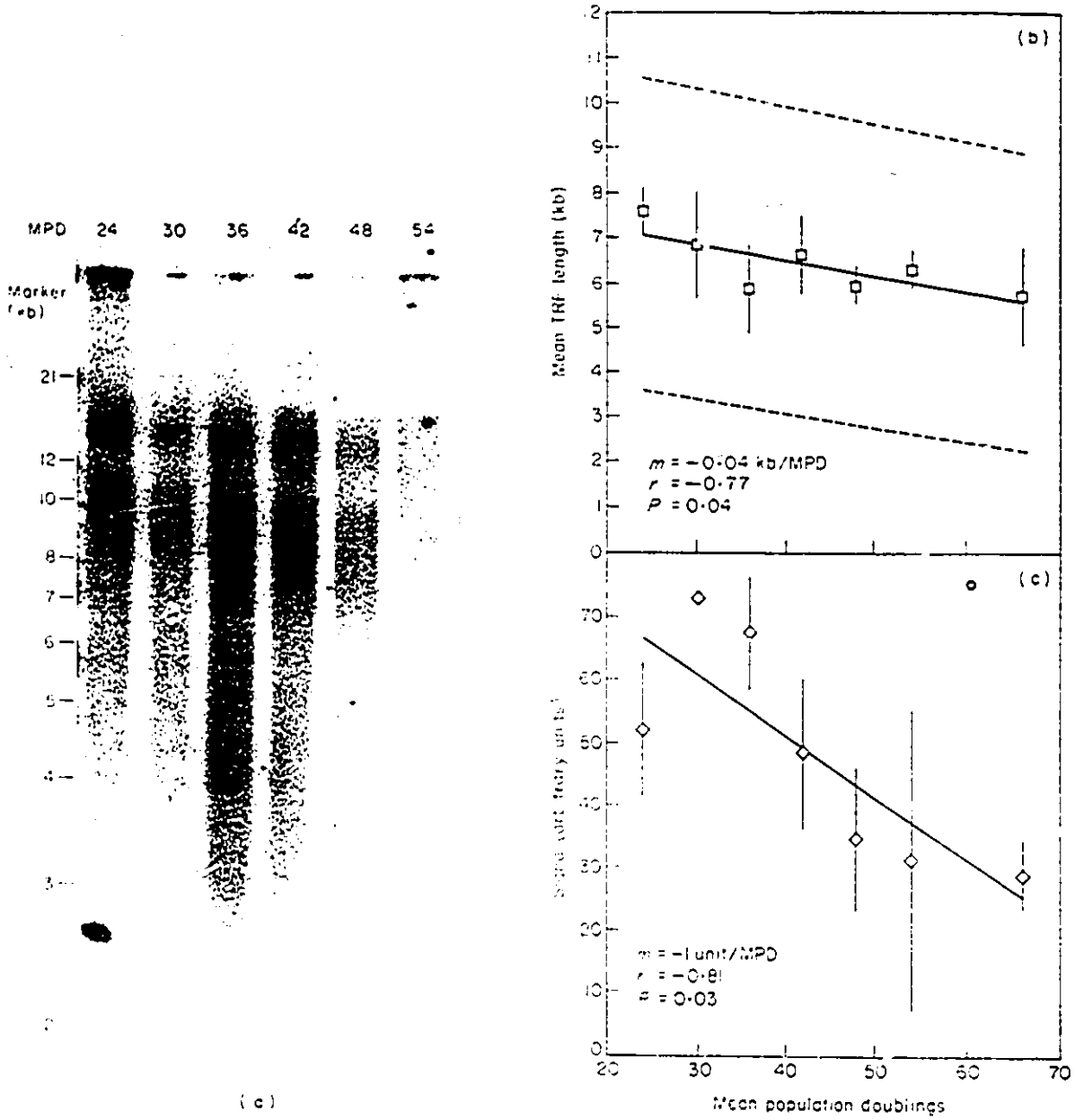


**Figure 5.** Loss of telomeric DNA in cultured human fibroblasts. (a) Autoradiogram showing the decrease in mean TRF length and total telomeric signal during aging *in vitro*. Cells were cultured as described (Harley *et al.*, 1990) and DNA isolated at indicated mean population doubling (MPD) levels, digested to completion with *Hin*fI and *Rsa*I, and quantitated by fluorometry. Following electrophoresis of 1·0 μg of each DNA in 0·5% agarose, the gel was dried, probed with end-labeled d(CCCTAA)$_3$ oligonucleotide and exposed to pre-flashed Kodak XAR film. (b) Mean TRF length and standard deviation of the distribution were calculated for each sample by densitometric scanning of the autoradiograms (Harley *et al.*, 1990). Symbols and error bars represent mean and standard deviation for analysis of DNA from 3 experiments. Linear regression analysis indicates TRF (kb) = 0·04·MPD + 8 kb, with a standard error in the slope of 0·01; *i.e.* the TRF shrinks by 100 ± 100 bp/MPD. Broken lines reflect the standard deviation of the TRF distribution. (c) The total amount of telomeric DNA per cell is represented by a plot of the integrated densitometer signal (arbitrary units) as a function of MPD. The linear regression line ($S = n \cdot 90$) was derived from data representing the mean of 3 experiments. Slope ($m$), regression coefficient ($r$) and the significance level of the slope ($P$, 2-tailed $t$-test) are shown on the graphs.

shown). Figure 6(a) shows the family of curves for $P(d_c, n)$ in a population of cells as a function of generation for various values of $d_c$ when $k = 10$. An important prediction of the model is the rapid decline in the fraction of dividing cells as the population approaches the terminal divisions. Moreover, we can estimate the critical number of deletions that have occurred on at least one telomere before cells become unable to divide further. For example, consider a clonal, uniformly dividing cell population that senesces after about 60 cell doublings. For such a population, the critical number of deletions ($d_c$) is

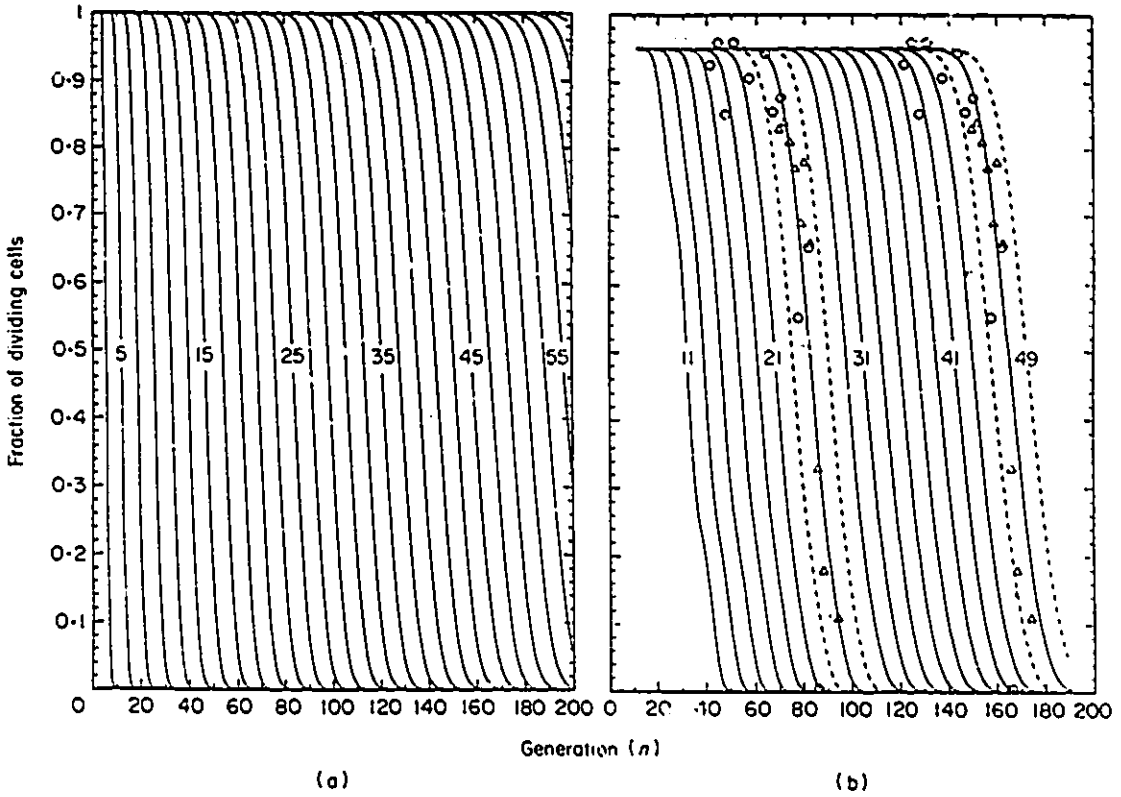


**Figure 6.** Fraction of dividing cells $F(d_c,n)$ as a function of generation ($n$) for (a) a clonal or (b) heterogeneous population of cells. Each line represents the curve obtained for a fixed critical number of deletions ($d_c$) as defined by eqn (3) with $k = 10$. Values of $d_c$ are indicated on every 5th curve. In (*b*), the population is assumed to be heterogeneous with respect to doubling time: $F'(d_c,n) = 0{\cdot}95\,[\frac{1}{4}F(d_c,n-10)+\frac{1}{2}F(d_c,n)+\frac{1}{4}F(d_c,n+10)]$. Experimental points ((△) and (○)) for 2 independent cultures of a human fibroblasts strain (Harley & Goldstein, 1980) are shown after correction for the difference between population doublings and generations (Harley, 1990). These points are superimposed on the theoretical data at 2 locations to reflect no prior *in vivo* doublings (left set of points), or 80 prior *in vivo* doublings (right set of points).

about 19 and the fraction of dividing cells should fall to zero between generations about 50 to 70 (Fig. 6(a)). Similar values are obtained whether one assumes all chromosomes have the same initial telomere length ($k = 46$) or only one chromosome has an unusually short initial telomere length ($k = 1$).

The range of generations over which a clonal population declines, as shown in Figure 6(a), is due solely to the distribution of telomere deletions in the ten relevant chromosomes. Other factors which might effect heterogeneity in longevity of cells within a non-clonal population of tissue culture cells include variable *in vivo* history of cells from which the culture was derived, and variations in generation level between individual cells that arise *in vitro* from variable doubling times (Harley & Goldstein, 1978; Smith *et al.*, 1978). For a fetal cell strain, we can assume that this variation is fairly narrow, perhaps as small as 20 generations (Cristofalo & Sharf, 1973; Harley & Goldstein, 1978, 1980).

Heterogeneity in generation level should be treated as a continuous variable. However, in the absence of information about the nature of this distribution, we have simply assumed that at any given mean generation $n$, 25% of the cells are at $n-10$, 50% are at $n$, and 25% are at $n+10$. There is also a small, apparently constant fraction (about 5%) of non-dividing cells even at early generations in culture (Harley & Goldstein, 1980) which should be accounted for in the model. With these refinements we can compare the predicted senescence of a fetal culture to previously reported data (Harley & Goldstein, 1980; Fig. 6(b)). The purpose of this is not to confirm the theory, since other independent models of cell cycle exit can also adequately explain these data. Rather, the comparison of experimental data and theory was done to estimate one of the parameters of the model: the number of deletions before cells reach replicative senescence. To bracket a reasonable range of doublings that fetal cells might undergo *in vivo*, the experimental points are shown at two locations within the family of theoretical curves. For the first set, generation 0 of the culture corresponds to generation 0 of the model population. This is the lower limit of the number of *in vivo* generations. For the second set, generation 0 of the culture corresponds to generation 80 of the model. This should be a reasonable upper limit to the number of *in vivo* generations. In either case, the data fall within a narrow range of critical deletion

events ($23 \leqslant d_c \leqslant 27$ for the first case and $45 \leqslant d_c \leqslant 49$ for the second case) suggesting that the model adequately explains the kinetics of cell senescence in these cultures. In the second scenario, telomeres would accumulate 20 deletions, on average, in their 80 *in vivo* generations (Fig. 6(a)), and thus the predicted $d_c$ value for a telomere *in vitro* would be 25 to 29. Therefore, our model predicts that the critical number of deletions occurring before senescence *in vitro* in cultured fetal cells is about 25 to 30. If a deletion event is 200 nucleotides, this corresponds to 5 to 6 kb of telomeric DNA lost from the end of at least one telomere in the cell at senescence. Note that in 80 population doublings the mean telomere length decreases by only 4 kb. In accord with this, we measured a loss of about 2 kb in about 25 to 60 generations for several fibroblast strains (Harley *et al.*, 1990), and here report a loss of about 1·5 kb in 40 generations of another cell strain (Fig. 5(b)). Since the length distribution of TTAGGG repeats on different chromosomes is not known, the structure of the telomere(s) containing a critical number of deletions is uncertain; it is possible that no TTAGGG or TTAGGG-like DNA remains at the threshold limit, but it is also possible that cells arrest due to a signal mechanism that recognizes a minimum length of TTAGGG.

### (c) *Estimating average lengths of TTAGGG and non-TTAGGG DNA in TRFs*

Comparison of the loss of TRF length and telomeric (TTAGGG) signal intensity as a function of generation (Fig. 5(b) and (c)) provides information about the length of TTAGGG repeats in the TRF. Since the decrease in TRF length (in kb/generation) corresponds to loss of TTAGGG repeats, we know that an appropriate scaling factor ($c$) exists such that the linear regression line for the total telomeric signal (S) (Fig. 5(c)) can be expressed with the same units as that for TRF length (Fig. 5(b)):

$$\text{TRF (kb)} = -0{\cdot}04 \text{ (kb/division)} \times n \text{ (divisions)} + 8 \text{ kb}, \quad (3)$$

$$\text{T (kb)} = cS = -1c \text{ (kb/division)} \times n \text{ (divisions)} + 90c \text{ (kb)}. \quad (4)$$

Since the slope of these two lines must be identical, we can calculate the scaling factor ($c = (-0{\cdot}04)/(-1) = 0{\cdot}04$) and hence the length of the non-TTAGGG portion of the TRF is:

$$\text{TRF} - \text{T} = 8 \text{ kb} - 90(0{\cdot}04) \text{ kb} \approx 4 \text{ kb}. \quad (5)$$

Conversely, the mean length of telomeric (TTAGGG) DNA is 4 kb less than the length of the TRF. Analysis of several other human cell strains (Harley *et al.*, 1990; K. Prowse *et al.*, unpublished results; Counter *et al.*, 1992), shows a similar length of non-TTAGGG DNA (range 2·5 to 6 kb) by this method of analysis. Consistent with this, Allshire *et al.*, (1989) estimated that the most proximal 3·6 kb of the TRF does not hybridize with probes that detect TTAGGG, TTGGGG or TGAGGG repeats.

### (d) *Other models*

We assumed that a single strand deletion was equivalent, functionally, to a double strand deletion. The effect of assuming that a single strand deletion has no effect on telomere function, or has 0·5 of the effect of a double strand deletion does not significantly alter the deductions from the model presented (not shown). We also assumed that single strand overhangs resulting from incomplete replication are not degraded. If they were, then the rate of loss of telomeric DNA would double, but the predicted size of a full deletion event would then be halved (from 200 bp to 100 bp) so that the net effect would essentially be the same.

## 4. Discussion

Although it has not been proven that telomere loss contributes to senescence of multicellular organisms, several lines of evidence suggest a causal relationship may exist. First, mutations that lead to loss of telomeric DNA cause cell death in single cell eukaryotes (Lundblad & Szostak, 1989; Yu *et al.*, 1990). The gradual shortening of telomeres during aging of human cells both *in vitro* and *in vivo* (Harley *et al.*, 1990; Hastie *et al.*, 1990) may reflect a similar process resulting from developmental inactivation of telomerase. Second, sperm telomeres are longer than somatic telomeres (Cooke & Smith, 1986; Allshire *et al.*, 1989) and do not decrease in length during aging *in vivo* (R. Allsopp *et al.*, unpublished results), lending support to the hypothesis that long telomeres are actively maintained in the germ line but not in normal body cells. Third, telomerase activity has been detected in an immortal tumor cell line (Morin, 1989) and in immortal, virally transformed human cell lines, but has not been detected in normal human cells O. (A. Avilion & C. W. Greider, unpublished results; Counter *et al.*, 1992). Finally, the most prevalent chromosomal abnormality that develops in old cells both *in vivo* and *in vitro* is one that presumably involves telomeres, i.e. the formation of dicentric chromosomes (Saksela & Moorhead, 1963; Benn, 1976; Sherwood *et al.*, 1989). These data suggest that critical telomere shortening may precede cellular senescence. The structure of the critically shortened telomeres, however, is uncertain.

Our results show that telomere loss by incomplete replication is a plausible explanation for the kinetics of cell cycle exit if certain shortened telomeres are capable of signalling a checkpoint in regulation of cell growth. It is unlikely, however, that this checkpoint represents an irreversible block, since SV40 T antigen allows cultured fibroblasts to bypass senescence; such extended lifespan clones continue to divide until a second "crisis" or checkpoint is reached, at which time most cells die (Wright *et al.*, 1989). Consistent with this, we have found that the mean TRF length continues to decrease during the extended lifespan phase of SV40 T-antigen trans-

formed human embryonic kidney cells (Counter *et al.*, 1992). These cells lack telomerase; only those cells that survive crisis and are immortal have a stable telomere length and express telomerase.

The mean TRF length of cultured fibroblasts at senescence is about 6 kb (Harley *et al.*, 1990; and data presented here). Since the subtelomeric, non-TTAGGG component of the TRF is about 4 kb (Allshire *et al.*, 1989; and data presented here), we predict that the mean telomere TTAGGG length at senescence is only about 2 kb. When the mean telomere length reaches this level, there may be very little, if any, TTAGGG repeats on some telomeres since incomplete replication itself is predicted to generate about 1 kb variation in telomere length during aging *in vitro* (S.D. ≈ 500 bp at 80 doublings) and interchromosomal variation in initial TTAGGG repeats may be substantially greater.

Although other mechanisms for telomere loss cannot be excluded, they involve additional *ad hoc* assumptions. For example, simple degradation of ends may occur with age, but degradation would have to be associated with mitotic age (i.e. the number of times a cell divides) and not chronological age since telomere shortening occurs in replicating, but not stationary cultures of cells (K. Prowse *et al.*, unpublished results). Similarly, unequal recombination can lead to a gradual loss of telomeres if cells with shorter telomeres have a growth advantage. However, it was shown that unequal recombination could account for loss of a similar amount of repetitious DNA (about 0·5% per generation) only with relatively high rates of recombination and a large influence on interdivision times (Harley *et al.*, 1982).

Kipling & Cooke (1990) have shown that most TRFs of mice are much longer than those of humans (≈ 50 to 150 kb *versus* ≈ 5 to 10 kb) and that many TRFs contain very large arrays of TTAGGG repeats. Since the lifespan of mice is relatively short, they conclude that telomere length has little to do with aging. However, it is possible that inter- and/or intrachromosomal variation in the length of TTAGGG (or non-TTAGGG) repeats obscures telomere loss which may only be significant on one or a few chromosomes. A decrease in 1 or 2 kb in telomere length would not be detected in very large TRFs, while short TRFs with few TTAGGG repeats would have a relatively weak signal that becomes weaker with age. Loss of this signal would be very difficult to detect. It is also possible that telomere loss with age is significant in humans, but not in mice.

Analyses of chromosome shortening in yeast that are defective for telomere maintenance (Lundblad & Szostak, 1989), and in *Drosophila* strains with a telomeric deletion on the X chromosome (Biessmann *et al.*, 1990), suggest that incomplete replication might account for 4 to 8 bp of DNA lost per cell doubling in these species. Our theoretical analysis is based on an observed loss of about 50 bp per cell doubling in human fibroblasts. The difference between human cells and the lower eukaryotes might reflect species differences or the existence of other factors (in addition to incomplete replication) operating in different systems. For example, during DNA replication, polymerase may be able to come closer to the end of the template on the lagging strand in yeast and *Drosophila* compared to human cells. Alternatively, there may be residual elongation of telomeres due to recombination or telomerase in the yeast mutants, or some exonuclease degradation of telomeres in human cells.

Finally, a model in which telomere shortening is causally involved in either replicative senescence of normal somatic cells or the crisis of transformed cells must be reconciled with hybrid cell data, indicating the dominance of senescence (for a review, see Norwood *et al.*, 1990) and the existence of complementation groups among transformed cell types (Pereira-Smith & Smith, 1988). One way to do this is to postulate a *trans*-acting repressor of telomerase expression, and the presence of multiple components or pathways for telomerase repression. Thus, mortal cells (with repressor) fused to immortal cells (without repressor) could yield hybrids that repress telomerase and thus senesce. Fusion of immortal cells with different, defective repression systems for telomerase could complement one another, thus repressing telomerase and generating mortal hybrids. Direct evidence that telomeres and telomerase play a role in senescence or transformation, however, requires probes that permit experimental manipulation of telomerase expression in mortal and immortal cells.

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## 2.3 Examination of the dependence of telomere shortening on cell division.

If telomere shortening is a cause of replicative senescence, loss of telomeric DNA should be dependent on cell division and not on chronological time. Furthermore, as mentioned earlier, telomere shortening is speculated to occur because of the end replication problem. This also implies that telomere shortening should be dependent on cell division. The work in this Section examines the dependence of telomere shortening on cell division. This was accomplished by comparing the rates of telomere shortening for actively dividing HDFs and quiescent HDFs in culture. Also, to determine if telomere shortening occurs in non-dividing cells *in vivo*, telomere length was measured for brain tissue from donors of various ages.

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# Telomere Shortening Is Associated with Cell Division *in Vitro* and *in Vivo*

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**In humans, the amount of terminal $(TTAGGG)_n$, telomeric DNA decreases during aging of various somatic cell types *in vitro* and *in vivo*. While the factors accounting for telomere shortening have not been thoroughly established, the inability of the DNA replication machinery to completely copy chromosomal termini (the "end replication problem") and the absence in somatic cells of telomerase, the enzyme that synthesizes telomeric DNA *de novo*, is a likely mechanism. One prediction of this hypothesis is that telomere shortening should be dependent on cell division. Thus we analyzed telomere length in actively dividing and quiescent cells *in vitro* and *in vivo*. In circular outgrowths of cultured human diploid fibroblasts (HDF), cells at the outer periphery had a significantly lower mean terminal restriction fragment (TRF) length ($P$ = 0.011) and telomeric signal intensity ($P$ = 0.024) than cells at the center. Also, the rate of telomere shortening over time for HDFs held quiescent was not statistically significant ($m$ = −12 bp/day, $P$ = 0.16) while that for serially passaged cells was significant ($m$ = −34 bp/day, $P$ = 0.017). To examine the rate of telomere shortening for quiescent cells *in vivo*, we measured mean TRF length in brain tissue from adult donors ranging in age from 32–75 years. No significant decrease was observed as a function of donor age ($P$ = 0.087), in contrast to the shortening of telomere length that occurs during *in vivo* aging of mitotically active cells ($P$ = 0.0001). These observations show that telomere shortening is largely, if not entirely, dependent on cell division and support the end replication problem as a mechanism for this process and the use of telomere length as a biomarker for replicative capacity.** 

## INTRODUCTION

Telomeres are genetic elements located at the end of all eukaryotic chromosomes and are essential for normal cell viability [1–4]. The function of telomeres is to prevent aberrent recombination and degradation at the ends of chromosomes. By associating with the nuclear matrix, telomeres may also play a role in the organization of the subnuclear architecture and be involved in the transcriptional suppression of genes at distal loci. Telomeres are composed of simple repetitive G/C-rich DNA complexed with specific telomere binding proteins as well as histones [reviewed in 5–7]. At least in ciliates and slime molds, the termini of telomeric DNA are capped by a 3′ overhang (G-rich DNA) that apparently forms intra- and interstrand folded complexes stabilized by non-Watson/Crick base pairing [8, 9].

In humans, the telomeric DNA sequence is $(TTAGGG)_n$ [10] and the length of the terminal restriction fragment (TRF) containing the $(TTAGGG)_n$ tract varies from 10–15 kbp in germ line cells to 5–12 kbp in peripheral blood leukocytes [11–16]. The amount of telomeric DNA has been shown to decrease during aging of various types of human somatic cells *in vitro* and *in vivo* [13–21]. Loss of telomeric DNA could be deleterious to the cell and thus has been proposed to be a cause of cell senescence [22, 23]. It is possible that cell senescence involves a checkpoint arrest in response to damaged DNA due to critically shortened telomeres on one or more chromosomal ends [24, 25]. Mortality of somatic cells may have evolved to reduce the chances of unlimited proliferation of neoplastic cells [reviewed in 26]. During growth of germline cells and immortal tumor cells, telomere length is maintained or extended by telomerase [19, 27–30]. Telomerase is a ribonucleoprotein which synthesizes telomeric DNA *de novo* [31]. This is facilitated by the RNA component of telomerase which contains a short motif complementary to the telomeric DNA sequence, thereby allowing it to serve as a template for the extension of the 3′ termini [32].

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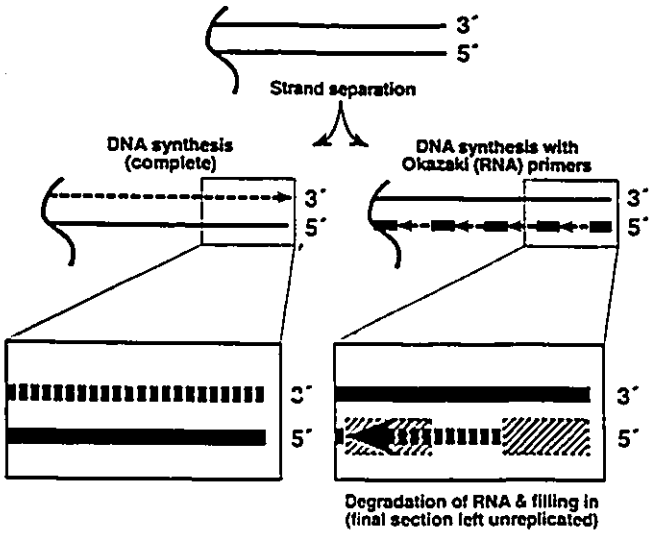


**FIG. 1.** The end replication problem. Schematic of DNA replication at the end of a linear DNA molecule showing strand separation followed by leading and lagging strand synthesis. The RNA primer (filled block), newly replicated DNA (dashed lines), and parental DNA (solid lines) are shown. Leading strand synthesis allows complete replication of the 5′ ends of the parental strand. However, a gap remains at the newly synthesized 5′ ends due to degradation of the most terminal RNA primer used in lagging strand synthesis.

However, the cause of telomere shortening in mortal cells is not fully understood.

One hypothesis explaining the cause of telomere shortening is the end replication problem [33–36] (see Fig. 1), i.e., the inability of the normal DNA replication machinery to complete lagging strand synthesis at sites opposite to the 3′ end of the template DNA. If this hypothesis is correct, then loss of telomeric DNA should be dependent on cell division. Although evidence exists in support of this prediction [14, 17], the extent that other mechanisms independent of cell division also contribute to this process is unknown. Thus the goal of this study is to determine to what degree telomere shortening is dependent on cell division by (i) comparing telomere length at the center and outer periphery of radial outgrowths of human diploid fibroblasts (HDF); (ii) comparing the rate of telomere loss in serially passaged HDFs and quiescent HDFs; and (iii), determining the change in telomere length of human brain tissue as a function of donor age. Together, our observations demonstrate that telomere loss is cell division-dependent and support the end replication problem as a mechanism for telomere shortening.

## MATERIALS AND METHODS

*Cell culture.* Radial outgrowths of HDFs were established as previously described [37]. Cells from a fetal lung fibroblast strain (BJ) at population doubling level (PDL) 24 were suspended in media (DMEM-M199 supplemented with 15% bovine calf serum) to a final concentration of approximately $6.75 \times 10^5$ cells/ml, and $4 \times 10^4$ cells were seeded in the center of a 100-mm tissue culture dish. Cells were allowed to attach for 8 h, after which time the unattached cells were washed off and 15 ml of media was added to the dish. All outgrowths established in this manner formed an initial confluent colony with a diameter of approximately 1 cm. The outgrowths were allowed to expand for a period of 75 days. During this period, the rate of cell turnover was monitored by etching concentric circles in the bottom of the tissue culture dish to mark the advancement of the outer boundary of the outgrowth. Any clonal colonies which established in the area of the tissue culture dish surrounding the outgrowths were removed periodically with a sterile plastic scraper. When the outgrowth expansion period was over, cells from the center and the outer periphery of the outgrowths were harvested using cloning cylinders and DNA was isolated.

The serially passaged and quiescent HDFs were cultured as follows. Starting at PDL 34 (Time 0), cells from an adult skin fibroblast strain (S36a) were either passaged at a 1:8 split ratio or held quiescent by contact inhibition with weekly refeeding. A subgroup of the quiescent cells were trypsinized and reseeded into the same dish (i.e., 1:1 split) every time the serially passaged cells were split. The serially passaged cells were harvested for extraction of DNA every 6 PDLs. At those times, one 100-mm dish of quiescent cells and quiescent-trypsinized cells was also harvested. All cells were grown in α-MEM supplemented with 15% fetal bovine serum.

*Brain tissue samples.* Human brain tissue was obtained as autopsy samples from the Laboratory of Molecular Brain Genetics, Moscow, Russia, the Center for Neurodegenerative Diseases in Toronto, Canada, the Henderson General Hospital in Hamilton, Ontario, or the Southwestern Medical Center at Dallas. A total of 81 different samples from various regions of the brain from 33 different adult donors (2 months to 76 years of age) and 8 fetuses (9–18 weeks) as well as blood samples from 20 donors (71–95 years of age) were utilized in the study. Postmortem times ranged from 2–12 h. Except for one brain sample, all DNA preparations were obtained by one of us (E.I.R.) using a common protocol (see below). All of the fetal and schizophrenic adult samples as well as 80% of the normal and Alzheimer adult samples originated from the Moscow site. Except for a sample from a 32-year-old male, the remaining samples were processed at Toronto. A glioblastoma tumor sample was kindly provided by Dr. Clifford S. Schold (Department of Neurology, University of Texas Southwestern Medical Center at Dallas).

DNA was extracted from the brain tissue by standard methods [see Telomere Length Analysis section and 38]. The DNA from human brain was taken from gross sections of the frontal, parietal, occipital, temporal, and cerebellar cortex. Since there was no significant difference in averaged mean TRF lengths between the different regions of the brain, data shown under Results represent an average of these measurements. For the adults, the brain tissues were taken from normal individuals as well as those suffering from Alzheimer's disease or schizophrenia.

*Telomerase activity assays.* Normal brain cortex and tumor tissue samples of 50–150 mg were homogenized in 250 μl of ice-cold Chaps lysis buffer [30, 39]. After a 25-min incubation on ice the lysate was centrifuged at 16,000*g* for 20 min at 4°C and the protein extract in supernatant was frozen in liquid nitrogen and stored at −80°C. Telomerase activity was measured in protein extracts by the TRAP (telomeric repeat amplification protocol) assay as described previously [30, 39]. Briefly, the assay was performed in Hot Start PCR tubes (Gibco/BRL, Gaithersburg, MD) which contained 0.1 μg of CX primer (complementary to the telomerase products) separated under a wax barrier from the reaction solution. The telomerase reactions were started by pipeting 2-μl aliquots of extracts (6 μg of protein) into 48-μl reaction solutions containing 20 m*M* Tris–HCl (pH 8.3), 1.5 m*M* MgCl2, 68 m*M* KCl, 1 m*M* EGTA, 0.05% Tween 20, 50 μ*M* dNTPs, [$^{32}$P]dCTP, [$^{32}$P]TTP (2 μCi each), 0.5 μ*M* T4 gene 32 protein,

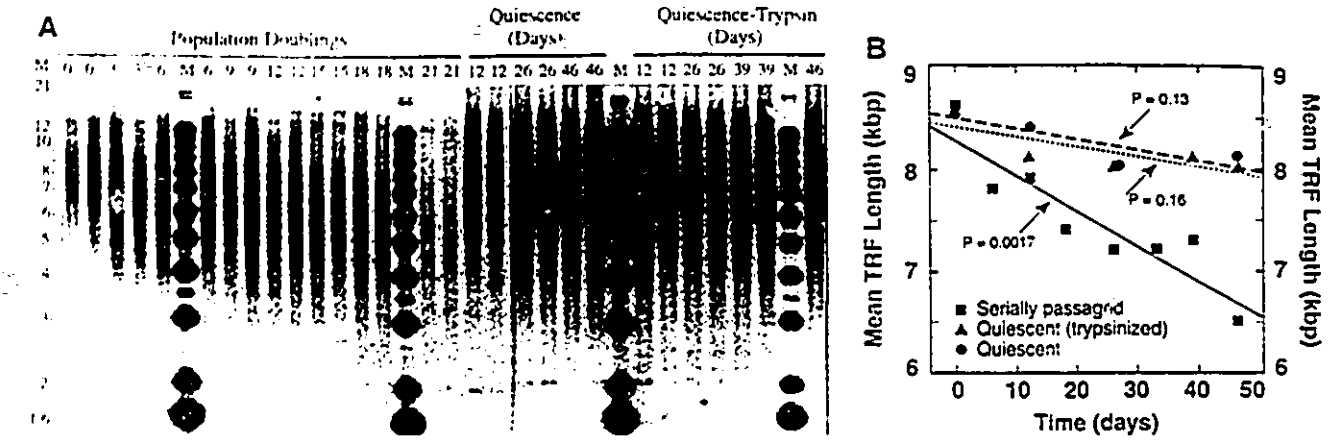


FIG. 2. Mean TRF length analysis during culturing of serially passaged and quiescent HDFs. (A) Autoradiogram showing the size distribution of TRFs at various PDLs and time points for the serially passaged cells and quiescent cells, respectively. All quiescent cells were held stationary at PDL 34 by contact inhibition. The PDL or time of mean TRF length analysis is indicated above each lane. Molecular weight standards are indicated to the left. (B) Line graph showing the change in mean TRF length during culturing of serially passaged, quiescent, and quiescent-trypsinized cells. The best fit line by linear regression is shown for each group of cells. Each data point is the mean value from two experiments. The $P$ value ($H_0$: slope = 0) is indicated for each line.

2 units of *Taq* DNA polymerase, and 0.1 μg of TS (telomerase substrate) oligonucleotide. After a 30-min incubation at room temperature to allow for telomerase-mediated extension of TS oligonucleotide, the reaction mixtures were heated at 90°C for 90 s and then subjected to 31 PCR cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s. The labeled PCR products were then separated on nondenaturing 10% polyacrylamide gels and visualized by phosphorimaging.

*Telomere length analysis.* DNA isolation, restriction enzyme digestion, and mean TRF length analysis were done as previously described [14, 15, 17, 19]. Briefly, high molecular weight genomic DNA was isolated by phenol/chloroform/isoamyl alcohol (25:24:1) extraction of SDS lysed and proteinase K-digested cell and tissue samples. Purified DNA was then digested with restriction enzymes *Hin*fI and *Rsa*I and electrophoresed in 0.5% agarose gels. Gels were dried, and the TRFs were detected by hybridization to 5′-$^{32}$P-$(CCCTAA)_3$. The gel was subsequently washed and exposed either to preflashed autoradiographic film or to a PhosphorImager (Molecular Dynamics) screen.

## RESULTS

### *Analysis of the Rate of Telomere Shortening in Actively Dividing and Quiescent HDFs*

A dependence of telomere shortening on cell turnover implies that a reduction in telomere length should be observed in serially passaged cells but not in cells that are held quiescent. To test this prediction, we analyzed mean TRF length for a HDF strain passaged at a 1:8 split ratio starting at PDL 34 and every other passage thereafter. Mean TRF length was also analyzed for cells that were held confluent (i.e., quiescent) at PDL 34. We observed a significant rate of loss for serially passaged cells ($m$ = −34 bp/day; $P$ = 0.002) and a small, statistically insignificant rate of loss for the quiescent cells ($m$ = −12 bp/day; $P$ = 0.13) (Fig. 2). To control for the possibility that trypsin treatment may have induced an accelerated rate of telomere loss in the serially passaged HDFs, we also analyzed mean TRF length in cells which had been passaged at a split ratio of 1:1 (i.e., trypsinized and reseeded into the same dish). The rate of telomere loss for these cells was similar to that observed for the cells held at quiescence and was not statistically different from 0 ($m$ = −10 bp/day; $P$ = 0.2) (Fig. 2), indicating that trypsinization did not accelerate the rate of telomere loss. Furthermore, similar results were observed in experiments performed independently and with different fibroblast strains [40].

### *Telomere Length Analysis in Radial Outgrowths of HDFs*

When a small, confluent colony of HDFs is established in the center of a culture dish, only cells within a narrow ring (approx. 0.7 mm) around the outer periphery will proliferate [37]. Consequently, as cells begin to divide, the colony expands radially outwards, forming a linear gradient of PDLs from the outer periphery of the initial colony to the advancing front of growing cells [37]. Thus if loss of telomeric DNA is dependent on cell division, telomere length should be unchanged at the center of the outgrowth compared to that in the original culture, but shorter at the outer periphery. Consistent with this prediction, both cells pooled from the center of four outgrowths which were seeded at PDL 24 and expanded for 2.5 months, and cells from the original culture at the time of seeding had a mean TRF length of 9.6 kbp, whereas cells pooled from the periphery of the expanded outgrowths had a mean TRF length of 8.6 kbp. The calculated difference in population doublings between central and peripheral cells of the outgrowths [37] was 31 population dou-

TABLE 1

Telomere Length Analysis in Radial Outgrowths

| Outgrowth | Mean TRF length (kbp) | | Signal intensity (units × $10^{-5}$) | | N[a] | r[b] (mm) | PDL[c] |
|---|---|---|---|---|---|---|---|
| | Center | Periphery | Center | Periphery | | | |
| 1 | 9.7 | 9.3 | 6.4 | 4.6 | 39 | 11.5 | 16 |
| 2 | 9.6 | 8.9 | 5.6 | 3.6 | 43 | 13.0 | 18 |
| 3 | 9.5 | 9.0 | 6.3 | 5.6 | 43 | 13.3 | 19 |
| 4 | 9.7 | 9.4 | 5.5 | 4.0 | 43 | 12.7 | 18 |
| Mean | 9.7 ± 0.1 | 9.2 ± 0.3 | 6.0 ± 0.5 | 4.5 ± 0.9 | | | |

[a] Number of days outgrowths were expanded before analysis.
[b] Difference between final and initial radius of outgrowth.
[c] Calculated difference in population doubling level between cells at the periphery versus the center of the outgrowth.

bling indicating that telomere loss occurred at a rate of ~33 bp/population doubling, similar to that observed in parallel cultures in which the cells were serially passaged by trypsinization (37 bp/population doubling).

To avoid the need for pooling samples from separate outgrowths, central and peripheral cells from four new outgrowths were further cultured, after removal from the expanded outgrowth, until confluence was reached in a 100-mm dish (Table 1). Both mean TRF length and signal intensity were observed to be significantly less for cells at the outer periphery than for cells from the center ($P = 0.011$ and $P = 0.023$, respectively). The calculated rate of telomere shortening (~30 bp/per day) was again similar to that observed for the mass culture during serial passage.

### *Analysis of Telomere Length as a Function of Donor Age in Human Brain Tissue*

Telomere length of PBLs has previously been shown to decrease as a function of donor age [13, 15], consistent with the active turnover of blood cells *in vivo*. To examine the extent of telomere shortening during aging of nonmitotic cells *in vivo*, we analyzed mean TRF length of brain tissue samples obtained from embrionic donors and adult donors (32–76 years). Figure 3A is a representative Southern blot of telomeric DNA obtained from individual brain and blood samples. Figure 3B is a graphical representation of the averaged mean TRF lengths for the brain samples from adult and fetal donors. As shown in Fig. 3B, there is no significant loss of mean TRF length with respect to donor age for the adult cohorts ($m = 3$ bp/yr, $r = 0.05$, $P = 0.90$). However, the inclusion of the fetal samples leads to a plot that does display a potentially significant loss of mean TRF length with respect to donor age. The rates of loss vary from 17 ($P = 0.06$) to 29 bp/year ($P = 0.04$) depending on whether the adult samples from the diseased donors are included; however, both rates are lower than that reported for PBLs ($m = -42$ bp/yr, $r = 0.83$, $P = 0.0001$; see Fig. 3B and [15]). The mean TRF lengths of PBLs from 30- to 80-year-old donors decreased from an average value of 8.0 kbp to approximately 6.0 kbp (Fig. 3B and [15]), thus demonstrating that for at least adult tissue, during *in vivo* aging, telomere length is stable in the brain, in contrast to the loss of telomere length in PBLs.

We examined the possibility of stable telomere length being attributable to active telomerase in brain tissue by assessing for telomerase activity in brain extracts (Fig. 4). There was no detectable telomerase activity in either fetal, neonatal, young, or adult cortex (Fig. 4, lanes 1–4), suggesting that stable telomere length in brain stems is attributable to lack of cell turnover. Telomerase was detected in human glioblastoma (lane 5) and in extracts from HPV16 E6/E7 immortalized human mammary epithelial cells. The presence of telomerase activity in mixed samples of normal human cortex and human glioblastomas indicates the lack of a telomerase inhibitor in normal cortical extracts (lanes 11–13).

Since the individual adult cohorts of brain did not show a change in telomere length with respect to age, we averaged the mean TRF lengths for each cohort and did cross-cohort statistical comparisons. There was no significant difference in mean TRF length among the adult (normal, Alzheimer, and schizophrenic) donors ($P > 0.2$ for all pair-wise comparisons). Although the difference in mean TRF length between fetal donors and adult Alzheimer or adult schizophrenic donors did not reach statistical significance ($P = 0.07$ and 0.08 respectively), that between normal adult and fetal brain tissue did ($P = 0.02$). The pooled mean TRF lengths of fetal brain DNA was 11.9 ± 1.5 kbp while that for all adult donors was 10.5 ± 0.5 kbp. The difference between the two values was not statistically significant ($P = 0.087$).

## DISCUSSION

In humans, telomere shortening occurs during replicative aging of various normal mitotic cells *in vitro* and

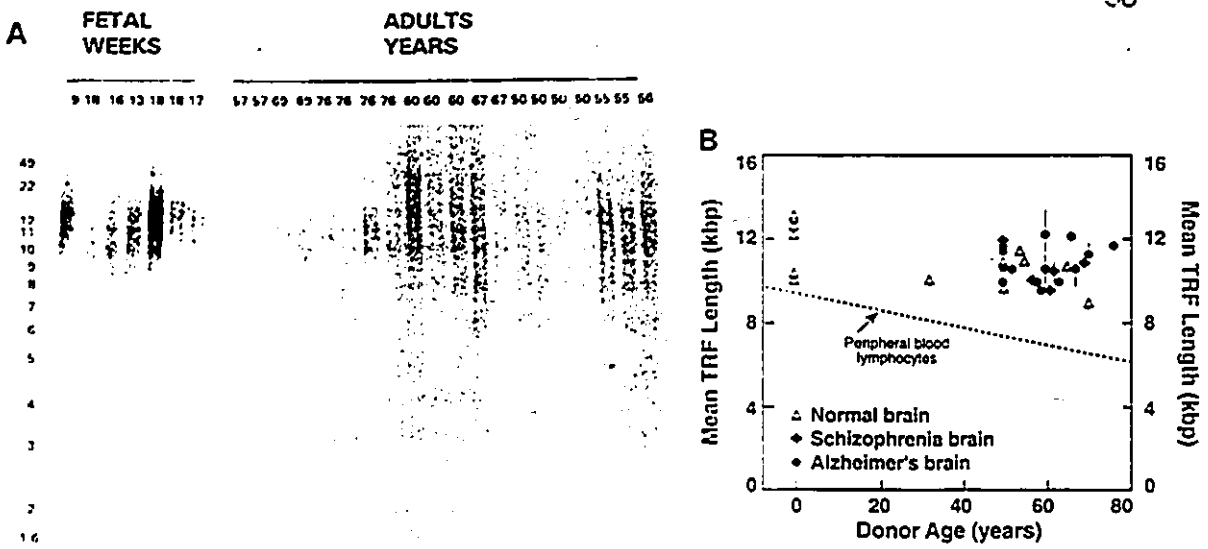


FIG. 3. Mean TRF length analysis of human brain and human PBLs from adults and fetuses. (A) Representative autoradiogram showing the size distribution of TRFs from adult and fetal brain tissues. Molecular weight standards are indicated on the left. (B) Graphic representation of the mean TRF lengths from fetal and adult brain, as well as adult blood. Each data point is the mean value from two or more experiments. Also shown is the best fit line for PBLs from donors ranging in age from 0 to 92 years [15]. Error bars (standard deviation) are shown, and *P* values ($H_a$: slope < 0) are summarized under Results.

*in vitro*, presumably due to the end replication problem in the absence of telomerase (Fig. 1). However, the degree to which telomere shortening is associated with cell division was not established in earlier reports. To address this question we compared the rate of telomere shortening in nondividing and dividing cells *in vitro* and *in vivo*. For HDFs maintained in culture for the same period of time, telomere length was observed to be significantly shorter for actively dividing cells than for quiescent cells (Table 1 and Fig. 2). Also, we observed no significant decrease of telomere length in adult brain tissue as a function of age, whereas telomere shortening is known to occur during *in vivo* aging of various mitotic cell types [13–16, 18, 21]. Together, these observations show that telomere shortening is largely dependent on cell division *in vitro* and *in vivo*.

A small but statistically insignificant rate of telomere shortening was observed for quiescent HDFs ($P = 0.16$; Fig. 2), indicating that there may be a small rate of telomere loss during culturing of quiescent HDFs. This slow rate of telomere loss in quiescent cells is probably due to a slow rate of cell turnover and cell death that normally occurs during culturing of contact-inhibited HDFs [41]. In addition, our data indicate that telomere length in fetal brain tissue is significantly higher than that in normal adult brain tissue ($P = 0.02$). Although telomere length in fetal brain tissue was also longer than that in schizophrenic or Alzheimer's brain tissues, the *P*-value did not reach statistical significance ($P = 0.07$ and 0.08, respectively). It is possible that these differences reflect the relatively small sample sizes or other technical variations which we could not control. However, it is also possible that the generally shorter telomere length in adult versus fetal tissue reflects some turnover within certain populations of cells in the brain during early development.

The most important conclusion from the analysis of the adult brain is that telomere length does not differ significantly as a function of donor age or as a function of normal versus pathological state in this primarily postmitotic tissue. Since there was no detectable telomerase activity in any normal cortex studied (Fig. 4), the general stability of telomeres in adult cortex is most likely due to the lack of cellular turnover. Thus, we believe that telomere loss occurs as a function of replicative but not chronological aging both *in vitro* and *in vivo*.

Although the reason for telomere shortening is still unknown, the association of loss of telomeric DNA with cell division implies that the end replication problem is a probable cause [14, 17]. Two additional lines of evidence supporting the end replication problem as a mechanism for telomere shortening also exist. First, the end replication problem predicts the occurrence of a 3′ overhang at chromosomal termini (Fig. 1). Guanine-rich overhangs have been shown to be present at chromosome termini in normal somatic human cells (CBH, unpublished data). Second, models based upon the end replication problem for telomere shortening in actively dividing cell populations have shown that the predicted kinetics for the appearence of a critical telomere length (i.e., the length which all telomeres in a

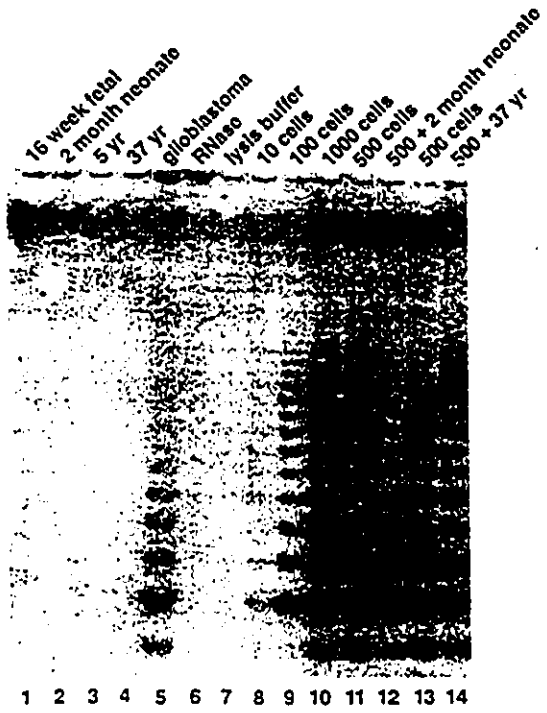


**FIG. 4.** Telomerase activity assays on extracts from normal brain cortex and a glioblastoma brain tumor. Normal brain samples including fetal brain exhibit no detectable telomerase activity (lanes 1–4) under the TRAP assay conditions allowing detection of the telomerase in 10 cell equivalents of a positive control. As a positive control, serial dilutions of extract from an HPV16 E6/E7 immortalized human mammary epithelial (HME) cell line having telomerase activity were assayed (lanes 8–10). Telomerase is readily detectable in a human glioblastoma tumor sample (lane 5) when the same amount of protein extract (6 $\mu$g) is used, as for assays of normal brain extracts. Pretreatment of this extract with RNase prior to the TRAP assay results in loss of telomerase activity signal (lane 6) consistent with the fact that the telomerase activity is RNase-sensitive. To exclude the possibility that the normal brain tissue contains an inhibitory factor(s) preventing detection of telomerase by the TRAP assay, the extracts from 500 cell equivalents of the positive control cell line (HME) were mixed with aliquots of extracts (6 $\mu$g of protein) from normal brain cortex (lanes 12, 14) or with identical aliquots of lysis buffer (lanes 11, 13) and assayed for telomerase. The mixing experiments demonstrate that it is unlikely that the normal brain tissues contain inhibitors sufficient to cause a complete loss of detection of telomerase activity.

cell must be above to allow cell division to continue) is similar to the observed kinetics for the appearence of senescent (nondividing) cells [36]. However, additional mechanisms for telomere shortening, such as degradation by exonucleases or unequal recombination between telomeres followed by growth selection of cells inheriting the shorter compliment of telomeres, have not been ruled out [17, 36]. For example, telomeres may only be susceptible to degradation by nucleases and/or recombination during S phase. Further work is required to determine what mechanisms contribute to telomere shortening and their relative contributions to this process.

The rate of telomere shortening varies significantly between cell types and, more noticeably, between species. For different species, the rate of telomere shortening ranges from a few bp/population doubling in Drosophila [42] to 140–200 bp/population doubling in human endothelial cells [21]. In terms of the end replication problem these differences in rates of shortening may be accounted for by interspecies variation in: (i) the length and positioning of the most terminal RNA primer during lagging strand synthesis; (ii) modification of the overhang length; and (iii) level of telomerase expression. Additionally, differences in exonuclease activity and frequency of recombination at telomeres may also contribute to interspecies variation in rate of telomere shortening. Differences in the rate of telomere shortening between cell types in humans has thus far been observed only for cells in culture due to the difficulty in directly measuring the turnover rate of cells *in vivo*. In particular, HDFs lose telomeric DNA at a rate of 30–100 bp/population doubling [17, 36], whereas the rate of telomere shortening in human endothelial cells is estimated to be 140–200 bp/population doubling. This difference in the rate of loss of telomeric DNA may be partially explained by an underestimation of the number of doublings per passage in endothelial cell cultures due to a lower plating efficiency and growth fraction in endothelial cells compared to that in fibroblasts [21 and unpublished data].

In conclusion, the data presented here show that, in somatic cells which lack telomerase, telomere shortening is dependent upon cell division. Thus loss of telomeric DNA is expected to be an inherent problem for actively dividing cells *in vivo*, but not for stationary cells. It may be possible to extend the limited replicative capacity of actively dividing somatic cells by slowing or stopping the telomere shortening process. One avenue of approach to achieve this goal may be through the controlled expression of telomerase in these cells or their progenitor stem cells.

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## 2.4 Examination of the existence of a critical telomere length in senescent HDFs.

The telomere hypothesis predicts that the shortening of telomeres below a critical length will cause cell senescence. According to this prediction, telomere length should be roughly the same for all senescent cells. In contrast, considerable variability in telomere length at early passage is expected for HDF clones which exhibit a broad range of replicative capacities. The observation that telomere length predicts replicative capacity for cultured HDFs (Section 2.1) supports the possibility of convergence of telomere lengths at senescence, however telomere length at senescence was not analyzed directly in this study. Therefore, the existence of a critical telomere length at senescence was examined by comparing the interclonal variability in telomere length at early passage and at senescence for a number of HDF clones established from the same mass culture. The size of the critical telomere length was estimated by calculating the mean telomere length at senescence (also see Appendix B).

EXPERIMENTAL CELL RESEARCH 219, 130–136 (1995)

# Evidence for a Critical Telomere Length in Senescent Human Fibroblasts

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**Telomeres, the G/C-rich DNA sequences capping the ends of all eukaryotic chromosomes, have been shown to shorten during replicative aging of normal cells *in vitro* and *in vivo*. Moreover, variation in the initial length of terminal restriction fragments (TRF) accounts for much of the variation in replicative capacity of fibroblast cultures from different donors. Since replicative capacity also varies significantly between clones in a mass culture of fibroblasts from a single donor, we wished to further test the hypothesis that the shortening of telomeres to a critical or threshold length acts as a signal for cell senescence. Thus, we measured TRF length and total telomeric signal intensity for 35 clonal fibroblast populations at early passage and at senescence. Replicative capacity was found to be directly proportional to mean TRF length ($m$ = 7.2 population doublings/kbp, $r$ = 0.65, $P$ = 0.0004) and total signal intensity ($m$ = 25.0 population doublings/unit, $r$ = 0.63, $P < 0.003$) at early passage. More importantly, the variability in both mean TRF length and signal intensity ($F$ = 2.0 and 2.9; $P$ = 0.02 and 0.03, respectively) at senescence was markedly less than that at early passage. Although initial telomere length cannot account for all of the interclonal variability in replicative capacity, our observations support the existence of a critical telomere length in senescing cells and a causal role of telomere shortening in cell senescence.** 

## INTRODUCTION

The thorough demonstration that normal human fibroblasts have a finite life span *in vitro* by Hayflick and Moorhead [1] established the cell senescence model of aging. However, despite the large body of evidence in support of this model [2–8] the events responsible for the finite proliferative capacity of normal somatic cells are still uncertain. Although many factors have been proposed to account for the mechanism(s) of cell senescence, including damage from external or epigenetic forces such as radiation or oxidation (reviewed in reference [9]), it is hard to account for the relatively precise limit on replicative, but not chronological, life span of these cells without invoking a mitotic clock [10, 11].

Olovnikov [12, 13] proposed a plausible mitotic clock limiting the replicative capacity of normal somatic cells based upon the inability of the normal DNA replication machinery to completely replicate the 3′ end of linear duplex DNA [14]. Olovnikov suggested that the cause of cell senescence is the gradual truncation of chromosomal ends (telomeres) due to this "end-replication problem."

Telomeres are essential genetic elements [15, 16]. They prevent degradation and aberrant recombination of the natural termini (reviewed in references [17] and [18]) and may contribute to the subnuclear localization of chromosome ends [19, 20]. Telomeres are characterized by tandem arrays of short DNA repeats ($(T_2AG_3)_n$ in vertebrates) and associated proteins (reviewed in reference [21]). To overcome incomplete replication of telomeres, most immortal eukaryotic cells express telomerase, a novel DNA polymerase containing an internal RNA component which elongates telomeric repeats by extending the 3′ end of telomeres (reviewed in reference [21]). Thus, in cells expressing telomerase, telomere loss is balanced by telomerase-catalyzed synthesis of telomeric DNA *de novo*.

While it is very likely that aging occurs at many levels and is pleiotropic [10, 11], a number of observations suggest telomere shortening may effect replicative senescence of cells. (i) Telomeres shorten during *in vitro* and *in vivo* aging of various cell types including fibroblasts [22–24] and peripheral blood leucocytes and colon mucosa epithelia [25]. Telomere loss occurs during replicative aging, but not chronological aging (reference [22] and unpublished data). (ii) Initial telomere length in fibroblasts from different aged donors is proportional to replicative capacity [24]. (iii) Telomere loss in mitotically active cells seems to be associated with the absence of telomerase [26, 27]. (iv) Detection of telomerase activity coincides with stabilized telomere lengths in immortal cell lines, thus suggesting that telomerase expression is required for cell immortalization [26]. (v) Telomere length is greater in sperm than in somatic cells [28, 29]. The telomere hypothesis of cell aging [11] is based

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upon these observations and suggests telomere shortening due to the end-replication problem provides a mitotic clock that ultimately causes replicative senescence, thus limiting the proliferative capacity of somatic cells.

One of the predictions of the telomere hypothesis is that the shortening of telomeres below a critical length could lead to the signalling of cell senescence. To examine the validity of this prediction we have compared interclonal variability in telomere length for clonal fibroblast populations at early passage and senescence.

## MATERIALS AND METHODS

*Cell culture and cloning.* A skin biopsy from a 24-year-old donor was cut into pieces 1-mm$^3$ or less in size and used to establish a primary culture as described previously [30]. When the first 100-mm dish reached confluence (continuous monolayer) the cell population ($\sim 3 \times 10^6$ cells) was assigned 16 population doublings. At population doubling level (PDL) 27 several 100-mm dishes were each seeded with approximately 100 cells. After 10–14 days in culture, 50 discrete clones ranging in size from ~50 to 5000 cells were randomly harvested and transferred to 35-mm dishes [30]. Those clonal populations showing good growth within a few days (~50% confluence or greater) were each seeded into a 100-mm dish and subsequently passaged 1:8 until confluence was no longer reached after 4 weeks with weekly refeeding (senescence). DNA was analyzed for each clonal population at 3 population doublings after the first 100-mm dish had obtained confluence and at senescence. Clones which senesced in the 35-mm dish or in the first 100-mm dish were not analyzed due to poor yield of DNA. In a separate study, the plating efficiency and percent dividing cells was determined for 8 clones. The data for both plating efficiency (>90%) and percentage of dividing cells (85–90%) was sufficiently high so as not to warrant correction of population doublings between passages.

*Analysis of DNA.* DNA was isolated as described previously [26] and quantified by fluorometry. After digestion with restriction enzymes *Hinf*I and *Rsa*I, 1.0 μg of each sample was resolved by electrophoresis in 0.5% agarose gels for 700–800 V-h. Hybridization of oligonucleotide probes to dried gels was based on a modification of Mather [31]. In brief, gels were dried under vacuum at 60°C for 45–60 min, soaked in 0.5 *M* NaOH, 1.5 *M* NaCl for 10 min, followed by 0.5 *M* Tris, pH 8, 1.5 *M* NaCl for 10 min, then incubated in 5× SSC at 37°C with $^{32}$P-end-labeled $(CCCTAA)_3$ for 8–12 h, and finally washed three times in 0.24× SSC at room temperature (8 min each) prior to exposure to preflashed Kodak XAR film or Phosphor Imager screen (Molecular Dynamics) for 1–2 days. Mean terminal restriction fragment (TRF) length (Fig. 1) was determined from densitometric analysis of autoradiograms as described previously [24].

For analysis of telomeric signal intensity, 0.5 μg of the *Hinf*I/*Rsa*I-digested DNA samples was blotted onto nylon membranes. The membranes containing the blotted DNA were then denatured and neutralized as indicated above except the incubation period was for 30 min at each step. The membranes were allowed to dry and the denatured DNA was then cross-linked to the membrane using a uv-stratalinker 1800 (Stratagene). The membranes were then hybridized and washed by a modification of the method described in reference 32. The membranes were prehybridized in a buffer containing 50% formamide, 5× SSC, 0.25% SDS, and 5× Denhardt's solution for 2 h at 42°C. A denatured [$^{32}$P]DNA telomeric probe labeled by nick translation/hexanucleotide primed synthesis [33, 34] was then added to the buffer and the membranes were incubated for a further 12–18 h at 42°C. Membranes were washed briefly in 1× SSC/0.1% SDS at room temperature and then twice at 65°C in 0.1× SSC/0.1% SDS for 30 min. After exposure to a Phosphor-Imager screen for 2 days, membranes were stripped by washing in boiling $ddH_2O$ which was subsequently allowed to cool at room temperature for 1 h. The stripped membranes were prehybridized once more and then hybridized to a denatured [$^{32}$P]-DNA probe specific for the c-fos gene, rinsed, and exposed to a Phosphor-Imager screen as described above.

*Statistical analysis.* To detect any change in interclonal variability in telomere length between young and senescent clones, the interclonal variance in telomere length was compared by the *F* test ($H_a$: variance (early passage) ≠ variance (senescence)). Although mean telomere length is significantly less for the clones at senescence than at early passage, other methods of comparison of variability were not warranted [35].

## RESULTS

Terminal restriction fragments (TRFs) are composed of a terminal $T_2AG_3$ tract (telomere) as well as nontelomeric sequences interspersed with telomeric or degenerate telomeric sequences [36] that occur between the most distal *Hinf*I/*Rsa*I restriction site and the terminal $T_2AG_3$ tract. The high degree of proportionality observed between mean TRF length at early passage and replicative capacity (the number of population doublings remaining from the PDL at which mean TRF length was analyzed until senescence) for fibroblast mass cultures [24] tends to support the existence of a critical telomere length (i.e., the length which all telomeres must be above to allow cell doubling to continue). However, a more direct test of this notion is to compare the interclonal variability in telomere length at early passage and senescence. Figure 1 illustrates the model which we wished to test in this analysis. It is expected that a significant amount of telomere length variability among clones may be due to differences in the previous number of cell divisions *in vivo* and *in vitro* [37, 38]. This contribution to telomere length variability would not be expected at senescence if a critical telomere length exists. Thus, a smaller variability in telomere length at senescence as compared to early passage would support the existence of a critical telomere length (Fig. 1).

In the following experiments we have used mean TRF length (Fig. 1) and total signal intensity as a relative estimate of the mean telomere length because currently it is not possible to directly measure the length of the terminal $T_2AG_3$ tract. We chose to analyze telomere length in fibroblast clones established from the same mass culture as opposed to the mass cultures to reduce the contribution of genetic factors affecting the variation in telomere length measurement. Fibroblast clones have previously been shown to exhibit a broad range in replicative capacities [37, 38], thus rendering them amenable to study the relationship between telomere length and lifespan *in vitro*. Also, we only compared interclonal variability in telomere length between cells at the earliest passage at which enough DNA could be obtained for telomere length analysis and senescent cells because a significant number of young clones were only 1–3 population doublings away from senescence. Therefore, the interclonal variability in remaining replicative capacity,

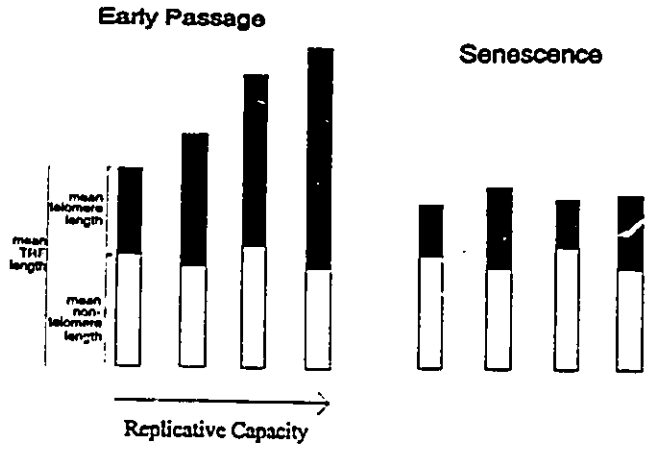


FIG. 1. A model of the relationship between interclonal heterogeneity in mean TRF length and replicative capacity. The mean TRF length consists of two components: (1) The average length of the telomeric (TTAGGG) repeats (solid bar), which bind to the telomeric probe, and (2) The average length of other proximal sequences (open bar) which do not bind the telomeric probe. The variability in mean TRF length among several hypothetical clonal cell populations is shown at early passage and senescence. At early passage, clones with longer telomeres will have proportionally longer proliferative capacities. Interclonal heterogeneity in mean telomere length will be affected by intercellular variability in PDL and by the intracellular distribution of telomere lengths. Interclonal heterogeneity in the mean length of the nontelomeric portion of the TRF reflects intercellular variation in sequences proximal to the telomere. In the simplest model, the interclonal variability in mean telomere length at senescence is reduced to that due to variability in the interchromosomal distribution of telomeric DNA. The length and sequence of the nontelomeric region of the TRF is assumed not to change during replicative aging of a cell.

and presumably telomere length, for clones at intermediate PDLs would be less than that for early passage clones. Thus comparison of interclonal variability in telomere length between clones at intermediate PDLs and senescent clones would not be expected to strengthen our results.

In this study, mean TRF length and telomeric signal intensity were measured for 35 fibroblast clones at early passage and senescence. A typical autoradiogram showing the TRF distributions for several of these clones is shown in Fig. 2A. Heterogeneity in mean length, signal intensity, and, surprisingly, the number of distinct TRF bands or modes can be seen among clones at equivalent PDLs. To determine if the modal heterogeneity among fibroblast clones originates *in vivo* or *in vitro*, the TRF profiles for 13 subclones were examined. Most of the subclones derived from the same parental clone had the same TRF profile, which also matched that of the parental clone (Fig. 2B). There may be some differences in the TRF profile between subclones and the parental clones as noted for subclones B1–B4 and the corresponding parental clone. However, these differences are difficult to quantify objectively. Therefore, these results suggest that the majority of the interclonal heterogeneity in the TRF distribution appears to be generated *in vivo*, although the possibility that some heterogeneity was generated subsequent to establishing the primary culture can not be excluded.

The correlation between replicative capacity and mean TRF length at early passage is shown in Fig. 3A. In agreement with our previous study on fibroblast cultures from different donors [24], a significant positive correlation was observed ($m$ = 7.2 population doublings (pd)/kbp, $P$ = 0.0004). Likewise, replicative capacity was also directly proportional to normalized signal intensity at early passage (Fig. 3B; $m$ = 25.0 pd/unit, $P$ = 0.003). Thus, the range in replicative capacity amongst clones from a single mass culture (20 pd; Fig. 3) was large enough to allow detection of a significant positive relationship with telomere length even though this range in replicative capacity is much smaller than that among mass cultures from donors ranging in age from 0 to 100 years of age (50 pd; [24]).

The variance in mean TRF length amongst the fibroblast clones at early passage was compared to the variance at senescence and was found to be significantly less at early passage than at senescence (Fig. 4A; $F$ = 2.0, $P$ = 0.02). The variance in normalized telomeric signal intensity for young and old cells, as determined by slot blot experiments, was also compared. Interclonal variability in signal intensity was also found to be significantly greater for young cells than for senescent cells (Fig. 4B; $F$ = 2.9, $P$ = 0.03). Although there is still considerable heterogeneity in mean TRF length and signal intensity at senescence which must be accounted for (Figs. 4A and 4B; see Discussion), this observation supports the existence of a critical telomere length.

## DISCUSSION

The telomere hypothesis of cell aging and immortalization predicts that the shortening of telomeres to a critical length on one or more chromosomes in human somatic cells could play a causal role in cell senescence [11]. We have previously shown replicative capacity to be highly proportional to telomere length [24], in support of the existence of a critical or threshold telomere length in senescent cells. In this study, we more closely examined the validity of the above prediction by comparing the variability in telomere length among clonal fibroblast populations at early passage and at senescence. For both measures of telomere length, mean TRF length and signal intensity, interclonal variability was less at senescence than at early passage (mean TRF length—$P$ = 0.02; signal intensity—$P$ = 0.03), further supporting the existence of a critical telomere length.

Values reported for mean TRF length at senescence range from 5 to 7.6 kbp ([27, 39, 40] and data presented here). The discrepancy in these values is at least partially accounted for by the different methodologies used,

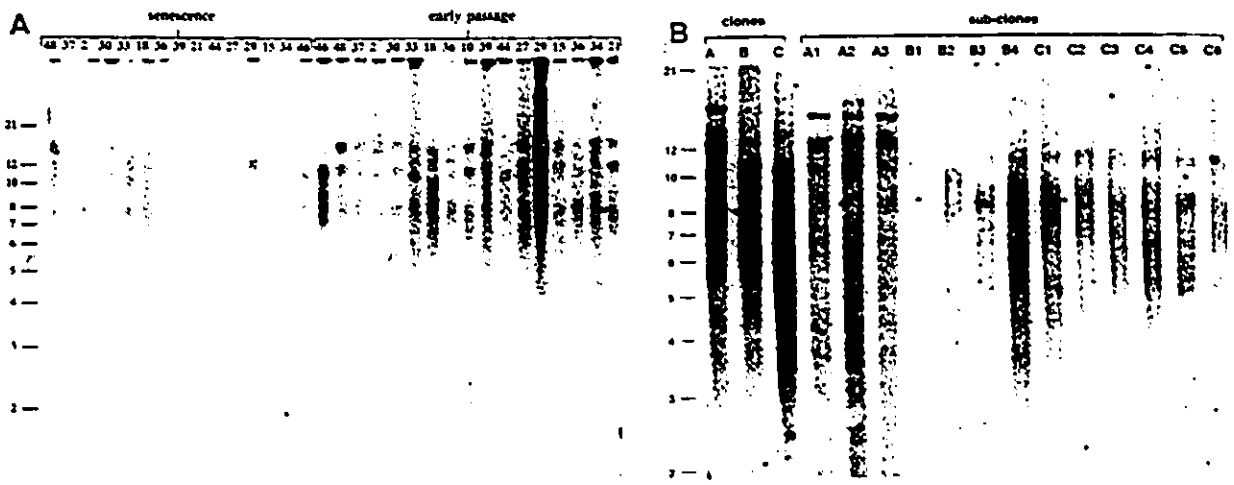


**FIG. 2.** TRF distribution for fibroblast clones at early passage and senescence. (A) Genomic DNA from clonal fibroblast populations was prepared as described and resolved in agarose gels by electrophoresis. TRFs were detected with a $^{32}$P-labeled telomeric oligonucleotide. Each clone from which the DNA was obtained is represented by the number above each lane. Size [in kilobase pairs (kbp)] and position of markers are indicated. (B) Genomic DNA from subclones and corresponding parental clones was analyzed as described in (A). Matching letters indicate the parental clone from which each subclone was established. The subclones have undergone 23–25 more population doublings than the parental clones.

and possibly the different cell types analyzed. However, the noticeable interclonal variability in mean TRF length and total signal intensity at senescence (Figs. 4A and 4B) cannot be explained by these factors. The significant interclonal heterogeneity in the size distribution of both the nontelomeric and telomeric portions of the TRFs may account in part for this interclonal variability. This possibility is supported by the observed heterogeneity in the number of telomeric modes amongst clones (Fig. 2A). Thus, although the total amount of telomeric DNA and mean TRF length at senescence varies from clone to clone, the shortest telomere within a se-

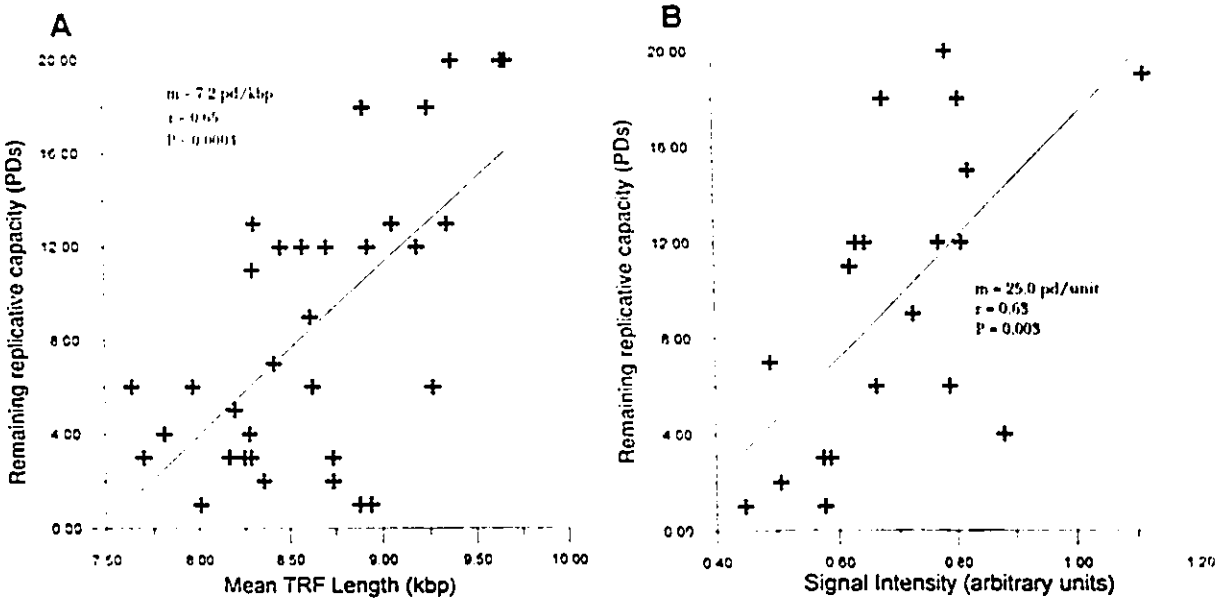


**FIG. 3.** Correlation between replicative capacity and telomere length at early passage. Replicative capacity is shown as a function of both mean TRF length and total telomeric signal intensity at early passage (A and B). Replicative capacity was measured starting at 3 population doublings after the first confluent 100-mm dish for each clone. Each point represents the mean of four or more determinations. Values for the slope ($m$), regression coefficient ($r$), and probability ($P$; $H_o$: slope = 0) of the linear regression lines are shown.

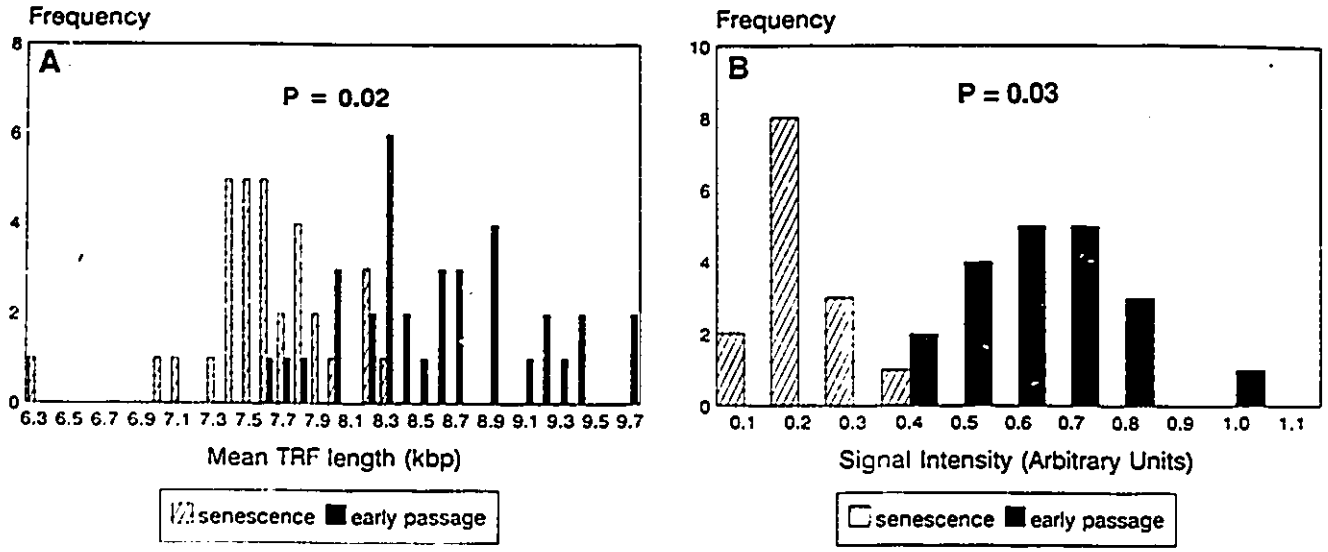


**FIG. 4** Variability in telomere length among fibroblast clones at early passage and senescence. Telomere length measurement data at early passage and senescence was used to construct the frequency distributions shown for mean TRF length (A) and telomeric signal intensity (B). Data for the clones at early passage are indicated by the solid bars and data for the clones at senescence are indicated by the dotted bars. The average mean TRF length values at early passage and senescence are 8.6 ± 0.55 and 7.6 ± 0.36 kbp, respectively. The average telomeric signal intensity values at early passage and senescence are 0.69 ± 0.15 and 0.28 ± 0.08 units, respectively. Values for the probability ($P$; $H_a$: variance (early passage) > variance (senescence)) of the distributions are shown.

nescent cell, which perhaps best represents the critical telomere length, may be much less variable. For example, if a critical telomere length exists, a clonal population in which a small fraction of the telomeres are significantly shorter than the rest would be expected to have a significantly higher mean TRF length and telomeric signal intensity at senescence than a clonal population in which the length of the telomeres is more homogeneous.

There are several possible explanations for the interclonal heterogeneity in TRF length distributions. First, subtelomeric DNA and telomeric DNA are known to be recombinogenic [41–43]. In our study, the majority of the interclonal variation in the TRF length distributions was apparently generated *in vivo*, although it is possible that some recombination occurred between telomeres *in vitro* in the clones analyzed in this study (see Results). Second, analysis of the end-replication problem predicts that random segregation of chromosomes with differing amounts of telomeric DNA deleted would lead to an increasing intercellular divergence in the telomere size distribution with increasing population doublings [27]. Third, interclonal variability in telomere length at senescence may also be partially attributed to termination of division as a result of factors other than loss of telomeric DNA. Factors which could result in cessation of growth before telomeres reach a critical length include unequal partitioning of cytoplasmic components as well as other epigenetic and stochastic processes [44–46]. Fourth, intercellular differences in DNA modifications affecting restriction enzyme accessibility may exist [47]. Therefore, the observed interclonal variability in telomere length at senescence does not preclude the existence of a critical telomere length.

The mean telomere length represents the average length of all telomeres within a cell population and therefore may be used to obtain an estimate of the upper limit for the critical telomere length. We have previously estimated mean telomere length for senescent human fibroblasts to be ~2 kbp [22, 27]. However, it is possible that some chromosomal ends in senescent cells lack telomeres entirely since the standard deviation of the TRF length distribution is large (Fig. 2, and [27]). Therefore, we estimated the putative critical telomere length to be between 0 and 2 kbp for the fibroblast strains analyzed in these earlier studies [27]. Using a similar approach, we found that the mean telomere length at senescence averaged over the clones used in this study was ~2.8 kbp (data not shown), implying that the putative critical telomere length for the clones analyzed here is between 0 and 2.8 kbp. Further evidence for the existence of a critical telomere length and a more accurate estimation of this length will require analysis of the lengths of individual telomeres within senescent cells.

Several theories have been proposed to explain how telomere shortening could cause cell senescence. Olovnikov proposed that telomere shortening would eventually lead to the deletion of a telomeric gene whose expression is required for ongoing cell proliferation [12, 13]. This hypothesis now seems unlikely since the amount of te-

lomeric DNA lost is relatively small compared to intergenic distances. Alternative theories assume a certain length of telomeric DNA is required to maintain a stable telomeric structure or telomere–protein complex. For example, telomere shortening could destabilize telomere structure leading to end-to-end fusion and dicentric formation, which could in turn act as a signal for checkpoint arrest or cell senescence. Indeed, a significant increase in the number of dicentrics has been observed to occur in the final growth phase of cultured human fibroblasts [48, 49]. Alternatively, telomere shortening could lead to the destabilization of an association between telomeres and a component of the nuclear envelope or nuclear matrix leading to a signal for checkpoint arrest. More recently, Wright and Shay proposed that telomere shortening causes changes in telomeric heterochromatin, thereby affecting the expression of telomere-associated genes which initiates cell senescence [50]. Some of these genes may be required for cell replication. Once engulfed in heterochromatin, these genes would become inactive and cell senescence would ensue. We favor a model in which loss of a critical amount of telomeric DNA on one or more chromosomes is recognized as damaged DNA, perhaps analogous to a double-strand break, and thus triggers a mechanism for checkpoint arrest following DNA damage [51]. This model is simpler than the others in that it does not require new pathways for signaling exit from the cell cycle; it only requires that a sufficient amount of DNA is lost from a chromosome end to render the telomere functionally inactive.

In conclusion, our observations further support the existence of a critical or threshold telomere length [11] in mitotically active, normal somatic cells and therefore support the hypothesis that the shortening of telomeres beyond a critical length may initiate replicative senescence. It should be possible to test whether telomere shortening is a true cause of cell senescence once methods for modifying telomere loss and/or telomerase expression in multicellular eukaryotes are developed.

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## 2.5 Analysis of changes in telomere length during replicative aging of hematopoietic stem cells *in vitro* and *in vivo*.

It is debatable whether stem cell populations are mortal or immortal. If stem cells are mortal (ie. undergo replicative senescence), then telomere length should decrease during replicative aging of these cells. If stem cells are immortal then telomere length would be expected to stay the same as PDL increases. To determine if telomere shortening occurs during division of stem cells *in vitro* and *in vivo*, telomere length was measured at various PDLs of long term cultures of $CD34^+/CD38^{lo}$ candidate hematopoietic stem cells, and in $CD34^+/CD38^{lo}$ cells obtained directly from bone marrow of adult donors or cord blood of fetal donors. The results from this analysis are presented in this Section.

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# Evidence for a mitotic clock in human hematopoietic stem cells: Loss of telomeric DNA with age

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ABSTRACT **The proliferative life-span of the stem cells that sustain hematopoiesis throughout life is not known. It has been proposed that the sequential loss of telomeric DNA from the ends of human chromosomes with each somatic cell division eventually reaches a critical point that triggers cellular senescence. We now show that candidate human stem cells with a $CD34^+CD38^{lo}$ phenotype that were purified from adult bone marrow have shorter telomeres than cells from fetal liver or umbilical cord blood. We also found that cells produced in cytokine-supplemented cultures of purified precursor cells show a proliferation-associated loss of telomeric DNA. These findings strongly suggest that the proliferative potential of most, if not all, hematopoietic stem cells is limited and decreases with age, a concept that has widespread implications for models of normal and abnormal hematopoiesis as well as gene therapy.**

The requirement for primers and the undirectional 5′ → 3′ nature of DNA synthesis by DNA polymerases results in incomplete replication of the terminal 3′ strands of linear chromosomes (1–3). Eukaryotic chromosomes end in specialized nucleoprotein structures called telomeres (4) and in vertebrates, including humans, telomeres terminate in tandem repeats of $(TTAGGG)_n$ (5). Telomeres have been shown to be critical for chromosome stability and function (4) and telomere loss has been shown to signal cell cycle arrest and chromosomal instability in yeast (6, 7). Telomeric repeats are synthesized by telomerase, a ribonucleoprotein capable of elongating telomeres *de novo* (8, 9). In the absence of telomerase, human telomeres shorten with cell divisions and such telomere loss may act as a mitotic clock to eventually signal cell cycle exit and cellular senescence (10, 11). Loss of telomeric DNA upon proliferation *in vitro* has been observed in fibroblasts (10, 12) and lymphocytes (13) but not in immortalized tumor cells, which were found to express telomerase activity (9, 14, 15). Shortening of telomeres during aging *in vivo* has been observed with skin dermal and epidermal cells (12, 16), peripheral blood leukocytes (13, 17), and colon epithelia (17) but not in sperm DNA (12).

Most mature cells of the hematopoietic system are relatively short-lived cells that need to be replaced continuously throughout life. A relatively small population of hematopoietic stem cells is ultimately responsible for producing the staggering numbers of cells estimated to represent the daily output of the hematopoietic system (i.e., $>10^{11}$ cells per day in human adults). Stem cells are usually defined as multipotential cells with self-renewal capacity—i.e., the capacity to give rise to more cells with indistinguishable properties and developmental potential. The maintenance of these properties is believed to be an essential feature of both steady-state hematopoiesis and the regeneration of hematopoiesis from a fraction of the number of stem cells present in the adult as occurs following the administration of myeloblative chemoradiotherapy protocols and injection of a marrow transplant (18–22).

Primitive hematopoietic cells in human bone marrow are contained in a very small subpopulation of cells that is characterized by cell surface expression of high levels of CD34 (23), low levels of Thy-1 (24, 25), and an absence or low levels of CD38 (26), CD45RA, and CD71 (27). Recent studies have shown striking ontogeny-related changes in the functional properties of cells with this phenotype (28). In view of these findings and the age-related loss of telomeric DNA observed in fractionated peripheral blood leukocytes (13) and other somatic cells (12, 16, 17), we undertook experiments to analyze the length of telomeric DNA in hematopoietic cells in relation to their stage of development.

## MATERIALS AND METHODS

**Small-Scale Cell Purification.** $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells from fetal liver (13–18 weeks of gestation), umbilical cord blood, and bone marrow (organ donors, 19 and 58 years, respectively) were purified by flow cytometry and cell sorting as described (27, 28). Purified cells were cultured for up to 4 weeks in serum-free medium supplemented with Steel factor, interleukin 6 (IL-6), granulocyte/macrophage colony-stimulating factor (GM-CSF)/IL-3 fusion protein (PIXY), macrophage CSF, granulocyte CSF, and erythropoietin as described (29). At various time intervals, the cells in the cultures were harvested, counted, and used for analysis of mean terminal restriction fragment (TRF) length, calculation of population doublings, and continuation of the cultures. Human material used in this study was obtained following protocols approved by local Institutional Review Boards as well as the Ethical Screening Committee of the University of British Columbia.

**Large-Scale Cell Purification.** Previously frozen bone marrow cells were depleted from cells expressing CD45RA, glycophorin, CD3, CD67, and an unknown platelet antigen (recognized by antibody 3H2 developed in our laboratory) using density separation and high gradient magnetic separation procedures (30). The magnetically preenriched $CD34^+$ cells (70–80% $CD34^+$) were stained for CD34 and CD38 antigens and sorted by fluorescence-activated cell sorting. A total of $0.65 \times 10^6$ and $0.41 \times 10^6$ $CD34^+CD38^{lo}$ cells and $4.2 \times 10^6$ and $2.3 \times 10^6$ $CD34^+CD38^+$ cells was recovered after sorting from $17 \times 10^8$ and $38 \times 10^8$ nucleated cells, respectively, from each organ donor prior to density separation and immunomagnetic selection.

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Abbreviation: TRF, terminal restriction fragment.

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**Telomere Length Analysis.** The methods for measurement of mean TRF length have been described elsewhere (12, 13). In brief, $10^6$ cells at different time points were washed twice in phosphate-buffered saline and the pellet was lysed. Genomic DNA was extracted and 2–5 μg was digested with excess *Hin*fI and *Rsa* I (BRL, 10–20 units each), ethanol precipitated, and quantified by fluorometry. One microgram of digested DNA was resolved in 0.5% agarose gels. Gels were dried and subjected to hybridization with 5' end-$^{32}$P-labeled $(C_3TA_2)_3$. Gels were washed and then exposed to a PhosphorImager screen (Molecular Dynamics).

## RESULTS

**Loss of Telomeric DNA in Hematopoietic Cells *in Vivo*.** Analysis of telomere length in the total nucleated cell population obtained from different bone marrow donors and different fetal liver and cord blood samples demonstrated a loss of telomeric DNA in these tissues at progressive stages of development (Fig. 1). The observed loss of telomeric DNA from early development (fetal liver or cord blood) to adulthood was highly significant ($P < 0.0001$, $t$ test). Telomere length also appeared to decrease with the age of the adult bone marrow donors (calculated loss of 9 bp per year), although considerable individual variation in the mean TRF length at any given age was observed. Most likely, these differences reflect inborn differences in TRF length between individuals. This is also suggested by the observed differences in mean TRF length between the various fetal liver and cord blood samples (Fig. 1). However, differences between individuals in the proliferative history of their hematopoietic cells is another explanation for the observed variations in mean TRF length. Findings in childhood leukemia that indicate that leukemic blast cells have shorter TRFs than normal hematopoietic cells from the same patient support this possibility (31).

**Loss of Telomeric DNA in the Cultured Progeny of Purified Hematopoietic Precursor Cells.** Primitive hematopoietic cells with a CD34$^+$CD45RA$^{lo}$CD71$^{lo}$ phenotype were purified from fetal liver, umbilical cord blood, and adult bone marrow by flow cytometry and cell sorting. The purified cells were cultured in serum-free medium supplemented with a mixture of cytokines to stimulate their proliferation and to obtain sufficient numbers of cells for telomere length analysis. Genomic DNA of the cultured cells was isolated and the mean length of TRFs [which contain the telomeric $(TTAGGG)_n$ repeats] was analyzed by Southern analysis. The results of these experiments are shown in Figs. 2 and 3. Cells produced in cultures of purified adult bone marrow precursors could readily be distinguished from those produced in cultures of purified fetal liver and cord blood cells by virtue of their shorter mean TRF length. Furthermore, the cells produced in the cultures showed a proliferation-associated loss of telomeric DNA. The calculated loss of telomeric DNA by the cultured cells was 45 and 35 bp per population doubling for bone marrow cells, 54 and 19 bp per population doubling for fetal liver cells, and 46 and 23 bp per population doubling for cord blood cells (calculated average loss for all cell types: 37 bp per population doubling). The cells produced in these cultures are known to be highly heterogeneous and contain a minority of primitive precursors amid an abundance of cells at various stages of erythroid and myeloid differentiation (27, 29). As a result, the calculated loss of telomeric DNA per population "doubling" is not indicative of the possible loss of telomeric DNA per cell division in the most primitive precursors.

**Loss of Telomeric DNA in the Most Primitive Hematopoietic Cells.** To exclude the possibility that the observed telomere loss in cells from adult bone marrow cultures was restricted to the progeny of a more mature subset of progenitors in the purified cell fraction we examined the mean TRF length of the purified cells themselves. This represented a considerable challenge as our current TRF analysis requires DNA from at least $0.5 \times 10^6$ cells. To purify sufficient candidate stem cells from CD34$^+$ bone marrow cells in these numbers, low-density cells from previously frozen organ donor bone marrow were first depleted from cells expressing lineage antigens using high gradient magnetic separation (30). CD34$^+$CD38$^{lo}$ cells (26) were then sorted from the magnetically preenriched cells by fluorescence-activated cell sorting.

The mean TRF length of purified CD34$^+$CD38$^{lo}$ cells (>99% pure) from the bone marrow of two different organ donors (aged 16 and 58 years) was compared to that of different cell fractions from the same donors and to that of (control) fetal liver cells (Fig. 4). As before (Figs. 1–3), bone marrow cells had a shorter mean TRF length than fetal liver cells and again an apparent decrease with the age of the marrow donor was observed. Small differences in mean telomere length between CD34$^+$CD38$^{lo}$, CD34$^+$CD38$^{lo}$, and total nucleated bone marrow cells were also found (CD34$^+$CD38$^{lo}$ > CD34$^+$CD38$^+$ > total nucleated bone marrow cells), in agreement with a higher proliferative potential

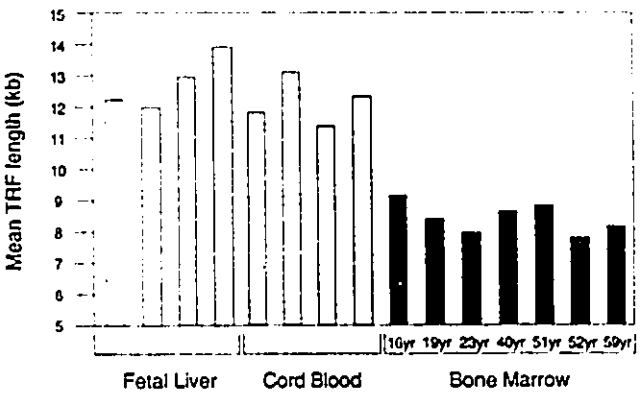


FIG. 1. Loss of telomeric DNA in hematopoietic cells with age. The mean TRF length in cells from the indicated tissue is shown. Fetal liver samples (13–18 weeks of gestation), umbilical cord blood (full-term pregnancies), and bone marrow (from donors of indicated ages) were separated by density centrifugation. DNA was extracted from low-density cells (>90% viable) for TRF analysis. The signal from the dried gels was detected and digitized with a PhosphorImager. The mean TRF length was calculated as described (12, 13).

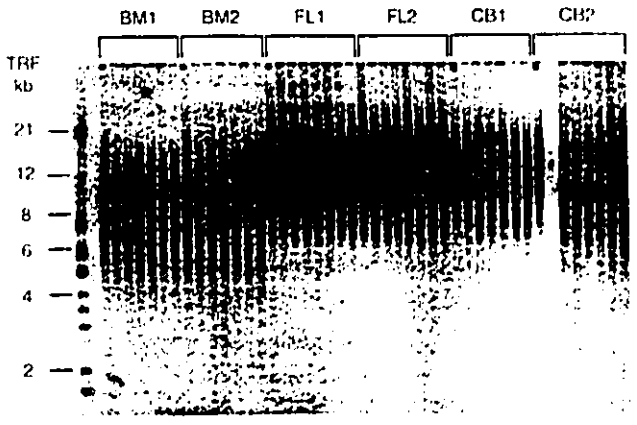


FIG. 2. Loss of telomeric DNA in human hematopoietic cells upon proliferation *in vivo* and *in vitro*. An autoradiogram showing the loss of telomeric DNA in the cultured progeny of primitive hematopoietic precursors purified from the indicated tissues is shown. BM, bone marrow; FL, fetal liver; CB, umbilical cord blood. DNA samples from total nucleated cells from each tissue before purification (indicated with an asterisk) are shown as a control. All subsequent lanes were loaded with digested DNA from cells present at increasing time intervals in cultures initiated with highly purified precursors from the indicated tissues (see also Fig. 3).

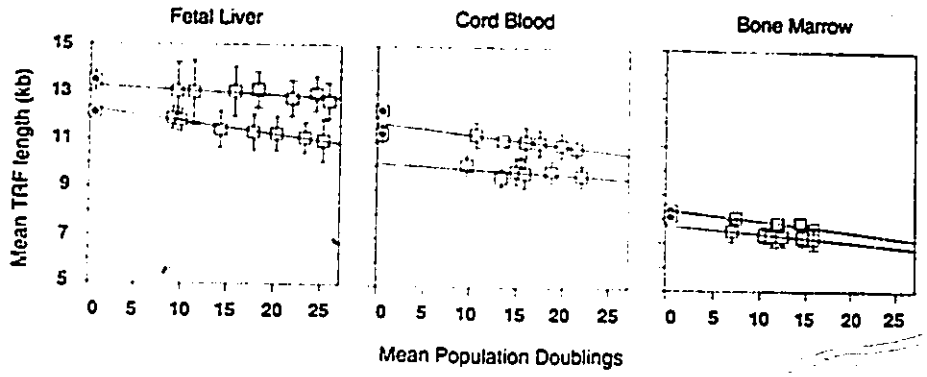


FIG. 3. Loss of telomeric DNA in cells from cultures of purified candidate hematopoietic stem cells. Quantitative analysis of experiments described in the legend to Fig. 2. ○, TRF data obtained with cultured cells from two different donors; □, total nucleated cells from the indicated tissues before purification and culture. The mean ± SE of two independent TRF measurements (different gels) for each DNA sample are shown.

of the $CD34^+CD38^{lo}$ cells. Although the presence of a minor subpopulation of cells within the population of $CD34^+CD38^-$ cells with long ("fetal length") telomeres or telomerase activity cannot be excluded by this experiment, the majority of adult bone marrow cells with this phenotype clearly appear to have shorter telomeres than unfractionated cells from fetal liver, presumably as a result of cell divisions *in vivo*.

## DISCUSSION

The observed age- and proliferation-related loss of telomeric DNA in hematopoietic cells confirms previous observations with DNA from whole blood [17] and lymphocytes as well as neutrophils [13]. Our data strongly suggest that fetal liver or cord blood cells have a significantly higher replicative potential than adult hematopoietic cells, a concept that would argue in favor of the use of such cells for transplantation purposes [32]. However, the importance of telomere length relative to other intrinsic and extrinsic factors controlling the proliferative potential and behavior of hematopoietic cells is currently not known.


FIG. 4. Age-related loss of telomeric DNA in primitive hematopoietic adult bone marrow cells with a $CD34^+CD38^{lo}$ phenotype. DNA was extracted from $CD34^+CD38^{lo}$ cells (lanes 3 and 6), $CD34^+CD38^+$ cells (lanes 2 and 5), and total nucleated bone marrow (BM) cells (lanes 1 and 4) from two different organ donors (age 16, lanes 1–3; age 58, lanes 4–6) for measurement of mean TRF length. DNA from fetal liver (FL) cells (15–18 weeks of gestation) was used as a control (lane 7). The mean TRF lengths (in kb) of the samples in this gel were as follows: lane 1, 9.2; lane 2, 9.1; lane 3, 10.0; lane 4, 8.0; lane 5, 8.4; lane 6, 9.3; and lane 7, 12.5.

If the most primitive hematopoietic cells lose telomeric DNA at a rate that is roughly comparable to other somatic cells [50–100 bp per doubling (12, 13)], our observations are compatible with limited mitotic activity and a limited proliferative potential in such cells from adult bone marrow. More extensive TRF analysis of larger numbers of samples and the development of more refined techniques to allow telomere length measurements in smaller numbers of cells or, ideally, single cells are required to increase the accuracy of estimates of the turnover rate and proliferation potential in various hematopoietic cells.

In previous studies, we found that the fraction of cells responding to a mixture of hemopoietic cytokines, the production of $CD34^+$ cells, as well as the proliferation rate of $CD34^+$ cells all decrease during development (28). This study, showing a decrease in mean telomere length with cell and donor age, suggests that replicative senescence within the hemopoietic lineage may be causally linked to some of these functional differences. Developmental changes in the turnover rate and cytokine responsiveness of primitive human hematopoietic cells (28) are most likely not directly related to changes in the mean telomere length of such cells. However, limitations in the proliferative potential of purified precursors from adult bone marrow could be directly correlated to the observed decrease in mean telomere length. A major obstacle to direct experimental support for this hypothesis is that human telomerase genes have not yet been cloned (33). However, a large body of evidence on telomere length in somatic cells *in vitro* and *in vivo* indicates that telomere length serves as a biomarker of the replicative history of cells (10, 12, 13, 17, 34).

The findings reported here have several implications for models of both normal and deregulated hematopoiesis. A finite life-span of primitive hematopoietic cells is in agreement with the observed loss of repopulating ability of murine bone marrow in serial transplantation experiments (35–37) or repeated cycles of cytotoxic treatment (38, 39). Our findings are also compatible with the higher proliferative potential of fetal versus adult hematopoietic cells observed in various assays (35, 40, 41). However, data documenting hematopoietic reconstitution of multiple recipients with the progeny of a single marked precursor support the notion that at least some hematopoietic cells in this species are capable of self-renewal (20, 42, 43). Although telomerase expression in regenerating stem cells and/or possible differences between

species cannot be ruled out, another explanation is that in these experiments telomere-related limitations in the proliferative potential of primitive murine precursors were simply not yet reached. A similar situation may exist in various forms of leukemia. At initial stages of the disease, altered signal transduction pathways and/or altered probabilities of differentiation events in a single cell will result in numerical expansion of the leukemic cells. In our current model, this expansion should eventually be limited by critical shortening of telomeric DNA, resulting in signals that trigger cell cycle arrest but also chromosomal instability. Further progression of the disease may involve selection of cells that ignore or can bypass the cell cycle arrest signal and, eventually, selection of cells that express the enzyme telomerase (14, 15, 33). A thorough analysis of telomerase expression in cells from normal hematopoietic tissues at various stages of development as well as cells from patients with various hematological disorders will be required to fully understand the pathophysiology of telomere length dynamics in hematopoietic cells. Further studies are also needed to explore the possibility of extending or maintaining the proliferative potential of adult stem cells by forced expression of exogenous or endogenous telomerase activity. The information derived from such analysis should help to guide the development of novel therapeutic strategies involving transplantation and genetic manipulation of primitive hematopoietic cells for the treatment of a variety of disorders.

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## 2.6 Analysis of changes in length of individual telomeres during replicative aging of cultured HDFs.

In the previous Sections, measurement of telomere length was accomplished by mean TRF length analysis. The telomere length and rate of shortening calculated by this approach represents the average length or rate of shortening for all telomeres in a cell population which itself may be heterogeneous. The method of fluorescent *in situ* hybridization (FISH) can be used to estimate the relative lengths of individual telomeres and requires minimal processing of the DNA. In this Section, FISH is used to examine the relative lengths and degree of shortening of individual telomeres during replicative aging of cultured HDFs.

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# *In situ* analysis of changes in telomere size during replicative aging and cell transformation

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## ABSTRACT

Telomeres in human somatic cells have been shown by Southern analysis to shorten during replicative aging. This study examines the shortening of individual telomeres at the single cell level by fluorescent *in situ* hybridization (FISH) analysis. FISH and confocal microscopy of normal human diploid fibroblasts (HDF) in interphase indicated that there may be interchromosomal heterogeneity in telomere length. FISH analysis of interphase HDFs at increasing population doublings showed a gradual decrease in detectability of telomeric fluorescence. To examine the relative lengths of individual telomeres in young and old cells in greater detail, FISH analysis was performed on metaphase spreads of HDFs at early and late passage. Heterogeneity in detection frequency was observed for telomeres on chromosomes 1, 9, 15 and the Y chromosome. Also, the intertelomeric distribution of detection frequencies for these chromosomes was similar for cells at early passage and senescence. These results provide evidence for interchromosomal heterogeneity in telomere length and indicate that all telomeres shorten at similar rates. FISH studies of two SV40 transformed HDF strains showed that at crisis, only a few telomeres remained detectable by FISH. Following crisis, in the immortal cell line, telomere size stabilized, and, in many cells, increased to sizes larger than those found in young HDFs. FISH analysis of metaphase chromosomes from these cells just prior to crisis revealed that the long arm of one copy of chromosome 9 was frequently involved in an end-to-end association with another smaller chromosome or chromosomal fragment, as indicated by the detection of telomeric signal at the junction site. This observation supports the hypothesis that the shortening of telomeres below a critical length causes telomeric instability, allowing the telomere termini to behave like a double stranded break.

## INTRODUCTION

Normal eukaryotic somatic cells can only undergo a finite number of divisions *in vitro*, also known as the Hayflick limit. This phenomenon was originally described as senescence at the cellular level over 30 years ago (Hayflick and Moorhead, 1961), and has now been established as a senescence process in higher eukaryotes (Martin et al., 1970; Dell'Orco *et al*, 1973; Goldstein, 1974; reviewed in Stanulis-Praeger, 1987; Finch, 1990; Goldstein, 1990). Numerous models have been proposed to explain the cause(s) of cell senescence (reviewed in Stanulis-Praeger, 1987; Finch, 1990; Goldstein, 1990). One of these models is based upon the loss of telomeric DNA that occurs during replicative aging of somatic cells which provides an intrinsic biological clock to explain cell senescence (Harley, 1991).

Telomeres are genetic elements located at the ends of all eukaryotic chromosomes and are essential for genetic stability (Muller, 1938; McClintock, 1941; Lundblad and Szostak, 1990; Sandell and Zakian, 1993; reviewed in Blackburn, 1991; Gilson *et al*, 1993). Telomeres cause the ends of chromosomes to behave differently than double stranded DNA breaks in that they protect chromosome ends from nuclease degradation and aberrant recombination (Muller, 1938; McClintock, 1941; Bourgain and Katinka, 1990; Henderson *et al.*, 1990). Telomeres may also play a role in the sub-nuclear organization of chromatin (reviewed in Gilson *et al*, 1993) and may facilitate chromosome pairing during meiosis (Sen and Gilbert, 1988). Telomeres are composed of specialized chromatin (Tommerup *et al*, 1994; reviewed in Blackburn, 1991), the DNA component of which consists of repetitive sequences that are G-rich in the 5' ---> 3' strand (Blackburn, 1991). The telomeric DNA sequence of vertebrates is (TTAGGG)n (Meyne *et al.*, 1989). Although the terminal restriction fragment (TRF) length of peripheral blood

leukocyte (PBL) DNA is typically in the 8-10 kbp range in adults (Hastie *et al*, 1990; Vaziri *et al*, 1993), about 4-5 kbp of the TRF in normal cells is estimated to be non-telomeric DNA (Fig. 1A; Levy *et al*, 1992; Allsopp and Harley, 1995). Thus, adult human telomeric DNA length ranges from 3-5 kbp in PBLs to 10-15 kbp in sperm (de Lange *et al*, 1990; Allsopp *et al*, 1992). In addition to non-telomeric DNA sequences, the portion of the TRF proximal to the terminal TTAGGG tract is composed of degenerate TTAGGG sequences as well as short blocks of TTAGGG sequences (Fig. 1A; Allshire *et al*, 1989; de Lange *et al*, 1990; Brown *et al*, 1990; Weber *et al*, 1990; Wells *et al*, 1990). Little is known about the protein components of mammalian telomeres, although they appear to include nucleosomal proteins (Makarov *et al*, 1993; Tommerup *et al*, 1994). Recently, a telomere-specific protein which binds to double stranded telomeric DNA has been identified in human cell extracts (Zhong, *et al*, 1992).

The need for a special mechanism to complete telomere replication was independently recognized by Olovnikov (1971 and 1973) and Watson (1972). This prediction was based upon the inability of the DNA replication machinery to completely replicate the ends of linear DNA molecules (the 'end-replication problem' (Olovnikov, 1971, 1973)) (reviewed in Blackburn, 1991). Recently, this prediction has been shown to be correct in studies by us and others which have revealed that the amount telomeric DNA decreases during replicative aging of various human somatic cell types both *in vitro* and *in vivo* (Harley *et al.*, 1990; Hastie *et al.*, 1990; Lindset *et al*, 1991; Allsopp *et al.*, 1992; Counter *et al.*, 1992; Vaziri *et al.*, 1993; Vaziri *et al.*, 1994; Chang and Harley, in press). These observations lead to the telomere hypothesis of cell aging which proposes that the shortening of 1 or more telomeres below a threshold length which is necessary for proper telomere structure and function (the critical telomere length or Tc) will induce irreversible cell cycle

arrest (cell senescence or the M1 checkpoint) (see Fig. 1B) (Harley, 1991).

However, there must exist some mechanism to maintain telomere length during species propagation. This mechanism is provided by telomerase, a protein/RNA complex capable of synthesizing telomeric DNA *de novo* (Greider and Blackburn, 1985; reviewed in Blackburn, 1991). The RNA component of telomerase contains a short motif which corresponds to the telomeric DNA sequence and which provides a template for the addition of telomeric DNA onto the 3' terminus of chromosomal ends (Greider and Blackburn, 1989; Feng *et al*, 1995). Telomerase has been detected in various human cell lines and tumor tissue (Counter *et al.*, 1992; Counter *et al.*, 1994a; Counter *et al.*, 1994b; Kim *et al.*, 1994) as well as human germ line tissue (Kim *et al.*, 1994) and appears to be either absent or present at very low levels in most adult somatic tissues (Counter *et al.*, 1992; Kim *et al.*, 1994; Broccoli *et al*, 1995; Counter *et al.*, 1995; Chiu *et al*, in press; Hiyama *et al*, in press). After transformed human cells have bypassed cell senescence, telomere length continues to shorten and the frequency of dicentric chromosomes increases until cells reach crisis (M2 checkpoint; Fig. 1B)(Counter *et al*, 1992; Counter *et al*, 1994b). In the rare transformed cells which have acquired the ability to bypass the M2 checkpoint, the expression of telomerase has been shown to coincide with the maintenance of telomere length, suggesting that telomerase is essential for immortalization (ie. the survival of crisis or the M2 checkpoint where most cells die) (Fig. 1B) (Counter *et al.*, 1992; Counter *et al.*, 1994b). These observations have lead to the suggestion that telomere shortening may also have a causal role in crisis (Harley, 1991; Counter *et al*, 1992; Wright and Shay, 1995).

All of our previous analyses of telomeric DNA length have used Southern hybridization with a telomere specific probe. However, it is not

possible to analyze the lengths of individual telomeres by this technique due to the heterogeneity in telomere length that exists in a cell population. In this study, we have used fluorescent *in situ* hybridization (FISH) to evaluate the relative loss of telomeric DNA from individual chromosomal ends during replicative aging of normal and transformed human fibroblasts.

## MATERIALS AND METHODS

### Cell Culture

BJ cells (fetal male human foreskin fibroblast), S2C cells (adult skin fibroblast), IMR90 cells (female human lung diploid fibroblast; ATCC CCL 186), SW26 cells (SV40 transfected IMR90 cells at pre-crisis stage) and SW26i cells (stable post-crisis immortal line from SW26) were cultured in DMEM/M199 (4:1) supplemented with either 10% FBS or 10% BCS and 1% penicillin/streptomycin or 1% gentamycin respectively.

WI-38 cells (female human lung diploid fibroblast; ATCC CCL 35), were cultured in α–MEM supplemented with 10% FBS and 1% penicillin/streptomycin. WI38-VA13 cells (SV40 transformed human WI-38 fibroblasts; ATCC CCL 75.1) were cultured in DMEM (Gibco/BRL, Gaithersburg, MD) supplemented with 10% FBS and 1% penicillin/streptomycin.

For interphase FISH analysis of HDFs, cells were plated and grown on 22 $mm^2$ acid-washed glass coverslips (1.5 thickness) in 35 mm culture dishes.

### Probes

The telomeric probe pHuR93/ ATCC #61076/ (Moyzis, 1988) labeled with either biotin-11-dUTP (Sigma Chemical Co., St. Louis, MO),

digoxigenin(DIG)-11-dUTP (Boehringer Mannheim) or FITC-dUTP (Boehringer Mannheim) by nick translation was used in FISH analysis of interphase cells. The telomeric probe pBLrep4 (this plasmid contains ~600 bp of PCR generated telomeric repeats cloned into pCMV (Stratagene)) labeled with either biotin-16-dUTP (Boerhinger Mannheim) or DIG-11-dUTP (Boerhinger Mannheim) by nick translation was used in FISH analysis of metaphase chromosomes.

**In situ Hybridization and Detection**

*Interphase Nuclei-* Cells were fixed in 2.5% formaldehyde plus 5 mM $MgCl_2$ in PBS, pH 7.4 for 10 min at room temperature (RT). Following fixation, the cells were washed 3 times (10 min each) with 0.3 M glycine in PBS, pH 7.4, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and then washed 3 times (10 min each) in PBS, pH 7.4. In order to preserve nuclear morphology, hypotonic swelling, cell lysing, ethanol dehydration and air drying (steps commonly employed during *in situ* hybridization protocols) were not used.

The cells were then washed in 2X SSC at RT prior to denaturation of cellular DNA in 70% deionized formamide in 2X SSC at 80°C for 10 min. Following denaturation, the cells were washed immediately with ice-cold 70 % deionized formamide in 2X SSC followed by a wash in 2X SSC at RT.

100 ng of telomeric probe was dried in a Speed-Vac (Savant DNA 100) and then resuspended in 10 µl of 100% deionized formamide. The probe was denatured by heating in a 80°C water bath for 10 min and then placed immediately on ice. Hybridization buffer was added to the probe to give a final volume of 20 µl and a final concentration of: 50% formamide, 10%

dextran sulfate, 2X SSC, 2X Denhardt's solution and 50 mM Tris-HCl, pH 7.5. The entire 20 µl volume was added to the cells on coverslips which were then inverted onto glass microscope slides and sealed with rubber cement. Following incubation overnight at 37°C, the coverslips were washed twice (30° min each) in 50% formamide/2X SSC at 37°C, once (30 min) in 2X SSC at 37°C and once (30 min) at RT in 2X SSC. The coverslips were then incubated for 60 min at RT in 2X SSC/1% BSA containing either fluorescein avidin DN (1:200) (Vector Laboratories Inc., Burlingame, CA) or RITC-conjugated anti-DIG antibodies (1:20) (Boerhinger Mannheim). Following labeling, the cells were washed (5 min. each) with 2X SSC, PBS, PBS/0.1% Triton X-100 and then 3 more times with PBS, pH 7.4. Coverslips were mounted in glycerol:PBS (at a 9:1 ratio) containing 0.1% p-phenylenediamine buffered to pH 8.0 with a 0.5 M carbonate/bicarbonate buffer (antifade).

Cells were imaged with a Zeiss confocal laser scanning microscope (CSLM) equipped with a 100X/1.3 NA oil immersion lens, an argon ion laser ( $\lambda$ = 488 nm) and a helium/neon laser ( $\lambda$ = 543 nm).

*Metaphase chromosomes-* Metaphase spreads were prepared using standard methanol/acetic acid fixation methods. DNA was denatured by immersing slides in 70% deionized formamide/2X SSC heated to 72°C for 2.5 min. Following denaturation, slides were immediately transferred through a series of ice-cold ethanol washes (70%, 95% and 100% solutions; 3 min each) and then allowed to air dry. 120 ng of telomeric probe was dried down and resuspended in formamide and denatured as described above.

When using a biotinylated telomeric probe, the hybridization protocol and subsequent washes were done essentially as described above for interphase cells, except that the post-hybridization washes were done at 44°C

and for 5 min each. Also, the fluorescent signal was amplified by incubating the slides at RT in PNM buffer (0.1 M phosphate, pH 8.0/0.1% Nonidet P40 plus 5% dried milk powder) containing 5 µg/ml biotinylated antiavidin (Vector Laboratories Inc., Burlingame, CA) for 20 min. The slides were then washed (5 min each) in 4X SSC, 4X SSC/0.1% Triton X-100, 4X SSC, PN buffer (PNM buffer without dried milk powder). The slides were incubated once more with FITC-avidin in PNM buffer (final concentration, 5 µg/ml) at RT for 20 min followed by the same wash steps used to remove unbound biotinylated antiavidin. DAPI/DA staining was then performed as described by Schweizer (1978). Slides were mounted in antifade (see above) containing 0.8 µg/ml propidium iodide.

When using a DIG-labeled telomeric probe, FISH was performed essentially as described by Parra and Windle (1993), except PNM buffer was used instead of PN buffer containing 4% goat serum.

Chromosomes were imaged with a Zeiss ICR-35 inverted fluorescent microscope equipped with a 100X/1.4 NA oil immersion lens and a UV filter set, a fluorescein filter set and a fluorescein/rhodamine dual filter set. Photomicrographs were taken with a Canon F12 camera using Kodak Ektachrome™ 400 ASA slide film. Photomicrographs of telomeric signals and DAPI/DA staining were taken at exposure times of 30 seconds and 10 seconds respectively.

**Southern analysis of TRF length**

For all HDF strains and cell lines, Southern analysis of terminal restriction fragment (TRF) length was performed as described previously (Allsopp *et al.*, 1992; Allsopp and Harley, 1995), except that the oligonucleotide $(TTAGGG)_3$ was used as a probe instead of $(CCCTAA)_3$.

## RESULTS.

### *Telomere size and distribution in interphase nuclei.*

Unlike biochemical analysis, *in situ* hybridization allows for the examination of changes in telomere size (and interchromosomal variability in telomere size) on an individual cell basis.

Serial optical Sectioning and 3-dimensional reconstruction by CSLM show that telomeres are uniformly distributed throughout the nucleus in cultured HDFs, in agreement with these previous studies (Fig. 2). The results in Figure 2 also indicate that there is interchromosomal heterogeneity in the telomere size. We have found similar results in a variety of cells in culture (e.g. normal fibroblasts, endothelial cells, transformed fibroblasts, cancer cell lines) as well as in Sections of human tissue (pancreas, thyroid, muscle) (data not shown). Furthermore, there is no apparent relationship between the spot size (ie. size of the telomere hybridization signal) and the distance of the telomere from the nuclear periphery, indicating that the variance in the size of the spot is not due to accessibility of the probe into the volume of the nucleus.

### *Changes in telomere size during replicative aging.*

Telomeres have been shown to shorten in a variety of human cell types (see introduction). Interphase FISH analysis of telomeres in the HDF strain BJ at various PDLs showed a decrease in signal detection frequency, signal intensity and spot size (measurements correlated with telomere length) during replicative aging (Fig. 3). These findings are consistent with the

decrease in TRF length and signal intensity with increasing PDL observed in Southern analysis of TRFs (Fig. 4). In late passage cells, only a minority of the telomeres are detected by FISH and have intensities less than the strongest signals observed in early passage cells (Fig. 3); these signals presumably correspond to the telomeres of chromosomes with the largest signals in early passage cells. Furthermore, the interchromosomal heterogeneity of telomere signal persisted during replicative aging. These observations are in agreement with a similar rate of loss of telomeric DNA for all chromosomal ends. Also, the remaining detectable telomeric signals in late passage and senescent cells were observed to occur any place within the nuclear volume, indicating that there is no relationship between the telomere position within the nuclear volume and telomere size and also possibly susceptibility to loss of DNA with replicative aging.

To more closely analyze the shortening of individual telomeres, FISH analysis of metaphase chromosomes from early and late passage BJ cells was performed. As was observed in the interphase FISH analysis, telomere signal was reduced at late passage relative to early passage (Fig. 5). Moreover, the detection frequency for telomeres on chromosomes 1, 9, 15 and the Y chromosome as identified by DAPI/DA staining were all less at late passage (Table I). Also, the frequency of detection of telomeric signal varied from telomere to telomere (Table I), which further supports the existence of intracellular heterogeneity in telomere size as indicated by the variability in telomere signal size and intensity observed in interphase cells (Fig. 2 and 3). The distribution in telomere size, as indicated by detection frequency, appears to be similar for late passage and early passage cells (Table I). A qualitatively similar distribution of detection frequencies for these telomeres in both early and late passage cells was also observed in a blind study. This observation

provides further evidence for a similar rate of shortening for all telomeres.

*Changes in telomere size following cell transformation.*

To assess interchromosomal changes in telomere size following transformation, FISH analysis was performed on early and late passage IMR90 cells and the SV40 T antigen transformed counterpart cells both pre- (SW26) and post-crisis (SW26-i). As was found in BJ cells, there was a heterogeneity in size and distribution of telomere signal within any nucleus as well as an apparent uniform decrease in signal size and intensity with replicative aging (Fig. 6). Following transformation, and prior to crisis, the telomere signal continued to decrease. Similar results were observed in FISH analysis of metaphase chromosomes from IMR90 cells at early passage and SW26 cells near crisis (Fig. 7 and Table II). As shown in Figures 6 and 7, only a few telomeres remain detectable in SW26 cells near crisis. This observation is in agreement with the short TRF length observed for these cells relative to IMR90 cells at early passage or senescence (Fig. 8).

In SW26 cells, one copy of chromosome 9 appeared to be associated in a dicentric at a frequency of ≈ 20-30%. This dicentric appeared to be formed by the end-to-end association, as indicated by the presence of telomeric signal at the junction site (Fig. 7), between the long arm of chromosome 9 and another smaller chromosome or chromosome fragment.

Post-crisis, the detectability of the telomeric signals in SW26-i cells was very heterogeneous (Fig. 6). There was a stabilization of the detectability of telomeric signal in interphase nuclei, and in many cells, the signal increased to sizes and intensities greater than those in the early passage IMR90 cells. A similarily large heterogeneity of telomeric signal was observed in WI38-

VA13 cells as well (data not shown). Southern analysis of TRF length of these cell lines also showed a large heterogeneity in telomere length relative to the pre-crisis and normal counterpart cells (Fig. 8). Furthermore, hybridization with a chromosome specific, subtelomeric probe showed no apparent clustering of subtelomeric signals (data not shown). These observations argue against the possibility that the large telomeric signals in these cell lines is due to telomere clustering. Also, as was the case for normal HDFs, there was no apparent relationship between an individual chromosome and the increase in size of the telomeric signal following immortalization, suggesting that the increase in signal is not telomere specific (data not shown).

## DISCUSSION

Previous analysis by Southern hybridization has shown that in somatic cells, mean TRF length decreases during cell division *in vitro* and *in vivo* (Harley *et al.*, 1990; Hastie *et al.*, 1990; Lindsey *et al*, 1991; Allsopp *et al.*, 1992; Counter *et al.*, 1992; Vaziri *et al.*, 1993; Vaziri *et al.*, 1994; Chang and Harley, in press). These observations are the basis for the hypothesis that, due to the absence of the enzyme telomerase in somatic cells, the shortening of 1 or more telomeres below a critical length during replicative aging will lead to cell senescence (Harley, 1991). However, these biochemical studies were performed on large and often heterogeneous populations of cells. Furthermore, what is measured by Southern analysis is a mean TRF length of all telomeres within the population and thus, makes no allowances for any potential intercellular variability, or more importantly, interchromosomal variability in telomere length or rate of telomere shortening. This study examines the changes in telomere length during replicative aging at the level

of a single cell.

FISH analysis of telomeres in interphase HDFs using confocal laser scanning microscopy (CLSM) shows that within the interphase nucleus, telomeres are generally distributed throughout the nuclear volume. These findings agree with previous findings by others (Manuelidis and Borden, 1988; Billia and De Boni, 1991; Ferguson and Ward, 1992; Vourc'h *et al.*, 1993) on the non-Rabl distribution of telomeres in mammalian nuclei. The distribution of telomeres is in all likelihood, not static. Vourc'h *et al.*. have shown a co-ordinated movement of centromeres and telomeres relative to the cell cycle in synchronized murine lymphocytes (1993). The significance of this movement remains to be determined. Possibly, the movement affects the placement of specific sub-chromosomal regions containing genes expressed in a particular environment in order to facilitate or enhance gene expression at given times during the cell cycle. It has been shown that telomeric DNA binds to nuclear matrix proteins (de Lange, 1992). Thus, telomeres may act as anchoring elements to the nuclear matrix and/or putative intranuclear motors that will allow for co-ordinated chromosomal movement.

FISH analysis of telomeres in interphase cells and metaphase chromosomes shows that the detectability of individual telomeres in a cell is heterogeneous, suggesting that different telomeres have different lengths. Analysis by FISH of interphase nuclei shows that there is a heterogeneity in the size of individual hybridized telomere spots and no relationship is apparent between size and distance from the nuclear periphery, at least in HDFs. The variability in the distribution of larger versus smaller spots within the nuclear volume countermands the argument that intranuclear differences in hybridization spot size may be related to the penetrability of the probe within the nuclear volume. The probe appears to be equally accessible

to all parts of the nuclear volume, suggesting that intranuclear differences in spot size reflect chromosomal differences in telomere size. However, we can not rule out the possibility of intranuclear and interchromosomal differences in telomeric chromatin structure which could interfere with probe accessibility. It is also possible that the variable detectibility is accounted for by interchromosomal differences in the amount of sub-terminal (TTAGGG)n and telomere-like DNA (Fig. 1A). Brown *et al.* (1990) and Wells *et al.* (1990) have examined the interchromosomal distribution of human genomic clones containing telomeric sequences and telomere-like sequences which map to sub-terminal loci. The distribution of these clones does not correspond to the distribution of detectable telomeric signals observed in this study, in support of the interpretation that the heterogeneity in detectability of individual telomeres reflects variability in telomere length. However, the distribution of subterminal sequences has also been shown to be polymorphic (Brown *et al.*, 1990). Further work utilizing improved methods of telomere length measurement will be required to resolve these issues.

It has been previously shown that the detectability of the sequence probed for using FISH correlates with the target size (Lichter *et al*, 1991). In this study, we have been able to detect by FISH, changes in telomere size (ie. changes in hybridization spot size, intensity and detectability) over as few as 4 population doublings in BJ cells. The overall rate of telomere shortening in these cells is ≈ 36 bp/pd (unpublished data). Thus, if the average rate applies to individual chromosomes, we are able to detect by FISH changes in telomere length as small as approximately 150 bp. Also, using a similar technique to stain individual telomeres (primed *in situ* labeling or PRINS), Therkelsen *et al.* (1994) have observed an inverse correlation between detection frequency of individual telomeres and donor age in human

lymphocytes. Together, these observations show that staining of telomeres by FISH and other *in situ* procedures using fluorescent tags can provide a reliable and sensitive method for detection of changes in telomere length.

Our results indicate that the heterogeneity of telomere length and the interchromosomal distribution of these lengths is maintained during replicative aging, which suggests that all telomeres shorten at similar, perhaps equivalent, rates. Also, Southern analysis of TRF length for some HDF strains has revealed that for many HDF strains, the distribution of TRF lengths is multi-modal, where each mode probably reflects a fraction of the telomeres in the genome. The rate of shortening of these modes and of the entire TRF length distribution appear to be similar (Allsopp and Harley, 1995; K. Prowse, personal communication; unpublished data), providing further evidence that the rate of shortening is similar for all telomeres. However, our study does not reveal whether the telomeres with a strong or weak signal in a specific cell at early passage give rise to telomeres with a correspondingly strong or weak signal in senescent cells . The simultaneous analysis of all 92 telomeres per normal human diploid cell in clonal cell populations will provide stronger evidence as to whether all telomeres shorten at equivalent rates.

Two predictions of the telomere hypothesis are, (i) the shortening of 1 or more telomeres below a critical length, Tc, will induce cell senescence and (ii), the accumulation of critically shortened telomeres to a threshold level will initiate crisis in transformed cells which have bypassed cell senescence (Harley, 1991). We have previously calculated the mean telomere length at senescence, which provides an upper estimate of Tc, to be approximately 2-3 kbp (Levy, *et al.*, 1992; Allsopp and Harley, 1995). However, if interchromosomal heterogeneity in telomere length exists as suggested in

this study, then Tc will be less than the mean telomere length. Since there is a large distribution of lengths for each individual telomere in a cell population at any given PDL (Harley *et al.*, 1990; Karen Prowse, personal communicaton), it is even possible that cell senescence is induced by the complete loss of telomeric DNA from the ends of 1 or a few chromosomes.

A critically short telomere may be analogous to a double stranded DNA break. Recently, it has been shown that the presence of a single double stranded DNA break in a cell is capable of inducing irreversible cell cycle arrest in human cells (di Leonardo *et al.*, 1994; Ishizaka *et al.*, 1995). Thus, replicative senescence may be initiated by the signaling of a DNA damage pathway. Furthermore, chromosome ends which lack telomeres are highly fusogenic (Muller, 1938; reviewed in Blackburn, 1991), and therefore the accumulation of critically shortened telomeres in senescent and transformed cells could explain the accumulation of dicentric chromosomes in these cells (Benn, 1976; Sherwood *et al.*, 1989; Counter *et al.*, 1992; Counter *et al.*, 1994b). This prediction is supported by the observations that the telomere on 9q appears to be relatively short in IMR90 cells and SW26 cells (Table II), and that one copy of chromosome 9 appears to be frequently engaged in an end-to-end dicentric involving 9q in SW26 cells .

In summary, FISH has been shown to be a useful method for detecting changes in length of individual telomeres and for qualitative analysis of the relative lengths of individual telomeres. The data confirms the previously observed loss of telomeric DNA during replicative aging as detected by Southern analysis of TRFs, and indicates that all telomeres shorten at similar, perhaps equivalent, rates and that different telomeres have different lengths. Analysis of telomere lengths by FISH may be particularily useful when dealing with small cell populations or heterogeneous cell populations,

including tissues. Thus possible diagnostic applications of FISH for detecting telomeres include analysis of telomere lengths in disease states and stem cells.

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Table I Quantitative analysis of telomeric signal on individual metaphase chromosomes for early and late passage BJ cells

| | % detectability[a, b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Chromosome | 1 | | 9 | | 15 | | Y | |
| | p | q | p | q | p | q | p | q |
| early passage[c] | 88 | 74 | 68 | 78 | 89 | 60 | 85 | 54 |
| late passage[d] | 58 | 43 | 32 | 44 | 76 | 40 | 48 | 27 |

a- values were calculated using data from 5 experiments in which either a biotinylated telomeric probe or a DIG-labeled telomeric probe was used (qualitatively similar results were obtained for both probes). The total number of chromosomes analyzed to obtain each value ranged from approximately 100-500.

b- although chromosome 16 is also identified by DAPI/DA staining, percent detectability of telomeric signals was not assessed for this chromosome due to difficulty in distinguishing between the long and short arms.

c- early passage cells were analyzed at PDL 24-27.

d- late passage cells were analyzed at PDL 75-82.

Table II Quantitative analysis of telomeric signal on individual metaphase chromosomes for early passage IMR90 cells and SW26 cells near crisis.

| | % detectability[a, b] | | | | | |
|---|---|---|---|---|---|---|
| Chromosome | 1 | | 9 | | 15 | |
| | p | q | p | q | p | q |
| IMR90[c] | 79[d] | 84 | 86 | 71 | 87 | 72 |
| SW26[d] | 39 | 39 | 37 | 26 | 58 | 30 |

c- early passage IMR90 cells were analyzed at PDL 24-27.

d- SW26 cells were analyzed at PDL 63. Crisis occurred at PDL 66.

For footnotes a and b, see table I.

## FIGURE LEGENDS

Figure 1. The telomere hypothesis of cell aging and immortalization. (A) A model of the sequence composition for a typical TRF is illustrated. The TRF is composed of a terminal (TTAGGG)n tract (T) and a proximal sequence devoid of cleavable restriction sites (X). The X portion of the TRF is likely composed of non-telomeric sequences (open boxes) as well as degenerate telomeric sequences (shaded boxes) and pure telomeric sequences (black boxes). (B) Changes in telomere length with increasing population doublings are represented for germ line, normal somatic and immortalized somatic cells. Events involved in regulation of telomerase expression during early embryonic development are uncertain. Telomere length is maintained in germ line cells by telomerase. In contrast, the observed decrease in telomere length in normal somatic cells is consistent with the absence or very low level of telomerase in these cells. At the Hayflick limit (M1), mean TRF length ranges from 6-8 kbp, depending on cell type and strain. We hypothesize that at M1 one or more telomeres have shortened below a critical length required for maintenance of proper telomere structure and function, signaling a check point in cell growth. Mean telomere length (ie. T) at this point is estimated to be 2-4 kbp. Partially transformed cells which bypass this checkpoint without activation of telomerase continue to lose telomeric DNA until crisis (M2), when cells have critically shortened telomeres on many chromosomes. At M2, the mean TRF length ranges from 2-4 kbp. The rare cells which survive crisis have acquired the ability to maintain or increase telomere length, presumably due to a mutation.

Figure 2. FISH analysis of the spatial distribution of telomeres in interphase cells. Interphase HDFs (strain S2c, established from adult skin) at PDL 38 were hybridized *in situ* with a FITC-tagged telomeric DNA probe. Serial optical Sections were taken at 0.5 μm intervals through the depth of the nucleus by CSLM. The 3-dimensional volume was reconstructed by superimposing the serial images and then the volume images were projected ± 6° as a stereo pair. The size of each hybridized telomere spot appears to be independent of its distance from the nuclear periphery or the depth along the z-axis of optical Sectioning.

Figure 3. FISH analysis of telomeric signal in interphase cells during replicative aging. BJ cells at PDLs a) 28, b) 34, c) 47, d) 63, e) 89 and f) 93 were hybridized *in situ* with a DIG-tagged telomeric DNA probe and subsequently labeled with RITC-conjugated anti-DIG antibodies. Single optical Sections were taken by CLSM through the midplane of each nucleus. FISH analysis was performed at the same time under identical conditions for cells at each PDL. All confocal microscope settings (ie. brightness, contrast, gain, offset, pinhole size) were maintained at identical values between each of the samples studied. Similar results were obtained for the fibroblast strain S2c (data not shown).

Figure 4. Southern analysis of TRF length at various PDLs for the HDF strain BJ. Genomic DNA was isolated from BJ cells and S2c cells at the PDLs indicated above each lane and digested with the restriction enzymes *Hinf*I and *Rsa*I. Southern analysis of TRF length was performed as described (Allsopp *et al.*, 1992; Allsopp and Harley, 1995). Size of molecular weight markers are indicated at the side.

Figure 5. FISH analysis of telomeric signal in metaphase spreads from BJ cells at early and late passage. Metaphase spreads prepared from BJ cells at (A) early passage (PDL 24), and(B) late passage (PDL 82) were hybridized *in situ* with a DIG-tagged telomeric DNA probe and the signal was amplified and subsequently detected with FITC-conjugated anti-DIG antibodies as described in materials and methods. FISH analysis was performed at the same time under identical conditions for cells at each PDL. Photomicrographs showing telomeric signal (bottom) and DAPI/DA staining (top) are shown. Chromosomes 1, 9, 15 and the Y chromosome as identified by DAPI/DA staining are indicated.

Figure 6. FISH analysis of telomeric signal in interphase cells prior to and following transformation and immortalization. a) IMR90 cells at early passage (PDL 25), b) IMR90 cells at late passage (PDL 46), c) SW26 cells near crisis (PDL 62) and d) post-crisis SW26-i cells were hybridized *in situ* with a DIG-tagged telomeric DNA probe and subsequently labeled with RITC-conjugated anti-DIG antibodies. FISH analysis was performed at the same time under identical conditions for all cells. Single optical Sections were taken by CLSM through the midplane of each nucleus.

Figure 7. FISH analysis of telomeric signal in metaphase spreads from IMR90 cells and SW26 cells. Metaphase spreads prepared from (A) IMR90 cells at early passage (PDL 24) and (B) SW26 cells near crisis (PDL 62) were hybridized *in situ* with a DIG-tagged telomeric DNA probe and the signal was amplified and subsequently detected with FITC-conjugated anti-DIG antibodies as described in materials and methods. FISH analysis was performed at the same

time under identical conditions for both cell strains. Photomicrographs showing telomeric signal (bottom) and DAPI/DA staining (top) are shown. Chromosomes 1, 9, and 15 as identified by DAPI/DA staining are indicated. The end-to-end telomeric association (TA) of one copy of chromosome 9 and another smaller chromosome as well as the telomeric signal at the junction (arrow) are also shown.

Figure 8. Southern analysis of TRF length for normal, transformed and immortal HDFs. (A) Genomic DNA was isolated from IMR90 cells, SW26 cells, SW26-i cells, WI38 cells and WI38-VA13 cells at the PDLs indicated above each lane and digested with the restriction enzymes *Hinf*I and *Rsa*I. Southern analysis of TRF length was performed as described (Allsopp *et al.*, 1992; Allsopp and Harley, 1995). Size of molecular weight markers are indicated at the side. (B) Southern analysis of TRF length was performed on 293 cell DNA as described in (A). Size of molecular weight markers are indicated at the side.

Fig. 1

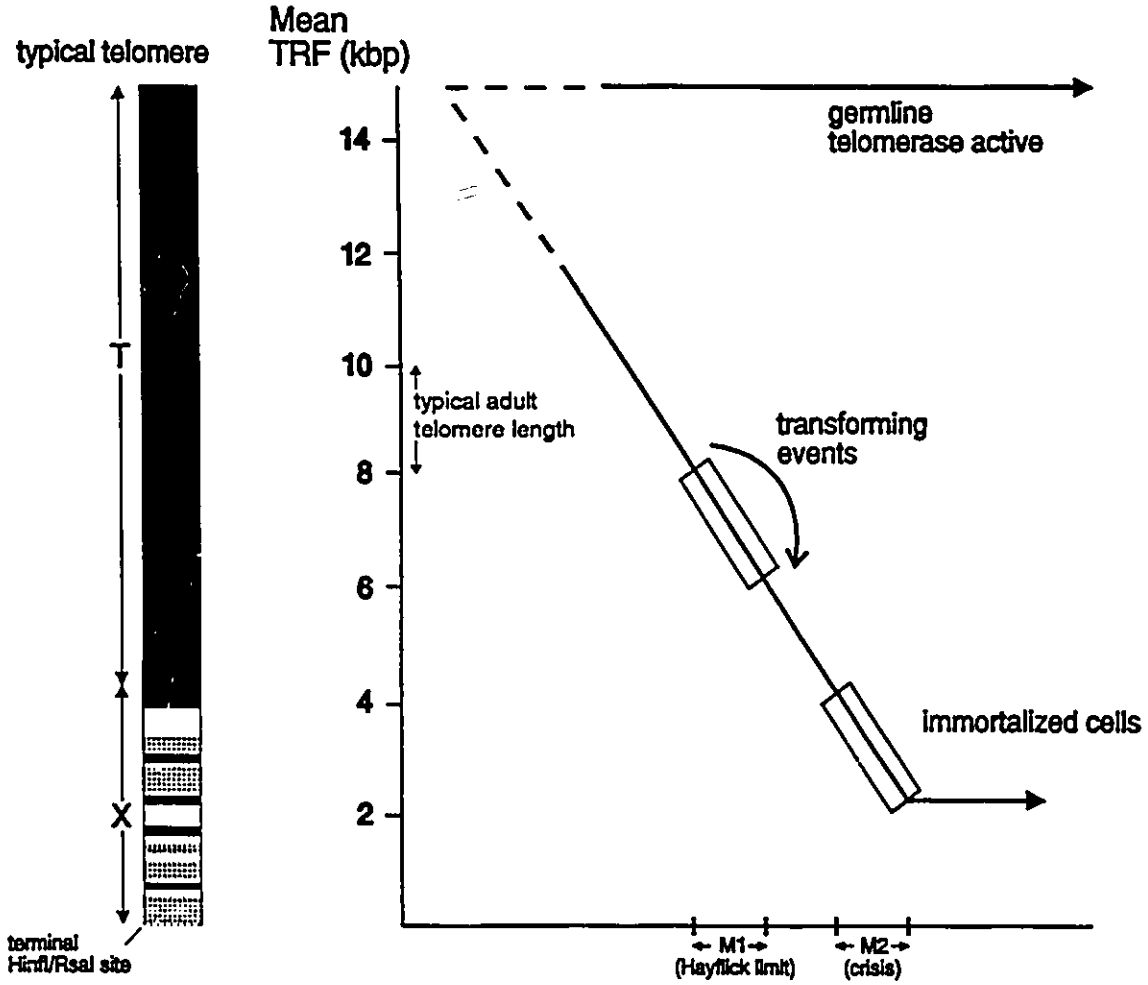

typical telomere
terminal
HinfI/RsaI site
Mean
TRF (kbp)
14
12
10
8
6
4
2
typical adult
telomere length
germline
telomerase active
transforming
events
immortalized cells
M1
(Hayflick limit)
M2
(crisis)

Fig. 2



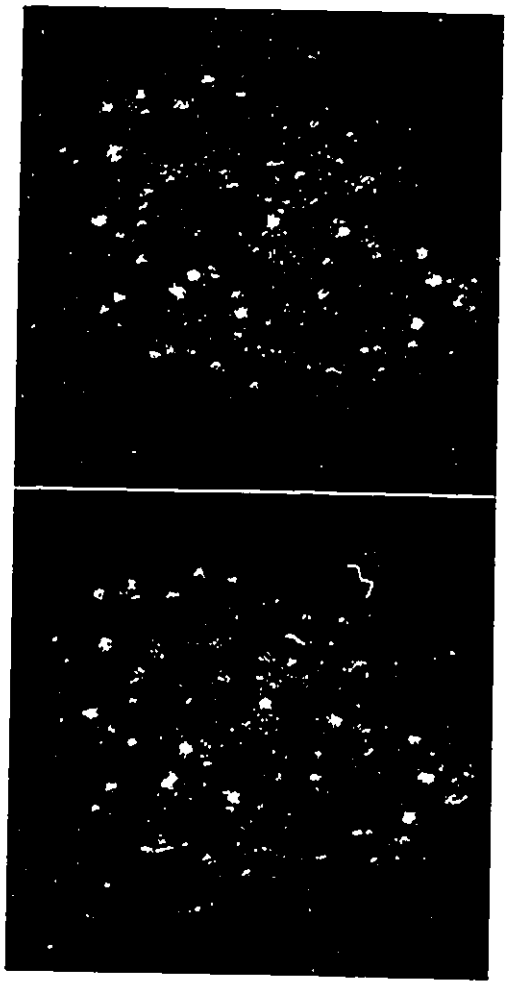


Fig. 3

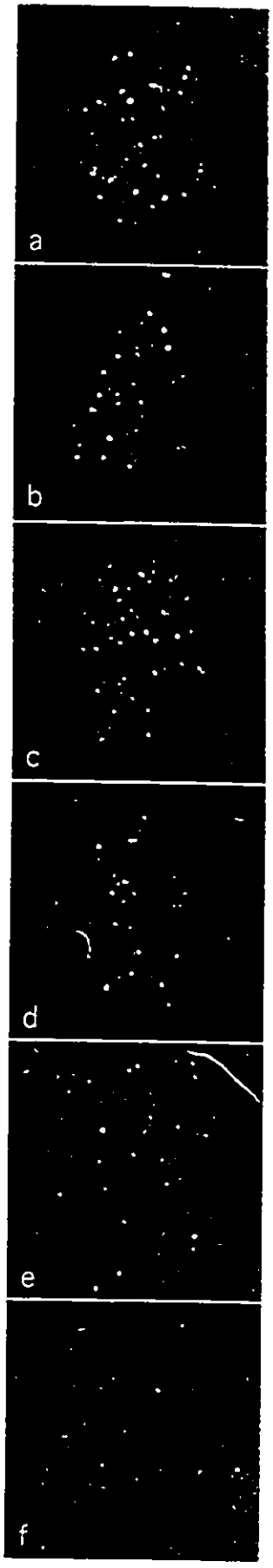


Fig. 4

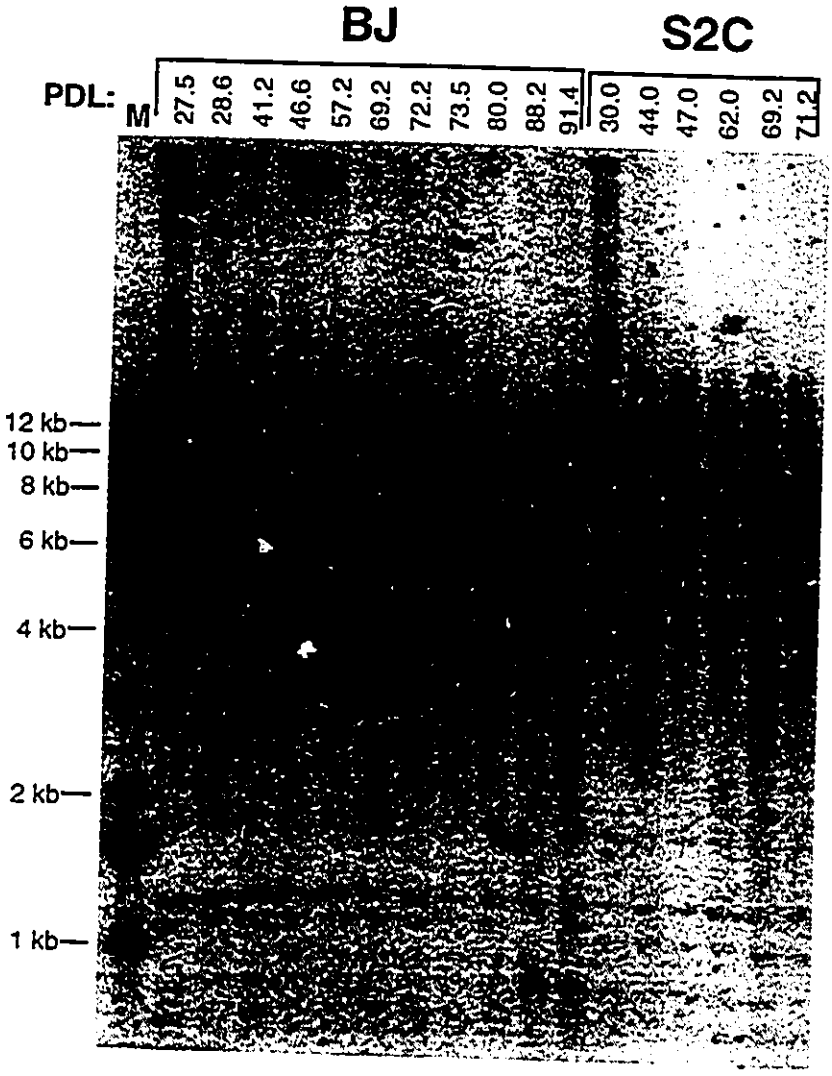

BJ
S2C
PDL:
M
27.5
28.6
41.2
46.6
57.2
69.2
72.2
73.5
80.0
88.2
91.4
30.0
44.0
47.0
62.0
69.2
71.2
12 kb
10 kb
8 kb
6 kb
4 kb
2 kb
1 kb

Fig. 5A

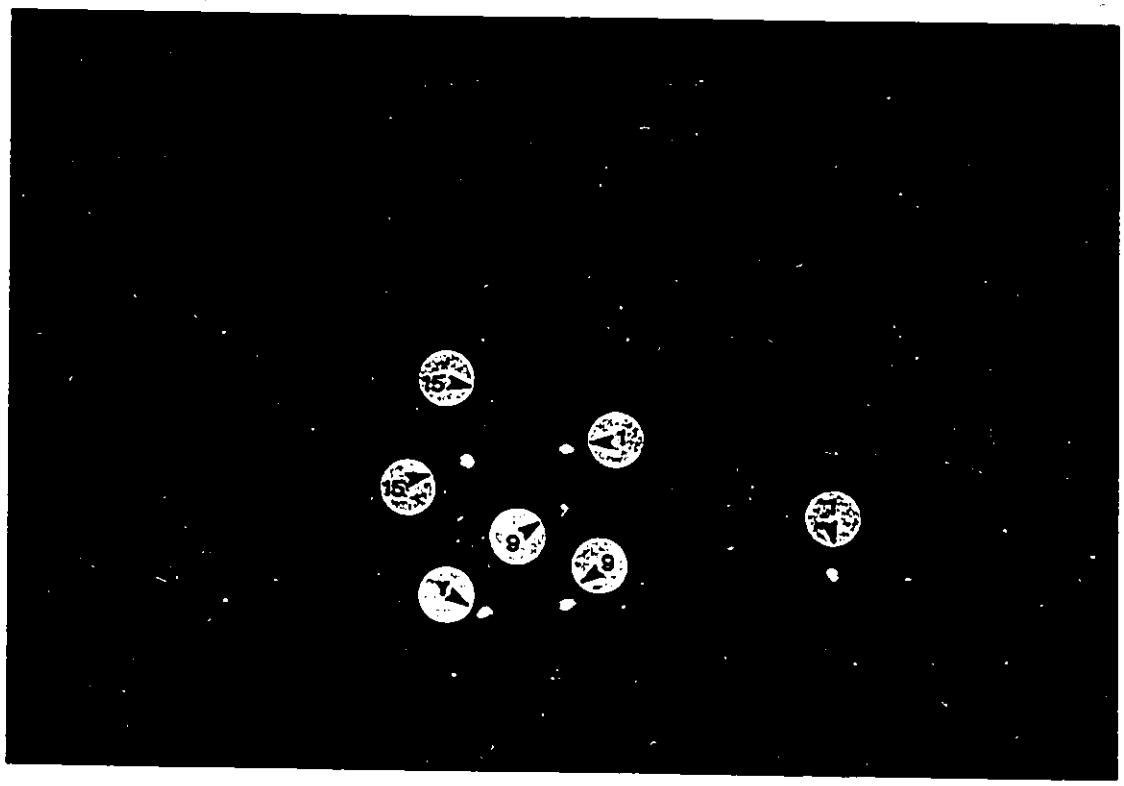


Fig. 5B


Fig. 6

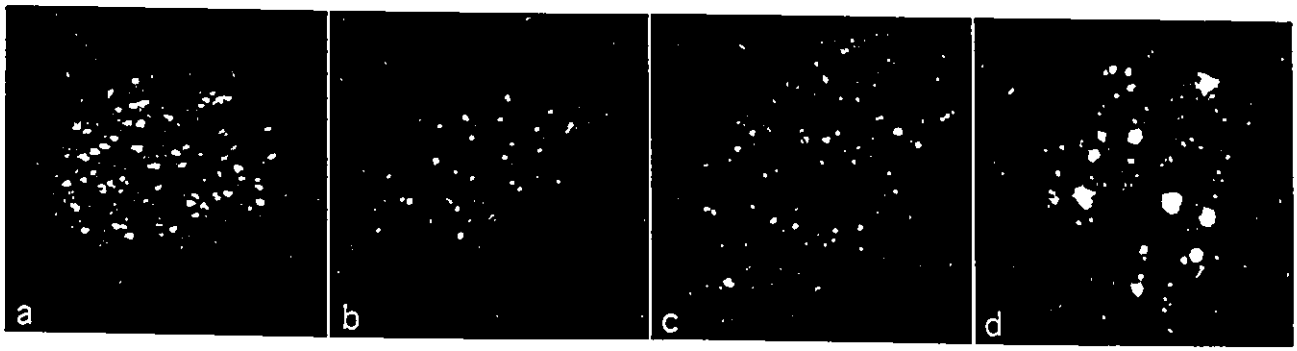


Fig. 7A

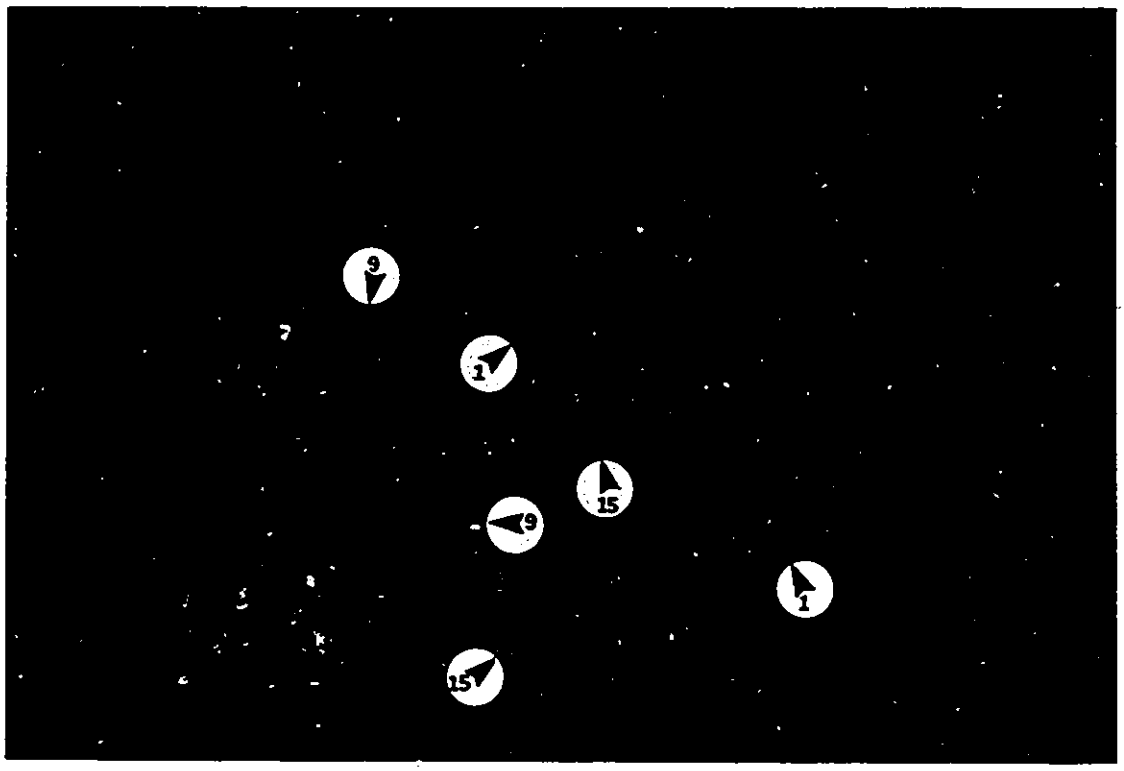



Fig. 7B

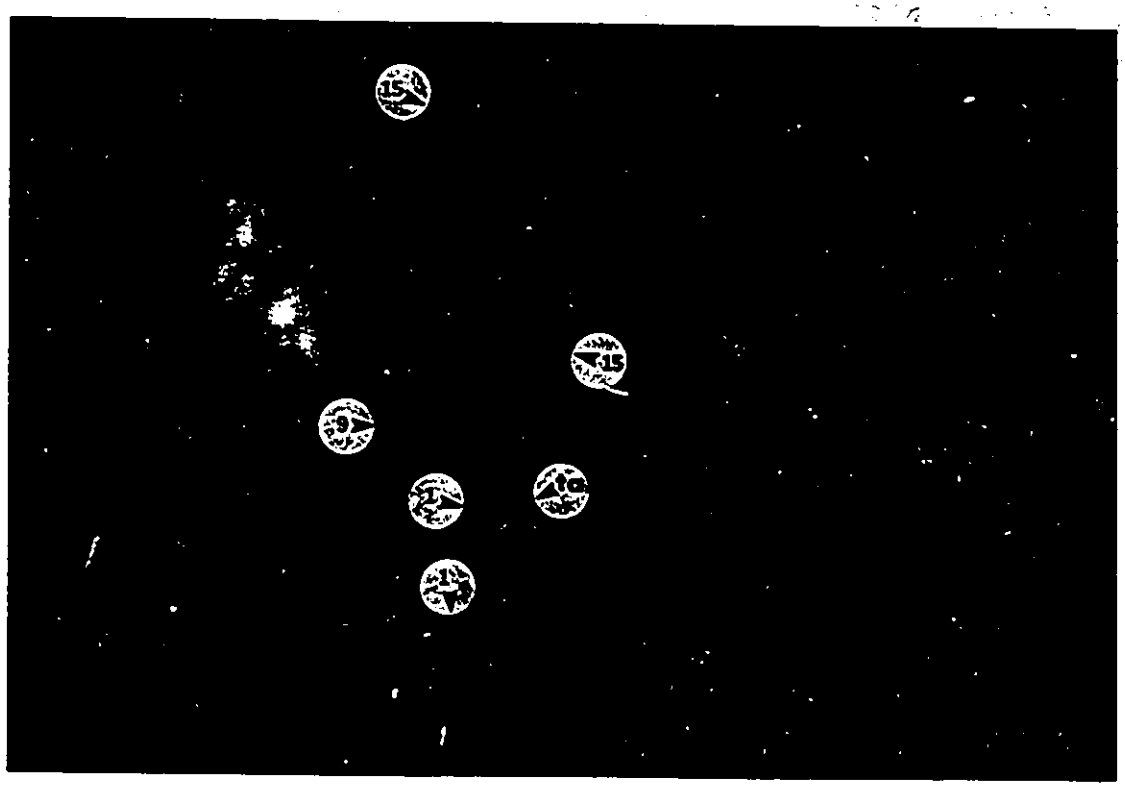
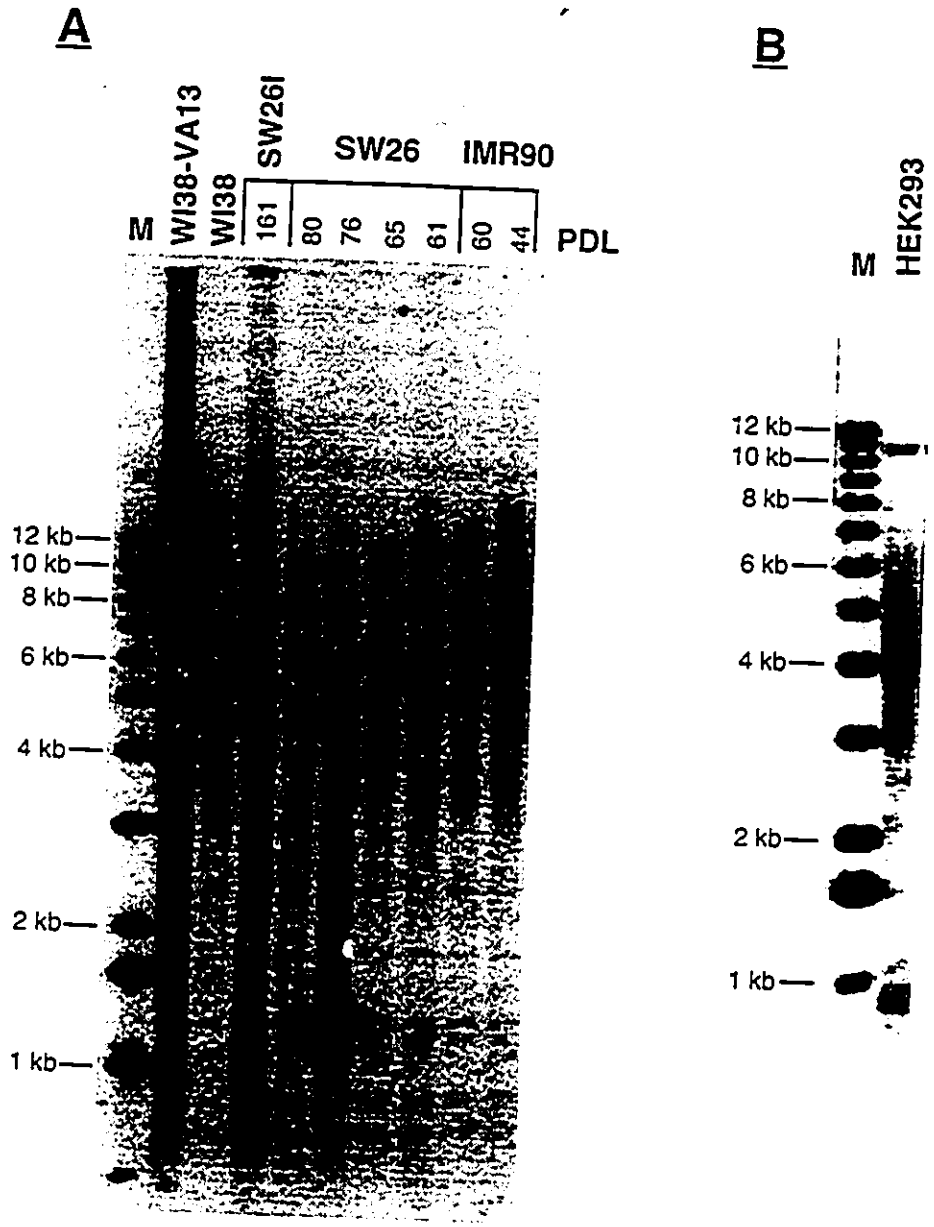



Fig. 8


A
M
WI38-VA13
WI38
SW26I
SW26
IMR90
161
80
76
65
61
60
44
PDL
12 kb
10 kb
8 kb
6 kb
4 kb
2 kb
1 kb
B
M
HEK293
12 kb
10 kb
8 kb
6 kb
4 kb
2 kb
1 kb

## 3. Conclusions

The results presented here provide evidence in support of a causal role of telomere shortening in cell senescence and shed insight on how cell senescence might be initiated by loss of telomeric DNA. Telomere length was shown to decrease significantly in HDFs at a rate of ≈ 15 bp/year during aging *in vivo*. Preliminary data also suggest that telomeres may shorten during replicative aging of hematopoietic stem cells *in vivo* as well. Telomere shortening was also shown to be largely, and possibly entirely, dependent upon cell division, in support of the end replication problem as a cause of telomere shortening.

Telomere length was shown to be a relatively good predictor of remaining replicative capacity in cultured HDFs, and intercellular variability in telomere length was reduced in senescent cells as compared to young cells. These observations imply that cell senescence might be caused by the shortening of one or a few telomeres below a critical or threshold length. This length may be considerably less than the mean telomere length at senescence (≈ 2-3 kbp), since the length of the terminal TTAGGG tract appears not to be equivalent for all telomeres as indicated by FISH analysis of individual telomeres. In support of this theory, a chromosomal end with a relatively short telomere length, as determined by FISH analysis, was frequently engaged in an end-to-end telomeric association with another chromosome or chromosomal fragment in a transformed HDFs near crisis.

3.1 Loss of telomeric DNA during organismal aging.

The first suggestion that cells might lose telomeric DNA with age came from early comparisons of sperm DNA and adult somatic tissue DNA (Cooke and Smith, 1986; de Lange *et al*, 1990). A more thorough analysis of the age effect on telomere length in humans revealed that the amount of telomeric DNA decreases during *in vivo* aging of various mitotically active cell types and tissues including skin fibroblasts (Section 2.1), PBLs (Hastie *et al*, 1990; Vaziri *et al*, 1993), vascular tissue and endothelial cells (Chang and Harley, 1995) and skin tissue (Lindsey *et al*, 1991). This shows that the loss of telomeric DNA observed during division of human cells *in vitro* is not due to loss of a biochemical process which maintains telomere length *in vivo*. Also, preliminary results in Section 2.5 suggest that telomere length decreases during replicative aging of candidate human hematopoietic stem cells *in vivo* having high and low levels of expression of the cell surface markers CD34 and CD38 respectively. This observation suggests that stem cells are mortal, however further work will be required to confirm this observation due to the limited number of adult samples (n = 2) analyzed. Additionally, $CD34^{+}CD38^{lo}$ cells may not be true stem cells, or actual stem cells may only constitute a small fraction of the total $CD34^{+}CD38^{lo}$ cell population. Recently, low levels of telomerase activity have been detected in candidate human hematopoietic stem cells, early progenitor cells and more mature lymphocytes (Broccoli *et al*, 1995; Counter *et al*, 1995; Chiu *et al*, in press; Hiyama *et al*, in press). However, it is difficult to interpret the role of telomerase in these cells in light of the loss of telomeric DNA that occurs during *in vivo* aging of PBLs (Hastie *et al*, 1990; Vaziri *et al*, 1993). Further work will be required to firmly establish whether telomere shortening occurs

during division of stem cells and the role that telomerase plays in these cells and in blood cells.

In old donors, the telomere length of PBLs (Vaziri *et al*, 1993) and of intimal tissue of the iliac artery (Chang and Harley, 1995) are similar to the telomere lengths of their cells at senescence. This observation is consistent with the relatively high rate of cell turnover for PBLs and the lining of the iliac artery due to repetitive immunogenic stimulation and high hemodynamic stress respectively. Also, most of the T lymphocytes in elderly individuals express low levels of CD28, similar to senescent T lymphocytes in culture (Effros *et al*, 1994). Furthermore, dicentrics formed from end-to-end associations of chromosomes, which may be caused by the shortening of telomeres below $T_c$ (see Section 3.5), accumulate in human T lymphocytes during aging (Bender *et al*, 1989). These observations indicate that a large fraction of the cells that make up the PBL population and intimal tissue of the iliac artery, and possibly in other tissues as well, are senescent in older individuals. This accumulation of senescent cells could in turn contribute to the manifestation of certain age-related diseases such as atherosclerosis as well as to the increased susceptibility of the elderly to infections.

In other organisms, including the mouse *mus musculus* (Kipling and Cooke, 1990), the yeast *S. cerevisiae* (D'Mello and Jaswinski, 1991) and the ciliate *Paramecium* (Gilley and Blackburn, 1994), telomere length does not appear to shorten during aging. In *S. cerevisiae*, telomere length appears to be maintained by telomerase as cells divide (Singer and Gottschling, 1994; Lin and Zakian, 1995; Cohn and Blackburn, 1995) and possibly, under certain circumstances, by other mechanisms involving recombination (Wang and Zakian, 1990; Lundblad and Blackburn, 1993). Telomere length may be maintained *in vivo* in *mus musculus* by telomerase since activity has been

detected in a number of different somatic tissues (Chadeneau *et al.*, 1995; Prowse and Greider, 1995). However, it is also possible that telomere shortening may occur in *mus musculus*, but is undetectable by Southern analysis due to the large size of telomeres in this mouse strain (Kipling and Cooke, 1990). In support of this latter possibility, telomere shortening has been shown to occur during replicative aging of cultured HDFs from the outbred mouse strain *mus spretus* in which telomere length is 25 kbp or less (Prowse and Greider, 1995). Further studies are required to determine if telomere shortening occurs during *in vivo* aging in mice.

## 3.2 Mechanistic models of telomere shortening

The mechanism(s) that account for the loss of telomeric DNA during replicative aging of human cells is debatable. The observation that little if any telomere shortening occurs in quiescent cells *in vitro* and *in vivo* (see Section 2.3) shows that telomere shortening is largely dependent on cell division, and therefore supports the end replication problem as a cause. Other evidence in support of the end replication problem as a cause of telomere shortening is that models for the timing of the appearence of a critically short telomere accurately correspond with the observed timing of the appearence of senescent cells in serially passaged HDFs (Section 2.2). Also, the large heterogeneity in telomere lengths in human cells, as indicated by the broad distribution of TRF lengths observed in Southern analysis (for example, see Appendix A), can be accounted for by the end replication problem which is expected to generate telomeres of varying lengths during successive rounds of DNA replication (see Section 2.2). The end replication problem is also

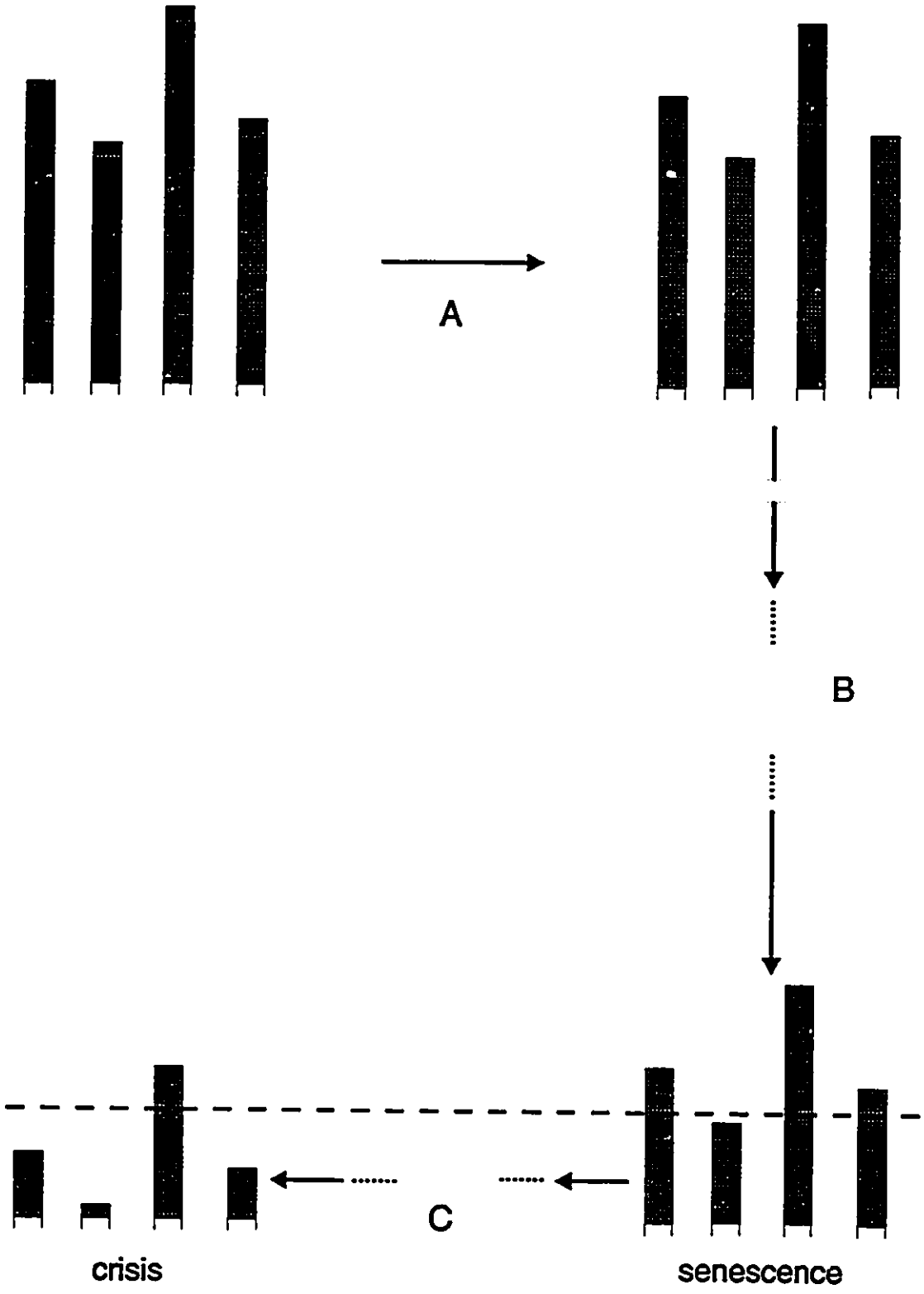
expected to cause an increase in the telomere length heterogeneity with increasing population doublings, however the standard deviation of the TRF length distribution was observed not to change with increasing population doublings in cultured HDFs (Section 2.2). Although this argues against the involvement of the end replication problem in telomere shortening, an increase in the heterogeneity of individual telomeres during cell division may be masked in Southern analysis of the overall TRF length distribution which is composed of the overlapping distributions of each of the 92 telomeres in normal human diploid cells.

Other mechanisms of telomere shortening are also possible, such as the gradual degradation of the ends of telomeres by nucleases, and unequal recombination followed by selection of the cells inheriting the shortest complement of telomeres. The observation that telomere shortening is dependent on cell division argues against nuclease degradation as a mechanism of shortening since nucleases would presumably be active at all phases of the cell cycle and in cells in $G_0$. However, it is also possible that telomeres may only be accessible to nucleases during DNA replication in S phase. Also, the observation that different cell types in humans have different rates of shortening as a function of population doublings, at least *in vitro* (Sections 2.2 and 2.5; Harley *et al*, 1990; Vaziri *et al*, 1993; Chang and Harley, 1995), is hard to account for by the end replication problem and suggests that there may be more than one mechanism of shortening. Further work is required to determine the exact cause(s) of telomere shortening and the relative extent that these cause(s) contribute to the loss of telomeric DNA in different cell types.

## 3.3 Telomere dynamics during replicative aging of human cells

Collectively, the results from Sections 2.2 and 2.6 as well as from other studies (Harley *et al*, 1990; Counter *et al*, 1992; Prowse personal communication) form the basis of a detailed model as to how telomeres shorten during replicative aging of a mortal clonal cell population (Fig. 3.3.1). That is, all telomeres shorten together at a constant or similar rate until the cells either senesce, in the case of normal cells, or reach crisis in the case of transformed cells (Fig. 3.1.1). The differences in initial length of the telomeres reflect the different telomere lengths of the progenitor cell which is accounted for by the telomere length heterogeneity that is generated as a result of the end replication problem and subsequent random segregation of the different sized telomeres during the previous life history of the progenitor cell. The detectability of individual telomeres as determined by FISH is less in cultured HDFs that are near senescence or crisis relative to HDFs at early passage, suggesting that all telomeres shorten during replicative aging (Section 2.6). Also, the similarity in the distribution of detection frequencies for individual telomeres for old HDFs and transformed HDFs near crisis as compared to early passage HDFs, provides preliminary evidence indicating that all telomeres shorten at the same or similar rates (see Section 2.6 for caveats). Some HDF strains and clones show a multi-modal distribution of TRF lengths, where each mode is most likely composed of a particular sub-set of the 92 telomeres in the human diploid genome. That the modal distribution observed in some HDF strains and clones does not appear to change during replicative aging also indicates that all telomeres shorten at the same rate (Section 2.2 and Appendix A). Furthermore, the rates of shortening of individual TRF modes has been measured for a number of different HDF

Fig. 3.3.1. Model of the dynamics of telomere shortening during replicative aging of human cells. Four hypothetical chromosomal termini of a clonal cell population are shown. The length shown for each telomere (shaded box) is the average length for that particular telomere in the cell population. (**A**) As cells undergo a single population doubling, the same amount of telomeric DNA (black boxes) is lost from each telomere. (**B**) As cells undergo further population doublings, telomeres continue to shorten by the amount indicated in **A** at each population doubling. Thus the distribution of telomere lengths at senescence is the same as at early passage. (**C**) If the cells are transformed, then they will bypass senescence and continue to lose telomeric DNA in the same manner as shown in **A** and **B** until crisis is reached. It is possible that other chromosomal termini entirely lack telomeric DNA at senescence and/or at crisis. The hypothetical critical telomere length (see Section 3.4) is indicated by the dashed line.


A
B
C
crisis
senescence

strains and clones. Within each HDF strain and clone, the rates of shortening was observed to be similar for each mode (K. Prowse, personal communication; Appendix A). Strong evidence in support of a constant rate of telomere shortening throughout replicative aging is the relatively high degree of linearity in the inverse correlation between mean TRF length and PDL (Harley *et al*, 1990). Further work will be required to determine if all telomeres shorten at exactly the same rate throughout the replicative lifespan of somatic cells.

Recent evidence suggests that telomeres undergo dynamic changes in length during division of immortal human cells (Murnane *et al.*, 1994). Specifically, results from Southern analysis using a sub-terminal probe specific for an individual telomere indicated that telomeres shorten in immortal cells until they reach a length of ~ 0 kbp, at which time there is a sudden and heterogeneous increase in telomere length. However, the cells examined in this study, an SV40 transformed HDF cell line, lacked detectable telomerase activity (Murnane *et al.* 1994). Other telomerase negative human cell lines have also recently been detected (Kim *et al.* 1994; Bryan *et al.* in press) and which also contain large, hetergeneous telomere lengths (Bryan *et al.* in press). Also, the 2 human cell lines which were observed to have large, heterogeneous telomere lengths by FISH and Southern analysis of TRFs in Section 2.6 also display an absence of detectable telomerase (Kim *et al*, 1994; Bryan *et al.*, in press, and unpublished data). Thus it is possible that large heterogeneity in telomere lengths and dynamic changes in length is specific to the rare immortal cell lines, and perhaps tumors as well (Kim *et al*, 1994), which maintain telomere length by a mechanism other than telomerase. In support of this prediction, the distribution of TRF lengths as determined by Southern analysis in 293 cells and human sperm is smaller than that

observed for SW26i cells, WI38-VA13 cells and other telomerase negative cell lines (Section 2.6 and Harley *et al*, 1992; Bryan *et al.*, in press). However, to confirm that this prediction is true, it will be necessary to more thoroughly compare the heterogeneity and dynamics of telomere length between immortal cell lines which express telomerase and those that do not.

The changes of telomere length that accompany terminal differentiation and apoptosis have yet to be analyzed. Recently, the formation of telomere associations has been shown to be an early event in cells undergoing apoptosis (Pathak *et al*, 1994). However, this likely reflects the activation of some sort of telomeric processing leading to a sudden reduction in telomere length or alteration of telomere structure to make the ends more fusogenic as opposed to sudden burst in proliferation in pre-apoptotic cells.

## 3.4 Models of initiation of cell senescence by telomere shortening.

Although it has yet to be established whether telomere shortening has a causal role in the induction of cell senescence, several lines of evidence in support of this theory exist (see Sections 2.1, 2.2 and 2.4)(Harley, 1991). A number of hypothetical scenarios, based on the known biochemical properties of telomeres, by which telomere shortening could cause senescence can be envisaged.

Wright and Shay (1992), have proposed a model base upon the telomeric position effect observed in *S. cerevisiae* (Gottschling *et al*, 1990). The telomeric position effect refers to the silencing of genes situated near telomeres as a result of their proximity to telomeric heterochromatin (Gottschling *et al*, 1990). In this model, telomere shortening is predicted to cause an alteration of the heterochromatin associated with telomeres such

that the expression of one or more genes at distal loci are affected. Cell senescence will occur when the expression of one or more distal genes involved in the regulation of cell division is affected. For example, telomere shortening could cause telomeric heterochromatin to recede, thereby allowing the expression of one or more inhibitors of the cell cycle (Fig. 3.4.1). Evidence in support of this particular scenario is that a decrease in telomere length in *S. cerevisiae* causes a decreased frequency of silencing of nearby genes (Renauld *et al*, 1993). However, it is unknown whether a telomere position effect exists in human cells.

The model that seems most likely at present is an elaboration of the model described in the telomere hypothesis (Harley, 1991). That is, cell senescence is predicted to be initiated by the shortening of telomeres below a certain length, $T_c$, that is critical for proper telomere structure and function (Fig. 3.4.2). When this occurs, the termini of critically shortened telomeres will no longer be distinguishable from a double-stranded break and a signal will be sent to initiate cell cycle arrest, perhaps similar to the mechanism by which damaged DNA induces cell cycle arrest (Dulic *et al*, 1994; di Leonardo *et al*, 1994).

In support of the existence of a critical telomere length as postulated in this model is the apparent convergence at senescence of telomere length in HDF strains (Section 2.1) and HDF clones (Section 2.4) exhibiting variable replicative capacities. Furthermore, the inter-donor variability in telomere length of apparently senescent or near-senescent PBLs from centenarian donors appears to be smaller than that for PBLs from young donors (Vaziri *et al*, 1993), which further suggests the existence of a critical telomere length in senescent cells. However, there is still a noticeable amount of variability in mean TRF length and telomeric signal intensity at senescence, at least for

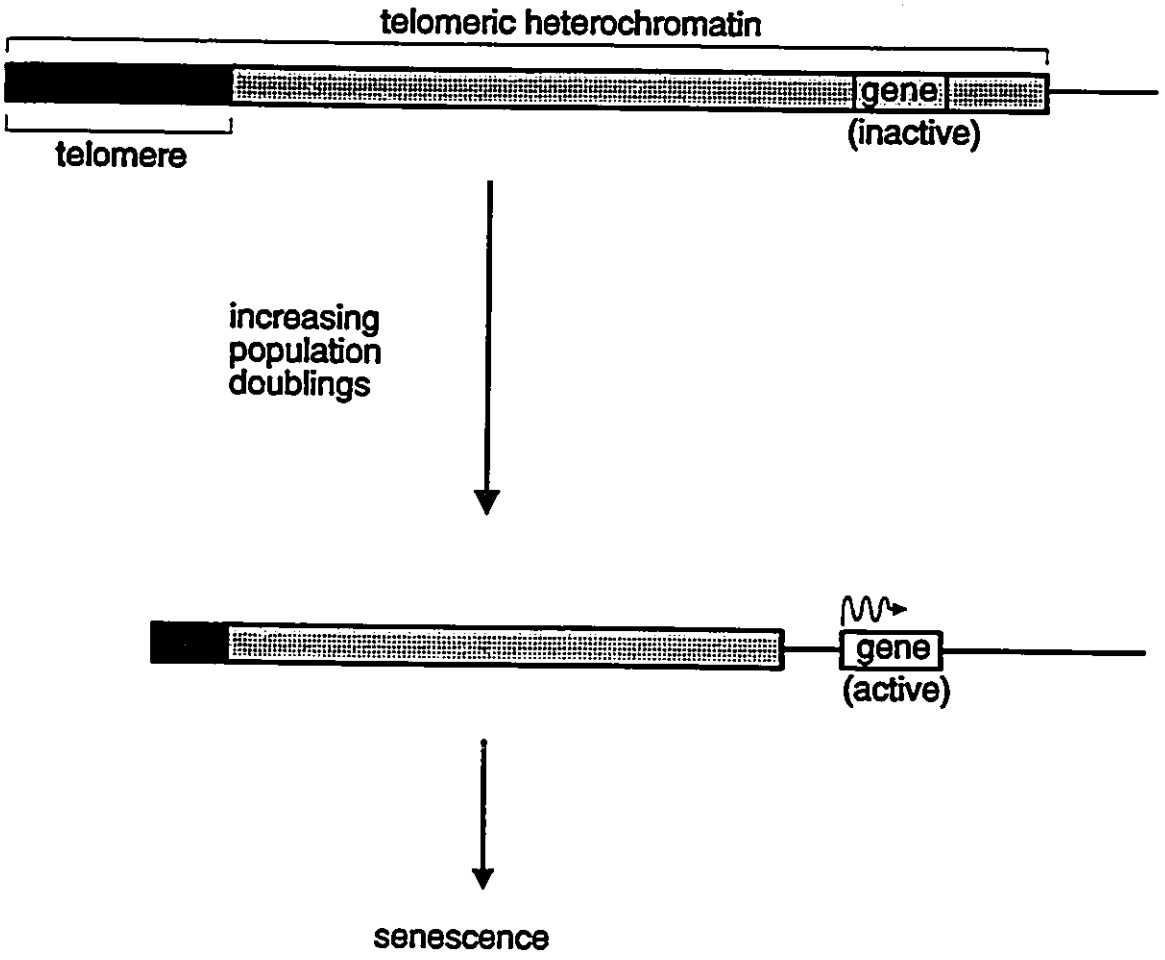



Fig. 3.4.1. Model for initiation of replicative senescence based on alteration of telomeric heterochromatin.

This model postulates that, in young cells, 1 or more genes which encode factors that initiate replicative senescence are engulfed in telomeric heterochromatin and are therefore silent. The gradual deletion of telomeric DNA during replicative aging will cause the proximal end of the telomeric heterochromatin to recede. Once the senescence initiating genes are freed from the heterochromatin block, they will become active and replicative senescence will occur.

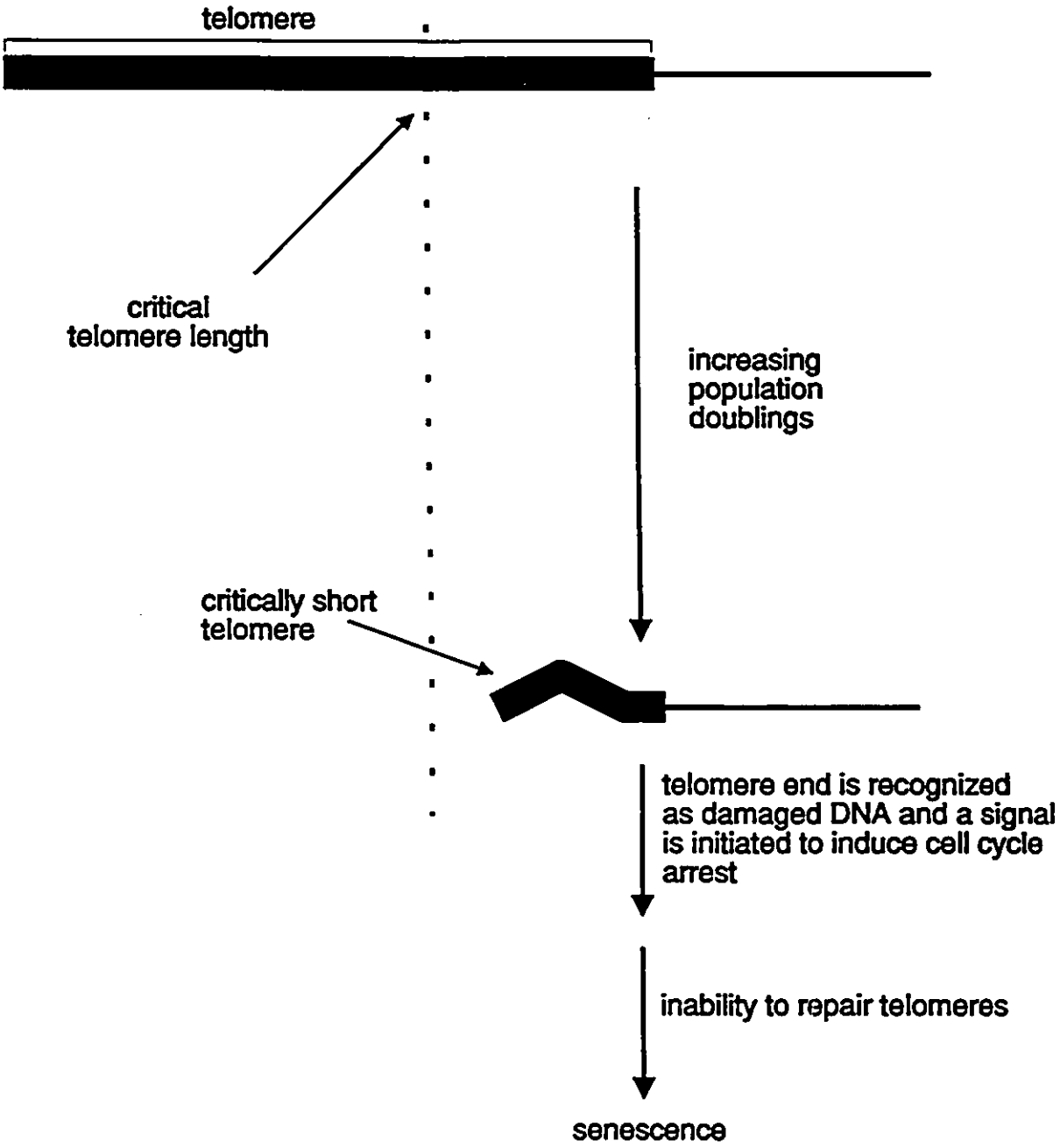



Fig. 3.4.2. Model for initiation of replicative senescence by acquisition of a critically short telomere.

This model postulates that the terminal telomeric DNA tract must be maintained above a critical length, $T_c$, to stably maintain telomere conformation. Cells will continue to divide until one or more telomeres have shortened below the length $T_c$. Once the presence of a critically short telomere is detected, a signal will be sent to initiate replicative senescence.

cultured HDFs (Section 2.4). There are a number of possible explanations for this variability, such as interclonal heterogeneity in the distribution of the lengths of the subterminal and telomeric portions of the TRFs (see Section 2.4 for further discussion). The development of methods for accurate measurement of the lengths of individual telomeres will allow a more stringent test for the existence of $T_c$. Such methods might include PCR or primer extension using oligonucleotides that correspond to chromosome specific sequences adjacent to the terminal $(TTAGGG)_n$ tract.

Presently, the mean telomere length at senescence provides the best estimate of the size of $T_c$. The mean telomere length can be calculated from the rates of loss of both mean TRF length and telomeric signal intensity as a function of population doublings of cultured HDFs (Section 2.2) or a function of time of BAL-31 nuclease digestion of high molecular weight DNA (Appendix B). From these analyses, the mean telomere length in senescent HDFs has been estimated to be ~ 2-3 kbp (Sections 2.2 and 2.4 and Appendix B). However, unless all telomeres are the same length, the mean telomere length, a measure of the average length of all telomeres in a cell population, will only provide an upper estimate of the size of $T_c$, which is best represented by the shortest telomere length in senescent cells. FISH analysis of the detectability of individual telomeres in cultured HDFs indicates that the length of each of the 92 telomeres in the a normal diploid human cell are not the same. However, differences in detectability of individual telomeres could also reflect different amounts of telomere-like DNA in the sub-terminal regions of chromosomes. Thus this result alone does not conclusively show that telomere length is variable from chromosome to chromosome. Other evidence for variability in the lengths of individual telomeres comes from mean telomere length analysis of different TRF modes

in HDF strains and clones which have a multi-modal distribution of TRF lengths (Prowse, personal communication; Appendix A). The results from these analyses suggest that although most of the variability in the size of each TRF mode is accounted for by differences in the mean length of the sub-terminal regions of the TRFs, there does exist some inter-modal variability in the mean telomere length as well. Also, the involvement of chromosomes in end-to-end telomeric associations observed in senescent HDFs (Benn, 1976; Sherwood *et al*, 1989) and immortal cells (Pathak *et al*, 1988) appears to be non-random. This observation provides some support for the existence of different telomere lengths on different chromosomes since, presumably, these end-to-end associations occur, or are more likely to occur, when the telomere length on a chromosomal end falls below the critical length required for telomere stability (Harley, 1991; Counter *et al*, 1992). Thus if the average length of all telomeres was the same then the frequency of telomeric association should be the same for all chromosomes. The above hypothesis is supported by the observation that in SW26 cells near crisis, the long arm of one copy of chromosome 9 is frequently engaged in an end-to-end telomeric association with another chromosome or chromosomal fragment (Section 2.6). In addition, the telomere on 9q appears to be relatively short in SW26 cells (Section 2.6). Together, these observations indicate that individual telomeres in the human genome have different lengths, implying that the size of $T_c$ is probably considerably less than 2-3 kbp. It remains possible that there are a small number of chromosomal ends which entirely lack a terminal $(TTAGGG)_n$ array at crisis and perhaps at senescence as well.

The following evidence provides additional support for a model of initiation of cell senescence involving acquisition of a critically shortened telomere: (i) Telomeres are essential genetic elements and therefore there

must be some lower limit to the length of the terminal $(TTAGGG)_n$ tract required to form a functional telomere. In this models shown in Figure 3.4.2, this length is equal to the critical telomere length, $T_c$. (ii) Studies have shown that as cultured HDFs approach senescence, there is an abrupt increase in dicentric chromosomes which appear to be formed by the association of chromosomal termini (Benn, 1976; Sherwood *et al*, 1989). Dicentric chromosomes and telomeric associations have also been observed in pre-crisis transformed cells (Section 2.6)(Counter *et al*, 1992; Counter *et al*, 1994b) and in immortal cell lines (Pathak *et al*, 1988). Since one of the functions of telomeres is to prevent chromosomes from undergoing aberrant end-to-end fusions and associations, these observations may be explained by the appearance of one or more critically shortened telomeres in cells just before replicative senescence. The observation that some human cell lines with very short telomeres (2-4 kbp) do not have increased levels of dicentric chromosomes while others do (Saltman *et al*, 1993) might be accounted for by differences in rates of repair of critically shortened telomeres by telomerase or some other mechanism in different cell lines. Other explanations are differences between cell lines in composition of telomeric chromatin which could affect the fusogenicity of the chromosomal ends or the size of the critical telomere length. (iii) The deletion of a telomere from a chromosomal end in *S. cerevisiae* has been shown to induce cell cycle arrest (Sandell and Zakian, 1993). Furthermore, the presence of a single double-strand DNA break, induced by exposure to gamma-radiation (di Leonardo *et al*, 1994) or breakage of a dicentric chromosome (Ishizaka *et al*, 1995), appears to be sufficient to induce cell cycle arrest in human cells by a p53-dependent mechanism. Thus the presence of critically shortened telomeres, which may resemble broken DNA, would be expected to induce cell cycle arrest by a

similar mechanism. However, the exact role p53 plays in replicative senescence remains to be established. (iv) Although oxidative DNA damage has been shown to be higher in senescent HDFs than in young HDFs (Chen *et al*, 1995) and mild hyperoxia (von Zglinicki *et al*, 1995) or hypooxia (Chen and Ames, 1994) can reduce or extend respectively the *in vitro* lifespan of HDFs, the telomere length at senescence is not affected by a change in the oxygen partial pressure of the atmosphere in which the cells are cultured, at least in the range of 20% to 40% (von Zglinicki *et al*, 1995). Instead, increasing the oxygen concentration of the atmosphere in which the cells are grown causes an increase in the rate of telomere shortening (von Zglinicki *et al*, 1995). Thus these observations provide additional support for a model of replicative senescence involving the shortening of one or more telomeres below a critical length.

A corollary to this model is that the pathway leading to cell cycle arrest in senescent cells must be blocked or inactivated in transformed cells which have an extended lifespan. The continued loss of telomeric DNA in transformed cells (Counter *et al*, 1992; Counter *et al*, 1994b) would be expected to lead to the further accumulation of damaged telomeres. There may be more than one type of damaged or aberrant telomeres in transformed, pre-crisis cells that have by-passed the M1 checkpoint. For example, telomeres below the critical length $T_c$ may have lost partial structure and function whereas telomeric DNA may have been completely lost from other chromosomal ends. In turn, the accumulation of damaged telomeres could lead to a progressive loss of sub-nuclear organization and nuclear function which could ultimately cause the signalling of the M2 checkpoint (crisis). In support of this prediction is the recent observation that the inactivation of human telomerase in antisense experiments results in telomere shortening

and cell death (Feng *et al*, 1995). Also, sub-terminal sequences, as well as telomeric sequences, are progressively lost during division of EBV-transformed B lymphocytes (Guerrini *et al*, 1993), indicating that chromosomal ends entirely lacking telomeric DNA accumulate as these cells approach crisis. Furthermore, dicentric frequency has been shown to increase sharply as virally-tranformed cells approach crisis (Counter *et al*, 1992; Counter *et al*, 1994b). Some of these dicentrics appear to be formed via telomere end-to-end associations (Section 2.6)(Pathak, 1988). To date, telomere end-to-end associations have not been observed to behave as other dicentrics in that they seem not to cause genomic rearrangements although it is possible that such rearrangements are rare and may have escaped detection (Saltman *et al*, 1993; Murnane *et al*, 1994). It is possible that formation of telomeric associations could lead to loss of heterozygosity via chromosome loss and non-disjunction of the remaining homologue(s) (Saltman *et al*, 1993) which could play an important role in tumor progression.

## 3.5 Implications of this work.

This Section provides speculation regarding cell immortalization and tumor progression, and cell senescence and organismal aging, under the assumption that loss of telomeric DNA is the primary cause of cell senescence.

If this is the case, and assuming that cell senescence has evolved as a form of protection against cancer in humans, then the mechanism(s) that regulate telomere length may have been selected for during evolution such that telomere length is maintained within a fairly narrow range in the germ line. That is, the telomere length in the germ line would need to be above a

certain length to allow growth and development into mature adults before cells senesce. Also, germ line telomere length would also have to be below a certain length in order for cell senescence to be an effective means of protection against growth of cancerous cells. Otherwise, in young individuals, cells which have acquired mutations to allow unregulated growth could form a tumor large enough to greatly reduce the chance of survival of the organism prior to production of offspring. However, it remains to be determined whether cell senescence has actually evolved as an effective form of protection against cancer (also see Section 1.1).

Also, if loss of telomeric DNA is the only obstacle limiting the proliferative capacity of normal somatic cells, an assumption which remains to be determined, then the observation that normal human somatic cells do not undergo spontaneous immortalization (McCormick and Maher, 1988) suggests that multiple hits are required for induction of telomerase expression. There are a number of possible reasons why multiple levels of repression of telomerase might be advantageous. For example, it would provide added protection against formation of malignant tumors. Also, each of the components of telomerase, the RNA component and the 2 or more (presumably) protein components, may have unique, independent functions depending on the cell type. Thus some cells may require the activation of one component of telomerase while maintaining repression of the other components in order to perform a particular function. Alternatively, telomerase may be activated by a single hit in normal somatic cells but with the no effect on telomere length, or even perhaps with the consequence of inducing cell death. For example, proper functioning of telomerase may require the coexpression of other factors to allow efficient synthesis of telomeric DNA, or to prevent telomerase from damaging the telomeres

which could be lethal.

Cell senescence may account for some of the degenerative changes that occur in some mitotically active somatic tissues in elderly individuals (see introduction). If telomere shortening causes cell senescence, then it may be possible to prevent these changes by preventing or postponing cell senescence via activation of telomerase in these tissues. Developing a method to accomplish this might be a major challenge of the future. It will probably require the cloning of the genes for all components of telomerase as well as an understanding of the mechanisms governing the regulation of telomerase expression and activity, and the structure and function of telomeric chromatin. Gene therapy may be an effective means to prevent telomere shortening by allowing the induction of high levels of telomerase expression in some cells, for example hematopoietic stem cells. However, gene therapy will probably not be very effective for many other cell types due to the difficulty of stably and efficiently introducing a gene into cells *in vivo*. For these cells, it may be necessary to develop drugs to specifically induce telomerase expression. In some cells, there may be one or more proteins present which inhibit, directly or indirectly, telomerase activity. Thus, in order to turn on telomerase, it may be necessary to develop methods to repress these inhibitors. Also, as mentioned above, direct activation of telomerase in somatic cells may have no effect or could even be deleterious or lethal to the cell. Furthermore, immortalization or extension of lifespan of cells may predispose them, relative to mortal cells, to tumor development. The benefits and deleterious consequences that will accompany the postponement or prevention of cell senescence via activation/expression of telomerase *in vivo* awaits further experimentation.

Appendices

## Appendix A: Analysis of rate of shortening and mean telomere length of TRF modes in HDF clones.

An alternative method to FISH for analysis of length and rates of shortening of individual telomeres is to measure the mean length and telomeric signal intensity of TRF modes, which are sometimes observed in Southern analysis of TRFs using a telomeric probe, at various population doublings of serially passaged HDFs. The results from this type of analysis done on some of the HDF clones described in Section 2.4 is presented in this appendix.

Southern analysis for all clones was done as described in Sections 2.1 and 2.4. A sample autoradiogram for one of the clones showing multi-modal TRF distribution is shown in Fig. A.1. As shown in this autoradiogram and others, there appears to be no large changes in the distribution of TRF modes during replicative aging, in support of a similar rate of telomere loss for all telomeres. To further examine the rates of loss of individual telomeres, the mean length of discrete, high molecular weight TRF modes and of the entire TRF distribution was measured at various population doublings for three clones, C2, C4 and C7 established from HDF strain S26a. The rates of loss calculated from this analysis are shown in Table A.1. In general, for a given clone, the rates of loss of individual TRF modes and the overall rate of loss are comparable. For clone C7, the overall rate of TRF shortening is considerably less than the rates of shortening for individual modes. However,

this is at least partially due to the large amount of lower molecular weight signal that is present in similar amounts at all PDLs (see Fig. A.1). Although general degradation of the high molecular weight DNA was not apparent at any PDL of clone C7 as determined by resolving the uncut DNA in 0.7% agarose gels and subsequently staining with ethidium bromide, it is possible that the lower molecular weight signal is due to small amounts of aberrant and/or sheared telomeric DNA. This is supported by the observation that the length of these telomeric fragments does not appear to decrease with increasing PDL. This low molecular weight signal appears to be present in other clones as well, although not as much as in C7, and may account for the slightly lower overall rates of shortening as compared to the rates of shortening of the high molecular weight TRF modes observed in clones C2 and C4 (Table A.1). The relatively poor resolution of the high molecular weight TRF modes greatly limits the accuracy in measurement of the mean length of these modes, which may also contribute somewhat to differences in the measured rates of shortening.

Using the measured values for the rate of TRF shortening and rate of decrease in telomeric signal intensity, the mean telomere length of the entire TRF distribution and of individual TRF modes was calculated at early passage for clones C2, C4 and C7 (Table A.1). The data for clones C2 and C7 indicate that there is intermodal variability in mean telomere length. This variability is somewhat, but not entirely, accounted for by the lower measured rate of shortening for the entire TRF distribution (Table A.1). Thus this data is consistent with the existence of heterogeneity in the length of individual telomeres.

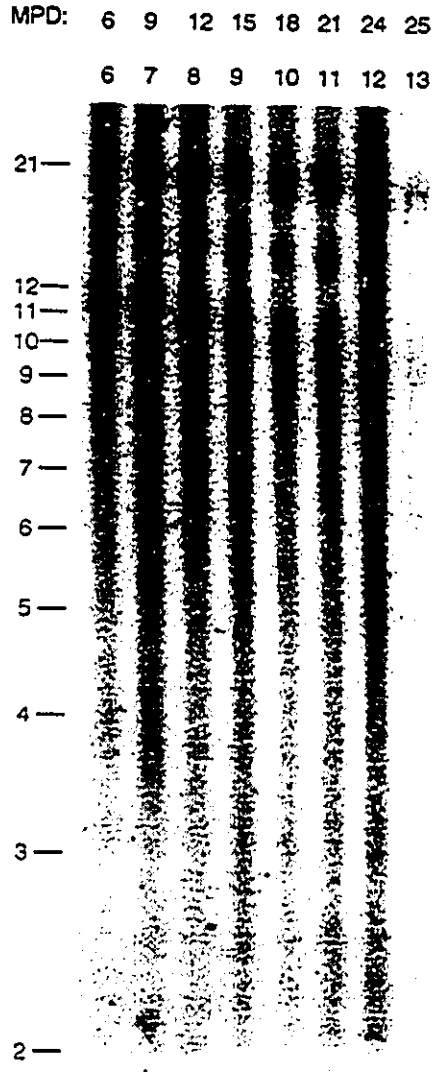



Fig. A.1 Southern analysis of TRF length and distribution for clone C7 at various population doubling levels.

Southern analysis was performed essentially as described in Sections 2.1 and 2.4. The PDL of each sample is indicated above each lane and size of the molecular weight markers and numbering of modes is indicated on the

right.

Table A.1. Telomere lengths and rates of shortening for TRF modes in HDF S26a clones.

| Clone | mean TRF length (kbp)[a] | mean telomere length (kbp)[a,b] | rate of shortening (bp/pd) |
|---|---|---|---|
| C2 | | | |
| overall[c] | 8.2 | 2.6 (3.0)[d] | 120 |
| mode 1 | 17.8 | 4.1 (4.1) | 150 |
| mode 2 | 14.1 | 3.1 (3.1) | 150 |
| C4 | | | |
| overall | 8.1 | 2.0 (2.4) | 70 |
| mode 1 | 15.9 | 2.0 (2.0) | 90 |
| C7 | | | |
| overall | 8.2 | 2.6 (2.7) | 80 |
| mode 1 | 21.0 | 6.0 (6.4) | 170 |
| mode 2 | 14.1 | 3.2 (3.4) | 140 |

a- Values are given for the earliest passage for which mean TRF length could be determined (see Section 2.4). Qualitatively similar results were obtained at other PDLs.

b- Mean telomere length was calculated as described in Section 2.2.

c- Values in these rows are for the entire TRF distribution.

d- Mean telomere length in parentheses was calculated using the average value for the rate of TRF shortening of the modes.

Appendix B: Analysis of mean telomere length at senescence for HDF clones.

In this appendix, analysis of the rate of BAL-31 nuclease digestion of uncut genomic DNA is used to estimate mean telomere length at senescence for the HDF clones described in Section 2.4. 38.5 µg of high molecular weight DNA from a clone at early passage was digested with 15 units of BAL-31 exonuclease (GIBCO-BRL). At various time intervals (see Fig. B.1) an aliquot containing 3.5 ug of DNA was removed and the BAL-31 nuclease was inactivated by adding EGTA to a final concentration of 20 mM followed immediately by phenol/chloroform extraction. Following phenol/chloroform extraction, all samples were ethanol precipitated, resuspended in TE buffer and digested with *Hinf*I/*Rsa*I. Mean TRF length analysis of these samples as well as samples from senescent clones established from HDF strain S26a (see Section 2.4) was performed as described in Section 2.4. 0.6 µg of each sample was loaded on the gel (Fig. B.1). The mean telomere length at senscence for each clone was then calculated using the following equation, $T = m_1/m_2 * ODsen.$, where T is the mean telomere length, $m_1$ and $m_2$ are the rates of mean TRF length shortening and loss of telomeric signal intensity respectively during the time course of BAL-31 digestion, and ODsen. is the telomeric signal intensity at senescence. For the analysis shown here, the average telomere length at senescence was calculated to be ≈2.0 kb. In a second analysis, an average mean telomere length at senescence of ≈3.4 kbp was obtained. Thus, the results from these 2 studies suggest that the mean telomere length at senescence for the HDF clones analyzed in Section 2.4 is ≈2.7 kbp.

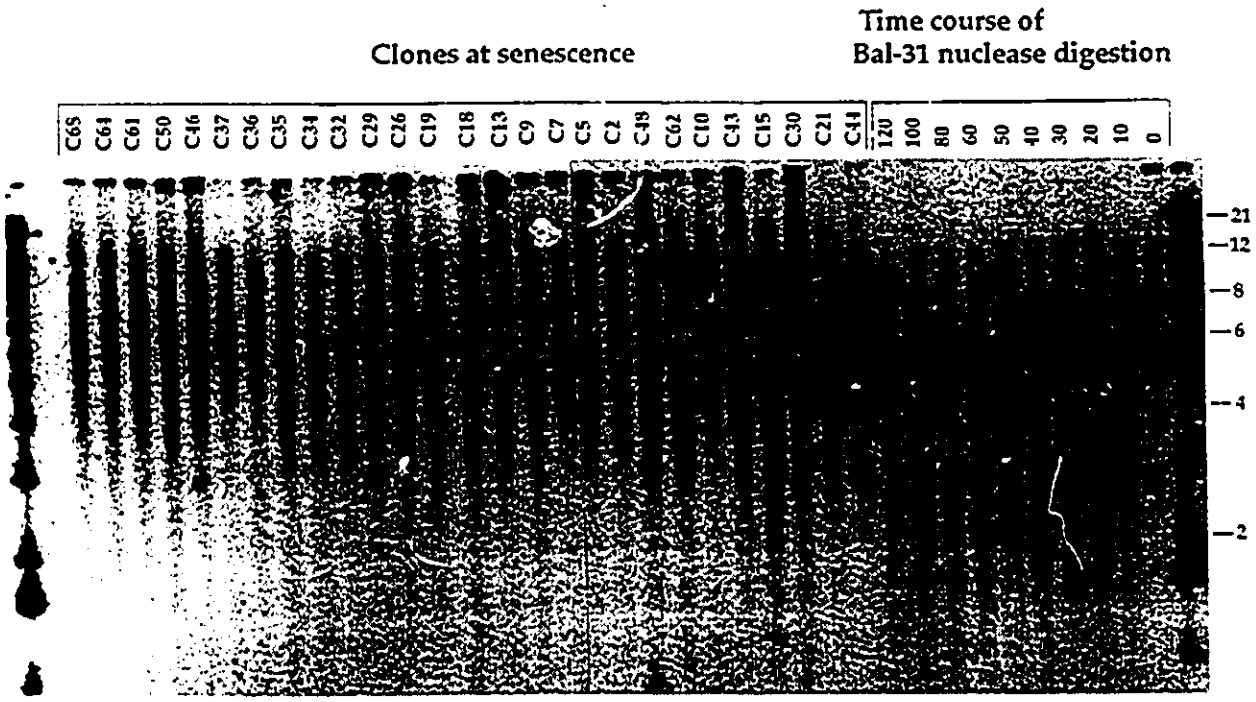



Fig. B.1. Mean telomere length analysis of HDF clones at senescence.

Southern analysis of mean TRF length was performed as described in Sections 2.1 and 2.4. For the BAL-31 nuclease time course analysis, the time that each sample was removed from the reaction is indicated. The code number assigned to each clone is also indicated. Size of molecular weight markers is shown on the left. See text for further details.

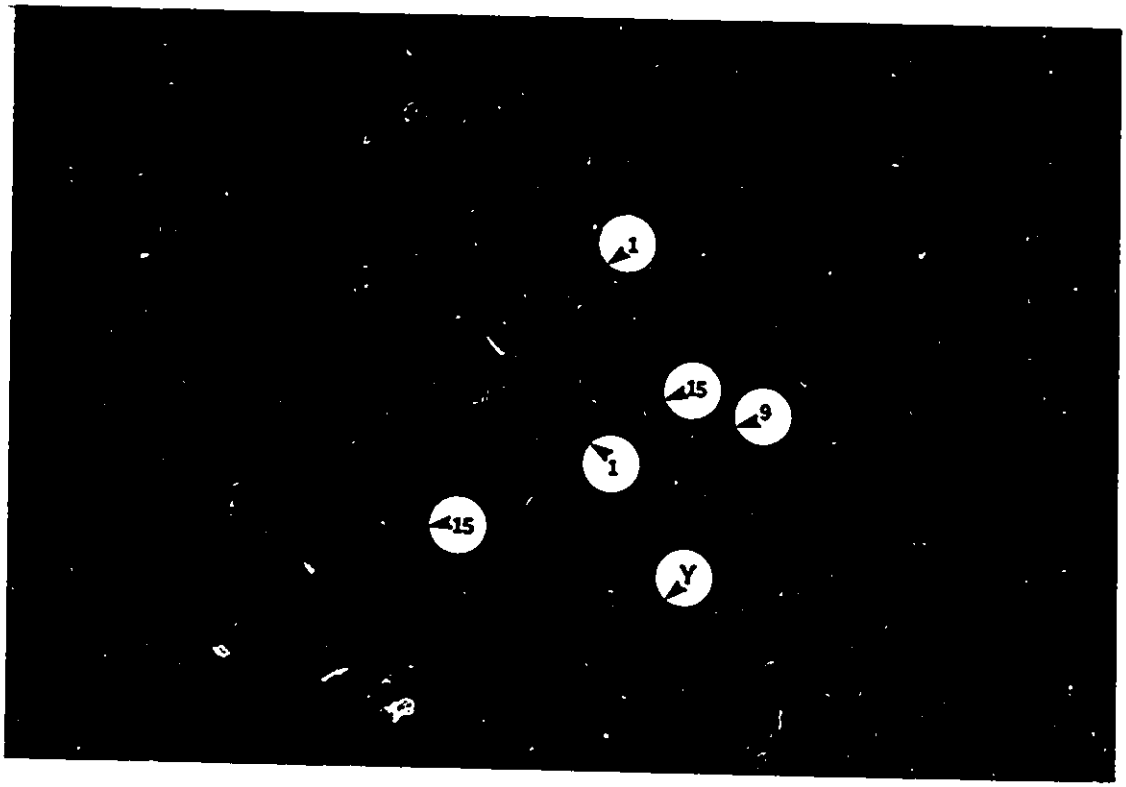
Appendix C: Sample metaphase spreads from early and late passage BJ cells, early passage IMR90 cells and SW26 cells near crisis showing telomeric signals and DAPI/DA staining.

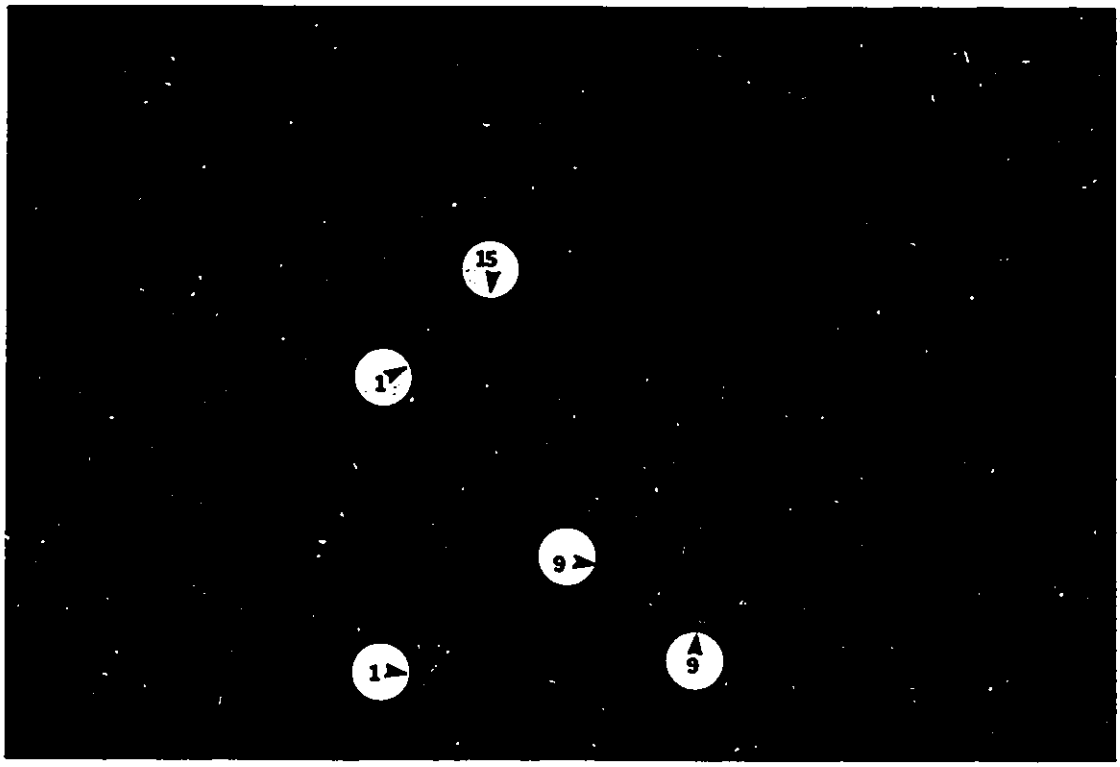
In this appendix, examples of metaphase spreads used in the FISH analysis of individual telomeres described in Section 2.6 are presented. FISH was performed with a DIG-11-dUTP labeled telomeric probe, which gives a somewhat stronger fluorescent signal than the corresponding biotin-16-dUTP labeled probe. Five different spreads showing fluorescent telomeric signals on individual chromosomes for young and old BJ cells and young IMR90 cells and near crisis SW26 cells are shown. In addition, images of the DAPI/DA staining for each spread are shown to allow identification of chromosomes 1, 9, 15 or the Y chromosome by size, morphology and position of the stained region. For each metaphase spread, only some of these chromosomes are identified because: (i) In any given metaphase spread, some chromosomes may not have fixed to the slide or may not have stained well; (ii) Some chromosomes may be clustered with others, making identification of the chromosome or its telomeres ambiguous; (iii) For chromosomes 1 and 9 it is sometimes impossible to distinguish the long and short arms; (iv) Sometimes faint staining of chromosomes other than 1, 9, 15 or the Y chromosome occurs, making it difficult to correctly identify some chromosomes.

Fig. C.1. Sample metaphase spreads showing fluorescent telomeric signal and DAPI/DA staining.

Metaphase spreads for BJ cells at early (PDL 24, pages 140-144) and late (PDL 82, pages 145-149) passage, IMR90 cells at early (PDL 24, pages 150-154) passage and SW26 cells (PDL 63, 3 population doublings before crisis, pages 155-159) were prepared and FISH and photomicrography were performed as described in Section 2.6. A DIG-labeled telomeric probe (pBLrep4) was used.

BJ early passage

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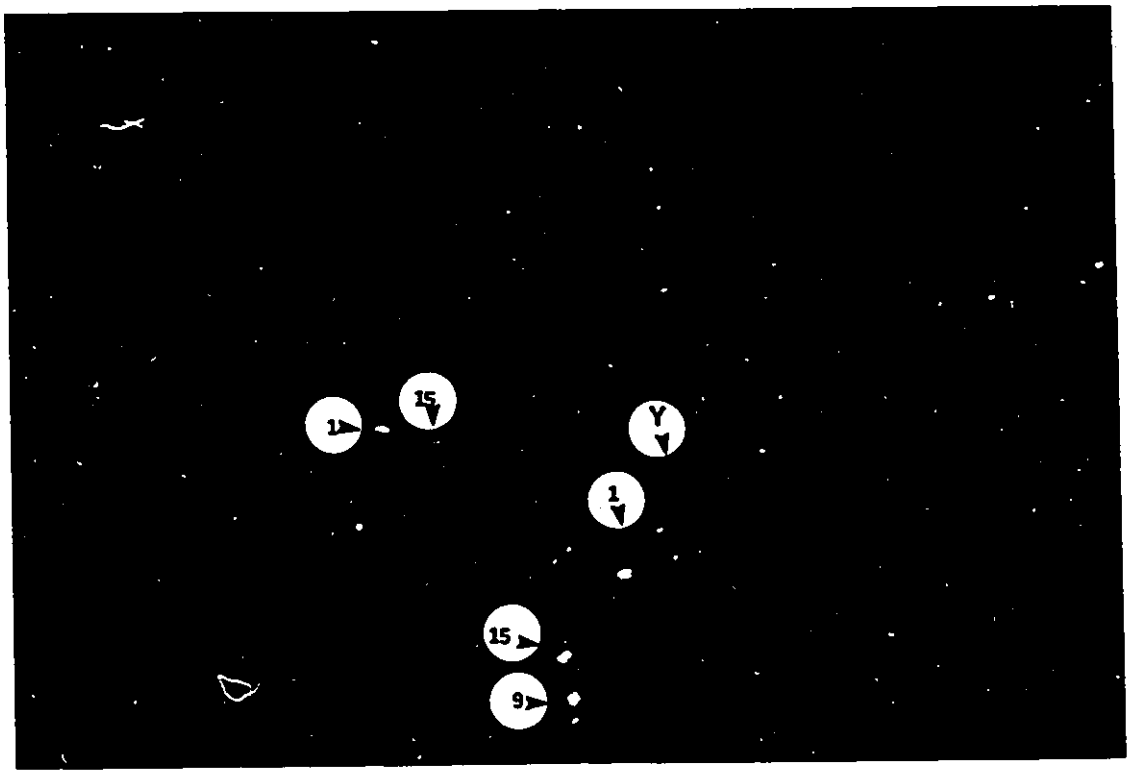
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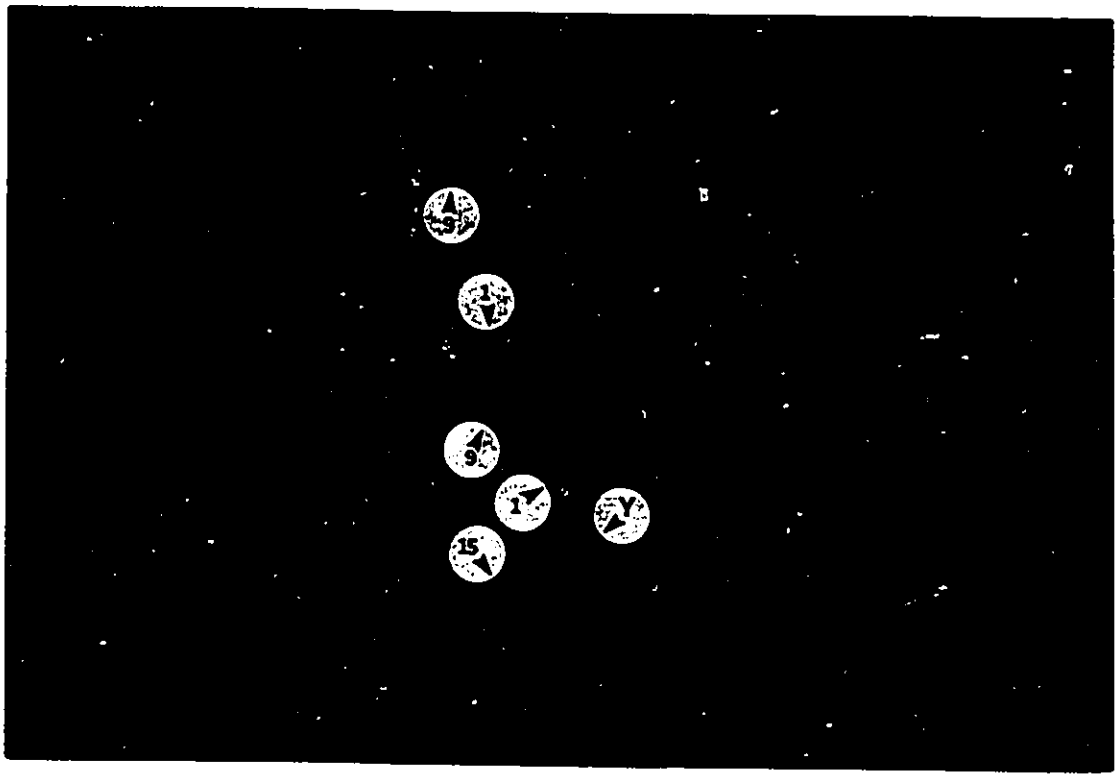
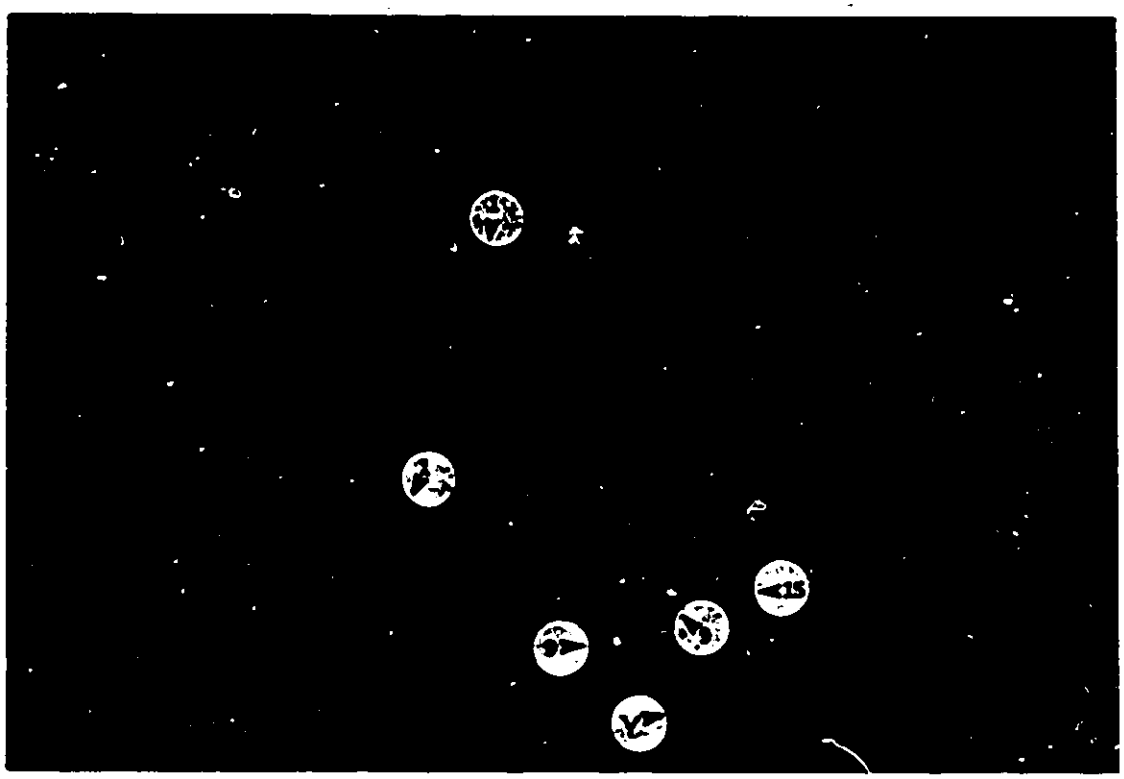
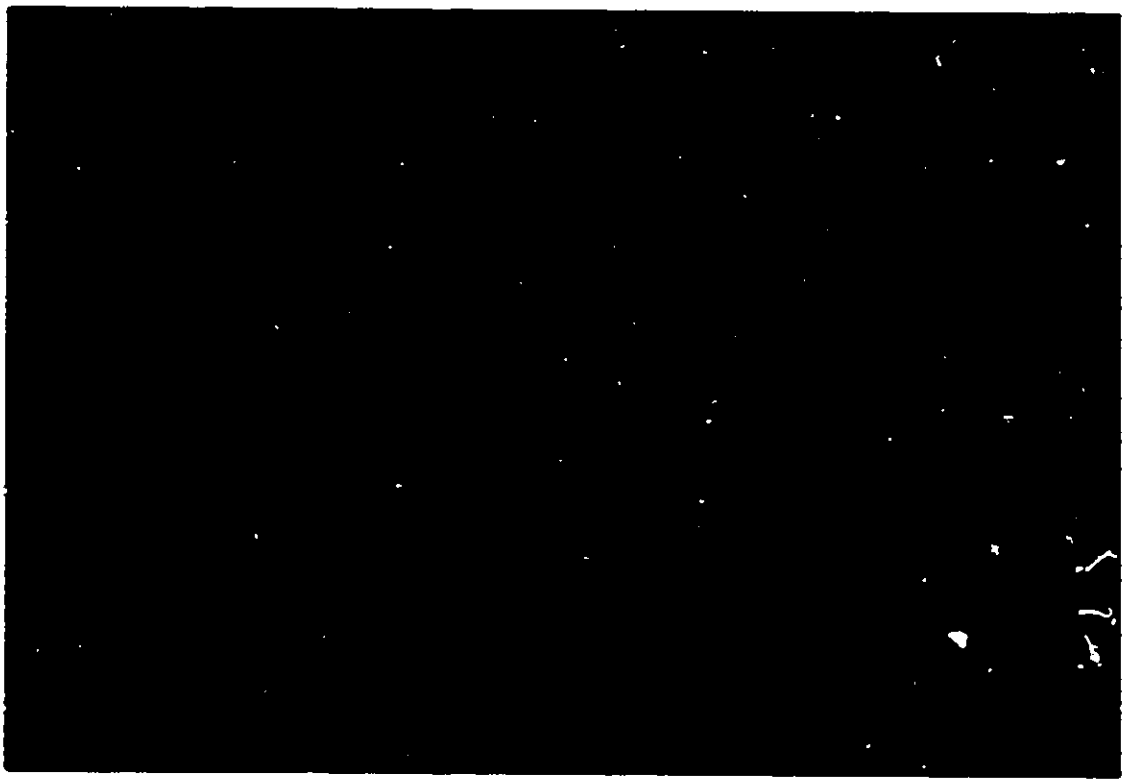


**Bj late passage**



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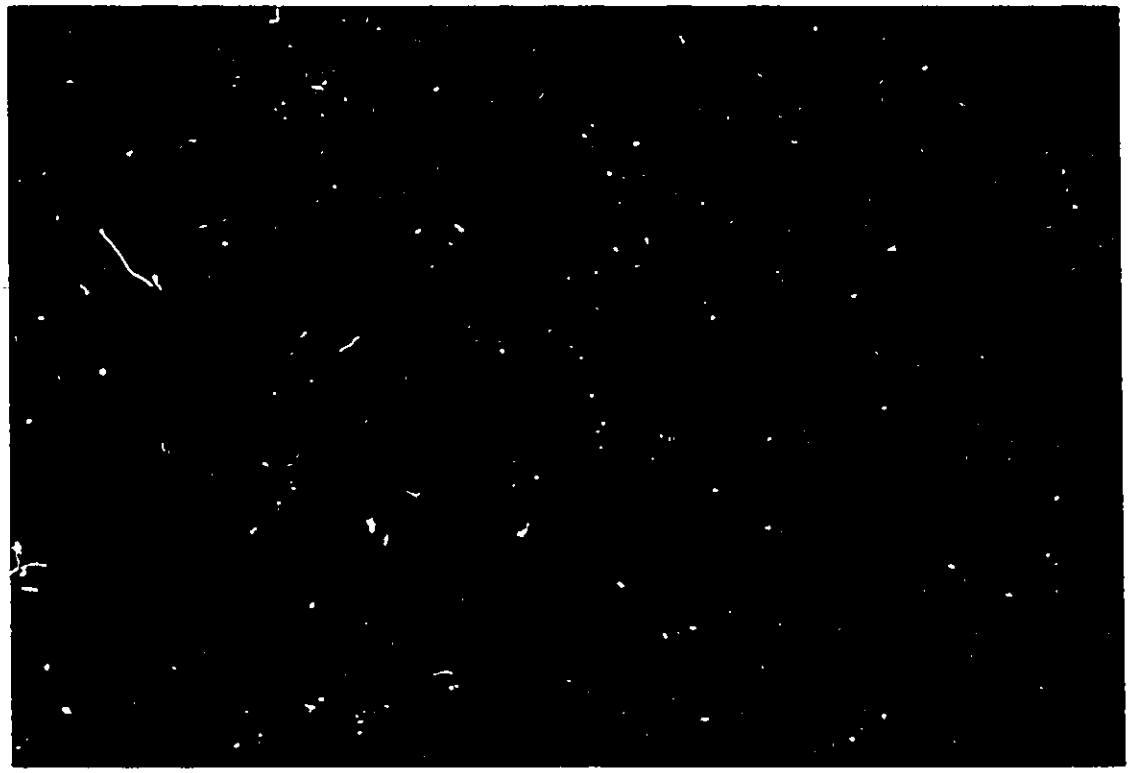


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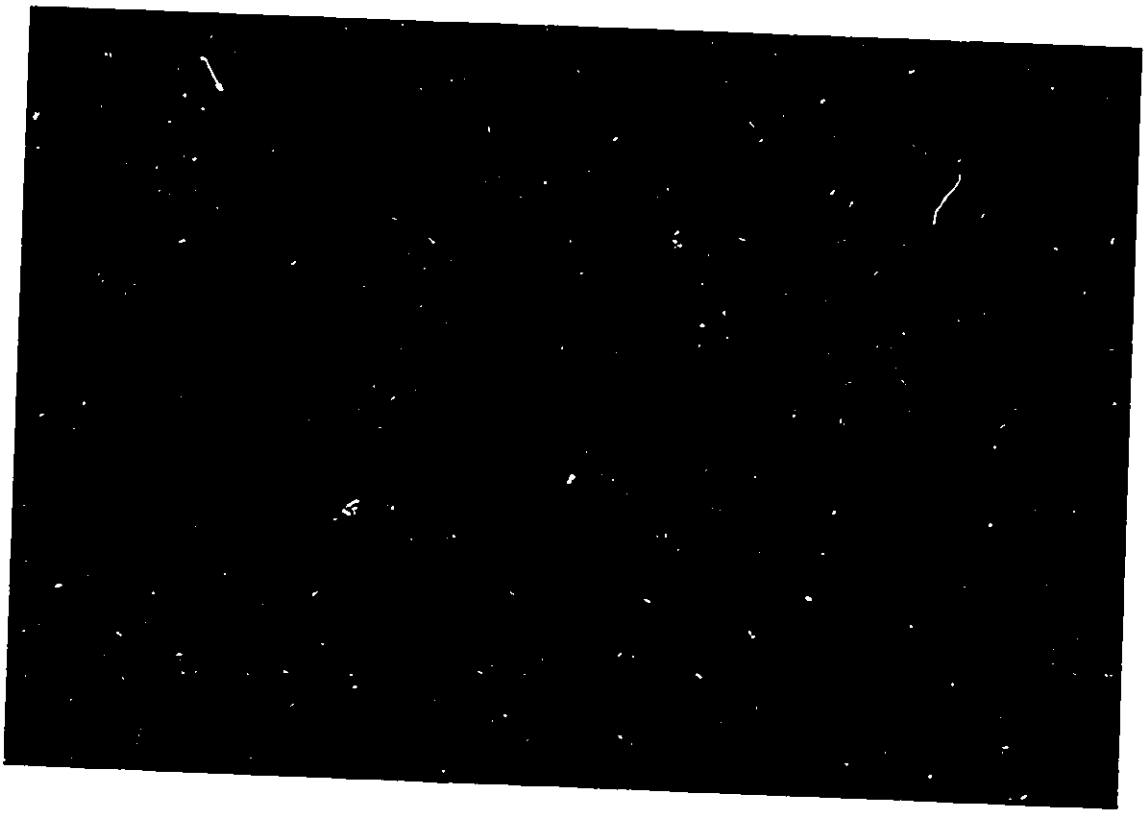

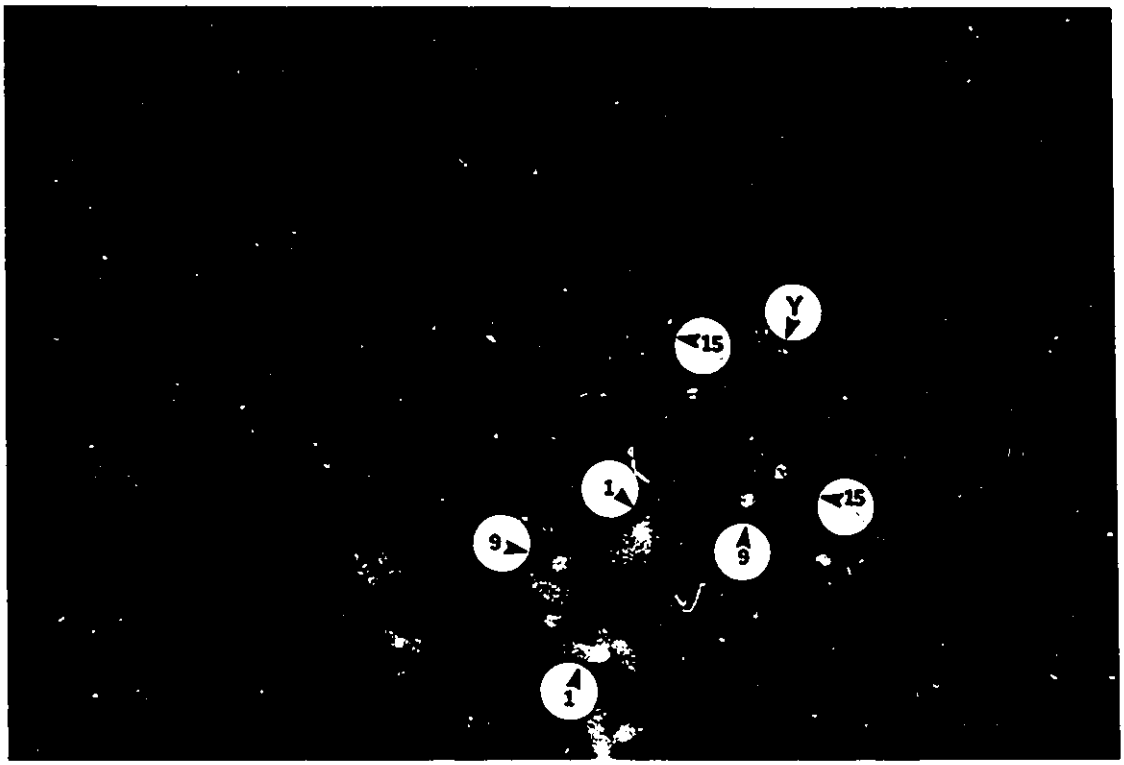
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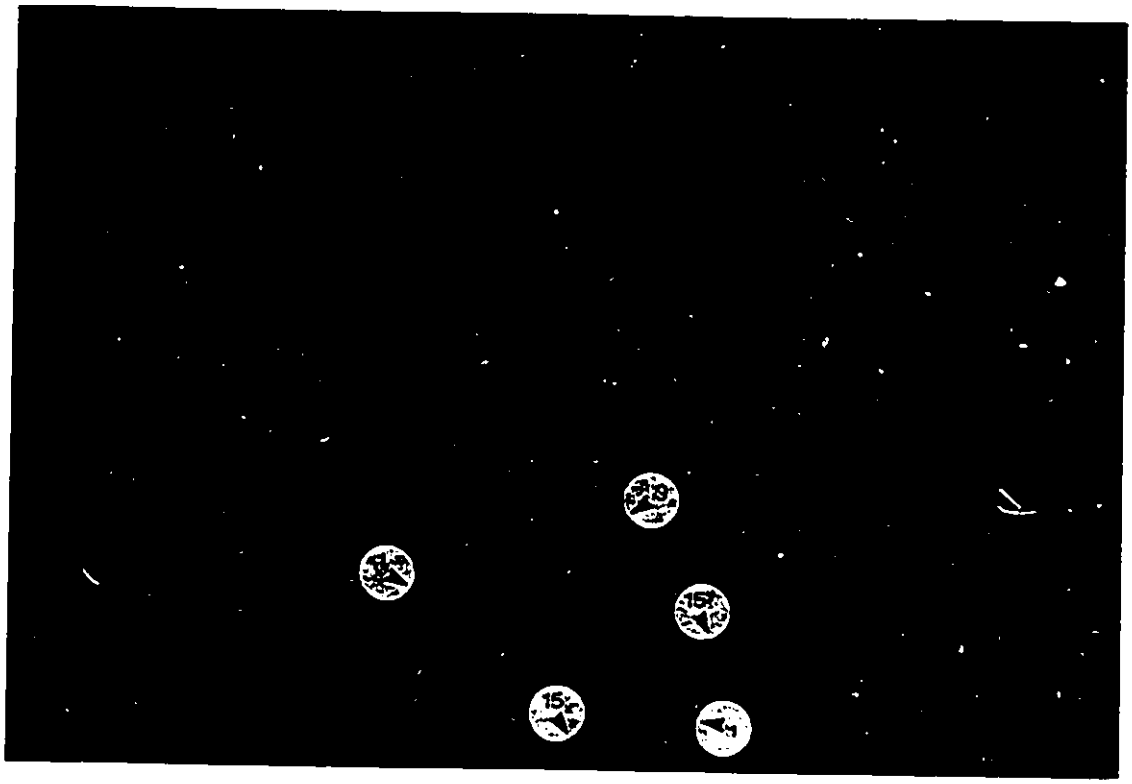
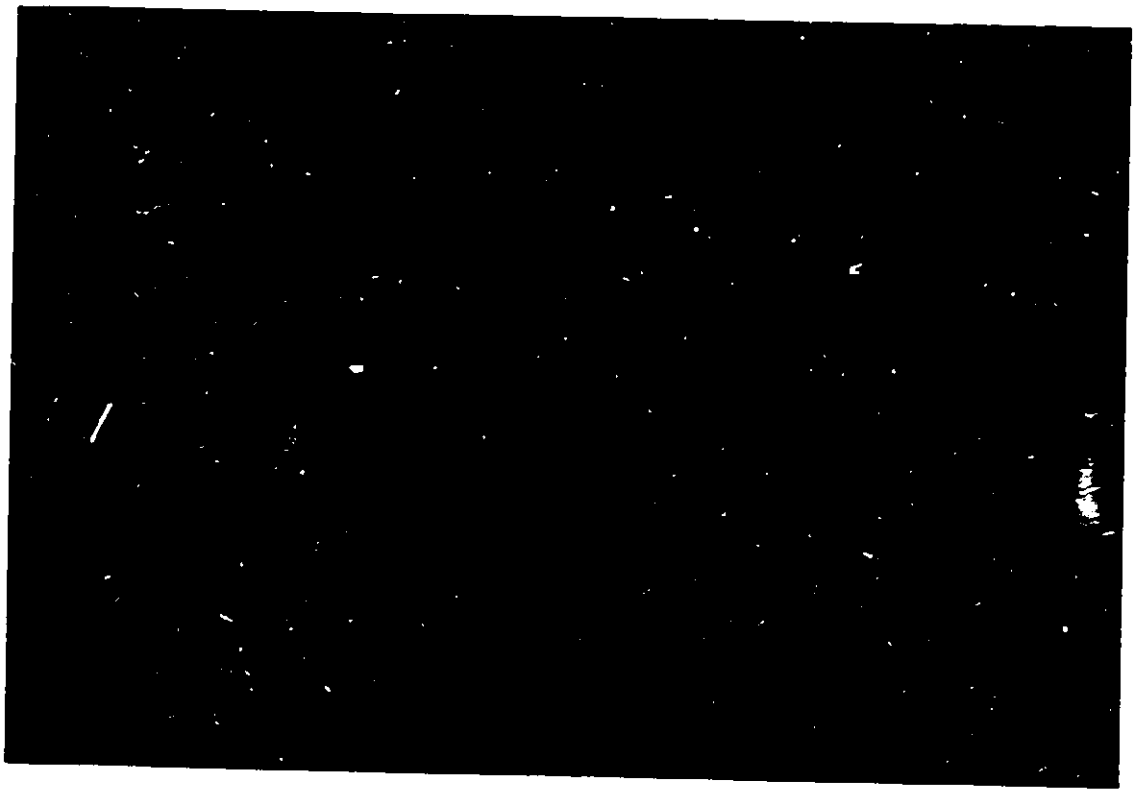
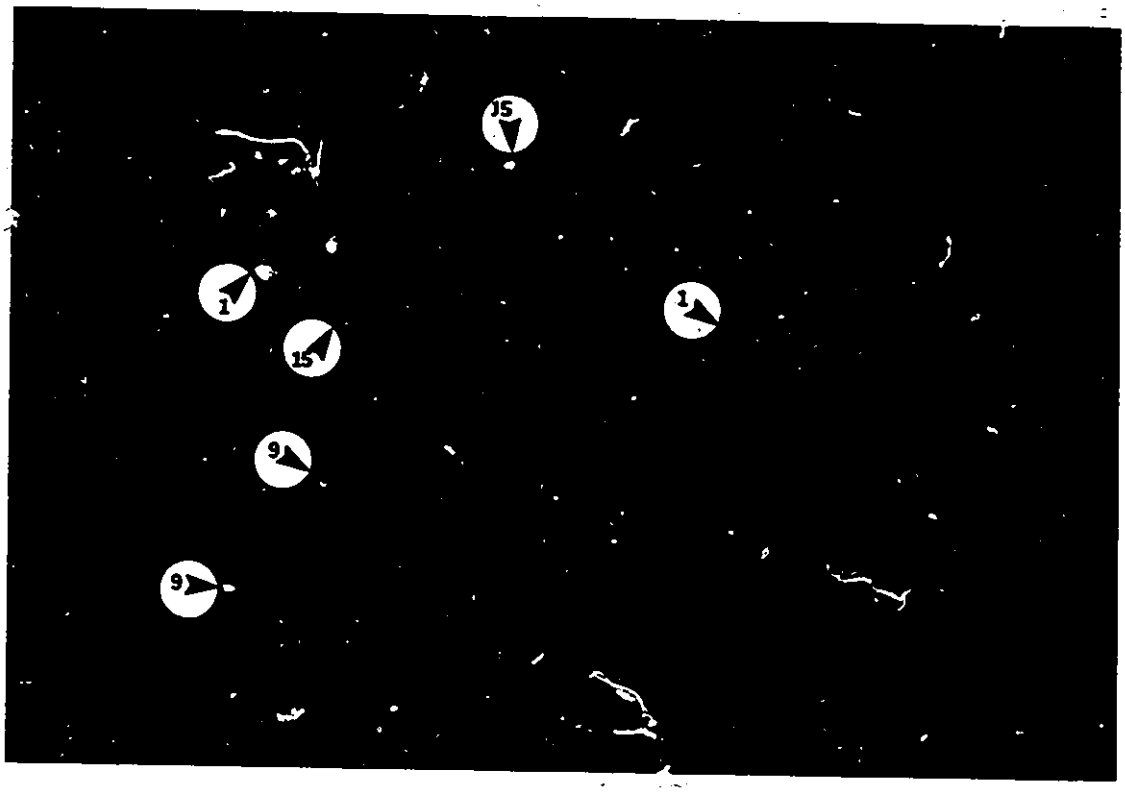
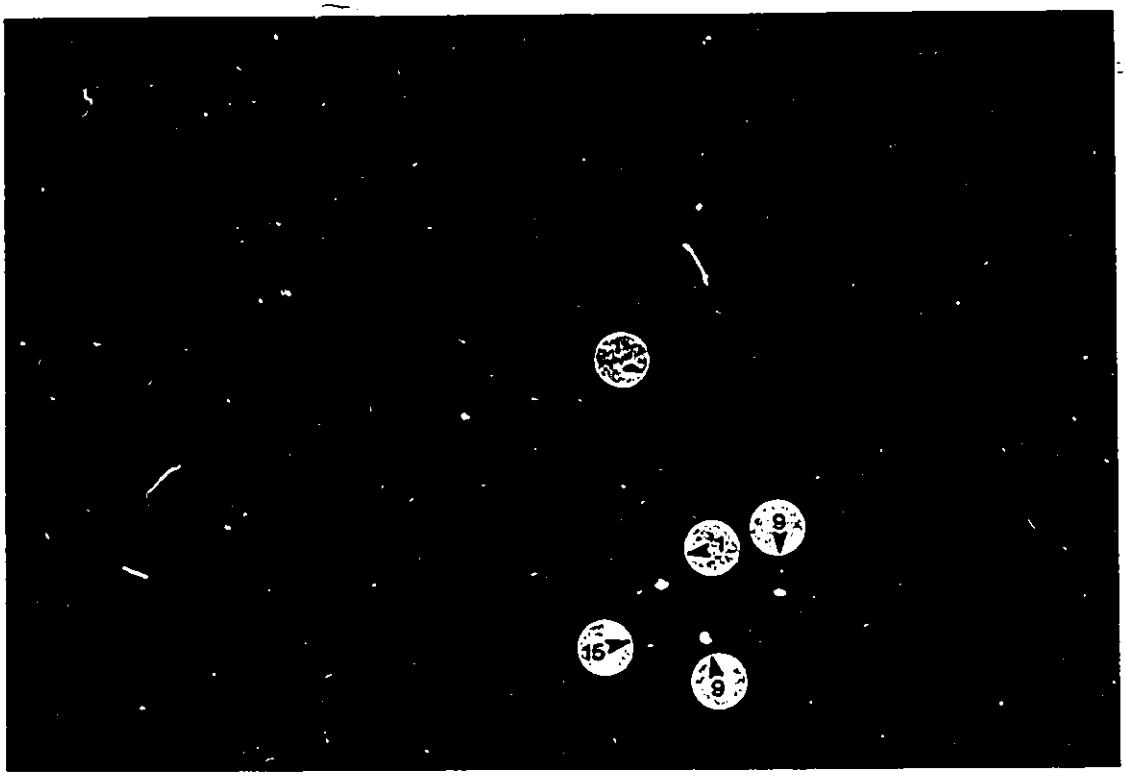




IMR90

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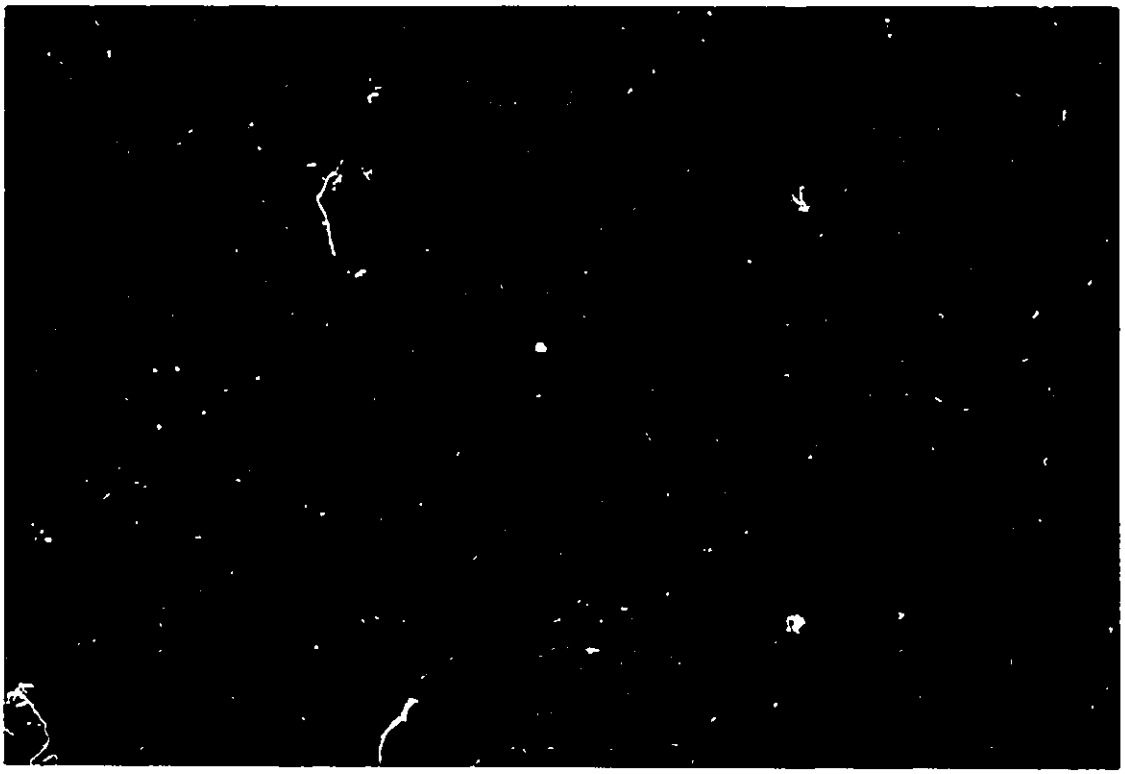

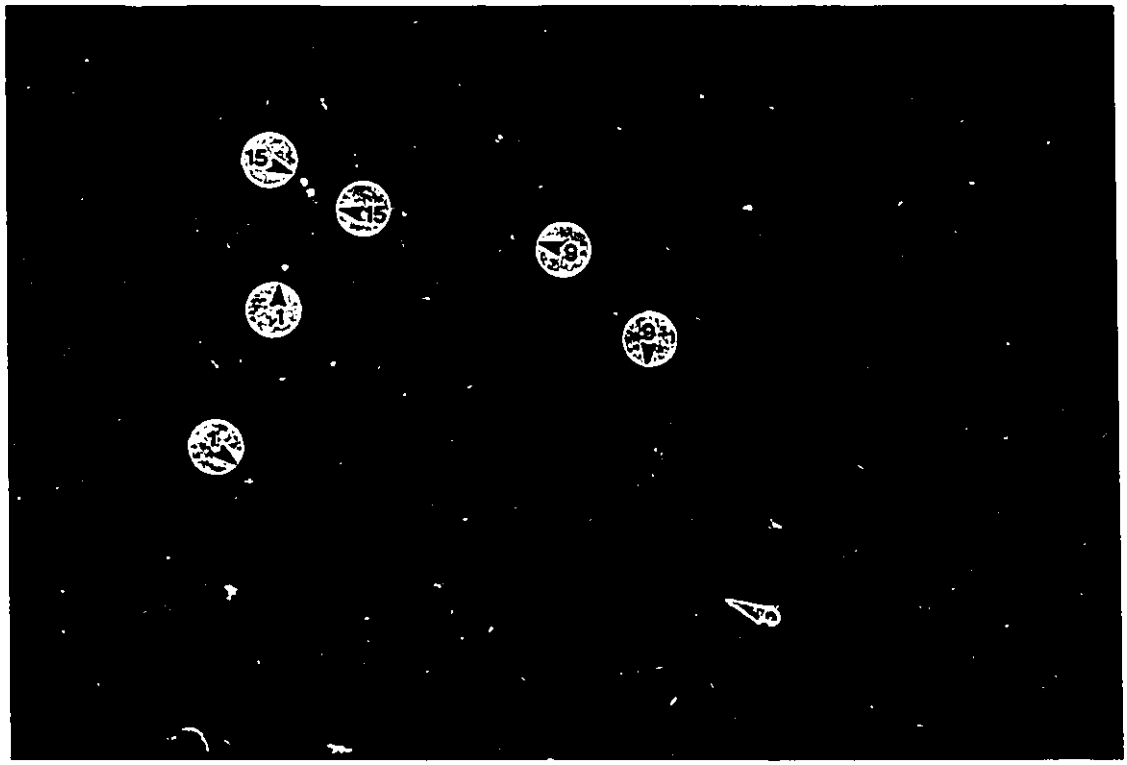

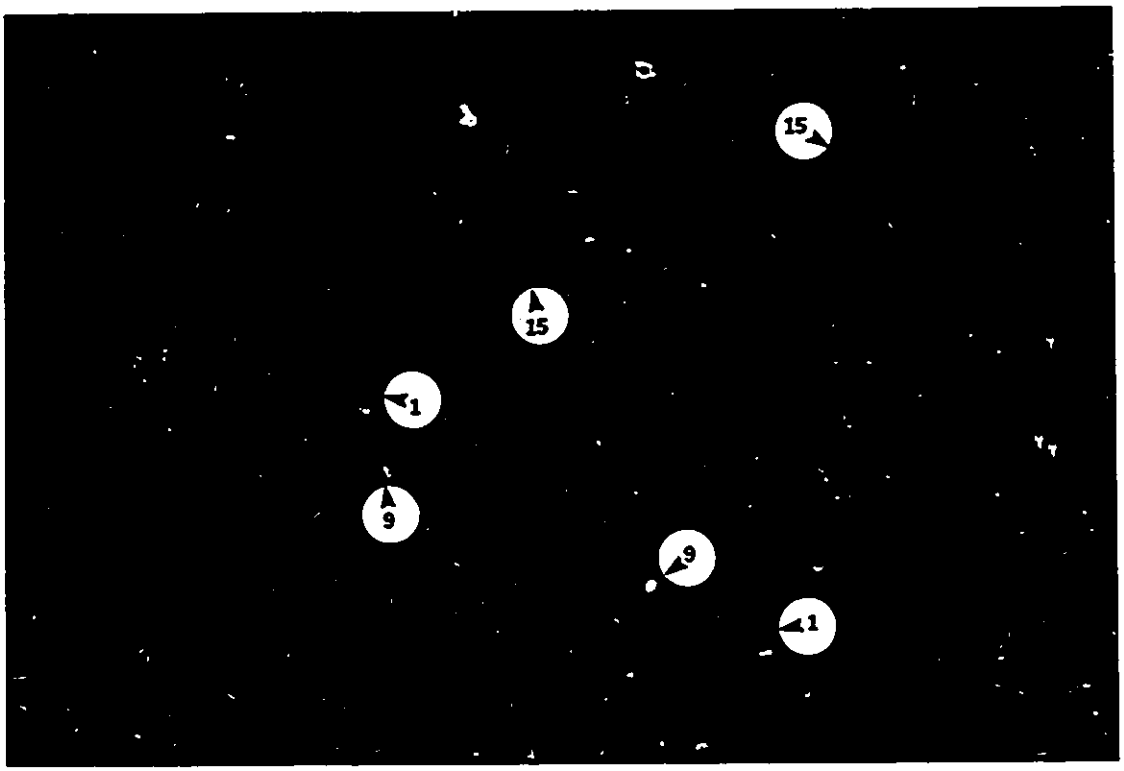
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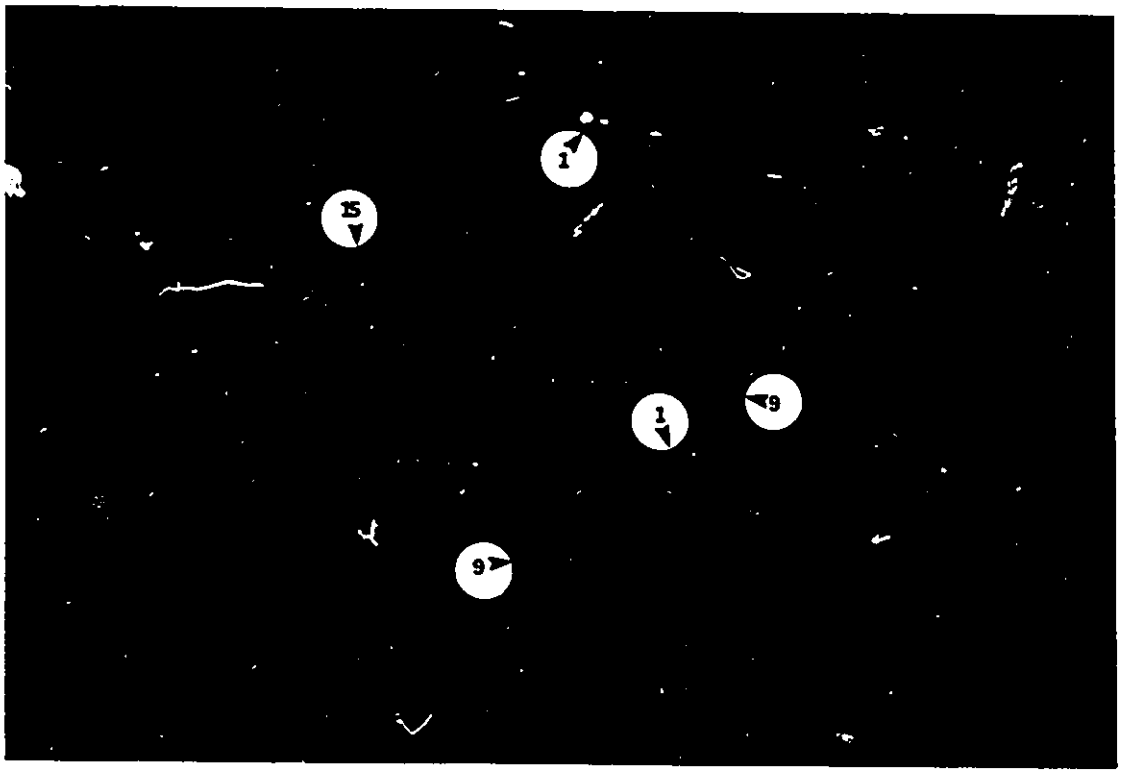
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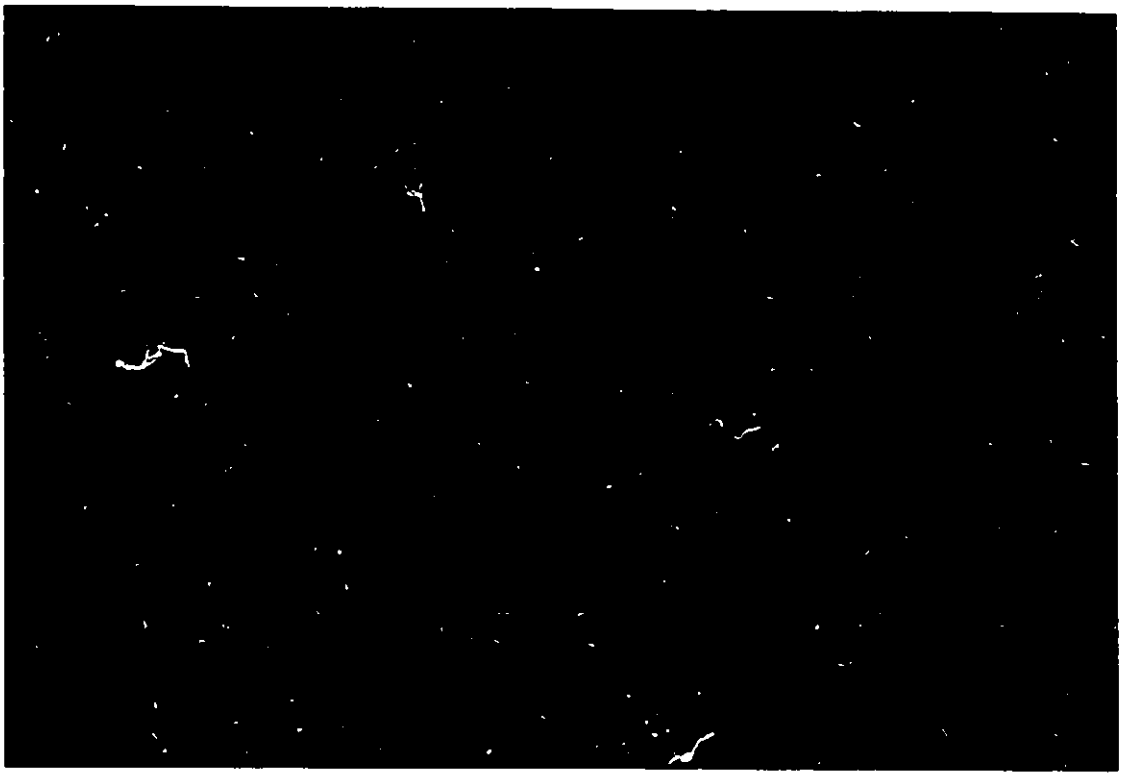
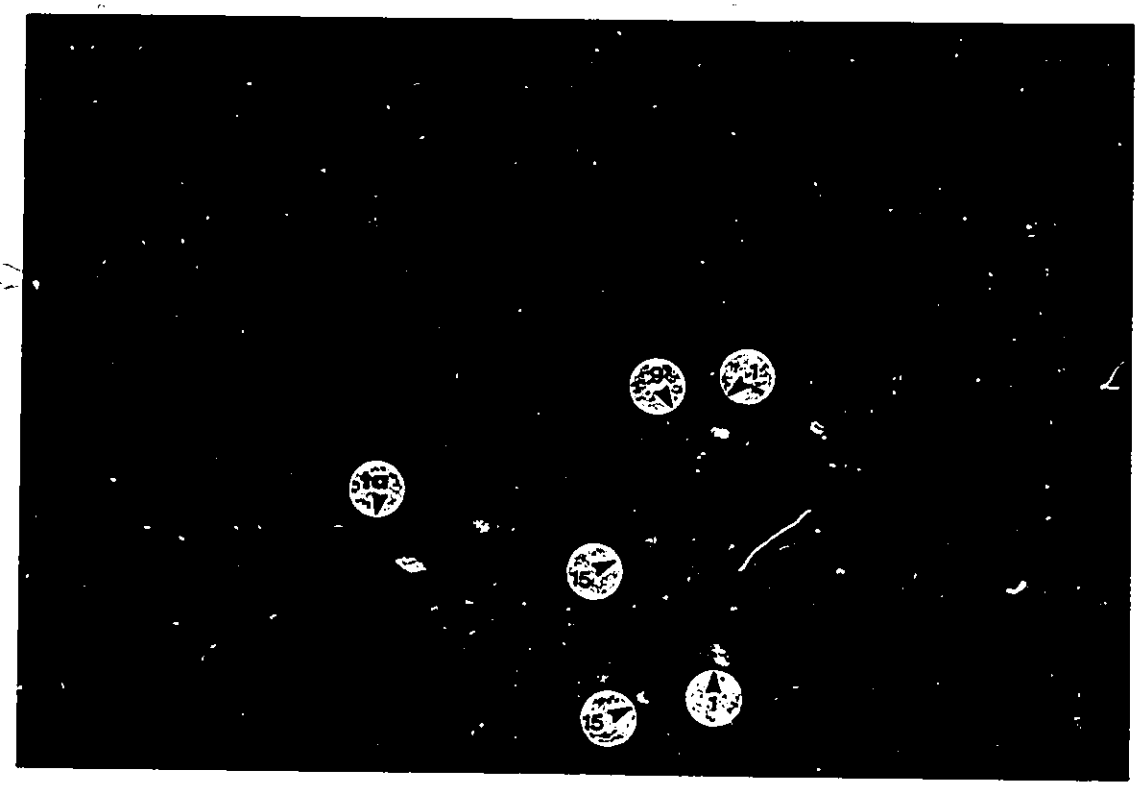


SW26

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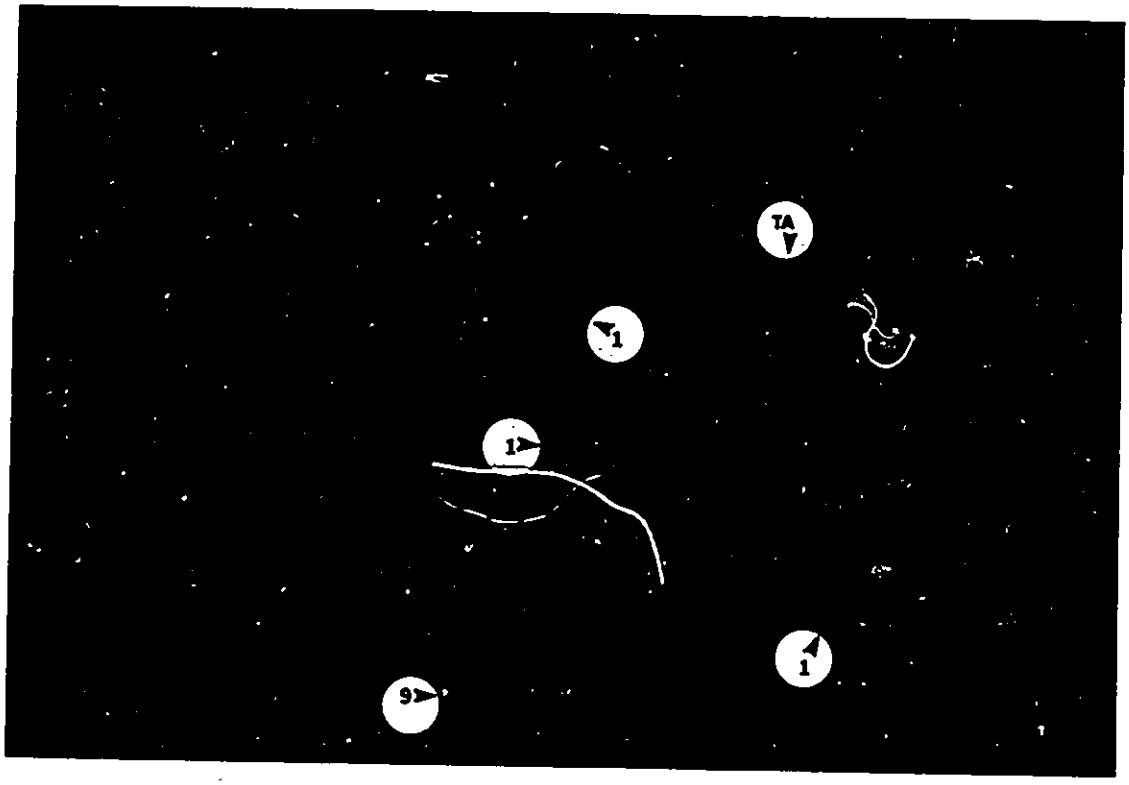
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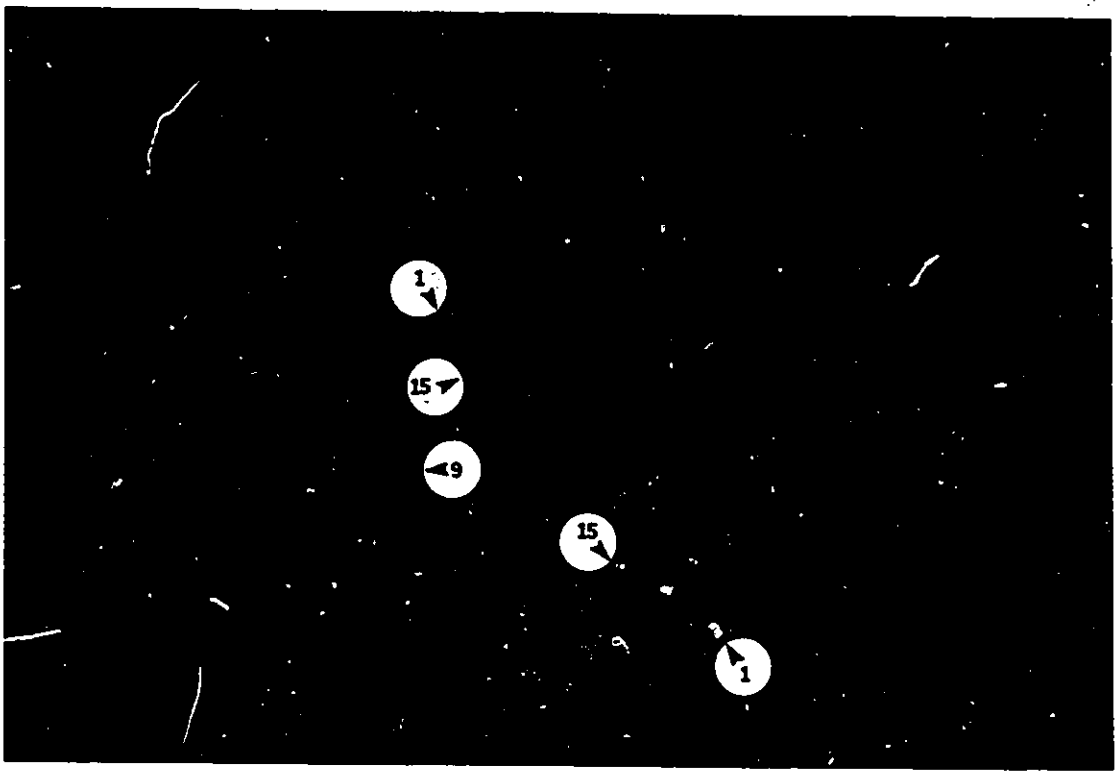
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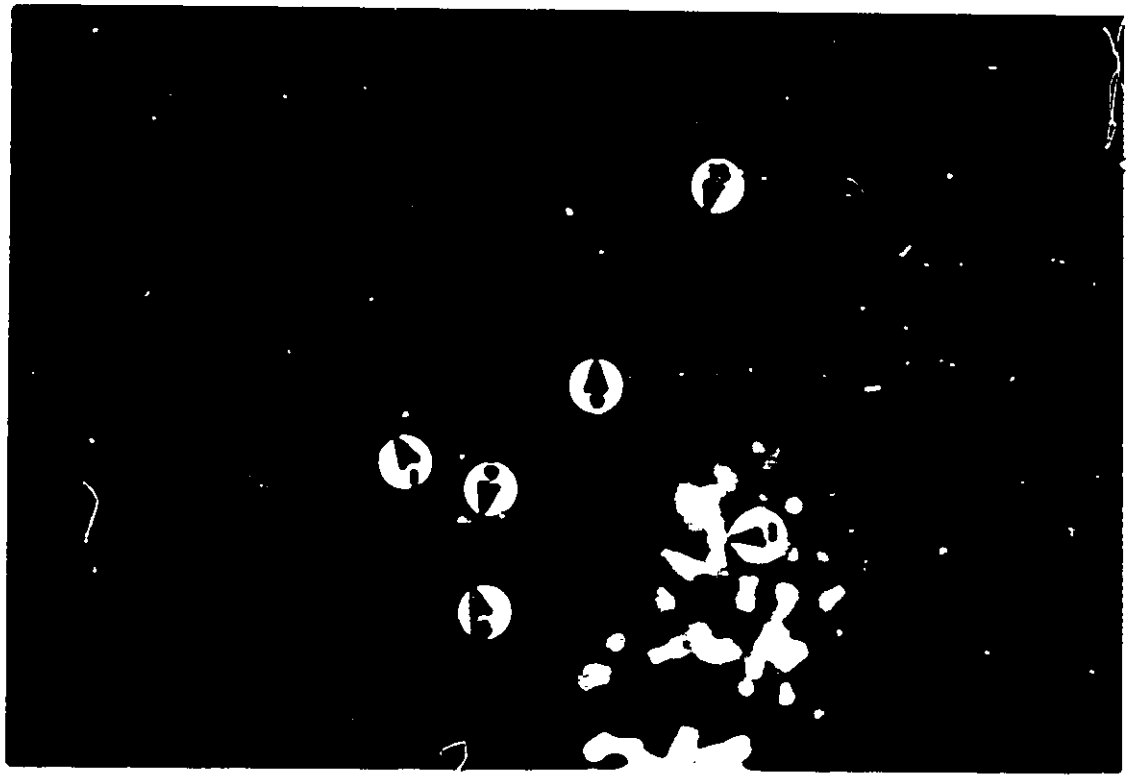

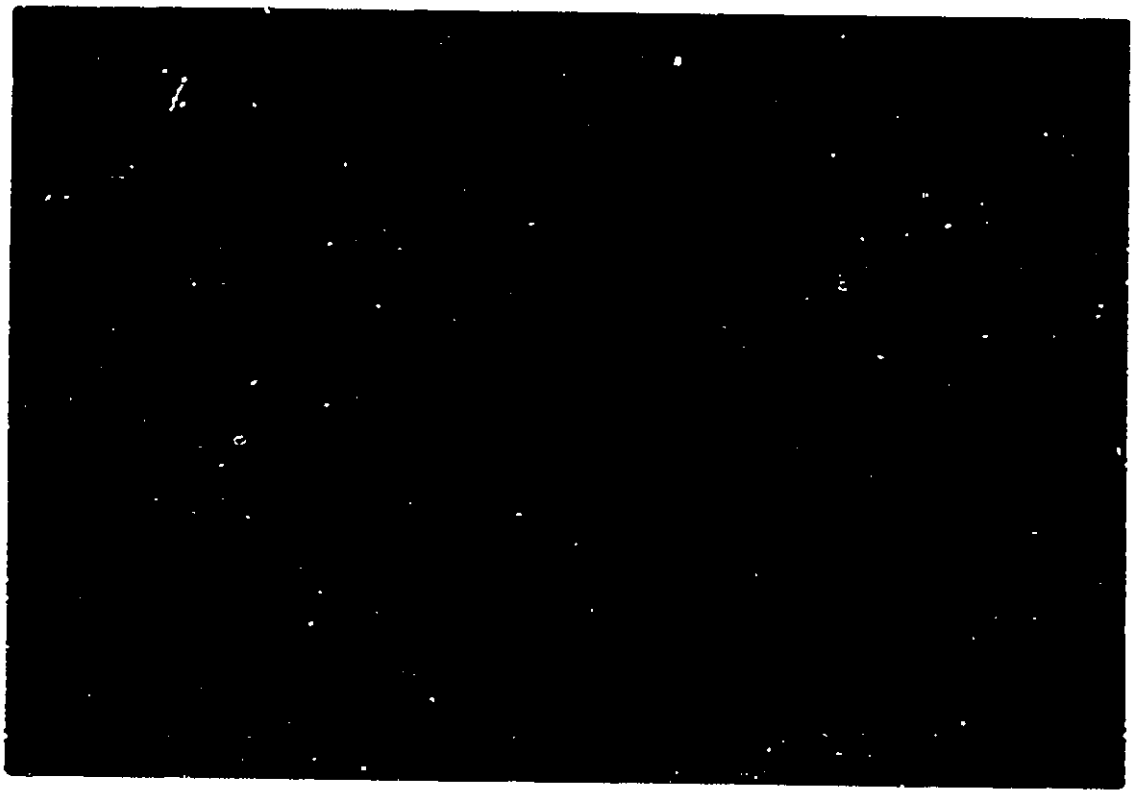

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