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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 ($\approx 3 \times 10^6$ 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. 1*C*), 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 *Hinf*I 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-

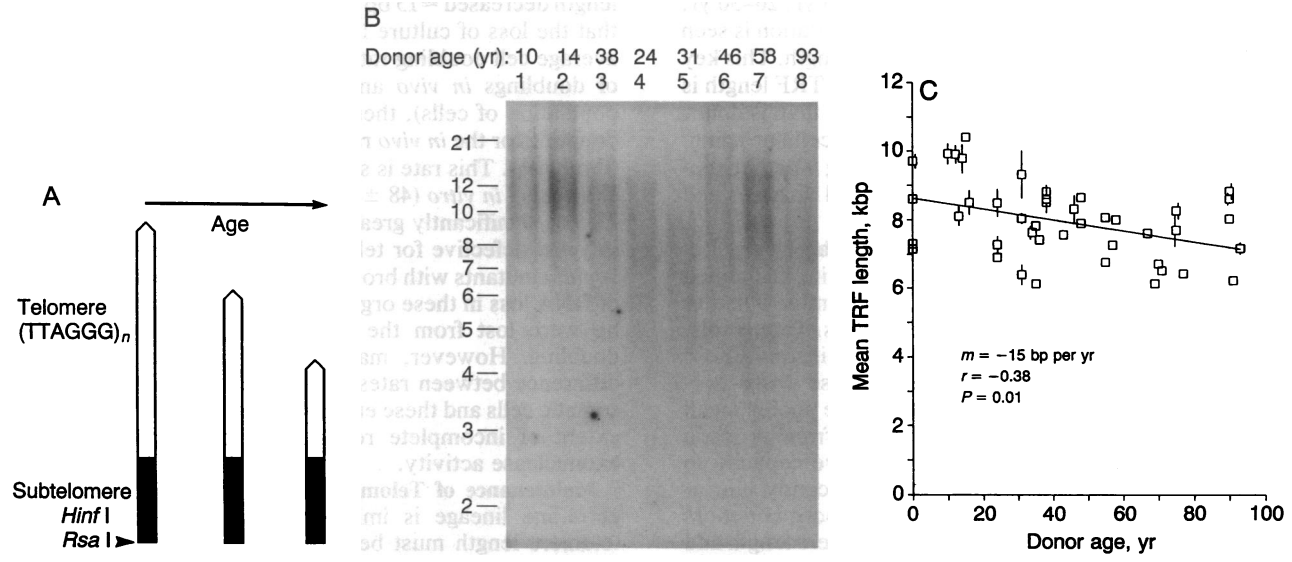

FIG. 1. Mean TRF length decreases in skin fibroblasts with increased age of human donors. (*A*) The TRF is composed of distal telomeric (TTAGGG) repeats (open bars), which hybridize to the telomeric probe, and other proximal sequences (solid bars), which do not. Arrowhead indicates most distal *Hinf*I 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.

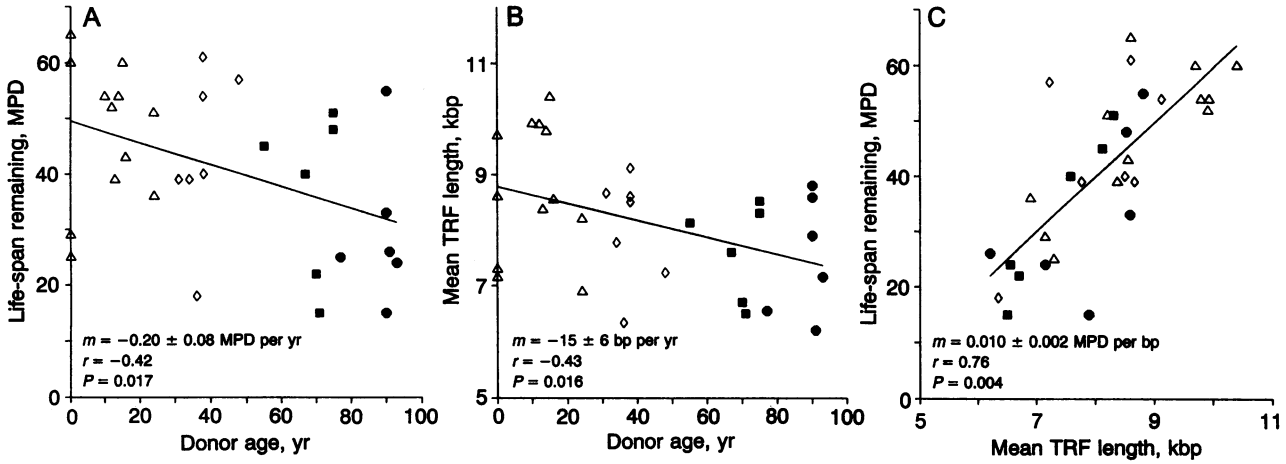

FIG. 2. Replicative capacity is proportional to TRF length. TRF length (*B*) and remaining replicative capacity (mean population doublings, MPD) after the initial 19 population doublings in culture (*A*) are shown as a function of donor age for a random subset of the strains tested in Fig. 1*C*. From 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,

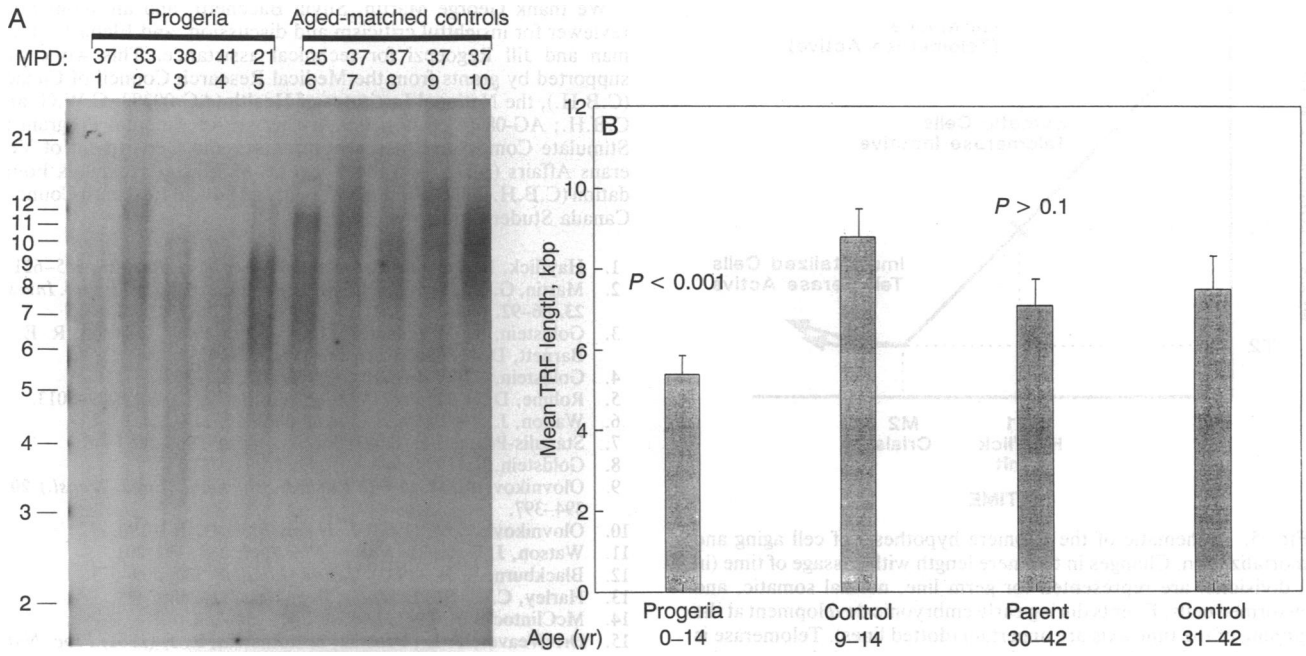

FIG. 3. Comparison of mean TRF length for fibroblast strains established from progeria donors and age-matched controls. (*A*) Genomic DNA prepared from skin fibroblast cultures established from Hutchinson–Gilford (progeria) and normal donors at the indicated MPDs was analyzed as described in Fig. 1*B*. Size and position of markers are indicated. The donor age and maximum replicative capacity of the strains in lanes 1–10 are 0, 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.

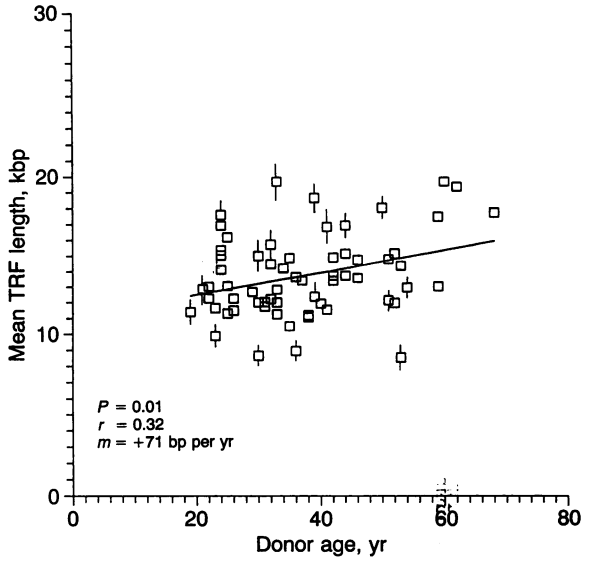

FIG. 4. Mean TRF length in sperm DNA increases as a function of donor age *in vivo*. Mean TRF length was determined for semen samples from 63 donors who ranged in age from 19 to 68 yr. SDs for points representing the mean of two or more determinations are indicated. The slope of the regression line is 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_0: 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,

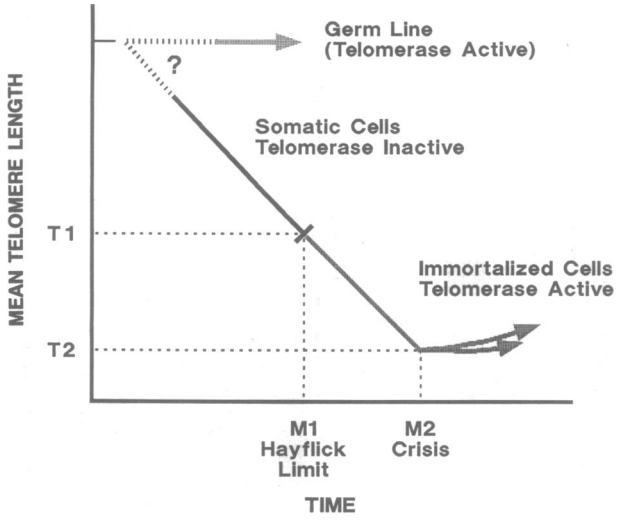

FIG. 5. Schematic of the telomere hypothesis of cell aging and immortalization. Changes in telomere length with passage of time (in cell divisions) are represented for germ line, normal somatic, and transformed cells. Events during early embryonic development at the beginning of the time axis are uncertain (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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