

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 over-

come 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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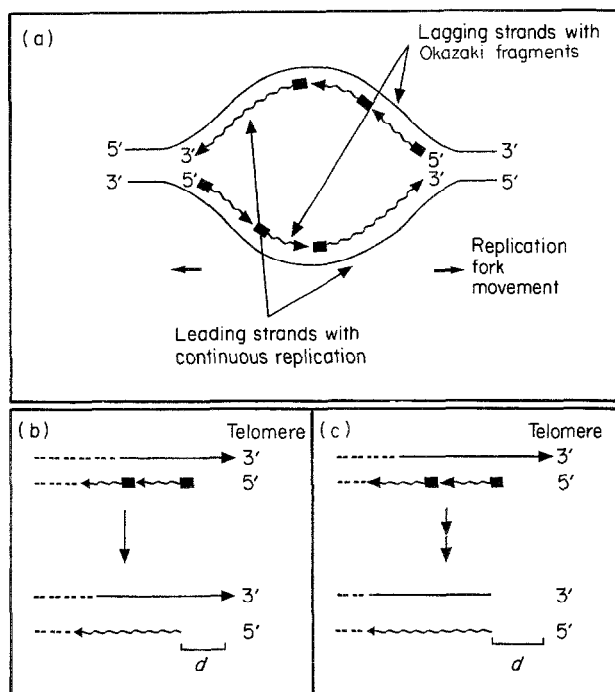


Figure 1. The end-replication problem. (a) Schematic of the DNA replication bubble at an origin of replication. The RNA primer (filled block) and newly replicated DNA (wavy lines) are shown. (b) and (c) Schematic of possible events on the lagging strand at either telomere. (b) After removal of the RNA 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 mm³ 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 *HinfI* and *RsaI*, and 1.0 µg 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 ([³²P]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, 10³ 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 $d(\text{CCCTAA})_3$ 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 $\text{TRF} = \text{T} + \text{X}$.

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

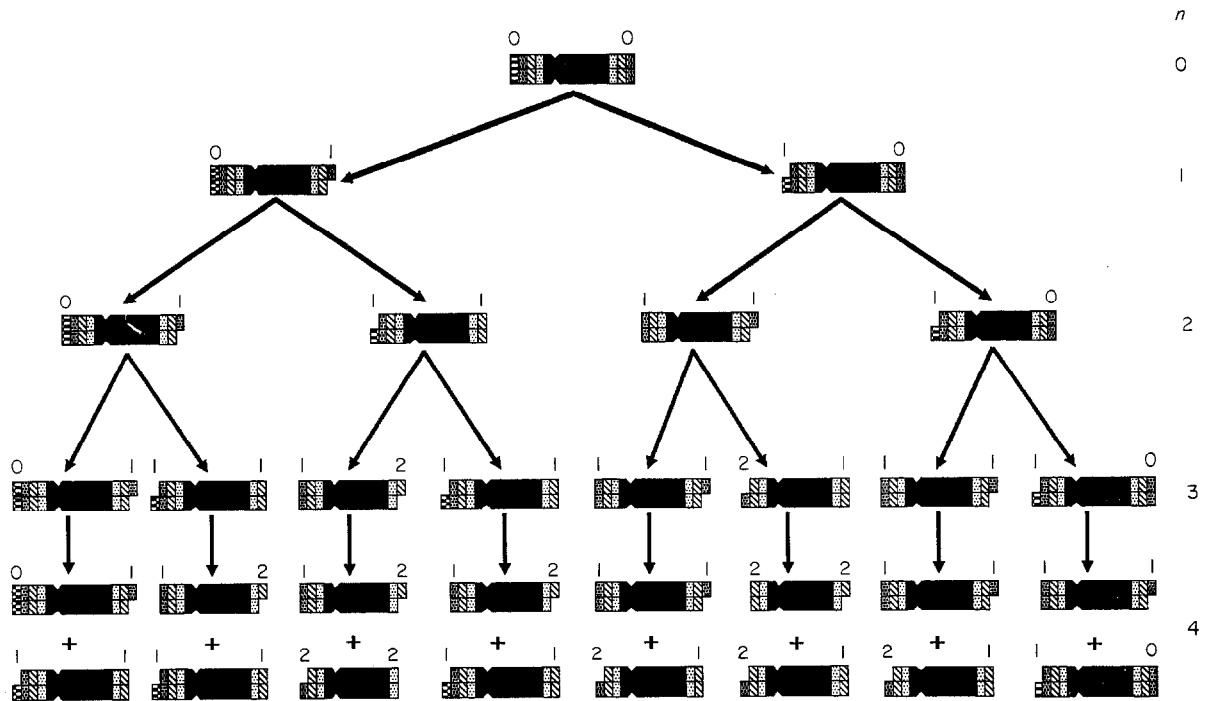


Figure 2. Lineage for a single chromosome showing telomeric deletions. Top strand of each chromosome is 5' to 3', left to right. Numbers above each telomere represent the number of deletions at that telomere. The initial telomere length on the long arm of this chromosome is arbitrarily shown shorter 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".

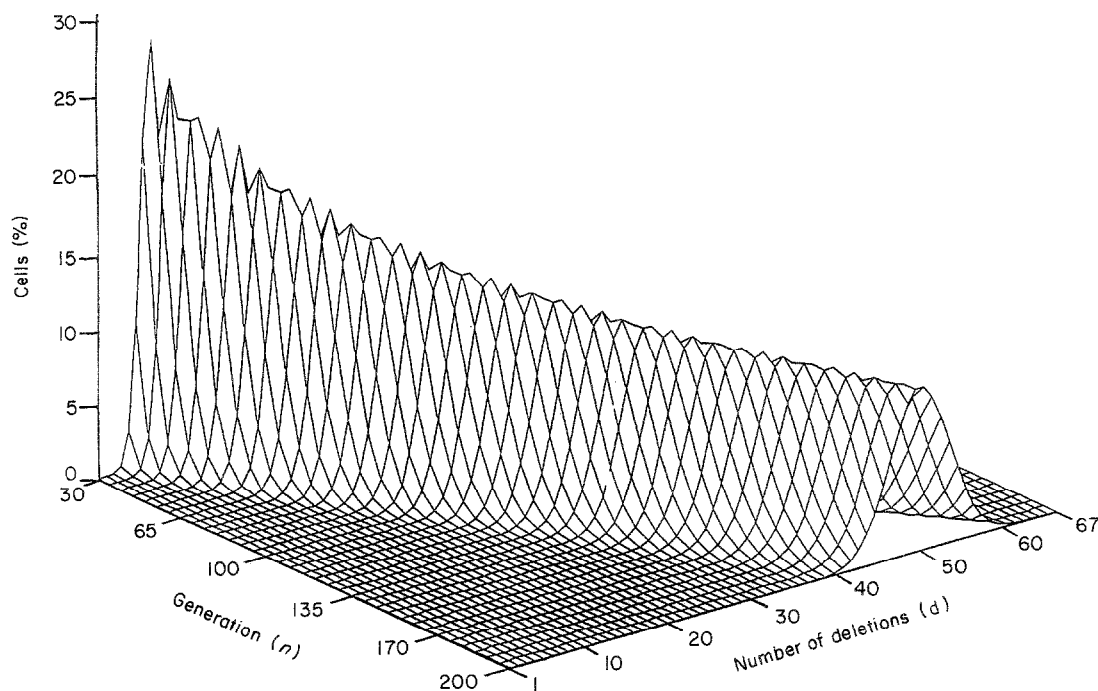


Figure 3. Theoretical distribution for the percentage of cells with d deletions on a given telomere at generation n . The area under each curve is 100%. Curves are derived from eqn (1) for $n = 30$ to 200.

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

Table 2
Distribution of deletions

Generations (n)	Deletions (d)			
	0	1	2	3
1	$\binom{2}{0}$ 1	$\binom{2}{1}$ 2		
2	$\binom{3}{0}$ 1	$\binom{3}{1}$ 3		
3	$\binom{4}{0}$ 1	$\binom{4}{1}$ 4	$\binom{4}{2}$ 6	
4	$\binom{5}{0}$ 1	$\binom{5}{1}$ 5	$\binom{5}{2}$ 10	
5	$\binom{6}{0}$ 1	$\binom{6}{1}$ 6	$\binom{6}{2}$ 15	$\binom{6}{3}$ 20

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.

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:

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

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

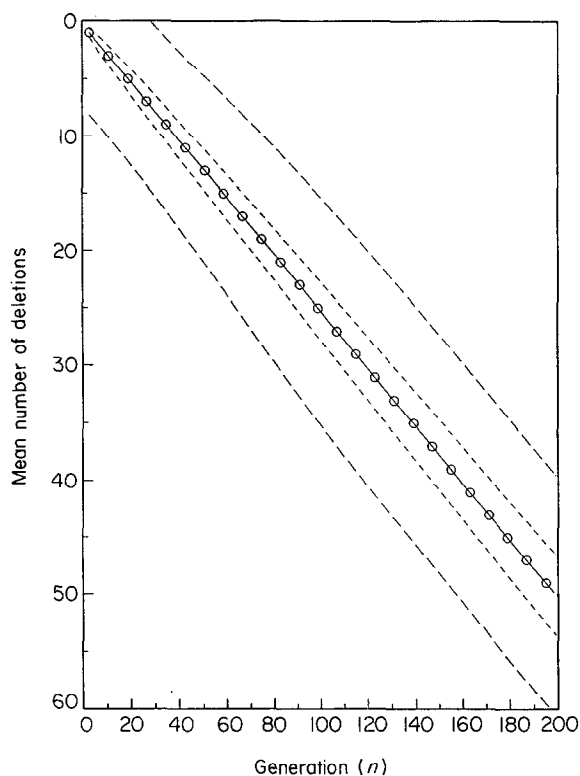


Figure 4. Mean number of telomeric deletions as a function of generation in a clonal population. Data were derived from the distribution shown in Fig. 3. Inner broken lines indicate ± 1 s.d. The outer broken lines represent the standard deviation of the distribution assuming variability in the initial 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 :

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

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:

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

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

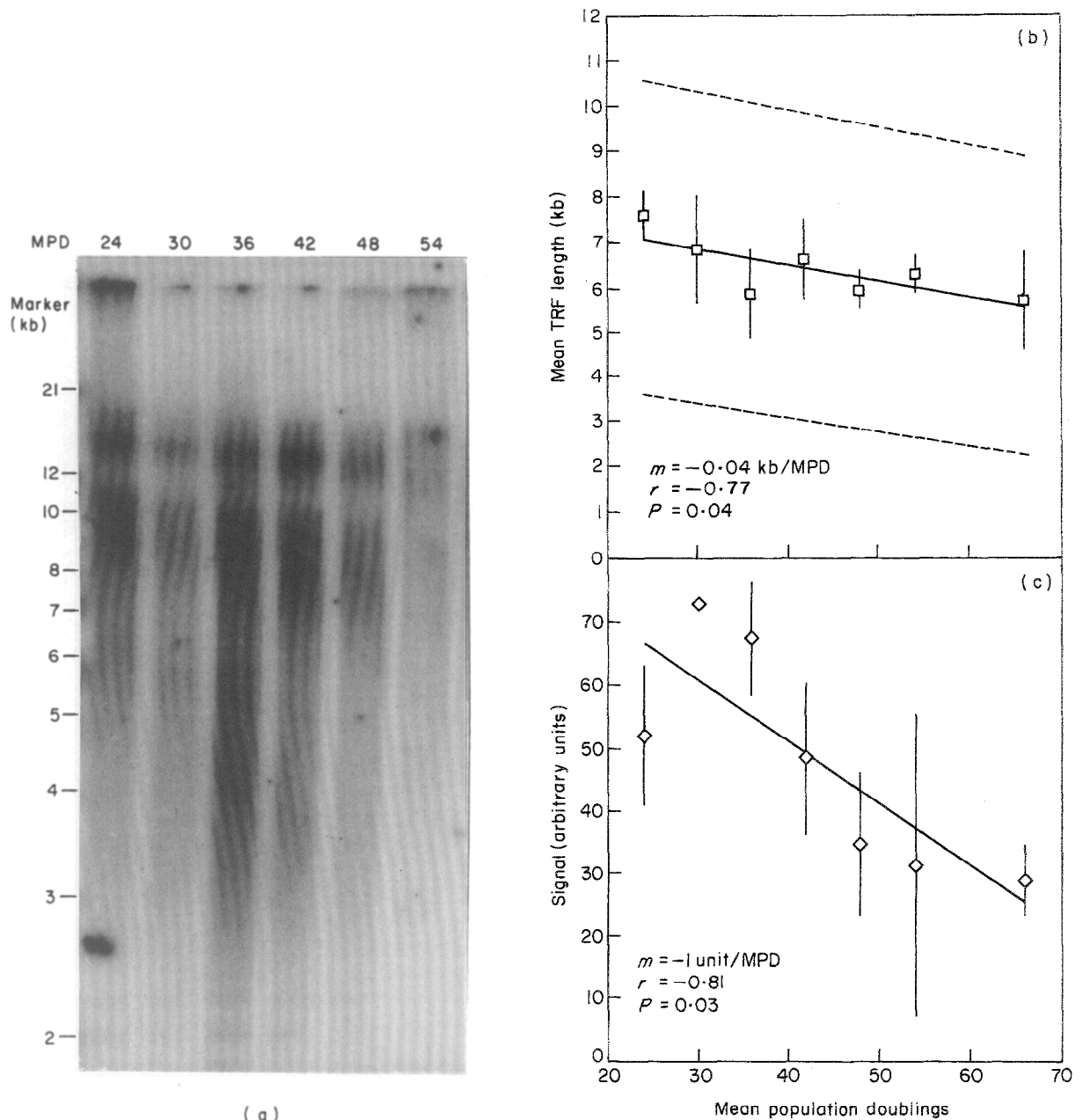


Figure 5. Loss of telomeric DNA in cultured human fibroblasts. (a) Autoradiogram showing the decrease in mean TRF length and total telomeric signal during aging *in vitro*. Cells were cultured as described (Harley *et al.*, 1990) and DNA isolated at indicated mean population doubling (MPD) levels, digested to completion with *HinfI* and *RsaI*, and quantified 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 $\text{TRF} = -0.04 \cdot \text{MPD} + 8 \text{ kb}$, with a standard error in the slope of 0.01; i.e. the TRF shrinks by $40 (\pm 10) \text{ bp/MPD}$. 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 ($S = -n + 90$) 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 $F(d_c, n)$ 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

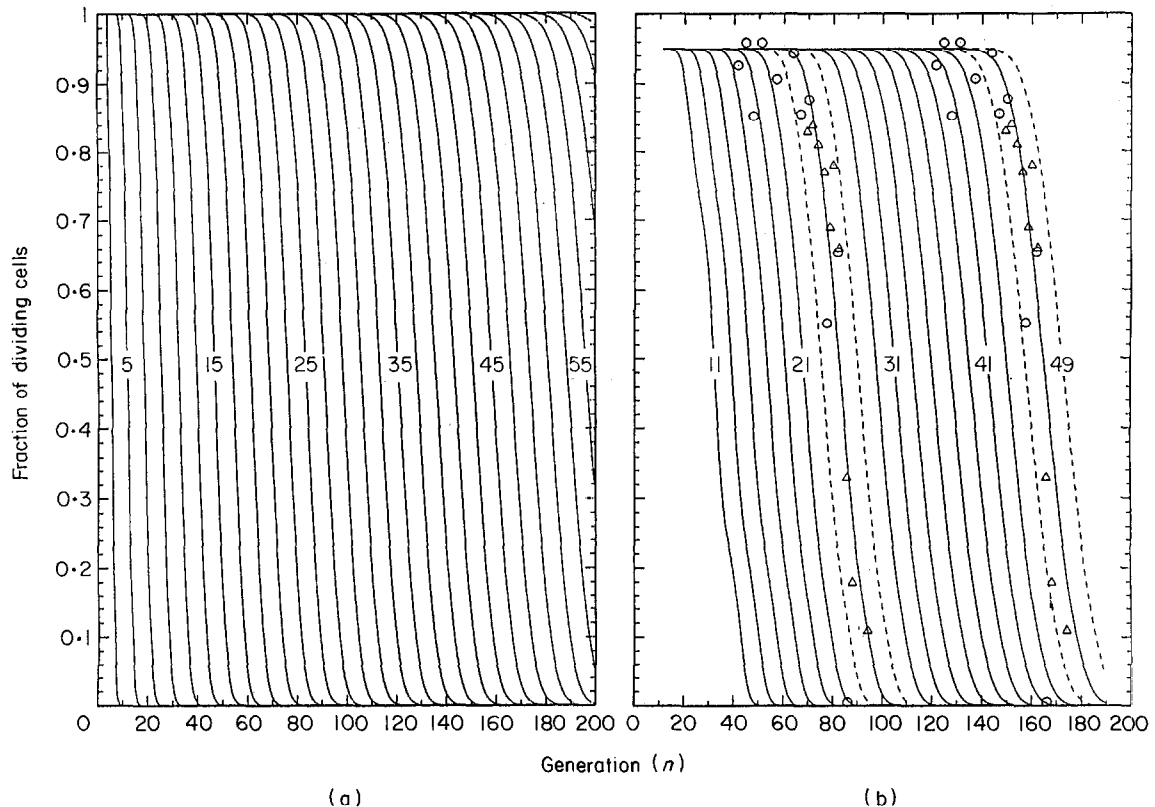


Figure 6. Fraction of dividing cells $F(d_c, n)$ as a function of generation (n) for (a) a clonal or (b) heterogeneous population of cells. Each line represents the curve obtained for a fixed critical number of deletions (d_c) as defined by eqn (3) with $k = 10$. Values of d_c are indicated on every 5th curve. In (b), the population is assumed to be heterogeneous with respect to doubling time: $F(d_c, n) = 0.95 [\frac{1}{4}F(d_c, n-10) + \frac{1}{2}F(d_c, n) + \frac{1}{4}F(d_c, n+10)]$. Experimental points (Δ) and (\circ) 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 ($23 \leq d_c \leq 27$ for the first case and $45 \leq d_c \leq 49$ 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)):

$$\text{TRF (kb)} = -0.04 \text{ (kb/division)} \\ *n \text{ (divisions)} + 8 \text{ kb}, \quad (3)$$

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

Since the slope of these two lines must be identical, we can calculate the scaling factor ($c = (-0.04)/(-1) = 0.04$) and hence the length of the non-TTAGGG portion of the TRF is:

$$\text{TRF} - \text{T} = 8 \text{ kb} - 90(0.04) \text{ kb} \approx 4 \text{ kb}. \quad (5)$$

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. \approx 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 (\approx 50 to 150 kb *versus* \approx 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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