Subsenescent Telomere Lengths in Fibroblasts Immortalized by Limiting Amounts of Telomerase*

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Human fibroblasts expressing the catalytic component of human telomerase (hTERT) have been followed for 250–400 population doublings. As expected, telomerase activity declined in long term culture of stable transfectants. Surprisingly, however, clones with average telomere lengths several kilobases shorter than those of senescent parental cells continued to proliferate. Although the longest telomeres shortened, the size of the shortest telomeres was maintained. Cells with subsenescent telomere lengths proliferated for an additional 20 doublings after inhibiting telomerase activity with a dominant-negative hTERT mutant. These results indicate that, under conditions of limiting telomerase activity, cis-acting signals may recruit telomerase to act on the shortest telomeres, argue against the hypothesis that the mortality stage 1 mechanism of cellular senescence is regulated by telomere positional effects (in which subtelomeric loci silenced by long telomeres are expressed when telomeres become short), and suggest that catalytically active telomerase is not required to provide a protein-capping role at the end of very short telomeres.

Normal human fibroblasts have a limited ability to proliferate in culture (1, 2). The use of conditionally expressed viral oncogenes led to the definition of two separate mechanisms regulating this phenomenon (3). Mortality stage 1 (M1) occurs when the functional activation of pathways requiring both p53 and pRB causes the growth arrest associated with cellular senescence (4, 5). Viral oncogenes that bind and inactivate p53 and pRB block M1 and permit continued cell division for an additional 20–40 doublings until an independent blockade to cell proliferation, the M2 mechanism, occurs. The balance of cell division and cell death at M2 (crisis) eventually tips in favor of cell death, so that the culture deteriorates and is generally lost. In human fibroblast cultures, some clones can spontaneously escape M2 and become immortal at a frequency of approximately 10−7 (6).

DNA polymerase a cannot replicate the very end of a linear chromosome (7, 8), and consequently the compensatory action of telomerase is required to maintain telomere length. Because telomerase is turned off in most human tissues during development (9) and cultured human fibroblasts lack telomerase activity (10, 11), telomeres shorten progressively with ongoing cell divisions. A causal relationship between telomere shortening and proliferative limits was firmly established by the demonstration that telomere shortening controlled M2 (12). Telomerase was repressed in hybrids between normal young fibroblasts with long telomeres and SV40 T-antigen immortalized fibroblasts whose telomeres had been experimentally manipulated to an average size of either 2.5 or 5 kb. The 20 extra population doublings obtained in the hybrids with the 5-kb starting telomere length established that telomere length was the limiting factor (12). Since T-antigen would have blocked the M1 mechanism in these hybrids, these results showed that telomere shortening controlled the onset of the M2 mechanism. The demonstration that inhibiting telomerase activity by antisense inhibition of the integral RNA component of telomerase caused proliferative failure in HeLa cells also suggested a causal relationship between telomere shortening and M2 (13). These conclusions were recently further extended by the observation that expressing an exogenous telomerase in cells infected with the viral oncoproteins that inactivate p53 and pRB prevented the occurrence of the M2 mechanism (14–16).

The ability of an exogenous telomerase to extend the lifespan of normal human diploid cells (17, 18) established that telomere shortening also controlled the onset of the M1 mechanism of cellular senescence. The current synthesis of the relationship of telomere shortening to M1 and M2 is that the repression of telomerase results in telomere shortening until the M1 mechanism occurs. There are two current hypotheses for the induction of M1. 1) One or a few of the 92 telomeres in a normal cell have shortened sufficiently so that their ends are no longer masked and they generate a DNA damage signal (19), and 2) there might be regulatory loci in subtelomeric regions that are silenced when telomeres are long but which are able to be expressed upon sufficient telomere shortening (20). If the consequent growth arrest is circumvented by blocking the actions of p53 and pRB/p16, cells can continue to proliferate and telomeres continue to shorten until they become so short that they are no longer hidden from the DNA repair apparatus and end-to-end fusions result in the M2 mechanism in which apoptosis balances cell division. Cells then escape the M2 mechanism only if they develop a method for maintaining telomeres, either through the derepression of telomerase (10) or by the
activation of an alternative pathway that probably involves recombination (21–23).

In this report, we describe the long term behavior of some of the human fibroblasts originally described as showing an extended lifespan following the introduction of an exogenous hTERT (18). Our results suggest there may be cis-acting mechanisms to preferentially recruit telomerase to maintain the shortest telomerens under conditions of limiting telomerase activity. The consequent reduction of average telomere length to sizes less than that observed in senescent cells argues against a role for telomere positional effects controlling subtelomeric loci that cause the growth arrest observed at M1. Additional observations suggest that telomerase does not have a capping function on very short telomerens that is independent of its catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Cells**—BJ normal human diploid foreskin fibroblasts expressing an hTERT cDNA were produced and maintained as described previously (18). The clones used in this study had been transfected with pZeoSV-hTERT, in which hTERT expression is driven by the SV40 promoter. The hTERT cDNA in this vector contains wild-type 5'- and 3'-untranslated regions, and gives lower levels of TRAP activity than other constructs in which an optimized Kozak sequence has been introduced and the 3'-untranslated region has been removed. The clones were subcultivated twice weekly and maintained in continuous log phase growth (were not allowed to become confluent) for the long term growth studies. Telomere Restriction Fragment Size Determination—A simplified method for isolating DNA that eliminated phenol-chloroform extractions and avoided alcohol precipitations was developed in order to avoid the large number of samples needed for this study. This also permitted DNA concentration to be calculated based on initial cell numbers, which proved much more reproducible than optical density measures. Cell pellets (fresh or frozen) were resuspended in 100 mM NaCl, 100 mM EDTA, pH 8.0, and 10 mM Tris, pH 8.0, using 30 μl per million cells. Once the cells were well dispersed, Triton X-100 (0.1% final concentration) and protease K (2 mg/ml final concentration) were added. After digesting at 55°C for 2 h and inactivating the protease K at 70°C for 30 min, the samples were dialyzed overnight against TE (10 mM Tris, pH 8.0, 1 mM EDTA). Triton X-100 rather than SDS was used so that the residual detergent present after dialysis did not inhibit restriction digestion. 100 mM EDTA was needed during the initial digestion to very rapidly inhibit nucleases in these concentrated cell suspensions. Heat inactivation of the protease K avoided the need to phenol-chloroform extract the DNA, and the residual protein present after dialysis did not inhibit enzyme digestion.

In-gel hybridization analysis of telomere restriction fragment (TRF) length was performed as described (24) with the following modifications. Agarose gels (0.7%) were denatured for 20 min in 0.5 M NaOH, rinsed in distilled water for 10 min, and then dried for 1 h at 50°C. Denaturing the DNA before rather than after drying the gel increased the signal intensity approximately 3–5-fold, presumably by permitting a much greater diffusion of the denatured DNA strands in the 0.7% agarose gel than in the very high percentage dried and rehydrated gel, and thus inhibiting the reannealing of the parental strands which would compete with the probe. Some loss of lower molecular weight DNA (particularly less than 1 kb) occurs during drying. Because most of the DNA samples did not fill the wells and were thus in the bottom half of the gel, the gels were flipped and dried with the upper surface against the filter paper support. This increased the distance between the DNA samples and the filter paper, and significantly reduced the loss of lower molecular weight DNA.

Mean TRF lengths were calculated from PhosphorImager scans of gels hybridized to kinased (TTAGGG)6 probes using the program TELORUN generously provided by C. Harley, R. Allsup, and H. Vaziri. A grid of 30 boxes was positioned over each lane, and the signal intensity and size (kb) corresponding to each box was determined. The mean TRF length was then calculated as the average of the weighted and unweighted means (20). The weighted mean assumes that there is no subtelomeric contribution to TRF length, so that the signal intensity is directly proportional to the number of repeats, which entirely determines the apparent size of the TRF. The unweighted mean assumes that there can be a substantial contribution of subtelomeric DNA, so that at a given population doubling all of the telomerens have approximately the same number of repeats regardless of their apparent migration on the gel. The signal intensity is thus not adjusted for length before determining the mean. In most cases, these two approaches give only modest differences. Because of uncertainties as to which method is more accurate, we determined both and present the average of the two values.

Telomere Fractional Size Distribution—The distribution of telomere lengths within a population of cells was determined by plotting the cumulative fraction of telomeres versus size. The signal from each of the 30 image quantitation boxes in the grid over each lane in the TRF gel was first divided by the position of each box converted to kb, so that the calculated signal intensity of, for example, a telomere with 6 kb of repeats would be the same as the signal from a telomere with 1 kb of repeats. The results were then divided by the sum of all of the normalized signals, so that each represented a fraction of the total. This simultaneously adjusts for variations in signal intensities due to different amounts of DNA actually loaded or different hybridization efficiencies/probe specific activities between gels. Finally, the results were added together starting with the smallest to obtain a cumulative fraction of telomeres that were at least a given size. The size at which the cumulative fraction equals 0.5 thus represents the median length, where 50% of the telomerens are longer and 50% are smaller than that size.

Telomerase Assays—Telomerase activity was determined using a TRAP assay kit (Intergen) as described previously (11, 26–29). In this assay, telomerase activity is determined by the PCR amplification of the ladder of 6-nucleotide extension products produced by the processive elongation of an oligonucleotide primer. An oligonucleotide with appropriate sequences at each end is included as an internal TRAP assay standard to monitor the efficiency of PCR amplification. This not only allows the identification of potential PCR inhibitors in cell extracts, it also permits a much higher degree of quantitation over a much greater range of activities between samples (26).

Retroviral Infections—A dominant negative hTERT cDNA containing the mutation D869A was subcloned into the retroviral vector pBABE-puro, and viral supernatants from the mouse amphotropic packaging cell line PA317 were used to infect human fibroblasts as described previously (35).

**RESULTS**

Stabilization of Telomere Size at Subsenescent Average Lengths—Four clones of BJ foreskin fibroblasts expressing a transfected hTERT cDNA that were previously reported to exhibit an extended lifespan (18) were followed for over 20 months of continuous culture (Fig. 1). These clones have maintained a steady growth rate and have accumulated 250–400 population doublings, compared with the approximately 60–70 doublings of the telomerase-negative control colonies (18). This extension of lifespan is so great that we now consider these
cells to be functionally immortal (30).

Telomerase activity was followed at multiple time points using the PCR-based TRAP assay (11). Activity fell progressively over the first 100 doublings following transfection, and then stabilized at relatively low levels (Fig. 2). This decrease in expression is expected for plasmid-based expression systems which are thought to become methylated over time (31). On average, activity decreased from 20–80% of the activity present in the control lung adenocarcinoma reference cell line H1299 to 1–5%. The progressive decrease in telomerase levels was accompanied by decreased telomere lengths. Fig. 3 shows TRF sizes for clone B34 between population doubling levels 82 and 302, while Fig. 2 summarizes the data for all of the four clones.

Mass cultures and clones of BJ foreskin fibroblasts both have average telomere lengths (TRF lengths) of approximately 6–8 kb when they become senescent (15, 32). These telomerase-expressing BJ cells exhibited progressive telomere shortening that eventually stabilized at an average length of only 4 kb. The first clone to develop 4-kb telomeres has maintained these short telomeres for over 150 doublings with no change in growth rate (B14; Figs. 1 and 2). Although most of the cells appeared small and elongated, some exhibited the enlargement and flattening typically seen in senescent cells. Dark blue SA-β-galactosidase staining, as observed in cells expressing a stress/senescence-associated phenotype (30, 33, 34), was seen in 2–10% of the cells. This suggests that, while the culture as a whole was capable of extended proliferation, some of the cells may have been unable to maintain their telomeres, were dropping out due to senescence, and were being overgrown by those cells able to maintain a minimally adequate telomere length.

Analysis of the telomere sizes indicates that the limiting amounts of telomerase still present in the cells after long term culture were likely to be preferentially maintaining the smallest telomeres while the larger telomeres continued to shorten, thus resulting in a decrease in average length. This is best seen if the data are replotted to show the fraction of telomeres as a function of size (see “Experimental Procedures”). At earlier population doubling levels, when average telomere sizes were about 6–8 kb, the 92 telomeres in a normal diploid cells varied in apparent size from about 1.5 kb to up to 10 kb. In contrast, when telomeres had shortened to about 4 kb, the size distribution was much narrower, ranging from about 1.5 kb to only 6 kb (Fig. 4). Importantly, there was little significant decrease in the length of the shortest telomeres, while the larger telomeres had decreased in size.

Lack of Protein Capping by Telomerase—It has recently been proposed that telomerase has a capping function independent of its maintenance of appropriate telomeric repeats at the ends of the chromosomes (14). These authors found that the average telomere size in cells expressing the papilloma virus proteins E6 and E7 and immortalized with hTERT was less than that normally found at M2/crisis. Because this effect was not found with a catalytically inactive mutant of telomerase, the authors proposed that the telomerase protein, binding to the ends of the telomeres, was providing a capping function that was dependent on catalytic activity but different from simply maintaining telomere sequences, so that this capping function was protecting the ends at sizes shorter than would normally be tolerated. This model predicts that if catalytically active telomerase was displaced by a catalytically inactive dominant-negative mutant, cells should immediately stop dividing due to displacement of the required telomerase protein cap, particularly in normal diploid cells containing perfectly normal checkpoint activities (30). To test this hypothesis, cells with subsenescent telomere lengths were infected with the dominant-negative D869A mutant, in which the aspartic acid to alanine mutation in reverse transcriptase motif C abolishes telomerase activity. Normal fibroblasts infected with this mutant do not express telomerase activity or maintain telomere length and do not show an extended lifespan (15, 35). Clones were isolated in which telomerase activity had been almost entirely abolished (Fig. 5A). Fig. 5B shows that significant additional telomere shortening had occurred during the approximately 20 doublings between the introduction of the dominant-negative mu-
FIG. 4. Maintenance of the smallest telomeres by limiting amounts of telomerase. The size distribution of telomeres in a given population of cells was calculated and expressed as a cumulative fraction versus size (see “Experimental Procedures”). The size at which the cumulative fraction equals 0.5 is the median value, in which half of the telomeres are smaller and half are larger. Data are presented for two different population doubling levels for each clone. In all four clones (A4, B14, B34, and B52), the 1.5–2-kb telomeres show very little shortening over hundreds of doublings while the longer telomeres decrease in size by many kb.

FIG. 5. Dominant-negative mutant hTERT does not block cell division in cells with very short telomeres. A, telomerase activity in infected clones. The 6-base pair elongation ladder characteristic of telomerase activity is present in the vector only B14 control cells, and absent in the lysis buffer control to which no cell extract was added. Infection of B14 cells with a retrovirus encoding an hTERT cDNA with a mutation (D869A) that eliminates catalytic activity produces clones telomeric sequences.

DISCUSSION

These results show that normal human fibroblasts expressing a transfected hTERT cDNA gradually showed reduced telomerase activity and decreasing telomere lengths. After 150–300 population doublings, the telomeres stabilized at subsenescent lengths and in some cases have remained at that size for over 150 additional doublings, and thus the cells are still functionally immortal. Analysis of these cells suggests several important interpretations. 1) The observed change in the distribution of telomere sizes implies the presence of cis-acting factors that preferentially recruit telomerase to act on the shortest telomeres; 2) the ability of cells with subsenescent telomere length to proliferate for 20 doublings following the abolition of telomerase activity argues against telomerase having a “capping” function independent of catalytic activity; and 3) the proliferation of normal cells with subsenescent telomere lengths provides evidence against the induction of growth arrest by subtelomeric regulatory loci silenced by long telomeres.

The cells used in the present study had been transfected with a plasmid-based hTERT expression vector and showed a progressive decrease in telomerase activity over time. Although the resumption of telomere shortening was thus expected, the stabilization of telomeres at lengths approximately 2–4 kb shorter than that normally observed in senescent cells was surprising. The size of the shortest telomeres was maintained in multiple different clones over many months during which the longest telomeres continued to shorten. Despite the fact that all of the telomeres were sufficiently short to be expected to provide cis-acting signals, under conditions of limiting telomerase activity the shortest telomeres were preferentially maintained. Possible explanations include a more efficient recruitment of telomerase to the shortest telomeres, and loss of cells with the shortest telomeres and selection of the survivors.

A very large number of proteins have been found to influence telomere length in yeast (reviewed in Ref. 36), and many of them are telomere-binding proteins. The most compelling evidence for cis-regulation of telomere length is for Rap1, where it has been shown that length is controlled by the number of Rap1 binding sites (37, 38). Preferential action of telomerase on the shortest telomeres has recently been demonstrated in yeast (39). Results using hTRF1, the human orthologue of Rap1, have also implicated it as a cis-acting factor influencing human telomere length control (40). Our results suggest that the cis-acting telomere-binding proteins present in normal human cells are not only able to cause telomerase to act on the telomeres, but do so in a quantitative fashion that preferentially recruits it to the shortest telomeres despite the presumed presence of signals from other very short but nonetheless longer telomeres.

An alternate interpretation is that telomerase is randomly acting on all telomeres, and that selection is producing the observed result. Cells in which telomerase acted on long but not short telomeres would become senescent and be lost from the population, while cells in which telomerase acted on short telomeres would continue to divide. When analyzing the entire population, the effect of this selection would be the apparent preservation of short telomere lengths while long telomeres shortened. Experiments in which chromosomes are broken by insertion of a plasmid with telomeric repeats on one end have
shown that the telomere on the “healed chromosome” elongates while the length of the endogenous telomeres remain unaffected (41). Under conditions in which telomerase is not limiting, this shows that in human cells telomerase can preferentially be recruited to act on a telomere that is too short. We believe that it is likely that the same mechanisms that recruit telomerase to act on these “too short” healing chromosomes would act to preferentially recruit limiting amounts of telomerase to the shortest chromosomes, and thus prefer recruitment rather than selection as an explanation for these observations.

Telomerase has been proposed to perform a capping function on short telomeres that requires catalytic activity (14). Telomerase activity became undetectable in two clones following the introduction of the D869A hTERT mutant in B14 cells. These cells with very short telomeres divided for 20 additional doublings in the presence of the mutant hTERT before undergoing a growth arrest. The telomere shortening that occurred during these 20 doublings demonstrates that catalytically active telomerase was not present for a significant fraction of time on most of the telomeres. The replacement of wild-type telomerase with the dominant-negative mutant argues against a “capping” role for the telomerase protein on short telomeres that requires catalytic activity but is independent of the actual addition of TTAGGG repeats to the ends of the chromosomes. The ability of limiting amounts of catalytically active telomerase to preferentially maintain the shortest telomeres, so that average size decreases while minimum size does not, provides a sufficient explanation for the presence of sub-senescent (this report) or sub-crisis (14) telomere lengths.

We have previously proposed that genes regulating cellular senescence might be located in subtelomeric regions, and that their expression might be controlled by changes in telomeric positional effects as telomeres shortened (20). The present results demonstrate that these cultures continue to proliferate vigorously even though telomere sizes decreased to well below their normal lengths at senescence. This provides evidence against the re-expression of previously silenced genes that induce a growth arrest when telomeres become sufficiently short, and favors the hypothesis that it is the generation of a DNA damage signal from an insufficiently long telomere(s) that causes M1 (19). Several previous reports have failed to find evidence of telomere positional effects in vertebrate cells (42, 43). Although we now think it unlikely that telomere positional effects regulate the onset of M1, we continue to entertain the possibility that telomere shortening might regulate gene expression in ways that permit the counting of cell divisions to be used as a mechanism for timing decades-long processes during the human life span (44).

The concept that short telomeres increase the efficiency with which they recruit telomerase leads to the speculation that very efficient inhibition of telomerase might be required for anti-telomerase cancer therapy to be successful. It also raises the possibility that a combination of interventions inhibiting both the catalytic activity of telomerase as well as its ability to be recruited to telomeres might be much more successful than either alone. It is important to remember that (in contrast to germline cells) adult human somatic cells are not biologically programmed to maintain telomere length, and that the expression and function of an unknown number of accessory factors may have been altered in different somatic cells that have repressed telomerase. We anticipate that the factors that modify telomerase, recruit it to the telomeres, cause it to catalyze the addition of telomeric repeats, and regulate the number of repeats added at one time will show significant variability in levels and efficiencies between different normal cell types, and that this variability will be compounded in cancer cells. The consequences of expressing telomerase in an “inappropriate” biological context, either via an exogenous cDNA or through the mutational inactivation of repressive pathways, are thus likely to be diverse as well. Disentangling these multiple mechanisms should increase our ability to alter telomere length regulation for modifying the time course of replicative aging and in the treatment of cancer.
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