Extension of Life-Span by Introduction of Telomerase into Normal Human Cells

Andrea G. Bodnar,* Michel Ouellette,* Maria Frolkis, Shawn E. Holt, Choy-Pik Chiu, Gregg B. Morin, Calvin B. Harley, Jerry W. Shay, Serge Lichtsteiner,† Woodring E. Wright†

Normal human cells undergo a finite number of cell divisions and ultimately enter a nondividing state called replicative senescence. It has been proposed that telomere shortening is the molecular clock that triggers senescence. To test this hypothesis, two telomerase-negative normal human cell types, retinal pigment epithelial cells and foreskin fibroblasts, were transfected with vectors encoding the human telomerase catalytic subunit. In contrast to telomerase-negative control clones, which exhibited telomere shortening and senescence, telomerase-expressing clones had elongated telomeres, divided vigorously, and showed reduced staining for β-galactosidase, a biomarker for senescence. Notably, the telomerase-expressing clones have a normal karyotype and have already exceeded their normal life-span by at least 20 doublings, thus establishing a causal relationship between telomere shortening and in vitro cellular senescence. The ability to maintain normal human cells in a phenotypically youthful state could have important applications in research and medicine.

Normal human diploid cells placed in culture have a finite proliferative life-span and enter a nondividing state termed senescence, which is characterized by altered gene expression (*1, 2*). Replicative senescence is dependent upon cumulative cell divisions and not chronologic or metabolic time, indicating that proliferation is limited by a "mitotic clock" (*3*). The reduction in proliferative capacity of cells from old donors and patients with premature aging syndromes (*4*), and the accumulation in vivo of senescent cells with altered patterns of gene expression (*5, 6*), implicate cellular senescence in aging and age-related pathologies (*1, 2*).

Telomere loss is thought to control entry into senescence (*7–10*). Human telomeres consist of repeats of the sequence TTAGGG/CCCTAA at chromosome ends; these repeats are synthesized by the ribonucleoprotein enzyme telomerase (*11, 12*). Telomerase is active in germline cells and, in humans, telomeres in these cells are maintained at about 15 kilobase pairs (kbp). In contrast, telomerase is not expressed in most human somatic tissues (*13, 14*), and telomere length is significantly shorter (*15*). The telomere hypothesis of cellular aging (*16*) proposes that cells become senescent when progressive telomere shortening during each division produces a threshold telomere length.

The human telomerase reverse transcriptase subunit (hTRT) has been cloned (*17*). We recently demonstrated that telomerase activity can be reconstituted by transient expression of hTRT in normal human diploid cells, which express low levels of the template RNA component of telomerase (hTR) but do not express hTRT (*18*). This provided the opportunity to manipulate telomere length and test the hypothesis that telomere shortening causes cellular senescence.

Introduction of telomerase into normal human cells. To determine if telomerase expression increases cell life-span, we transfected hTRT$^-$ normal cells with two different hTRT expression constructs. One construct was engineered for increased translational efficiency by removal of the 5′ and 3′ untranslated regions of hTRT and creation of a Kozak consensus sequence. This engineered hTRT cDNA was cloned downstream of the MPSV promoter (*19*). The second construct consisted of the complete (native) hTRT cDNA cloned downstream of the SV40 promoter in pZeoSV (*19*). In the first experiments, we compared the life-span of stable clones transfected with MPSV-hTRT versus "vector only" clones, and in the second, we compared the life-span of activity-positive and activity-negative stable clones containing integrated SV40-hTRT constructs.

hTRT$^-$ normal retinal pigment epithelial cells (RPE-340) were transfected with the MPSV-hTRT vector at population doubling (PD) 37, and 27 of the 39 resultant stable clones (69%) expressed telomerase activity. BJ foreskin fibroblasts were transfected with the MPSV-hTRT vector at PD 58, and 3 of the 22 stable clones (14%) expressed telomerase activity. Reverse transcriptase–polymerase chain reaction experiments demonstrated that the hTRT mRNA originated from the transfected cDNA and not the endogenous gene (*20*). Telomerase activity, measured relative to that in the lung cancer–derived human cell line H1299, ranged from 65 to 360% in the RPE clones (Fig. 1) and 86 to 95% in the BJ clones. This range of telomerase activity is similar to that observed for tumor cell lines (*13*). Thirty-three RPE clones and 24 BJ clones transfected with the control plasmid were also isolated; RPE clones that generated sufficient cells for the TRAP assay (n = 15) (Fig. 1) and control BJ clones (n = 15)

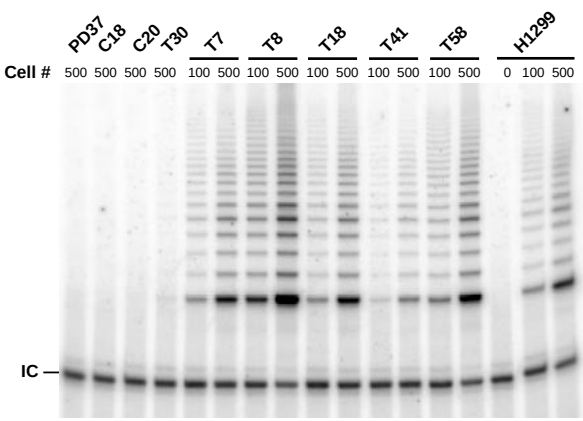

Fig. 1. Telomerase activity in stable RPE clones. Stable human RPE clones obtained by transfection with a control vector (clone numbers prefixed with "C") or with a vector expressing the hTRT cDNA ("T" clones) were analyzed for telomerase activity by the TRAP assay (*19*). "PD37" represents the cell population at the time of transfection. The number of cells assayed for each clone is indicated above each lane. "IC" is the internal control in the TRAP assay. The positive control was the telomerase activity extracted from H1299 cells (*20*).

A. G. Bodnar, M. Frolkis, C.-P. Chiu, G. B. Morin, C. B. Harley, and S. Lichtsteiner are at Geron Corporation, 230 Constitution Drive, Menlo Park, CA 94025, USA. M. Ouellette, S. E. Holt, J. W. Shay, and W. E. Wright are in the Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235–9039, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: slichtste@geron.com; wright@utsw.swmed.edu

were negative for telomerase activity. BJ fibroblasts were also transfected with the pZeoSV-hTRT construct at PD 44. Six of 76 clones (8%) expressed telomerase activity ranging from 10 to 30% of that in the reference H1299 cell line. As assessed by a ribonuclease protection assay, hTRT mRNA was undetectable in activity-negative BJ cells but readily observed in $hTRT^+$ clones (*20*).

Telomere lengthening in $hTRT^+$ normal cells. We then measured telomere lengths to determine if the hTRT-reconstituted telomerase acts on its normal chromosomal substrate (*21*). Telomeres in the $hTRT^-$ cells decreased by 0.4 to 1.3 kbp (Fig. 2), comparable to the shortening seen in mass cultures at equivalent PDs, whereas telomeres in the $hTRT^+$ RPE and BJ clones transfected with the MPSV-hTRT vector increased by 3.7 kbp (± 1.4 kbp, $n = 26$) and 7.1 kbp (± 0.3 kbp, $n = 3$), respectively. Telomeres in six $hTRT^+$ clones transfected with the pZeoSV-hTRT vector, increased by 0.4 kbp (± 0.3 kbp, $n = 6$). Because two $hTRT^+$ clones expressing only 5 to 7% relative telomerase activity (RPE clone T30 and BJ clone B13) did not maintain telomere length, they were considered to be functionally $hTRT^-$ (Fig. 2B). These results demonstrate that hTRT-reconstituted telomerase extends the endogenous telomeres in a normal cell.

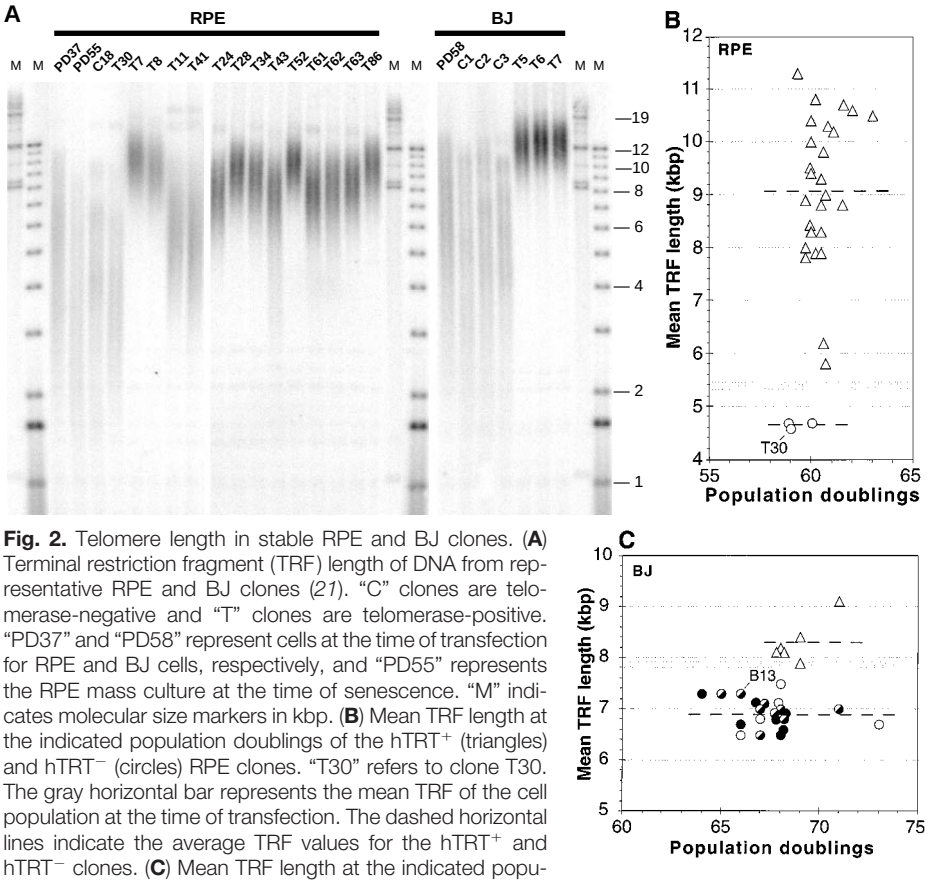

Fig. 2. Telomere length in stable RPE and BJ clones. (**A**) Terminal restriction fragment (TRF) length of DNA from representative RPE and BJ clones (*21*). "C" clones are telomerase-negative and "T" clones are telomerase-positive. "PD37" and "PD58" represent cells at the time of transfection for RPE and BJ cells, respectively, and "PD55" represents the RPE mass culture at the time of senescence. "M" indicates molecular size markers in kbp. (**B**) Mean TRF length at the indicated population doublings of the $hTRT^+$ (triangles) and $hTRT^-$ (circles) RPE clones. "T30" refers to clone T30. The gray horizontal bar represents the mean TRF of the cell population at the time of transfection. The dashed horizontal lines indicate the average TRF values for the $hTRT^+$ and $hTRT^-$ clones. (**C**) Mean TRF length at the indicated population doublings of the BJ clones transfected with pZeoSV-hTRT; designations are as in (B). "B13" refers to clone B13. Closed symbols represent cells that senesced; half-filled symbols correspond to cells near senescence (dividing less than once per week).

Life-span, karyotype, and phenotype. To investigate the effect of telomerase expression on the life-span of normal cells, we compared the growth of $hTRT^+$ and $hTRT^-$ clones. $hTRT^-$ RPE clones showed the expected slowing of growth that is associated with aging in vitro, and 30 out of 33 senesced (*22*) by an age typical for mass RPE cultures (Fig. 3). In contrast, $hTRT^+$ RPE clones transfected with MPSV-hTRT exceeded the mean life-span of the $hTRT^-$ clones by ~20 doublings ($P < 10^{-24}$; Student's T test). These clones have exceeded the maximal RPE life-span (PD 55 to 57), and continue to divide at the rate of young RPEs (Fig. 3). Similarly, most of the $hTRT^-$ BJ fibroblast clones senesced or are near senescent (64 of 70 clones), whereas all six of the $hTRT^+$ clones transfected with the pZeoSV-hTRT vector exceeded the maximal BJ life-span (85 to 90 PD) (Fig. 3). The average PD of these six rapidly dividing $hTRT^+$ clones is already 36 doublings beyond the average life-span of the 70 $hTRT^-$ clones ($P < 10^{-6}$). Similar results were obtained with human vascular endothelial cells (*23*). Thus, expression of functional hTRT in normal cells extends their life-span.

Senescence-associated β-galactosidase (SA–β-Gal) is an established biomarker associated with cellular aging (*6*). We stained $hTRT^-$ RPE clones at or near senescence and compared the level of SA–β-Gal staining to that in $hTRT^+$ clones that had undergone a similar or greater number of cell divisions (Fig. 4, A and B). A majority of the cells in the $hTRT^-$ clones showed strong staining; by contrast, few of the cells in $hTRT^+$ clones at equivalent or greater PD showed staining. The cells of the $hTRT^-$ clones that had stopped dividing exhibited SA–β-Gal staining levels equivalent to that observed in senescent mass

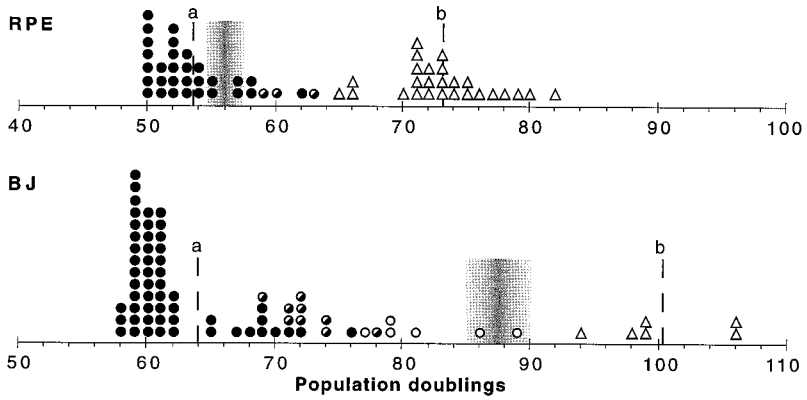

Fig. 3. Effect of telomerase expression on cell life-span. The proliferative status of each RPE (upper panel) and BJ (lower panel; pZeoSV-hTRT experiment) clone is shown. The $hTRT^+$ clones (triangles) and the $hTRT^-$ clones (circles) are plotted (*35*). Closed symbols represent senescent clones (dividing less than once per 2 weeks); half-filled symbols correspond to cells near senescence (dividing less than once per week); open symbols represent clones dividing more than once per week. The shaded vertical area indicates the typical PD range where the mass population of cells senesce. Dashed vertical lines represent the mean PD of: (a) the $hTRT^-$ and (b) the $hTRT^+$ clones.

cultures. Their large size and increased ratio of cytoplasm:nucleus also indicates that the clones had senesced (Fig. 4A). The remainder of the slowly dividing hTRT$^-$ clones exhibited SA–β-Gal staining typical of cells close to senescence. The same result was found for fibroblasts: Six of six hTRT$^+$ clones showed low levels of staining typical of young fibroblast cultures, whereas all of the hTRT$^-$ clones showed elevated SA–β-Gal staining (Fig. 4C). Detailed G-banding of two hTRT$^+$ RPE clones and two hTRT$^+$ BJ clones revealed that the cells had the normal complement of 46 chromosomes and no abnormalities (*24*). hTRT$^+$ cells with an extended life-span therefore appear to have a normal karyotype and phenotype similar to young cells.

Implications. Our results indicate that telomere loss in the absence of telomerase is the intrinsic timing mechanism that controls the number of cell divisions prior to senescence. The long-term effects of exogenous telomerase expression on telomere maintenance and the life-span of these cells remain to be determined in studies of longer duration.

Telomere homeostasis is likely to result from a balance of lengthening and shortening activities. Although certain proteins in yeast are thought to facilitate the interaction of telomerase with the telomere (*25*), our results indicate that if analogous mammalian factors are required, they are already present in hTRT$^-$ human cells. The telomerase catalytic subunit produces the lengthening activity, but other factors including telomere binding proteins such as hTRF-1 and -2 (*26*) might be involved in establishing a telomere length equilibrium. Very low levels of telomerase activity, such as that exhibited by RPE clone T30 and BJ clone B13, are apparently insufficient to prevent telomere shortening. This is consistent with the observation that stem cells have low but detectable telomerase activity, yet continue to exhibit shortening of their telomeres throughout life (*27*). Thus, we believe that a threshold level of telomerase activity is required for life-span extension. Promoter strength, structure of untranslated regions, site of integration, levels of hTR and hTRT, and telomere- or telomerase-associated proteins in specific cell types are all factors that may affect the functional level of telomerase. This hypothesis is supported by our finding that hTRT$^+$ clones derived from different cell types and transfected with different vectors showed marked differences in telomere lengths.

Certain stem cells or germline populations are telomerase positive (*13*, *27*, *28*) and have long or indefinite life-spans, illustrating that telomerase expression per se is not oncogenic. Cellular transformation with viral oncoproteins can also extend cell life-span, but through mechanisms that reduce checkpoint control, increase genomic instability, and fail to prevent telomere loss (*29*, *30*). We have not observed any gross phenotypic or morphological characteristics of transformed cells (such as loss of contact inhibition or growth in low serum) that might account for the extended proliferative capacity of the hTRT$^+$ cells. The normal karyotype and the absolute correlation between extended life-span and telomerase activity suggest that stochastic mutagenesis does not account for the life-span extension.

Cellular senescence is believed to contribute to multiple conditions in the elderly

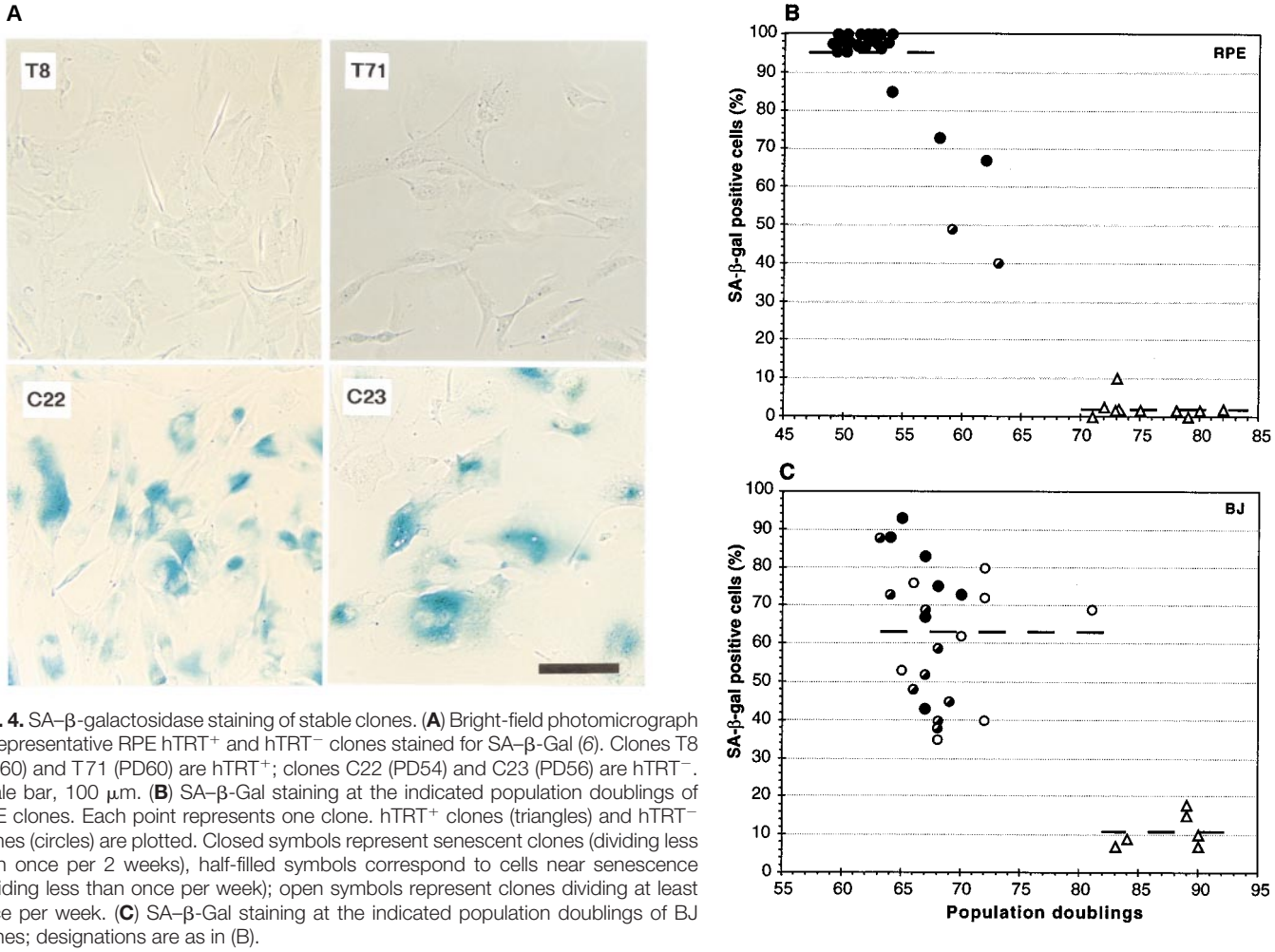

Fig. 4. SA–β-galactosidase staining of stable clones. (**A**) Bright-field photomicrograph of representative RPE hTRT$^+$ and hTRT$^-$ clones stained for SA–β-Gal (*6*). Clones T8 (PD60) and T71 (PD60) are hTRT$^+$; clones C22 (PD54) and C23 (PD56) are hTRT$^-$. Scale bar, 100 μm. (**B**) SA–β-Gal staining at the indicated population doublings of RPE clones. Each point represents one clone. hTRT$^+$ clones (triangles) and hTRT$^-$ clones (circles) are plotted. Closed symbols represent senescent clones (dividing less than once per 2 weeks), half-filled symbols correspond to cells near senescence (dividing less than once per week); open symbols represent clones dividing at least once per week. (**C**) SA–β-Gal staining at the indicated population doublings of BJ clones; designations are as in (B).

that could in principle be remedied by cell life-span extension in situ. Examples include atrophy of the skin through loss of extracellular matrix homeostasis in dermal fibroblasts (*31*); age-related macular degeneration caused by accumulation of lipofuscin and downregulation of a neuronal survival factor in RPE cells (*32*); and atherosclerosis caused by loss of proliferative capacity and overexpression of hypertensive and thrombotic factors in endothelial cells (*9, 33*). Extended life-span cells also have potential applications ex vivo. Cloned normal diploid cells could replace established tumor cell lines in studies of biochemical and physiological aspects of growth and differentiation; long-lived normal human cells could be used for the production of normal or engineered biotechnology products; and expanded populations of normal or genetically engineered rejuvenated cells could be used for autologous or allogeneic cell and gene therapy. Thus the ability to extend cellular life-span, while maintaining the diploid status, growth characteristics, and gene expression pattern typical of young normal cells, has important implications for biological research, the pharmaceutical industry, and medicine.

Note added in proof: As of the time of galley proofs, virtually all of the $hTRT^-$ clones were senescent or near senescent, whereas all of the $hTRT^+$ clones continued to divide rapidly.

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19. RPE-340 cells and BJ fibroblasts were cultured as previously described (*18*). In one set of experiments, RPE and BJ cells were subjected to electroporation with control vector (pBBS212) or vector encoding hTRT with a consensus Kozak sequence downstream of the myeloproliferative sarcoma virus (MPSV) promoter (pGRN145) (*18*). After 48 hours, transfected cells were placed into medium containing Hygromycin-B (50 μg/ml) for 2 to 3 weeks, at which time the concentration was reduced to 10 μg/ml. Individual stable clones were selected and analyzed for telomerase activity by the telomeric repeat amplification protocol (TRAP) (*13, 18*). In a separate experiment, BJ fibroblasts were transfected with pZeoSV-hTRT, a derivative of pZeoSV (Invitrogen, Carlsbad, CA) encoding hTRT downstream of the simian virus 40 (SV40) promoter. After electroporation, the BJ cells were cultured in zeocin (200 μg/ml). $hTRT^+$ and $hTRT^-$ clones from each transfection were obtained and expanded.
20. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed on cells transfected with pGRN145. Endogenous hTRT mRNA was detected with the primer set RA58 (5′-GGCTGAAGTGTCACAG-3′) and hTRT3′UTR (5′-GGCTGCTGGTGTCTGCTCTCGGCC-3′). Exogenous hTRT mRNA was detected with the primer set RA58 and RA55 (5′-TCCGCACGTGAGAAT-3′). RT-PCR showed that the hTRT mRNA derived from the transfected cDNA, but not the endogenous hTRT mRNA, was present in telomerase-positive RPE ($n = 4$) and BJ ($n = 3$) clones (*34*). Ribonuclease protection assays were performed on BJ cells transfected with pZeoSV-hTRT and on H1299 cells (NCI-H1299, American Type Culture Collection). The probe corresponded to hTRT sequences spanning amino acids 541 to 647. The abundance of the catalytic subunit was comparable to that in H1299 cells (*34*).
21. DNA isolation and TRF analyses for the RPE and BJ clones were performed essentially as described (*7*), except that in some cases the DNA was resolved on 0.6% agarose gels and electroblotted to nylon membranes (*18*), and for mean TRF calculations, the average of the weighted and unweighted means was used [M. Levy, R. C. Allsopp, A. B. Futcher, C. W. Greider, C. B. Harley, *J. Mol. Biol.* **225**, 951 (1992)].
22. Senescence is defined as less than one PD in 2 weeks; near-senescence is defined as less than one PD per week. Young BJ and RPE cells typically double in 1 to 2 days.
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35. To accumulate doublings as rapidly as possible, we shifted all six $hTRT^+$ BJ clones and the six fastest growing $hTRT^-$ BJ clones from 10% to 20% serum and maintained them in continuous log growth as of PD 66 to 78 ($hTRT^-$) or PD 74 to 80 ($hTRT^+$). Neither increased serum nor exponential growth conditions significantly extends life-span [T. Ohno, *Mech. Ageing Devel.* **11**, 179 (1979); J. R. Smith and K. I. Braunschweiger, *J. Cell Physiol.* **98**, 597 (1979)] particularly if instituted near the proliferative limit of the culture.

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