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Telomerase Maintains Telomere Structure in Normal Human Cells

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Summary

In normal human cells, telomeres shorten with successive rounds of cell division, and immortalization correlates with stabilization of telomere length. These observations suggest that human cancer cells achieve immortalization in large part through the illegitimate activation of telomerase expression. Here, we demonstrate that the rate-limiting telomerase catalytic subunit hTERT is expressed in cycling primary presenescent human fibroblasts, previously believed to lack hTERT expression and telomerase activity. Disruption of telomerase activity in normal human cells slows cell proliferation, restricts cell lifespan, and alters the maintenance of the 3′ single-stranded telomeric overhang without changing the rate of overall telomere shortening. Together, these observations support the view that telomerase and telomere structure are dynamically regulated in normal human cells and that telomere length alone is unlikely to trigger entry into replicative senescence.

Introduction

Eukaryotic chromosomes terminate in nucleoprotein complexes termed telomeres, composed of arrays of G rich sequences and telomere binding proteins (Blackburn, 2001). Loss of telomeres leads to end-to-end chromosomal fusions, facilitates increased genetic recombination, and triggers cell death through apoptosis (Counter et al., 1992; Blasco et al., 1997; Artandi et al., 2002). In mammalian cells, telomeres contain several thousand double-stranded repeats of the sequence TTAGGG and terminate with a single-stranded 3′ extension of the G rich strand. This 3′ overhang, together with specific telomere binding proteins, participates in forming a terminal loop structure termed the T loop (Griffith et al., 1999).

Telomeres are synthesized by telomerase, an enzyme composed of RNA and catalytic protein subunits called hTERC and hTERT, respectively, in humans (Feng et al., 1995; Nakamura and Cech, 1998). Telomerase, acting as a reverse transcriptase, produces telomeric repeats using a template provided by hTERC (Greider and Blackburn, 1989). In the absence of telomerase—a state reported in most types of human cells—telomeric DNA erodes progressively with each round of cell division (Harley et al., 1990). In contrast, the majority of nonmalignant, immortalized human cells and human cancer cells express detectable telomerase activity and exhibit stable telomere lengths upon extended propagation in culture (Kim et al., 1994). This enzymatic activity correlates with hTERT expression, implicating this catalytic subunit as the rate-limiting component of the telomerase holoenzyme (Nakamura and Cech, 1998).

Together, these observations serve as the basis of a model that predicts that telomeres function as a molecular counting device that registers cell divisions and triggers a proliferative arrest when telomeres erode to specific lengths (Harley et al., 1994; Wright and Shay, 2002). Consistent with this hypothesis, ectopic expression of hTERT in normal human cells confers telomerase activity, stabilizes telomere lengths, and directly immortalizes some of these hTERT-expressing cells (Bodnar et al., 1998).

Several lines of experimentation suggest, however, that the control of replicative capacity in human cells is subject to a more complex regulatory program (Blackburn, 2001). Indeed, two barriers limit the lifespan of cultured human cells. After explantation, most primary human cells proliferate for a limited number of cell divisions and then enter replicative senescence, a state characterized by continued cell viability without further cell division (Wei and Sedivy, 1999). Concomitant inactivation of the retinoblastoma and p53 pathways by expression of viral oncoproteins such as the simian virus 40 large T antigen (SV40 LT) or by other means permits human cells to bypass replicative senescence (Shay and Wright, 1989; Hara et al., 1991). Such postsenescent cells proliferate until they encounter a second barrier-termed crisis, a state characterized by widespread apoptosis (Wei and Sedivy, 1999).

Although telomeres shorten progressively as cells approach replicative senescence (Harley et al., 1990), only at crisis do telomeres reach lengths that fail to provide protection to chromosome ends (Counter et al., 1992).

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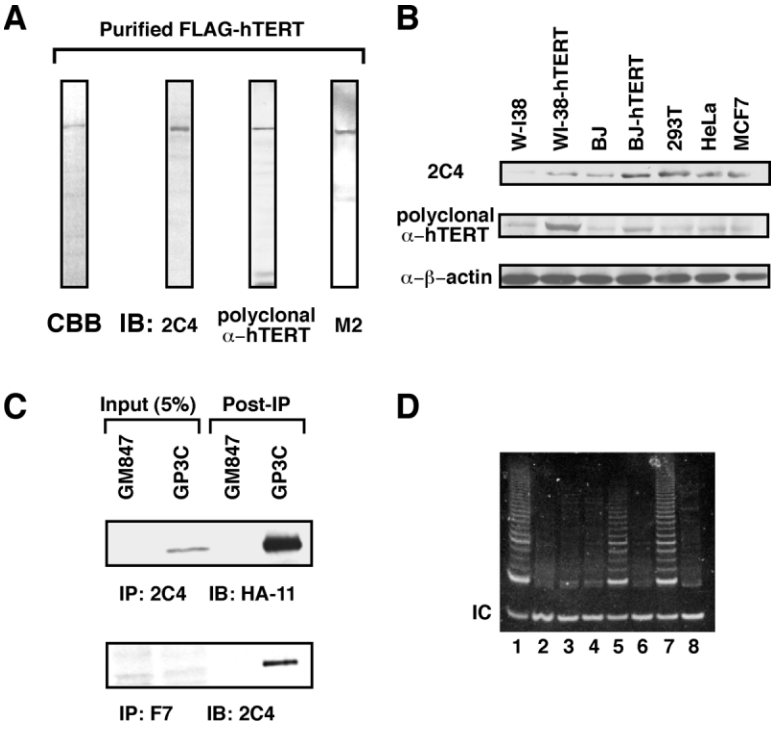


Figure 1. Characterization of an Anti-hTERT mAb (2C4)

(A) Purified FLAG-hTERT was fractionated by SDS-PAGE and stained with Coomassie brilliant blue (CBB) or detected by immunoblotting with the 2C4 mAb or a polyclonal anti-hTERT antibody. The position of hTERT was confirmed by reprobing with the anti-FLAG M2 antibody. 50 ng of FLAG-hTERT was loaded per lane. α = anti.

(B) Detection of hTERT in human cells. Whole cell lysates (100 μg) were immunoblotted with the 2C4 mAb or a polyclonal anti-hTERT antibody.

(C) hTERT IP. Whole-cell lysates of hTERT negative (GM847) or HA-tagged hTERT-expressing (GP3C) cells (Counter et al., 1998b) were immunoprecipitated using the 2C4 mAb or an anti-HA antibody (F7), subjected to SDS-PAGE, and immunoblotted with an anti-HA mAb (HA-11) or with the 2C4 mAb.

(D) IP of endogenous hTERT from HeLa cells. Lane 1, diluted HeLa lysate used for IP (input 2%); Lane 2, diluted HeLa lysate used for IP (input 2%) + RNase; Lane 3, IP with anti-HA antibody; Lane 4, IP with anti-mouse IgM; Lane 5, IP with 2C4 and anti-mouse IgM; Lane 6, IP with 2C4 and anti-mouse IgM + RNase treatment; Lane 7, HeLa whole-cell lysate (0.5 μg); Lane 8, reaction buffer.

Cells that survive crisis are typically aneuploid; these observations provide evidence that increased karyotypic instability and fusions occur during crisis (Maser and DePinho, 2002). While some have proposed that telomere shortening triggers both replicative senescence and crisis (Wright and Shay, 2002), others have proposed that a change in some aspect of telomere state rather than overall telomere length activates replicative senescence (Blackburn, 2001; Karlseder et al., 2002). This altered state is reflected in the configuration of the telomeric 3′ overhang, since much of this single-stranded overhang is lost in senescent human cells (Li et al., 2003; Stewart et al., 2003a). Thus, although it is clear that telomere length, telomere structure, and telomerase each affect replicative lifespan in human cells, the relationships among these elements remain largely correlative.

Furthermore, some types of normal human cells, notably keratinocytes (Harle-Bachor and Boukamp, 1996; Yasumoto et al., 1996), lymphocytes (Liu et al., 1999), and CD34+ hematopoietic progenitor cells (Broccoli et al., 1995; Hiyama et al., 1995) exhibit telomerase activity, yet telomere lengths continue to shorten in such cells with propagation in culture, indicating that telomerase activity alone is not sufficient to maintain stable telomere length (Son et al., 2000; Allsopp et al., 2001). The physiologic role of telomerase activation in these cells is unclear but may prove to be of significance, since therapeutic strategies that target telomerase are under development, which may cause undesired toxicity in telomerase-expressing cell lineages.

Here, we investigate the relationships among telomere attrition, telomerase activity, and the timing of replicative senescence in human cells. To facilitate these studies, we have developed a monoclonal antibody (mAb) that recognizes catalytically active, full-length hTERT (Masutomi et al., 2000). Use of this reagent has revealed, quite unexpectedly, that the maintenance of telomere structure by telomerase occurs in normal human fibroblasts, hitherto believed to be devoid of telomerase activity, and that this low-level expression of active telomerase plays an important role in controlling replicative lifespan in human cells.

Results

Characterization of a mAb Specific for hTERT

By immunizing mice with a recombinant version of the hTERT protein (FLAG-hTERT) (Masutomi et al., 2000), we generated a mAb directed against hTERT (2C4), which recognizes purified FLAG-hTERT in immunoblotting (Figure 1A). In addition, this mAb detects ectopically expressed hTERT in human cells as well as endogenous hTERT in several immortal cell lines that demonstrate telomerase activity as gauged by the telomere repeat amplification protocol (TRAP) (Figure 1B). This pattern of expression is similar to that recognized by a previously reported polyclonal antibody (Vaziri et al., 1999) (Figures 1A and 1B). We also noted that both of these antibodies identified a faint signal in normal human fibroblasts (see following section). By performing immunoprecipitation (IP) with the 2C4 mAb and lysates from a cell line that expresses a hemagglutinin (HA)-tagged version of hTERT (GP3C) (Counter et al., 1998b), we verified that this mAb forms immune complexes with hTERT (Figure 1C). Moreover, immune complexes consisting of 2C4 and endogenous hTERT from HeLa cell lysates retain assayable telomerase activity (Figure 1D), indicating that the 2C4 mAb recognizes enzymatically active telomerase.

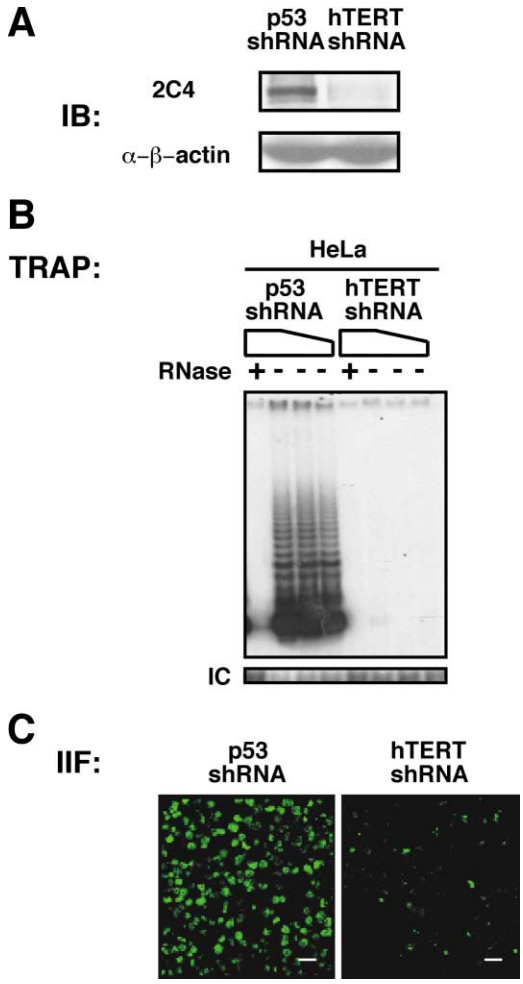


Figure 2. ShRNA-Mediated Suppression of hTERT

(A) ShRNA specific for hTERT or p53 were introduced into HeLa cells using the pMKO.1-puro retroviral vector. After selection and single-cell cloning, hTERT expression was assessed by immunoblotting of whole-cell lysates (100 μg) with the 2C4 mAb.

(B) Suppression of telomerase activity in HeLa cells with an hTERT-specific shRNA. IC = TRAP internal control. The amount of whole cell lysate used was 0.2 μg, 0.5 μg, and 1 μg. RNase treatment was performed on a parallel sample (1 μg).

(C) HeLa cells expressing a p53-specific shRNA (left) and the hTERT-specific shRNA (right) were stained with the 2C4 mAb and visualized with a FITC conjugated anti-mouse IgM (green). Panels are shown at 100× magnification. The bars represent 20 μm.

Although the 2C4 mAb recognized hTERT in immunoblotting and IP, we further determined the specificity of the 2C4 mAb by developing cell lines in which hTERT expression was suppressed by the introduction of a small interfering RNA (siRNA) complementary to sequences present in the hTERT mRNA. To introduce this siRNA stably into human cells, we used a retroviral vector (pMKO.1-puro) (Stewart et al., 2003b) to drive high-level expression of a small hairpin RNA (shRNA), which, when introduced into the human cervical cancer cell line HeLa, resulted in suppression of hTERT mRNA (data not shown) and protein expression (Figure 2A) and abolished telomerase enzymatic function (Figure 2B). In contrast, a control vector carrying a p53-specific shRNA failed to alter telomerase expression or function (Figures 2A and 2B). Using these cell lines, we performed indirect immunofluorescence (IIF) with the 2C4 mAb and identified readily detectable hTERT expression in HeLa cells expressing a p53-specific shRNA (Figure 2C). Importantly, the immunofluorescent signal identified by the 2C4 mAb was nearly completely abrogated in HeLa cells expressing the hTERT-specific shRNA (Figure 2C). Together, these observations indicate the 2C4 mAb recognizes hTERT.

Expression of hTERT in Normal and Cancer-Derived Human Cell Lines

To determine the subcellular localization of hTERT, we then stained a series of mortal and immortal human cell lines with the 2C4 mAb and studied these cells by IIF. Immunofluorescent staining of human fibroblasts (BJ-hTERT) immortalized by the introduction of hTERT or of human cancer cells that constitutively express telomerase (HEp2, HeLa, 293T) revealed easily detectable hTERT expression, primarily localized to the nucleus, consistent with prior reports that used ectopically expressed green fluorescent protein (GFP)-hTERT fusion proteins (Etheridge et al., 2002; Wong et al., 2002) (Figures 2C, 3A, and 3C and data not shown). This pattern was similar to that obtained when we costained these BJ-hTERT cells with the rabbit anti-hTERT antibody (Figure 3B). Although in each case we detected hTERT in the nucleus, in human cancer cells we noted a diffuse nucleoplasmic staining pattern (Figure 3C), while the nuclear staining of hTERT in immortalized human fibroblasts showed a punctate pattern, reminiscent of the pattern reported in prior studies (Figure 3A) (Martin-Rivera et al., 1998; Wong et al., 2002).

We also analyzed asynchronously dividing, early passage human cells that have been reported to lack detectable telomerase activity (Kim et al., 1994). Unexpectedly, we reproducibly observed that a subset of normal BJ and WI-38 fibroblasts showed the same punctate pattern of nuclear staining as fibroblasts expressing ectopically introduced hTERT (Figures 3D, 3G, 3J, and 3M). These cells were often arranged at the edges of the cultures (see Supplemental Figure S1 online at http://www.cell.com/cgi/content/full/114/2/241/DC1). To determine if the proliferative status of these cells correlated with hTERT expression, we costained asynchronously dividing human fibroblasts with the 2C4 mAb and a mAb that recognizes the proliferating cell nuclear antigen (PCNA) (Figures 3E, 3H, 3K, and 3N) under conditions where there was no crossreactivity between the secondary antibodies (Supplemental Figure S2). In both BJ and WI-38 fibroblasts, we found that the 2C4 mAb and the PCNA-specific antibody identified the same cells (Figures 3F, 3I, 3L, and 3O), suggesting that the expression of hTERT in normal human cells is associated with cell proliferation.

To address whether telomerase expression was correlated with any specific phase of the cell cycle, we synchronized BJ fibroblasts and analyzed the expression of hTERT mRNA and protein. When cells were treated with agents, which prevent entrance into S phase or halt further progression of those cells already in S phase (such as aphidicolin, hydroxyurea, and thymi-

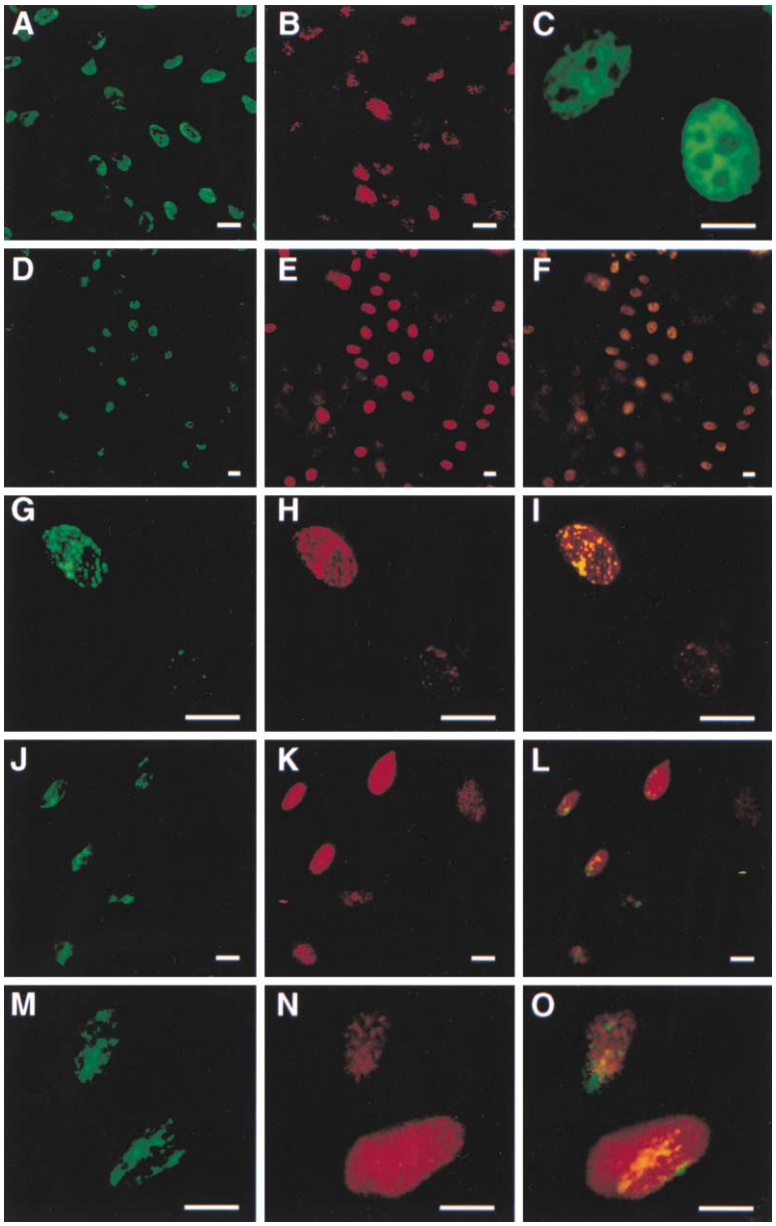


Figure 3. Localization of hTERT in Cycling Human Cells

(A and B) BJ-hTERT cells were costained with the 2C4 mAb, visualized with a FITC-conjugated anti-mouse IgM (green, A), and a rabbit anti-hTERT antibody, identified with an AlexaFluor 568-conjugated anti-rabbit IgG (red, B). (C) HEp2 cells were stained with the 2C4 mAb and visualized with a FITC-conjugated anti-mouse IgM.

(D–I) BJ and (J–O) WI-38 fibroblasts were costained with the 2C4 mAb, visualized with a FITC-conjugated anti-mouse IgM (green in [D], [G], [J], and [M]) and with an anti-PCNA mAb, visualized with an AlexaFluor 568-conjugated anti-mouse IgG (red in [E], [H], [K], and [N]). Merged signals (yellow) are shown in (F), (I), (L), and (O). (A), (B), and (J)–(L) are shown at 400× magnification; (C), (G)–(I), and (M)–(O) are shown at 1000× magnification; and (D)–(F) are shown at 200× magnification. The bar represents 10 μm. For (D)–(O), a peripheral section of the slide is shown.

dine), we found that we could detect hTERT mRNA (Figure 4B). Using primers that distinguish full-length hTERT from alternative splice forms of the hTERT mRNA (Kilian et al., 1997; Ulaner et al., 1998), we confirmed that the hTERT present in these cells derived from transcription of the endogenous gene (Figure 4A and data not shown). The level of hTERT observed correlated with the percentage of cells trapped in S phase (Figure 4B). In contrast, treatment of cells with colcemide or nocodazole, which arrest cells in M phase, failed to yield cells with detectable hTERT mRNA expression (Figure 4B). Similar results were obtained with two other human fibroblast lines, WI-38 and TIG-3 (Figures 3, 4E, and data not shown).

To determine with greater precision the schedule of hTERT expression during the cell cycle, we monitored hTERT expression in synchronized BJ fibroblasts. When cells were synchronized by serum starvation followed by the addition of serum, hTERT mRNA expression was upregulated within 24 hr (S phase index: 47.8%) and persisted until at least 32 hr after release from serum starvation (Figure 4C). When these cells were treated with the additional application of aphidicolin for 16 hr after serum starvation and release to prolong S phase (S phase index: 49.9%), hTERT mRNA expression was upregulated and persisted a further 16 hr after release from the aphidicolin treatment (56 hr after release from serum starvation; Figure 4C). We also noted that hTERT protein levels correlated with these changes in hTERT mRNA, as assessed by immunoblotting whole-cell lysates with either the 2C4 mAb or the polyclonal anti-hTERT antibody (Figure 4D). In each case, the expression of hTERT corresponded temporally with the entrance and transit of a large percentage of these synchronized cells through S phase. At the end of S phase, we were no longer able to detect hTERT mRNA and

protein expression. In contrast, in several immortal cell lines, hTERT mRNA expression and hTERT protein were constitutively expressed after treatment with several cell cycle blockers (data not shown).

To ascertain whether this upregulation of hTERT in S phase led to the formation of functional telomerase, we synchronized human fibroblasts and then performed telomerase activity assays on whole-cell lysates and on immune complexes formed with the 2C4 mAb (Figure 4E). Even with synchronization, we failed to detect telomerase activity in whole-cell lysates using the conventional TRAP assay, confirming previously reported observations (Kim et al., 1994; Bodnar et al., 1998; Hahn et al., 1999a). In contrast, when we used the 2C4 mAb to IP hTERT, we observed detectable telomerase activity in synchronized cells but identified only trace telomerase activity in lysates from asynchronously dividing cultures (Figure 4E). The level of this activity was significantly lower than that observed in HeLa cancer cells. Together, these observations indicate that hTERT and enzymatically active telomerase is expressed at low levels in normal human fibroblasts during each cell division.

Biological Functions of hTERT in Normal Human Fibroblasts

To address whether this transient expression of hTERT in normal fibroblasts is physiologically significant, we disrupted hTERT function by stably expressing either a catalytically inactive hTERT mutant (DN-hTERT) that we previously demonstrated inhibits telomerase activity in telomerase-expressing cancer cell lines (Hahn et al., 1999b) or the hTERT-specific shRNA (Figure 2). Expression of either the DN-hTERT mutant or the hTERT-specific shRNA in two types of normal human fibroblasts (BJ and WI-38) completely inhibited the observed transient telomerase activity (Figure 5A). In addition, we noted that cells expressing the DN-hTERT mutant or the hTERT-specific shRNA proliferated more slowly than parallel cultures infected with a control retrovirus (Figure 5B). When we synchronized these human fibroblasts and analyzed their progression through the cell cycle, we noted that cells expressing the DN-hTERT mutant or the hTERT-specific shRNA exhibited delayed entry into S phase and accumulated in the G2/M phases (Figure 5C). We failed to detect evidence of cell death in such cultures, and such cells continued to proliferate for many population doublings (PDs), albeit more slowly than control cells. These observations indicate that inhibition or suppression of telomerase expression or function affects the transit of normal human cells through the cell cycle.

Moreover, upon extended culture, we found that while BJ fibroblasts infected at PD20 with a control vector ceased proliferating after 32 additional PD (PD52 overall), the cells expressing the DN-hTERT mutant stopped dividing after only 22 PD (PD42 overall) and cells expressing the hTERT-specific shRNA arrested after 18 PD (PD38 overall, Figure 6A). Similarly, another fibroblast strain, WI-38, expressing a control vector stopped proliferating after 11 additional PD (PD38 overall) after infection, while DN-hTERT-expressing or hTERT-specific shRNA-expressing cells ceased proliferating after eight additional PD (PD35 overall) and seven additional PD (PD34 overall), respectively (Figure 6B). In contrast, BJ or WI-38 cells expressing a p53-specific shRNA continued to proliferate, even when cells expressing a control vector entered senescence (Figures 6A and 6B), confirming that expression of shRNA alone fails to induce a growth arrest. At the time that they stopped dividing, fibroblasts expressing either the DN-hTERT or the hTERT-specific shRNA exhibited a vacuolated, flattened morphology compared to that of control cells and failed to achieve confluence, even after several weeks of further culturing (Figure 6C). These observations suggested that the cells expressing either the DN-hTERT or the hTERT-specific shRNA entered replicative senescence earlier than control cells. To confirm this hypothesis, we performed senescence-associated β-galactosidase (SA-β-Gal) staining (Dimri et al., 1995) and determined that a significantly higher percentage of SA-β4-Gal-positive cells were seen in fibroblast cultures expressing DN-hTERT or the hTERT-specific shRNA than were observed in parallel cultures expressing a control retrovirus (Figures 6C and 6D). These observations indicated that inhibition of hTERT function in normal human fibroblasts affects cell lifespan by accelerating entry into senescence.

Since it is widely believed that replicative senescence is triggered by telomere attrition, we determined whether the expression of the DN-hTERT mutant or the hTERT-specific shRNA in normal human fibroblasts accelerated telomere shortening. Surprisingly, we failed to detect significant differences in telomere length between fibroblasts expressing a control vector, the DN-hTERT mutant, or an hTERT-specific shRNA at two points in their replicative lifespan (Figure 7A). Since this assay was performed on genomic DNA isolated from cells that had proliferated for the same number of PD (2 PD and 18 PD after infection), we concluded that the rate of overall telomere shortening was not affected by the expression of either the DN-hTERT mutant or the hTERT-specific shRNA in these normal human fibroblasts.

We recently have found that the onset of replicative senescence in human cells correlates with loss of the telomeric 3′ single-stranded overhang (Stewart et al., 2003a). Since human fibroblasts expressing the DN-hTERT mutant or the hTERT-specific shRNA demonstrated premature entry into a senescence-like state, we speculated that the periodic expression of hTERT during each S phase was necessary to maintain this 3′ telomeric overhang. For these experiments, we isolated genomic DNA from BJ fibroblast cell lines expressing the DN-hTERT mutant or an hTERT-specific shRNA at several points in their replicative lifespan including the time just before the majority of these cells entered senescence (2, 10, and 18 PD after infection). In addition, we also isolated genomic DNA from BJ cells expressing a control vector (2, 10, and 18 PD after infection) or from BJ cells expressing WT-hTERT (20 and 84 PD after infection), both of which exhibited exponential growth at the time of DNA isolation.

Using an assay specific for 3′ telomeric overhangs (T-OLA, Figure 7B, left) (Cimino-Reale et al., 2001; Stewart et al., 2003a), we found that the telomeric 3′ overhang was markedly diminished in cells expressing the DN-hTERT mutant or the hTERT-specific shRNA, at a

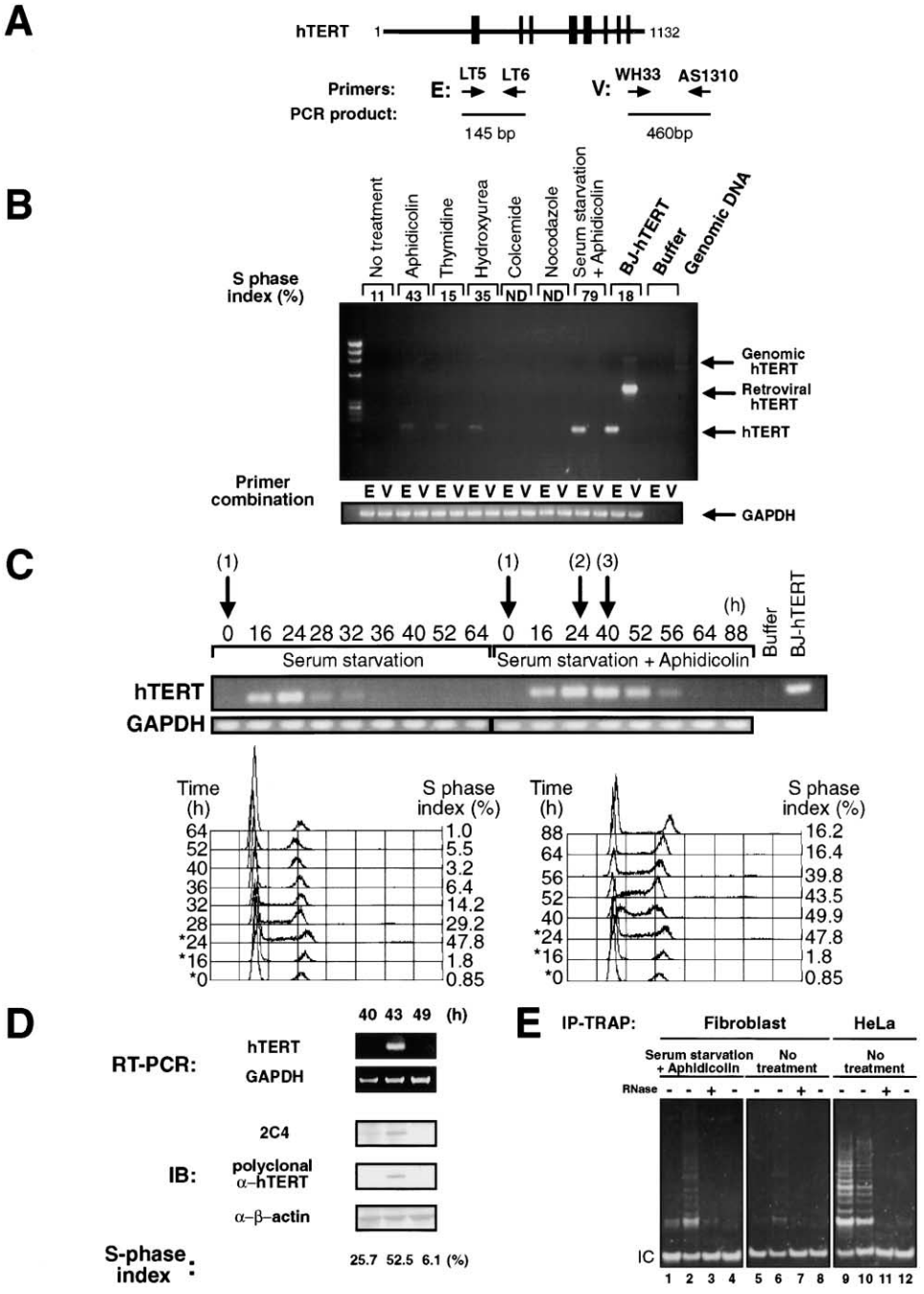


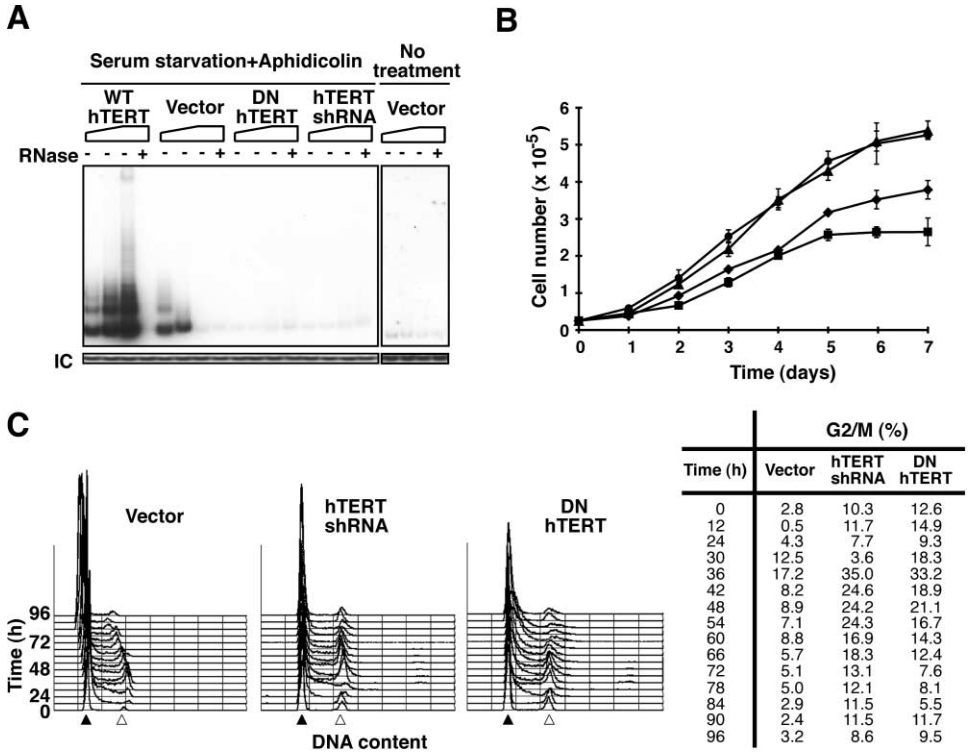
Figure 4. Expression of hTERT in Normal Human Fibroblasts

(A) Conserved telomerase-specific motifs are represented by boxes. Primers used for RT-PCR and predicted PCR product sizes are shown. The primer combination E (LT5/LT6) detects endogenous and ectopically introduced hTERT, while the primer combination V (WH33/AS13) detects only hTERT encoded by a retrovirus.

(B) Expression of hTERT mRNA in normal BJ fibroblasts. Cells were synchronized as indicated and total RNA isolated. Parallel samples were analyzed by flow cytometry to determine the S phase fraction. The primer combinations used for RT-PCR are indicated. BJ-hTERT, buffer, and genomic DNA denote total RNA isolated from BJ-hTERT cells, RT-PCR buffer only, and genomic DNA from BJ fibroblasts as templates for RT-PCR, respectively. ND = not determined.

(C) Time course of hTERT mRNA expression levels and cell cycle analysis in BJ fibroblasts. RT-PCR and flow cytometry analysis were performed with parallel samples. At time point (1), cells were released from serum starvation. Aphidicolin was added to half of the cultures at time point (2), and cells were released from aphidicolin treatment at time point (3). Since parallel cultures were used in these experiments, the flow cytometry plots for 0, 16, and 24 hr (*) are identical for the left and right panels. After time point (2), separate cultures were used for the remainder of this experiment. This panel is representative of four independent experiments.

(D) Time course of hTERT expression in BJ fibroblasts. Parallel cultures of serum-starved cells were stimulated with serum (0 hr), aphidicolin was added after 24 hr, and cells were released from aphidicolin after 16 hr (40 hr). hTERT mRNA was detected RT-PCR, and hTERT protein was detected by immunoblotting either with the 2C4 mAb or a rabbit anti-hTERT antibody. The cell cycle distribution at each time point (reported as



Time (h)	G2/M (%) Vector	hTERT shRNA	DN hTERT
0	2.8	10.3	12.6
12	0.5	11.7	14.9
24	4.3	7.7	9.3
30	12.5	3.6	18.3
36	17.2	35.0	33.2
42	8.2	24.6	18.9
48	8.9	24.2	21.1
54	7.1	24.3	16.7
60	8.8	16.9	14.3
66	5.7	18.3	12.4
72	5.1	13.1	7.6
78	5.0	12.1	8.1
84	2.9	11.5	5.5
90	2.4	11.5	11.7
96	3.2	8.6	9.5

Figure 5. Effects of Suppressing hTERT on the Proliferation of Normal Human Fibroblasts

(A) BJ fibroblasts expressing WT-hTERT, the DN-hTERT mutant, an hTERT-specific shRNA, or a control vector synchronized by serum starvation and aphidicolin treatment. Telomerase activity was then assessed by IP-TRAP. IC = TRAP internal control.

(B) Proliferation of BJ fibroblasts expressing a control vector (circles), a nonfunctional mutant hTERT-specific shRNA (triangles), the DN-hTERT mutant (diamonds), or an hTERT-specific shRNA (squares). For (B) and (C), cells were analyzed 6 PD after infection (PD26 overall). Error bars indicate the mean ± SD for each time point.

(C) Cell cycle analysis of BJ fibroblasts expressing a control vector, an hTERT-specific shRNA, or the DN-hTERT mutant. Parallel cultures were serum starved for 72 hr, treated with aphidicolin for 16 hr, stimulated with 15% IFS, and collected as indicated. The percentage of cells in G2/M at each time point is shown at right. Peaks indicate cell number. Filled arrowheads indicate 1N DNA content (G1), and open arrowheads denote 2N DNA content (G2/M).

time (18 PD after infection) when the growth of the majority of such cells had slowed, particularly when compared to the 3′ overhang seen in cells expressing WT-hTERT or cells expressing a control vector (Figure 7B, right). These observations demonstrate that expression of hTERT in normal human fibroblasts correlates with the maintenance of the 3′ telomeric overhang and suggests that periodic expression of hTERT facilitates cell proliferation by delaying the onset of replicative senescence. Moreover, we were unable to recover telomerase activity even by IP with the 2C4 mAb in BJ human fibroblasts that had entered replicative senescence (Figure 7C), indicating that senescent cells fail to express telomerase. These observations add further evidence to support the view that the timing of entrance into replicative senescence is regulated by factors that operate independently of telomere length. Taken together, we conclude that the periodic expression of telomerase in normal human cells participates in the regulation of cell proliferation and replicative lifespan.

Discussion

Telomerase Expression in Normal Human Cells

Telomeres control two distinct biological functions in mammalian cells. In addition to protecting the integrity of chromosomes (Blackburn, 2001), several lines of evidence implicate telomere maintenance as a critical regulator of cell lifespan (Wright and Shay, 2002). Since telomere attrition correlates with cell division, it has been proposed that this telomere shortening occurs in most normal human cells because telomerase is strongly repressed (Harley et al., 1990, 1994). However, the expression of hTERT in certain types of normal human cells

hr after release from serum starvation) was assessed in parallel cultures. For immunoblotting, 100 μg of whole-cell lysate was loaded.

(E) Telomerase activity in the normal human fibroblast TIG-3 (lanes 1–8) and HeLa (lanes 9–12). Cells were treated with serum starvation and aphidicolin as described in (C) and (D). IP of whole-cell lysates (lanes 1, 5, and 9) was performed with the 2C4 mAb (lanes 2, 6, and 10) or an anti-β-actinin mAb (lanes 4, 8, and 12), and TRAP was performed on the immune complexes. 3% and 0.1% of the input lysate is shown for TIG-3 and HeLa, respectively. Samples in lanes 3, 7, and 11 were treated with RNase after IP with the 2C4 mAb. IC = TRAP internal control. Similar results were obtained with BJ fibroblasts.

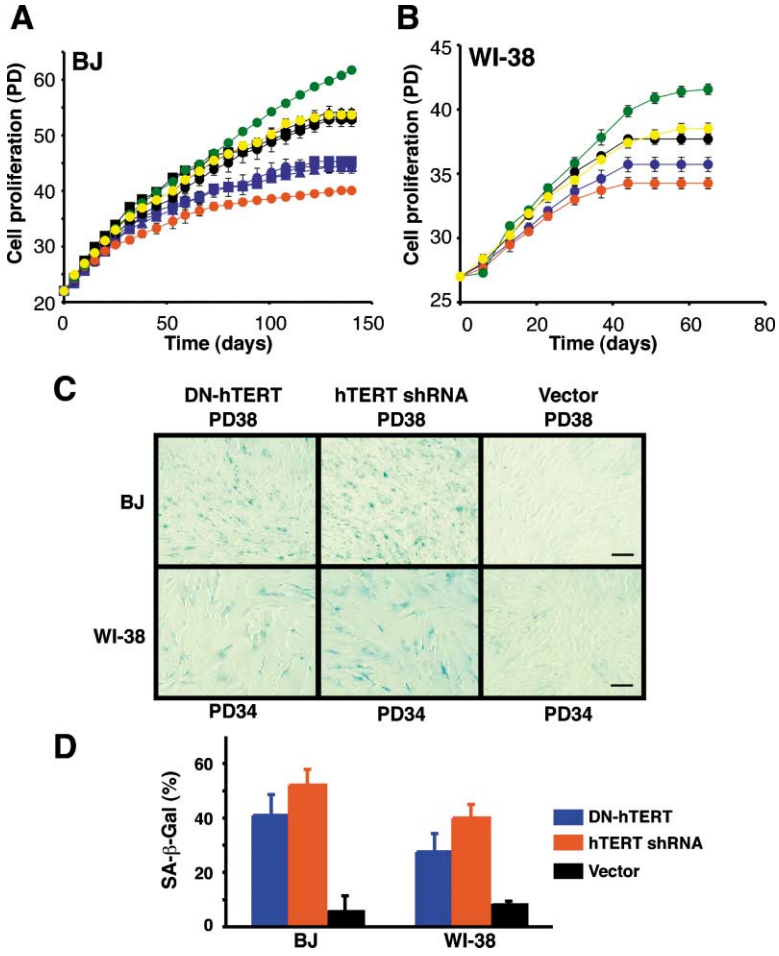


Figure 6. Effects of Suppressing hTERT on the Replicative Lifespan of Human Fibroblasts

(A and B) Effects of DN-hTERT and hTERT shRNA on the long-term proliferation of normal BJ (A) and WI-38 (B) human fibroblasts. Cells expressing a control vector (black symbols), the DN-hTERT mutant (blue symbols), or the hTERT-specific shRNA (red symbol) are shown. Three independently generated cell lines expressing control vector (black circles, triangles, and squares) and DN-hTERT (blue circles, triangles, and squares) are shown. In addition, BJ and WI-38 cells expressing a mutant hTERT-specific shRNA that fails to suppress hTERT expression (yellow) or a p53-specific (green) shRNA are shown. Error bars indicate the mean ± SD.

(C) SA-β-Gal staining of BJ fibroblasts (top) and WI-38 fibroblasts (bottom) at the indicated PD. DN-hTERT, hTERT shRNA, or vector indicate cells expressing the DN-hTERT mutant, an hTERT-specific shRNA, or a control vector, respectively. Panels are shown at 100× magnification, and the bar represents 40 μm.

(D) Bar graph shows the percentage of cells that stained with SA-β-gal in each culture at the PD indicated.

(Broccoli et al., 1995; Hiyama et al., 1995; Yasumoto et al., 1996; Liu et al., 1999) as well as the demonstration that telomere length does not always correlate with entry into replicative senescence (Karlseder et al., 2002; Stewart et al., 2003a) indicate that the relationships among telomere length, telomerase expression, and replicative lifespan are more complex than previously believed.

Here, using a mAb that recognizes hTERT, we unexpectedly found that the expression of hTERT is upregulated in normal, early passage human fibroblasts during their transit through S phase. This regulated expression of hTERT results in functional yet transient expression of active telomerase, and disruption of this expression alters both the proliferative and replicative potential of these normal human cells without affecting the rate of overall shortening of the double-stranded portions of the telomere. These findings indicate that the regulation of hTERT and telomerase is a dynamic process even in normal human cells and suggest that active maintenance of the telomere is necessary for the proliferation of normal human cells.

Since we were unable to detect telomerase activity without using IP to concentrate telomerase, these findings explain why prior studies failed to identify telomerase expression in normal fibroblasts. Moreover, these observations unify prior reports that indicated that telomerase activity is expressed in other types of normal human cells (Harle-Bachor and Boukamp, 1996; Liu et al., 1999) and that suggested that telomerase activity correlated with proliferation (Broccoli et al., 1995; Zhu et al., 1996). While these previous reports suggested that telomerase expression is confined to a specific subset of normal human cell lineages, the current observations suggest physiologic regulation of hTERT occurs in most, if not all, human cells as they pass through S phase.

Telomere Structure, Telomerase, and Replicative Senescence

Inhibition of hTERT expression in normal cells results an early replicative arrest, and the active maintenance of the 3′ overhang appears to play an important role in the physiology of normal human cells (Figure 7D) (Li et al., 2003; Stewart et al., 2003a). While the DN-hTERT mutant used in these studies may exhibit a gain-of-function phenotype, we observed nearly identical results when we used an hTERT-specific shRNA to silence hTERT. Although a prior report noted that the expression of an antisense RNA complementary to hTERC failed to affect the short-term proliferation of a human fibroblast strain (Feng et al., 1995), our observations indicate that long-term inhibition of telomerase function induces premature entry into senescence. The present observations suggest that this telomeric 3′ overhang is a central constituent of the telomeric cap (Blackburn, 2001) and that

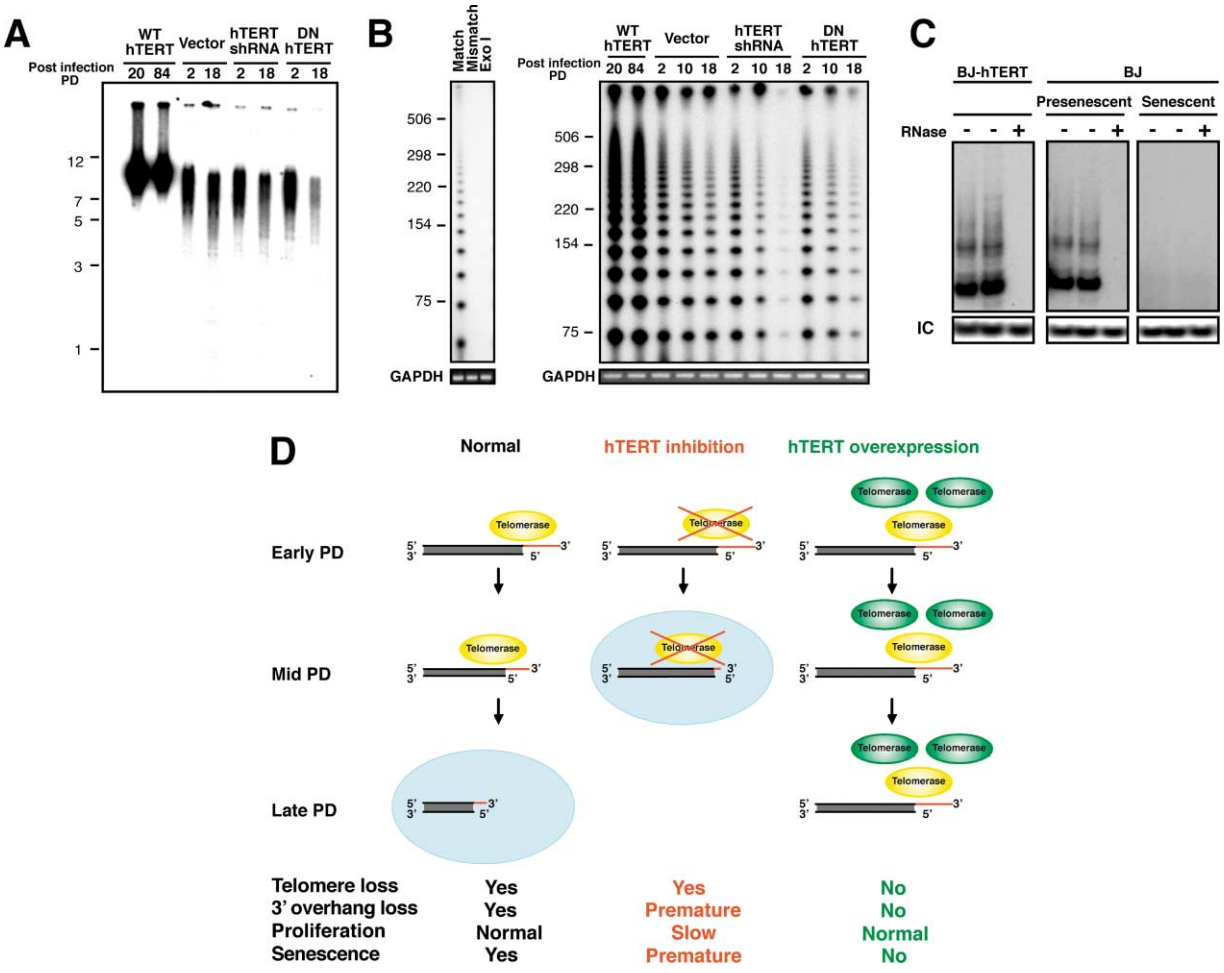


Figure 7. Effects of Inhibiting hTERT on Telomere Structure

(A) Telomere restriction fragments for BJ fibroblasts expressing WT-hTERT, a control vector, an hTERT-specific shRNA, or the DN-hTERT mutant were analyzed by Southern blot analysis at 2 PD and 18 PD after infection. WT-hTERT and vector-expressing cells were proliferating at each of these time points while DN-hTERT- and shRNA-expressing cells had slowed 18 PD after infection. Size markers in kilobases (kb) are indicated. BJ cells were infected at PD20; the overall PDs for the time points shown are PD22, PD30, and PD38.

(B) Loss of telomeric overhang in DN-hTERT and hTERT-specific shRNA-expressing cells. T-OLA analysis of DNA from the same cell lines as in (A) except that DNA from an intermediate time point (10 PD after infection) is also shown (right). To ensure that equal amounts of genomic DNA were present in each sample, PCR was performed using primers specific for genomic GAPDH (bottom). Product sizes in nucleotides (nt) are indicated. To confirm that the signals were derived from 3′ single-stranded DNA, results were obtained using the T-OLA assay performed on genomic DNA treated with Exonuclease I or performed using a mismatch oligonucleotide are shown (left).

(C) Senescent cells lack detectable telomerase activity. IP-TRAP was performed with synchronized BJ fibroblasts prior to senescence (left) and at senescence (right). The telomerase activity recovered from BJ-hTERT fibroblasts by IP-TRAP is also shown.

(D) A model for the physiological role of telomerase and telomere structure in normal human cells. Disruption of the transient expression of telomerase during S phase in normal human cells slows cell proliferation, leads to premature entry into senescence, and correlates with accelerated loss of the telomeric 3′ overhang. Constitutive hTERT expression results in stabilization of both the 3′ overhang and telomere length. Blue circles indicate senescent cells. Green circles represent telomerase formed from ectopically introduced hTERT, while yellow circles indicate endogenous telomerase.

disruption of telomerase function in S phase prevents telomerase from performing a physiological function that maintains the integrity of this telomeric cap. Alternatively, transiently expressed telomerase in normal human cells may preferentially elongate one or more critically short telomeres (Steinert et al., 2000).

Inhibition of telomerase expression or function in normal human cells alters their proliferative rate (Figures 5B and 5C). While we also note that serum-starved fibroblasts expressing the DN-hTERT mutant or an hTERT-specific shRNA exhibited delayed entry into S phase, such cells accumulate in the G2/M phases. This latter finding explains the slower proliferative rate of such cells prior to their entry into senescence and is similar to recent observations in yeast lacking telomerase (Enomoto et al., 2002; IJpma and Greider, 2003). The loss of this transiently expressed telomerase in human cells may reflect incomplete telomere or telomeric overhang replication or alterations in the integrity of telomere capping functions. Alternatively, since the introduction of mutant telomere sequences in *Tetrahymena thermophilia* disrupts telomere maintenance and results in the

accumulation of cells in anaphase (Kirk et al., 1997), loss of this periodic expression of telomerase may alter telomere integrity and activate a G2/M checkpoint.

Entry into senescence correlates with alterations to the telomeric 3′ overhang (Stewart et al., 2003a). While it remains possible that the transient expression of hTERT directly maintains this telomeric 3′ overhang, genetically altered yeast (Dionne and Wellinger, 1996) and mice (Nikaido et al., 1999; Goytisolo et al., 2001) lacking functional telomerase retain the ability to create single-stranded telomeric overhangs. In addition, inhibition of telomerase in normal human cells resulted in an increase in the rate of 3′ overhang loss but did not eliminate the single-stranded portion of the telomere immediately (Figure 7B). These observations indicate that telomeric overhangs can be created in the absence of telomerase and that telomerase only partially regulates the integrity of the telomeric overhang. Indeed, recent observations in *Tetrahymena* suggest that deletion of telomerase affects the precision of overhang processing by specific nucleases (Jacob et al., 2003). In consonance with these findings, we observed that entry into replicative senescence correlates with preferential loss of longer T-OLA products (Stewart et al., 2003a), while inhibition of telomerase results in accelerated loss of all T-OLA products (Figure 7B), further suggesting that transient expression of telomerase participates in the maintenance of the telomeric overhang in normal human cells. Loss or truncation of the telomeric overhang may destabilize the T loop (Griffith et al., 1999), thereby triggering the cellular responses that lead to altered cell proliferation and senescence. Alternatively, disruption of a telomeric cap or T loop when cells reach replicative senescence may render the telomeric overhang susceptible to active degradation in the absence of transiently expressed hTERT.

Since cells derived from mice deficient for the telomerase RNA component do not exhibit altered rates of entry into senescence (Blasco et al., 1997), it remains possible that transiently expressed telomerase is only required when some telomeres reach a critically short length, a state never achieved in inbred mice that harbor extremely long telomeres (Kipling and Cooke, 1990). However, such mice, even at early generation, show partially impaired tumor formation in response to chemical carcinogens, which may represent a phenotype attributable to telomerase deficiency in the setting of long telomeres (Gonzalez-Suarez et al., 2000). Moreover, the regulation of what has been termed senescence in murine cells differs from that observed in human cells (Smogorzewska and de Lange, 2002), suggesting that transiently expressed telomerase plays a more prominent role determining human cell replicative lifespan.

As demonstrated here for human fibroblasts and previously for lymphocytes (Son et al., 2000; Allsopp et al., 2001), the periodic expression of hTERT fails to stabilize overall telomere length. This failure to maintain telomere length may be related to the timing or expression level of telomerase in normal cells. Moreover, recent work in *S. cerevisiae* indicates that catalytically inactive telomerase associates with telomere heterochromatin at times when telomere elongation does not occur, suggesting that this telomere-associated telomerase plays a physical rather than catalytic role in telomere capping (Taggart et al., 2002). The observation that stable telomere length closely correlates with constitutive expression of hTERT and telomerase (Counter et al., 1992; Kim et al., 1994; Bodnar et al., 1998), suggests that such continuous expression of telomerase rather than the cyclic expression of telomerase seen in normal human cells is required for the stable maintenance of telomere length.

The observations presented herein indicate that telomeres play a role in regulating cellular lifespan, albeit through a mechanism quite different from that previously proposed and widely accepted (Harley et al., 1994; Wright and Shay, 2002). Instead, these findings are consistent with an alternative hypothesis for the role of telomeres in regulating cell lifespan (Blackburn, 2001; Karlseder et al., 2002). We conclude that telomere maintenance does indeed play an essential role in specifying replicative lifespan and that the present observations clarify the relationship of telomeres and the two barriers that limit human cell proliferation, replicative senescence, and crisis. In this revised formulation, entrance into replicative senescence occurs at a time when alterations of the 3′ telomeric overhang are observed (Figure 7D), while shortening of overall telomere length results in loss of chromosome protection, triggering cellular crisis. Thus, differential maintenance of two distinct aspects of telomere structure induces different cellular responses.

Telomerase Activation and Cancer

In contrast to normal cells, most human cancer cell lines exhibit constitutive hTERT expression and maintain stable telomere lengths (Kim et al., 1994). The findings reported here may help to explain contradictory reports regarding the expression of telomerase in clinical samples and at different histopathological stages of cancer development, where a variable percentage of histologically normal tissue has been reported to have detectable telomerase activity (Hiyama et al., 1996; Tahara et al., 1999; Matthews et al., 2001) or hTERT mRNA (Kolquist et al., 1998). The observations presented here suggest that these findings may represent those normal cells in S phase that express hTERT.

Furthermore, these observations have important implications for our understanding of telomerase activation during malignant transformation. Activation of telomerase is strongly associated with cancer (Kim et al., 1994) and has facilitated the experimental transformation of a variety of human cells (Hahn et al., 1999a). The findings reported here indicate that hTERT repression is not complete in normal human cells. Thus, the difference in telomere biology between normal and malignant cells is not determined by whether such cells lack or express telomerase but, rather, by the patterns of telomerase expression.

Finally, the observation that hTERT and telomerase expression occurs in normal human cells has important implications for ongoing efforts to develop diagnostic and therapeutic applications of telomerase. Since telomerase expression was believed to be restricted to cancer cells, strategies that detect telomerase held tremendous potential for the early diagnosis of cancer. Certainly, these applications will require critical reevaluation, as assays for telomerase will need to distinguish

between the low-level, transient expression of this enzyme in normal cells and the higher, constitutive expression in most human cancer cells. Moreover, these findings suggest therapeutic strategies that inhibit telomerase activity may induce undesired toxicity in normal proliferating human cells. Future studies will determine whether, in spite of this expression of hTERT in normal cells, the targeting of telomerase will nevertheless prove to have a useful therapeutic index.

Experimental Procedures

Generation of an Anti-hTERT mAb

Amino-terminal FLAG epitope-tagged hTERT purified from baculovirus vector-infected insect cells (Masutomi et al., 2000) was used as immunogen to stimulate the production of anti-hTERT mAb. The hybridoma clone described here, HJ123-2C4 (2C4), produces an IgM mAb and was grown in hollow fiber cultures.

Cell Culture

The human cell lines WI-38, TIG-3, 293T, MCF7, HeLa, GM847, and GP3C were maintained in DMEM with 10% heat-inactivated fetal bovine serum (IFS, standard medium). BJ fibroblasts were cultured as described (Hahn et al., 1999a). Amphotropic retroviruses were created using the pBABE series of retroviral vectors as described (Hahn et al., 1999a). PD were determined by the formula: PD = Log (Nf/Ni)/Log2, where Nf = the number of cells counted and Ni = the number of cells seeded.

Stable Expression of siRNAs

To introduce siRNA stably into human cells, we modified pQCXIN (Clontech) by introducing the puromycin resistance gene and the human U6 promoter to drive the expression of an shRNA (pMKO.1-puro). We introduced hTERT sequences from nucleotides 3114–3134, followed by 9 bp to form a loop and the corresponding antisense hTERT nucleotides, followed by five uridines into this vector. The sequences used for the hairpin were: forward, 5′-TTTCATCAGCAAGTTTGGATTCAAGAGATCCAAACTTGCTGATGAAATTTTTG-3′; and reverse, 5′-AATTCAAAAATTTCATCAGCAAGTTTGGATCTCTTGAATCCAAACTTGCTGATGAAA-3′, where the underlined letters represent hTERT sequences. This retroviral vector or control retroviral vectors encoding either a p53-specific shRNA (Stewart et al., 2003b) or an hTERT-specific shRNA that contains a single nucleotide substitution were introduced into cells as described (Hahn et al., 2002).

Immunoblotting, IP, IIF, and SA β-Galactosidase Staining

Cells were lysed in lysis buffer A (LBA, 20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% NP-40, 0.1 mM DTT) for 30 min on ice. After sonication, the lysate was pelleted by centrifugation at 16,000 × g for 20 min at 4°C. The resulting pellets were lysed in RIPA buffer (12.5 mM NaPO4 [pH 7.2], 2 mM EDTA, 50 mM NaF, 1.25% NP-40, 1.25% SDS, 0.1 mM DTT), sonicated, and centrifuged for 20 min. After 8% SDS-PAGE and transfer to nitrocellulose, samples were probed with a 1:1000 dilution of the 2C4 mAb RT. Reactive bands were identified using a 1:100,000 dilution of a peroxidase-conjugated goat anti-mouse IgM (Pierce). The rabbit polyclonal antiserum that recognizes hTERT was the gift of T. Pandita (Washington University, St. Louis) and was used at a 1:2000 dilution. Anti-FLAG (M2, Sigma) and anti-HA (HA-11, Covance) mAbs were used as suggested by the manufacturer.

For IP, cells (1×10^7) were lysed in 600 μl of LBA, sonicated, and precleared with 15 μl of 50% slurry of Protein A Sepharose (PAS, Amersham). The precleared cell lysate was first incubated with 50 μl of the 2C4 mAb and 20 μl of rabbit anti-mouse IgM (1:10; Pierce), followed by a second incubation with 30 μl of 50% slurry of PAS for 12 hr at 4°C. For IP using the anti-HA antibody, 600 μl of precleared total cell lysate was incubated with 10 μl of anti-HA (clone F7, Santa Cruz Biotechnology) and 15 μl of a 50% slurry of PAS. The bound proteins were visualized by immunoblotting with the HA-11 or 2C4 mAbs.

For IIF, cells were seeded on glass slides ($1–2 \times 10^4$ cells/cm^2) and incubated for 12 hr in standard medium. Cells were washed with PBS, fixed in chilled acetone (5 min), treated with 2 M HCl for 20 min, and neutralized with 0.1 M boric acid (pH 8.5) for 10 min at RT. After washing, cells were blocked with 1% BSA (Fisher), incubated for 12 hr (4°C) with 2C4 (1:5000), and then incubated with a FITC-conjugated anti-mouse IgM (1:400; Pierce) in 1% BSA for 1 hr at 37°C. For experiments in which we performed PCNA and hTERT double staining, we incubated cells fixed as described 12 hr (4°C) in the presence of an anti-PCNA antibody (1:100, PC10, DAKO) followed by incubation with a AlexaFluor568-conjugated anti-mouse IgG (1:400, Molecular Probes). Cells were then stained with the 2C4 mAb. SA-β-Gal activity was performed as described (Dimri et al., 1995).

RT-PCR

Total RNA was isolated using TRIzol (Invitrogen). The following primers were used: hTERT (LT5: and LT6) (Nakamura et al., 1997), hTERT encoded by the pBABE-puro-hTERT retrovirus (WH33, 5′-GTGGTGGAATTCTAGATTTGCAGGTGAACAGCCTC-3′ and AS1310, 5′-GACACACATTCCACAGGTCG-3′), and human GAPDH (5′-GAGAGACCCTCACTGCTG-3′ and 5′-GATGGTACATGACAAGGTGC-3′). The RT reaction was performed for 30 min at 42°C using 500 ng of total RNA, followed by PCR (35 cycles: 94°C, 45 s; 60°C, 45 s; 72°C, 90 s).

Cell Cycle Analyses

BJ, WI-38, and TIG3 fibroblasts were deprived of serum for 48–72 hr, treated with hydroxyurea (5 mM, Sigma), thymidine (5 mM, Calbiochem), aphidicolin (5 μg/ml, Calbiochem), colcemide (10 ng/ml, LIFE technologies), or nocodazole (40 ng/ml, Sigma) for 24 hr and then refed with standard medium. Cell cycle distributions were determined using 5-bromo-2′-deoxyuridine (BrdU)-labeled, propidium iodide-stained, ethanol-fixed cells, using an anti-BrdU mAb as described (Hahn et al., 2002).

TRAP, Telomere Structure, and Length Analysis

Cellular extracts were assayed for telomerase activity with the PCR-based TRAP (Kim et al., 1994). For IP-TRAP, GammaBind G Sepharose (Pharmacia Biotech) was preblocked in 10% nonfat dry milk in LBA. 20 μl of a 50% slurry of GammaBind G Sepharose was bound to a rabbit anti-mouse IgM (1.5 μl) and washed with LBA. This complex was incubated with the 2C4 mAb (15 μl) in a binding buffer containing 0.1% BSA, 0.1% casein, 100 ng/ml tRNA, and 100 ng/ml yeast total RNA for 1 hr at 4°C. HeLa cells were first lysed in a standard CHAPS lysis buffer (Kim et al., 1994), and 5 μg of this cell lysate were diluted 1:10 with LBA and used for IP with 2C4. The resulting immune complexes were assayed for telomerase activity by TRAP. For fibroblasts, 5×10^5 cells were lysed in 750 μl LBA, and IP was performed. An isotype-matched anti-α-actinin antibody (Clone BM75.2, IgM, Sigma) was used as a control.

Telomere length was measured as described (Counter et al., 1998a). The telomeric 3′ overhang was analyzed by a telomere 3′ overhang ligation assay (T-OLA) as described (Cimino-Reale et al., 2001). Oligonucleotides (matched $[CCCTAA]^4$ and mismatched $[CCCTTA]^4$) were end labeled with T4 polynucleotide kinase (New England BioLabs). For experiments using Exonuclease I, 10 μg of freshly isolated, nondenatured genomic DNA at a concentration of 0.05 μg/μl was treated with 1 unit/μl Exonuclease I at 37°C for 24 hr. 5 μg of high molecular weight DNA, 2 μl of 10× Taq ligase buffer (New England BioLabs), and 0.5 pmol of labeled oligonucleotide in a total reaction volume of 20 μl were incubated at 50°C for 12–14 hr, 0.5 μl of Taq ligase was then added, and the mixture incubated for 5 hr at 50°C. Reaction products were analyzed on 5% acrylamide/6M urea-TBE gels. The genomic GAPDH locus was amplified with the primers 5′-AAAGGGCCCTGACAACTCTT-3′ and 5′-GGTGGTCCAGGGGTCTTACT-3′.

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