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Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening

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ABSTRACT **Human fibroblasts whose lifespan in culture has been extended by expression of a viral oncogene eventually undergo a growth crisis marked by failure to proliferate. It has been proposed that telomere shortening in these cells is the property that limits their proliferation. Here we report that ectopic expression of the wild-type reverse transcriptase protein (hTERT) of human telomerase averts crisis, at the same time reducing the frequency of dicentric and abnormal chromosomes. Surprisingly, as the resulting immortalized cells containing active telomerase continue to proliferate, their telomeres continue to shorten to mean lengths below those in control cells that enter crisis. These results provide evidence for a protective function of human telomerase that allows cell proliferation without requiring net lengthening of telomeres.**

A central feature of cancer cells that distinguishes them from normal cells is the ability to divide indefinitely. Normal human fibroblast cells in culture have a limited proliferative lifespan, dividing 50–80 times (1) before they undergo a permanent growth arrest, known as replicative senescence or Mortality 1 (M1). When M1 growth arrest is bypassed by transformation with viral oncogenes such as simian virus 40 (SV40) large T antigen or by the loss of growth suppressors such as p53 and pRb, cells can gain an additional lifespan of 20–30 doublings and then undergo a crisis or Mortality 2 (M2) (2, 3). Only rare cells survive M2 and proliferate indefinitely as immortal cell lines. Although changes in genomic stability likely are involved, the mechanisms by which such cells enter and survive M2 crisis have not been elucidated.

Functional telomeres are essential for chromosomal integrity in eukaryotes, and, consequently, telomere maintenance is required for long-term cellular proliferation (4, 5). Human telomeric DNA consists of TTAGGG repeats, with one DNA strand synthesized by telomerase. Telomerase is a cellular ribonucleoprotein reverse transcriptase (6) with two components required for core enzymatic activity *in vitro*: telomerase RNA (TER) and a telomerase reverse transcriptase protein (TERT) (7–10). The expression of TERT correlates with the presence of telomerase activity in human and mouse cells (11–14). It has been hypothesized that shortening of telomeres in the absence of telomerase activity causes M1 replicative senescence of human cells, because ectopic expression of hTERT in human cells in culture before senescence elicits telomere lengthening and indefinite cell proliferation (15, 16).

Here we analyze the role of telomerase activation in the immortalization of pre-M2 human fibroblasts transformed by SV40 large T antigen. These cells are prone to immortalization, presumably because the presence of the large T antigen abrogates the function of two tumor suppressor proteins (pRB and p53). Telomerase expression normally remains fully suppressed in these cells, thus providing a model system for examining the biological effects of telomerase activation.

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MATERIALS AND METHODS

Cell Culture and Virus Infection. hTERT cDNA was kindly provided by Robert Weinberg, Massachusetts Institute of Technology, and subcloned into retroviral vector pBABE-puro. The C-terminal hemagglutinin (HA) tag in the original construct supplied was removed to restore the wild-type C-terminal region. An aspartic acid to alanine mutation at codon 868 (D868A) was introduced via PCR site-directed mutagenesis.

IMR90 cells were obtained from the American Tissue Culture Collection (Manassas, VA). PLR.HS.217 cells were a gift from John Murnane of University of California at San Francisco. Cell culture, retrovirus preparation, and infection were described previously (17). IMR90 cells were transfected with plasmid p6–1 (expressing SV40 large T and small t antigens with a defective replication origin) (18) together with a plasmid conferring hygromycin resistance. Cells were selected in medium containing 200 μg/ml hygromycin, and colonies were ring-cloned and expanded.

In the colony assay, pBABE-puro vector virus and pBABE-hTERT virus-infected cells before crisis were seeded at a density of 500–4,000 cells per 10-cm dish, based on the trypan blue exclusion assay. Puromycin-sensitive cells (10^5) also were added to each plate to enhance colony formation. Puromycin (0.5 μg/ml) was added into medium 3 days after plating to select puromycin-resistant colonies. Colonies were stained with methylene blue.

Telomerase Assays and Southern Analysis of Telomere Lengths. S100 of the cell lysate was made as described previously (19). Assays of telomerase in the S100 cell extract were based on a modified telomeric repeat amplification protocol (20). After the PCR step, reaction product was analyzed by denaturing PAGE (10%) followed by phosphorimaging (Molecular Dynamics). RNase pretreatment consisted of the addition of 0.5 μl of RNase A (0.25 μg/μl) to 5 μl of S100 cell extract and incubation at 30°C for 5 min followed by the addition of 60 units of RNasin (Promega).

Genomic DNA from cultured cells was isolated by using a Wizard genomic DNA-extraction kit (Promega). Each DNA sample was hydrolyzed with restriction enzymes (*Rsa*I and *Hin*fI), then fractionated by 0.5% agarose gel electrophoresis, and blotted onto a Hybond-N^+ membrane (Amersham). Hybridization was performed as described (21).

Chromosome Analysis. Exponentially growing cells were arrested for 30 min with 0.1 μg/ml of Colcemid (GIBCO/BRL). Metaphase spreads were prepared as described previously (22). C-band staining was used to unequivocally identify the dicentric chromosomes in the spreads (23). Microscopic

Abbreviations: M1 and M2, Mortality 1 and 2; SV40, simian virus 40; TRF, terminal restriction fragment; HA, hemagglutinin; TER, telomerase RNA; TERT, telomerase reverse transcriptase protein.

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A Commentary on this article begins on page 3339.

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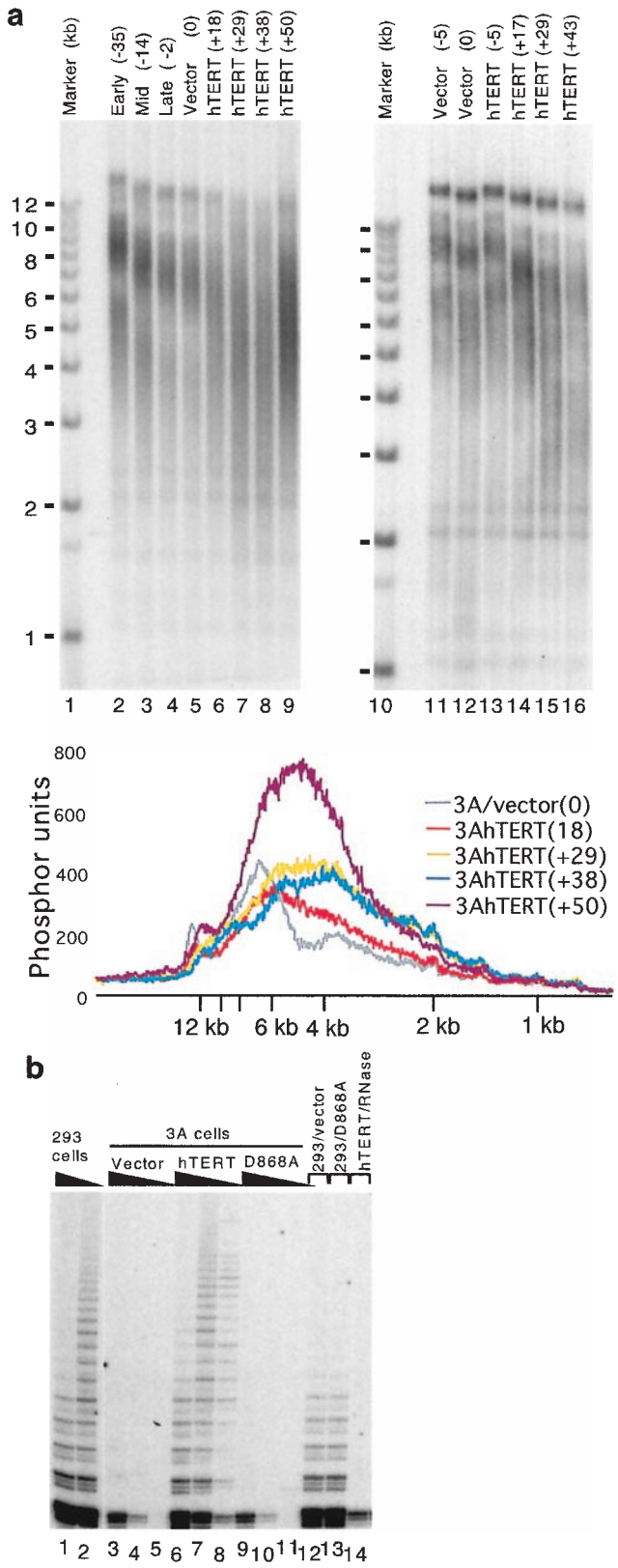

FIG. 1. Telomere length and telomerase activity in hTERT-expressing human fibroblasts. (*a*) Southern analyses of telomere length. Lanes 2–9 are 3A cells and lanes 11–16 are PLR cells. The numbers across the top indicate the relative population doublings at which DNA were made. The stage at which cells entered crisis was designated as population doubling 0. Early, Mid, and Late indicate precrisis cells at 35, 14, and 2 population doublings before they entered crisis, respectively. Vector indicates vector virus-infected cells. hTERT represents the cells transduced with hTERT virus. The numbers on the left are the sizes of DNA markers (kb) (lanes 1 and 10). (*Lower*) PhosphorImaging scans of telomere hybridization signals in lanes 5–9. (*b*) Telomerase activity in 3A cells. Telomerase assay was carried out using a modified telomeric repeat amplification protocol, followed by product separation on a 10% denaturing polyacrylamide gel. The triangles across the top indicate the relative amount of protein extracts used in assays. On the left of each set of assays, 2 μl of extract (0.5 μg/μl protein) was used. In the next two assays, 1/20 and 1/400 distributions of extract were used. In 293/vector and 293/D868A lanes, 293 cell extract was mixed with 3A cells extracts (0.5 μg each) transduced with vector and hTERT/D868A viruses. In the hTERT/RNase lane, 0.5 μg of 3A/hTERT cell extract was mixed with RNase.

images of metaphases were acquired by a video camera on a Leica microscope (Optronic cooled CCD, MDE1850).

RESULTS

Bypass of M2 Crisis by Ectopic Expression of hTERT. We tested the effects of ectopic expression of hTERT on M2 crisis in normal human lung fibroblasts (IMR90 cells) and normal skin fibroblasts. These cell types have been widely used to study cellular senescence and immortalization (24, 25). To bypass M1 senescence and to study the effects of hTERT on M2 crisis, we established IMR90 cell strains expressing the SV40 large T and small t antigens. Two such IMR90 cell strains, 3A and 3C, grew for ≈50 population doublings and then reproducibly underwent crisis, a period of no net growth characterized by continued proliferation accompanied by gradually increased levels of cell death. Consistent with published results (24), survivors of crisis arose at a frequency of about 10^{-7} (data not shown). Cell morphology and population growth rates were the same before and after crisis (data not shown). We also analyzed PLR.HS.217 cells, which are normal human skin fibroblasts transformed by the SV40 early gene (25). The PLR cells similarly underwent crisis, and survivors emerged at very low frequencies (data not shown). As shown previously (21), telomeres progressively shortened in the precrisis cells (Fig. 1*a*, lanes 2–4) and the cells did not express telomerase activity (Fig. 1*b*, lanes 3–5, and data not shown).

Using a retroviral vector we introduced three forms of the hTERT gene into 3A and PLR cells: wild type, a gene encoding a catalytically inactive form [hTERT(D868A)] (26), and hTERT tagged at its C terminus with HA (hTERT-HA) (27). Cells infected with these recombinant viruses and the control empty vector virus initially were selected as pools and propagated. *In vitro* telomerase activity was readily detectable in extracts of hTERT- and hTERT-HA-expressing cells at levels similar to that of the telomerase-positive immortal cell line 293 (Fig. 1*b* and data not shown). Telomerase activity was undetectable in cells transduced with hTERT(D868A) or with the empty vector.

To assess the effect of ectopic expression of hTERT on crisis, pools of cells transduced with wild-type hTERT were passaged, at 10^4–10^5 cells seeded per 10-cm plate, and allowed to propagate through crisis. Cells from strain 3A infected with the empty vector entered crisis in every plate after about 16 population doublings, and all the cells died over a 2-month period (Fig. 2 *a*, A, and *b*). In contrast, 3A cells expressing wild-type hTERT showed no crisis in any plates, even after 100 population doublings beyond the point at which crisis was expected (Fig. 2 *a*, B, and *b*; Table 1). The same results were obtained with another IMR90/T antigen-transformed cell strain, 3C, developed in parallel with strain 3A (Table 1). Similarly, PLR cells infected with the empty vector entered crisis 12–15 doublings later, and all eventually died. As with the 3A cells, PLR cells expressing hTERT proliferated continuously for 80 population doublings, the longest time measured. Like cells transformed by viral oncogenes, the lifespan of $p21^{-/-}$ human fibroblasts also was extended by expression of

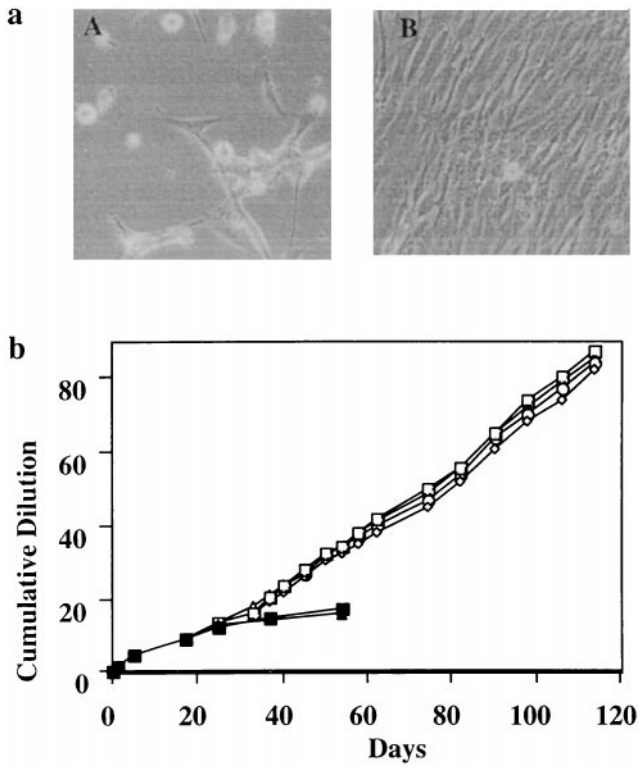

FIG. 2. Effects of hTERT expression in human fibroblast cells transformed by SV40 large T antigen. (*a*) Morphology of 3A cells 4 weeks after they were transduced with either vector (A) or hTERT virus (B). (*b*) Growth curves of 3A cells infected with either hTERT (open symbols) or vector pBABE viruses (solid symbols). Puromycin-resistant cell pools were grown in 10-cm dishes and passaged when they reached confluence.

hTERT (ref. 28; data not shown). All hTERT-expressing 3A, 3C, PLR, and $p21^{-/-}$ cells currently maintain the same proliferative rate with no sign of crisis (Table 1 and data not shown).

Expression of hTERT enhanced the colony-forming potential of SV40 T antigen-transformed precrisis human fibroblasts. Just before crisis, various numbers of puromycin-resistant cells, from pools of cells infected with hTERT virus or with empty vector virus, were seeded onto Petri dishes. For both 3A and PLR cells, empty vector cells formed very small, sparsely packed colonies (Fig. 3*a*, B and D), whereas significantly more and larger, denser colonies were formed by hTERT-expressing cells (Fig. 3*a*, A and C).

We assayed the frequency with which isolated hTERT-expressing clonal cells avoided crisis. Colonies were selected with puromycin immediately after retroviral infections and expanded into clonal lines. For 3A cells, 23 of 24 hTERT cell clones were able to grow continuously, with no detectable crisis (Fig. 3*b*). In contrast, all 23 control empty vector cell clones analyzed entered crisis before reaching population doubling 22. For PLR cells, 18 of 20 hTERT clones were able to proliferate without crisis, whereas all 20 empty vector clones analyzed failed to grow beyond population doubling 15 (Fig. 3*b*). Together, these results show that expression of hTERT is sufficient for fibroblasts with extended lifespan to overcome crisis, as manifested by both colony-formation potential and immortalization frequency.

To determine whether the catalytic activity of hTERT is necessary for its biological effects in this system, an aspartic acid (codon 868) in the conserved catalytic domain was mutated to alanine (D868A), completely eliminating telomerase activity (ref. 26 and Fig. 1*b*). None of the 3A, 3C, or PLR cell pools expressing the catalytically inactive hTERT(D868A) displayed any growth advantage compared with control cells infected with the empty vector (Table 1). Moreover, all 21 3A cell clones expressing hTERT(D868A) entered crisis and died before reaching population doubling 22, and none of the 24 PLR cell clones transduced with hTERT(D868A) grew beyond population doubling 18 (Fig. 3*b*). Therefore, the catalytic activity of hTERT likely is essential for its biological effects.

Table 1. Proliferative potential of cells transduced with hTERT virus

Cells	Exp.	Virus	No. plates	Cumulative dilution
3A	1	Vector	15	7–11
		hTERT-HA	36	8–12*
	2	Vector	2	16
		hTERT	4	>90
	3	Vector	4	18
		D868A	4	18–21
		hTERT	4	>60
PLR	1	Vector	6	13–15
		hTERT-HA	8	13–17
		hTERT	8	>70
	2	Vector	4	12
		D868A	4	12–14
		hTERT	4	>50
3C		Vector	4	8
		D868A	4	8
		hTERT	4	>50

The cells were infected with recombinant viruses and selected as pools with puromycin. The cells were passaged when they became confluent, and the numbers in the last column indicate total number of 2-fold dilutions. All hTERT cells were still proliferating when the manuscript was submitted.

*Colonies appeared in some plates 2–4 months after the cells entered crisis.

hTERT-HA was similarly tested for its ability to overcome M1 senescence and M2 crisis. In agreement with previous reports (15, 16), ectopic expression of wild-type hTERT overcame M1 senescence of IMR90 and NHF cells (data not shown). In contrast, the same cells transduced with hTERT-HA or with empty vector underwent senescence. The frequency of hTERT-HA-expressing 3A cells that overcame M2 crisis was only marginally higher than control levels (10^{-6} for hTERT-HA cells compared with less than 10^{-7} for control cells; data not shown), with only a few colonies apparent in some plates 2–4 months after the onset of crisis (Table 1). Thus, the catalytically active hTERT with the HA tag at its C terminus could not confer the capacity to bypass M1 senescence or avert crisis.

Association of hTERT Expression with Reduced Levels of Chromosome Abnormalities. Shortening of telomeres has been suggested to cause aberrant forms of chromosomes. To test the effect of hTERT expression on formation of chromosomal abnormalities, we analyzed metaphase spreads in hTERT-expressing and control cells. Levels of genomic instability were high in control PLR cells during crisis: about 60% of metaphase spreads contained at least one dicentric chromosome (average, 0.9 per metaphase cell; Table 2 and Fig. 4), with some cells exhibiting as many as six. In contrast, cells expressing hTERT, at the equivalent proliferative stage (population doubling 0, in the designation in Fig. 1) contained significantly fewer dicentric chromosomes (average, 0.1 per metaphase; Table 2 and Fig. 4) and other abnormalities (data not shown). Moreover, 61% ($n = 87$) of control cells were hyperploid compared with 24% ($n = 62$) of hTERT-expressing

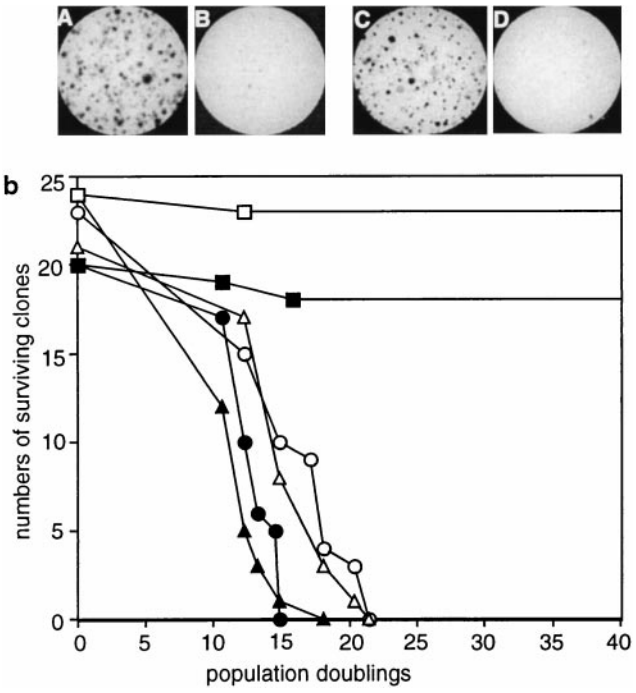

FIG. 3. Effect of hTERT expression on cellular proliferation. (*a*) Colony formation of 3A and PLR cells expressing hTERT. 3A (A and B) and PLR (C and D) cells infected by hTERT virus (A and C) or by vector pBABE virus (B and D) were seeded onto 10-cm dishes at densities of 2,000 cells per 10-cm dish. The colonies were stained with methylene blue 2 weeks after plating. (*b*) Growth of clonal 3A and PLR cells. 3A (open symbols) and PLR (solid symbols) cells were infected with hTERT (squares), hTERT (D868A) (triangles), or pBABE-puro (circles) viruses, and clonal cells were propagated. The stages at which cells were infected were designated as population 0. When a clone entered crisis, the majority of cells died and the culture was discontinued.

cells. In 3A cells, the overall frequencies of dicentric chromosomes were higher than in PLR cells: 22 of 26 metaphases of control 3A cells contained one or more dicentric chromosomes (average, 1.6 per metaphase cell), and 51% ($n = 31$) of spreads showed hyperploidy. However, spreads of 3A/hTERT cells contained significantly fewer dicentric chromosomes: less than half contained any dicentric chromosomes (average, 0.6 per metaphase; Table 2 and Fig. 4) and only 28% ($n = 40$) were hyperploid. Therefore, hTERT-mediated evasion of M2 crisis was accompanied by reduced frequencies of dicentric chromosomes and less aneuploidy.

Telomere Length and Expression of hTERT. It was proposed previously that critically shortened telomeres mediate massive genomic instability and contribute to M2 crisis (21). We examined telomere lengths in cells at different stages of proliferation (Fig. 1*a*). In precrisis 3A cells and 3A cells infected with the vector virus, the terminal restriction fragments (TRFs) had the heterogeneous length distribution with visible broad bands characteristic of cells lacking telomerase activity. As these cells approached crisis, TRFs became shorter (Fig. 1*a*, lanes 2–4).

Table 2. Expression of hTERT suppresses the formation of dicentric chromosomes

No. dicentric chromosomes per cell	PLR cells		3A cells	
	hTERT	Vector	hTERT	Vector
0	51/88%	21/41%	19/54%	0/0%
1	7/12%	20/39%	10/29%	7/58%
2	0/0%	4/8%	6/27%	3/25%
≥3	0/0%	5/10%	0/0%	2/17%
Total no. metaphase	58	51	35	12
No. dicentric chromosomes per cell	0.1	0.9	0.6	1.6

Exponentially growing 3A and PLR cells were arrested with Colcemid, and metaphase spreads were prepared on glass slides. The slides then were stained with Giemsa staining solution and examined. The number of dicentric chromosomes in each metaphase spread was scored.

Unexpectedly, in cells expressing wild-type hTERT, the TRFs continued to shorten for 30–40 doublings after their empty vector counterparts entered crisis (Fig. 1*a*, lanes 6–8 and 14–16, and Fig. 1*a*, *Lower*). The banding pattern of TRFs also became less distinct compared with precrisis cells and control cells in crisis. In 3A/hTERT cells, after 50 doublings beyond the expected crisis point, the bulk of the TRFs increased slightly in length and became more clustered around a mean length of 5–6 kb (Fig. 1*a*, lane 9). Thus, although homeostasis of telomere length was eventually achieved in 3A/hTERT cells, presumably by the balance between telomere synthesis by telomerase and the erosion of telomeres during proliferation, the telomeres were still shorter than in the control cells that had entered crisis. In PLR/hTERT cells, telomere length did not increase even after 43 doublings past the normal crisis point; average telomere length was about 7 kb at the last time point analyzed (Fig. 1*a*, lane 16). In summary, telomeres continued to shorten in hTERT-expressing cells as they proliferated well beyond the expected crisis point, with the average telomere length in these late-passage hTERT-expressing cells considerably shorter than in control cells in crisis.

DISCUSSION

A Proposed Requirement for Capping by Telomerase. Here, we report that rescue of human telomeric function by telomerase can be uncoupled from net telomere lengthening *per se*. Telomere function in the T antigen-transformed human cells was rescued by hTERT expression, as manifested by increased cell proliferation capacity and reduced dicentric chromosome frequencies. However, strikingly, telomeres in proliferating hTERT-expressing cells were, on average, no longer than telomeres in vector-control cells in crisis; indeed, the bulk of the telomeres became even shorter in later-passage hTERT cells that were fully viable and continued to proliferate. Telomere lengthening is currently the most well understood function of telomerase. Consistent with such a role, it was concluded previously that it is the lengthening of telomeres caused by ectopic expression of hTERT that enables cultured human cells to bypass M1 senescence and become immortalized (15, 16) and that telomere shortening in the absence of telomerase may be one signal that provokes M1 senescence. Previous models have proposed a simple correlation between telomere length and function in precrisis fibroblasts, and it was suggested that telomere length is an indicator of the well being of a human cell, including its genomic stability and long-term replicative potential (29).

One possible explanation of our results might be that the control cells that entered crisis contained as little as one telomere per cell, not detected in Southern analyses of TRFs, that was even shorter than all the telomeres in the proliferating hTERT cells and specifically caused cell death (Fig. 5*a*). The critical shortest length of a telomere in this system is unknown. However, we attempted to address this issue by PhosphorImager scanning of the telomeric profiles of Southern analyses from three independent experiments, including that shown in Fig. 5*a*. Such scanning revealed no consistent loss in the fraction of telomeric signal in TRFs below 1.5 kb in late-passage hTERT cells compared with control cells in crisis (data not shown), although very rare, short telomeres might not have been detected in these Southern blots.

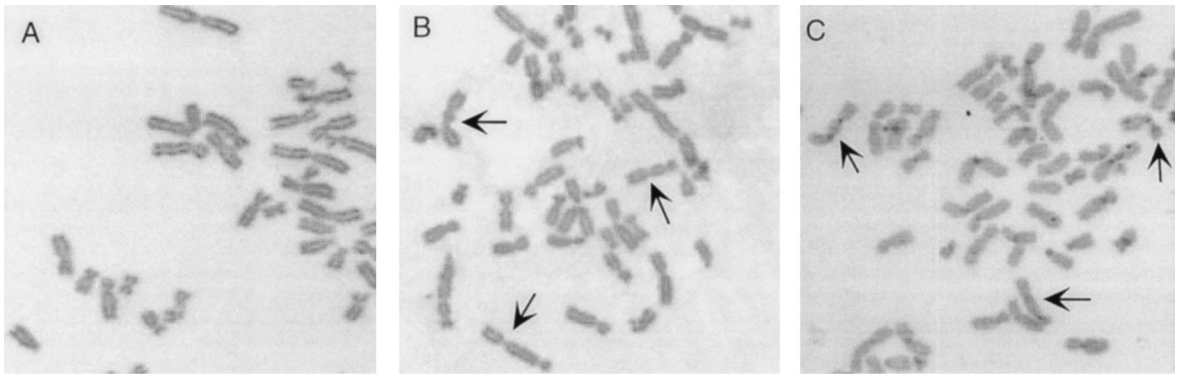

FIG. 4. Effect of hTERT expression on dicentric chromosome formation. Metaphase spreads of 3A cells were visualized with Giemsa staining (*A* and *B*) or C-band staining (*C*). *A* shows hTERT virus-transduced cells and *B* and *C* show vector-infected cells. Arrows point to dicentric chromosomes.

Results in other systems are consistent with a catalytically active telomerase ribonucleoprotein (RNP) at the chromosomal end contributing to telomere capping and, hence, stabilization. First, in two different yeasts, *in vitro* evidence indicates that catalytically active telomerase remains bound to its elongation product, the telomeric DNA (30, 31). Such a stable association of the *Saccharomyces cerevisiae* telomerase RNP with telomeric DNA is formed only after polymerization by telomerase has taken place. These results suggested that the capping function of telomerase may involve physical interaction of telomerase with the telomere. Second, *S. cerevisiae* cells containing only a catalytically inactive mutant telomerase RNP cease dividing after an initial period of telomeric shortening. At this point, all their telomeres were significantly longer than the short, stable telomeres in otherwise isogenic cells containing various mutant, but still catalytically active, telomerases. Notably, the latter cells continued dividing actively (30). We observed that strikingly similar results were obtained here with the D868A hTERT mutant and wild-type hTERT. Third, we note that the majority of human immortal cell and tumor cell lines are telomerase-positive yet have considerably shorter telomeres than those in fibroblasts at crisis. On the other hand, telomerase-negative tumor cells have much longer telomeres (32). Observations in the yeast *Kluyveromyces lactis* and immortalized human cells indicate that even long telomeres are uncapped in the absence of active telomerase. High rates of telomeric recombination events occur, and telomeres are hyperelongated in such telomerase-negative cells (5). Hence, cells with long telomeres may be selected in the absence of telomerase. Finally, in telomerase RNA knock-out mice, significant amounts of telomeric DNA are still present at the fusion point of a subpopulation of dicentric chromosomes, consistent with loss of a capping function provided by telomerase (M. Blasco and P. Landsorp, personal communication). Hence, under multiple circumstances, telomere function is lost in the absence of active telomerase despite the telomeres being of significant length.

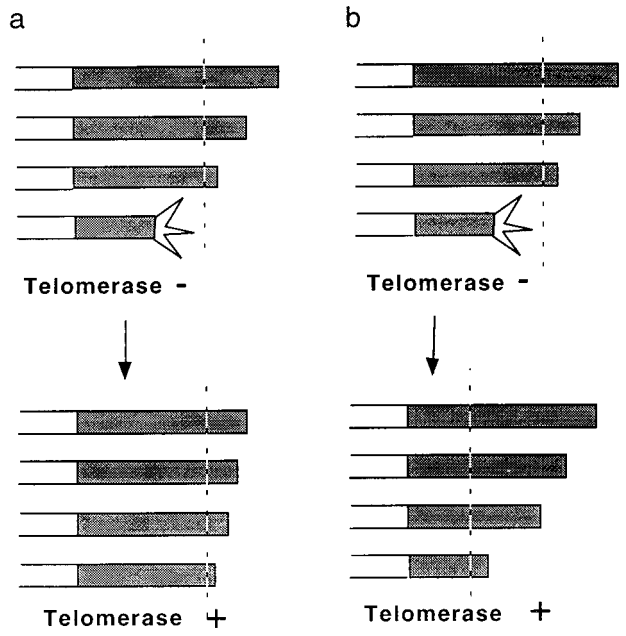

FIG. 5. Models for the telomere-capping function(s) of telomerase. (*a*) Preferential elongation upon hTERT expression of telomeres that are critically short. Dotted lines indicate a proposed threshold of telomere length below which telomeres may be compromised in their function. (*b*) Telomere protection by telomerase. In the absence of telomerase, telomeres lose their stabilizing function when they are shortened below a threshold length (dashed line). The presence of active telomerase lowers this threshold. Open and shaded portions of the horizontal bars indicate the interior of chromosomes and telomeric sequences, respectively.

The results reported here suggest a model that is reinforced by the findings in other systems outlined above: that human telomerase has a telomere-stabilizing or "capping" function that permits cells to proliferate by protecting telomeres even when they remain short. Thus, the stability of a short telomere depends on the capping function of telomerase. This function of telomerase is distinguishable from its effects on net telomere length. Our results further show that this proposed capping function of human telomerase requires not only the catalytic activity of hTERT but also an unknown property affected by the C-terminal HA tag.

We propose that this capping function of telomerase is required for telomere function only when the telomere falls below a threshold length and, specifically, that active telomerase lowers this threshold in otherwise isogenic cells. Thus, in the absence of telomerase, although telomeres are stable above this first threshold, when their length falls below it, capping is compromised (Fig. 5*b*). M1 senescent cells still contain telomeres of substantial length, and telomeres continue to shorten in cells that have circumvented M1 senescence until the M2 crisis is reached. Therefore, by this model, this first critical threshold for telomere length is different in M1 and M2, perhaps because M2 cells have lost molecular mechanisms that normally mediate the response to shortened telomeres. In the presence of telomerase, even further erosion of the telomere does not compromise telomere function as long as length is maintained above a second, lower threshold (Fig. 5*b*). Our results with the hTERT-expressing fibroblasts show that telomerase carries out this capping function without a requirement for net overall telomere lengthening.

Telomerase Activation and Cancer. Although telomerase activity becomes up-regulated in transgenic mouse tumor models (33, 34), embryonic fibroblasts from telomerase RNA knock-out mice can be immortalized, transformed by oncogenes, and form tumors in nude mice as efficiently as cells from wild-type animals (35). However, the long (30- to 40-kb) telomeres of mouse cells (35) may not limit cell proliferation in many situations.

In humans, the hTERT gene, in tight association with telomerase activity, is expressed in some proliferative lymphocytes (36) and a subset of normal tissues with long-term proliferative capacity (37, 38), but telomerase is also repressed in many normal adult tissues of humans (39). The strong correlation between telomerase activation and the malignant phenotype led to the view that telomerase activation might be required for human tumorigenesis (refs. 19, 39, and 40; reviewed in ref. 41). Most immortal human cells have telomerase activity (21, 32). However, previous work did not distinguish whether expression of telomerase has a selective value for cancerous cell proliferation or is an inessential consequence of other genetic and epigenetic changes during tumorigenesis (38). We have shown here that the acquisition of telomerase activity and rescue of telomere function can have two outcomes with opposite predicted effects on tumorigenesis. On the one hand, cells had reduced genomic instability. Conversely, cells overrode the M2 barrier, which is thought to restrict the tumor-forming ability of human cells that have lost normal cell-cycle control (42). Hence, active telomerase could confer a significant, proliferative advantage and thus increase the chance of malignant progression.

After this work was completed, Counter *et al.* (43) also reported that hTERT expression overcomes crisis in virus-transformed human embryonic kidney (HEK) cells. In contrast to our findings, significant lengthening of telomeres was observed in the proliferating hTERT-expressing HEK cells. We propose that the difference in telomere length between the two studies results from the different human cell types used (lung and foreskin fibroblasts, in the present work, compared with HEK cells).

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