Dissociation among *in vitro* telomerase activity, telomere maintenance, and cellular immortalization

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ABSTRACT The immortalization of human cells is a critical step during tumorigenesis. *In vitro*, normal human somatic cells must overcome two proliferative blockades, senescence and crisis, to become immortal. Transformation with viral oncogenes extends the life span of human cells beyond senescence. Such transformed cells eventually succumb to crisis, a period of widespread cellular death that has been proposed to be the result of telomeric shortening. We now show that ectopic expression of the telomerase catalytic subunit (human telomerase reverse transcriptase or hTERT) and subsequent activation of telomerase can allow postsenescent cells to proliferate beyond crisis, the last known proliferative blockade to cellular immortality. Moreover, we demonstrate that alteration of the carboxyl terminus of human telomerase reverse transcriptase does not affect telomerase enzymatic activity but impedes the ability of this enzyme to maintain telomeres. Telomerase-positive cells expressing this mutant enzyme fail to undergo immortalization, further tightening the connection between telomere maintenance and immortalization.

Tumors arise as the end products of a multistep process involving successive rounds of mutation, selection, and clonal expansion. A substantial obstacle to the completion of tumor progression may lie in the limited number of replication cycles allowed normal cell populations in humans (1). *In vitro,* normal human cells exhibit a limited replicative potential, eventually succumbing to senescence (2). Premalignant cell populations evolving *in vivo* may exhaust this allotment of doublings before they succeed in completing their agenda of malignant progression. To do so, such cells must disrupt the generational clock that determines their finite growth potential by acquiring replicative immortality.

Recently, the catalytic subunit of telomerase (human telomerase reverse transcriptase or hTERT) (3–6), the enzyme that elongates telomeres (7), has been implicated as an important participant in the immortalization process. In general, human somatic cells have little or no *hTERT* expression (4–6, 8, 9) or telomerase activity (10) and lose telomeric DNA every cell division (11, 12). These cells eventually enter a senescent state, which can be avoided by transformation with certain viral oncogenes (13). Transformed cells continue to proliferate until they reach crisis when telomeres become critically short; indeed, telomeric shortening may trigger crisis through chromosomal fusion and degradation (14). In rare cells that overcome crisis and become immortal, the *hTERT* gene and telomerase usually are spontaneously up-regulated and telomere shortening is arrested (4, 5, 14–17). Indeed, many types of tumor cells exhibit unlimited growth in culture, and 80–90% of tumors have been documented to express telomerase and hTERT (5, 6, 9, 10).

This succession of events suggests that telomere shortening represents the molecular device that tallies replicative doublings and induces senescence and then crisis. Hence, ectopic telomerase expression may derail the machinery responsible for triggering these two responses. Presenescent cultures of human retinal pigmented epithelial cells and fibroblasts that had acquired ectopic *hTERT* were able to continue proliferation at a time when their untreated counterparts senesced (18–20). This work did not address whether the subsequently occurring, quite distinct process of crisis also is averted by telomerase expression. We show here that this hypothesis is indeed the case. Moreover, we demonstrate that the addition of a carboxyl-terminal influenza virus hemagglutinin (HA) epitope tag results in a mutant of *hTERT* that is capable of inducing high levels of soluble telomerase activity but has lost the ability to maintain and extend telomeres. Telomerase-positive cells expressing this version of hTERT are unable to surmount crisis. Our results formally demonstrate that acquisition of hTERT expression is sufficient to immortalize human cells and validate the role of telomerase in rescuing cells from crisis.

MATERIALS AND METHODS

***hTERT-HA* and *hTERT* Expression Constructs.** The *Eco*RI–*Sal*I fragment containing the *hTERT-HA* cDNA was subcloned from plasmid pCI-Neo-*hTERT-HA* (21) into the *Eco*RI–*Sal*I site of pBabe-hygro, pBabe-puro (22), or the blasticidin-resistant vector pWZL-Blast (gift of J. Morgenstern, Millennium Pharmaceutical, Cambridge, MA). *hTERT* expression constructs lacking the HA tag were created by PCR of an *hTERT Eco*RV–*Sal*I fragment with an oligonucleotide that deleted the nucleotides encoding the HA tag.

***hTERT-HA*- and *hTERT*-Expressing Cell Lines.** The cell line HA1, a clonal population of human embryonic kidney (HEK) cells transformed with simian virus 40 large T antigen (T-Ag) (23), was transfected with the pBabe-hygro or pBH-*hTERT-HA* plasmid at population doubling (pd) 53 (SuperFect reagent, Qiagen). Stably transfected clonal populations were

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Abbreviations: hTERT, human telomerase reverse transcriptase; HEK, human embryonic kidney; T-Ag, simian virus 40 large T antigen; HA, hemagglutinin; pd, population doubling; TRF, terminal restriction fragments.

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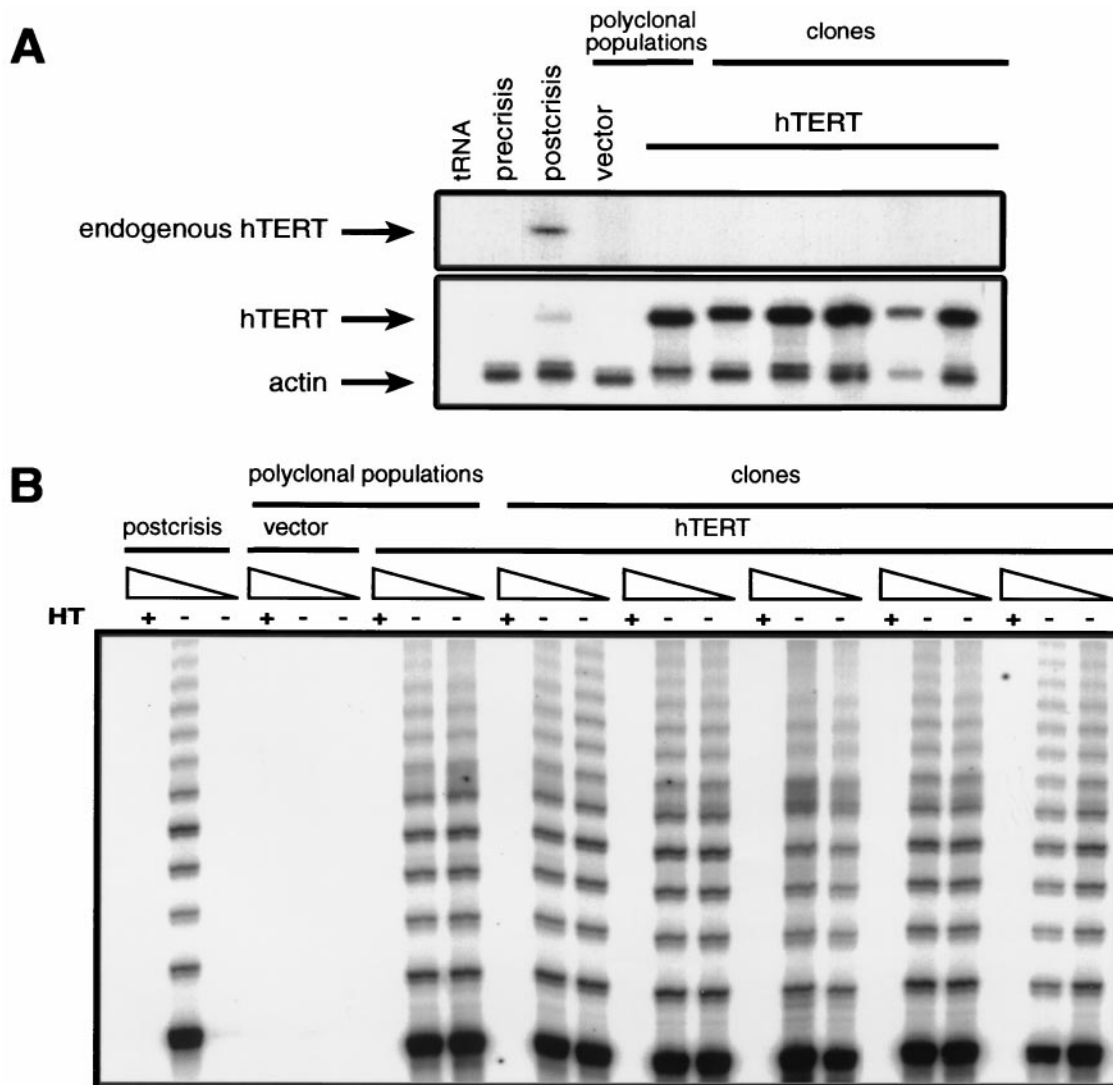

FIG. 1.   *hTERT* expression confers telomerase activity in transformed human cells. (*A*) T-Ag-transformed HEK cells were infected with control or *hTERT*-expressing retroviruses. Total RNA (40 μg) was isolated from the resulting polyclonal populations and clonal isolates and assayed for *hTERT* expression by an RNase protection assay using antisense probes specific for the 3′ untranslated portion region of hTERT (endogenous) or the terminal 275 bp of the *hTERT* cDNA (hTERT). The latter probe protects identical length RNA fragments when hybridized with both endogenous and ectopic hTERT mRNA. Yeast tRNA and a human β-actin probe demonstrate the specificity of the probe and the presence of equal amounts of RNA, respectively. (*B*) Cytosolic cellular extracts (0.2 or 0.02 μg) prepared from the parental line postcrisis, control, or *hTERT*-infected populations, or *hTERT*-infected clonal isolates were assayed for telomerase activity. As a negative control, 2 μg of all extracts tested was heat treated (HT) to inactivate telomerase before telomere repeat amplification protocol assay.

selected in 100 μg/ml of hygromycin. Amphotrophic control and *hTERT-HA* or *hTERT* retroviruses were created with the vectors pBabe-puro, pBP-*hTERT-HA,* or pBP-*hTERT,* respectively, using standard techniques (24). HA1 cells (pd 57) or LM216 (the gift of J. Murnane, University of California, San Francisco; ref. 25) were infected with pBabe-puro or pBP-*hTERT* retroviruses. HA5 cells, a different clonal population of HEK cells transformed with T-Ag (23), were infected with pBabe-puro or pBP-*hTERT-HA* retroviruses. Cells from each infection were selected in 0.5 μg/ml of puromycin. In all cases, pd 0 was assigned as the point when a culture reached confluence in a 10-cm culture dish. The frequency of immortalization was $\leq 10^{-7}$ unless the cells were infected with the *hTERT* retroviral construct. Normal or transformed cells were deemed to have entered either senescence or crisis, respectively, when they could no longer be passaged. Normal human lung embryo fibroblasts, strain LF-1, and the p21$^{-/-}$ derivative strain created by gene targeting were propagated as described (26). In all cases clonal cultures were established by ring cloning of drug-resistant colonies.

Telomere Analysis. DNA was isolated, digested with *Hin*fI and *Rsa*I, and hybridized with a ^{32}P-labeled telomeric (CCCTAA)$_3$ probe as described (14). Fluorescent *in situ* hybridization with Cy-3-labeled (CCCTAA)$_3$ peptide-nucleic acid and subsequent analysis of digital images were performed as described (27).

Telomerase Assays, RNase Protection Assays, and Western Blotting. Cellular extracts were isolated and assayed for telomerase activity by using a PCR-based telomere repeat amplification protocol assay described elsewhere (28). To quantitate telomerase activity, we serially diluted cytosolic extracts from 200 ng to 7 ng at 3-fold dilutions. The protein concentration at which enzyme activity was not detected in a sample was divided by the protein concentration at which a postcrisis, parental cell clone failed to support telomerase activity. The resulting value was multiplied by 100 to obtain a percent telomerase activity. RNase protection assays were performed as described (21). Immunoblotting was performed by using standard methodology (24) using the mAb 12CA5 (Boehringer Mannheim).
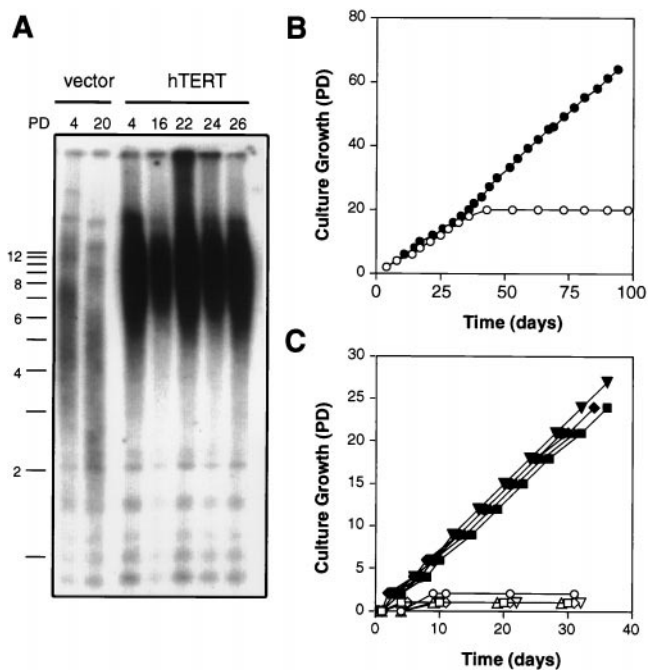
FIG. 2.   *hTERT* expression permits telomere maintenance and allows T-Ag-transformed HEK cells to overcome crisis. (*A*) Genomic DNA was isolated from the parental line and the control and *hTERT*-infected populations and hybridized with a telomeric probe to visualize the TRF. The relative migration of molecular weight markers are on the left. (*B*) Culture growth (pd) versus time (days) are shown for polyclonal populations of T-Ag-transformed HEK cells infected with the control retrovirus (○) or the *hTERT*-expressing retrovirus (●). (*C*) Culture growth (pd) versus time (days) are shown for clonal isolates infected with the control retrovirus (open symbols: ○, □, ◇, △, ▽) or the *hTERT*-expressing retrovirus (closed symbols: ●, ■, ◆, ▲, ▼).

RESULTS AND DISCUSSION

Expression of hTERT Permits Cells to Overcome Crisis. Previous reports have indicated that ectopic *hTERT* expression enables cells to circumvent senescence (18–20). However, cells that bypass senescence must surmount a second proliferative barrier termed crisis before becoming immortal (13, 26). To test whether expression of *hTERT* allows cells to overcome crisis, we studied HEK cells that had been transformed by T-Ag, which allowed these cells to bypass senescence (23) and progress to a precrisis state. These cells do not express the *hTERT* gene (5), are telomerase-negative, and continue to lose telomeric DNA until they reach crisis (14).

We infected a clonal population of these cells with either a control amphotropic retrovirus vector or one expressing an *hTERT* cDNA. Polyclonal, mass-infected populations as well as clonal isolates were obtained and tested for *hTERT* expression by an RNase protection assay with antisense probes that recognize the endogenous and retroviral versions of *hTERT* mRNA, respectively (Fig. 1*A*). In addition, we used the highly sensitive, PCR-based telomere repeat amplification protocol to measure telomerase enzyme activity (28). As anticipated, telomerase activity was restored only in the cells expressing the retroviral *hTERT* gene (Fig. 1 *A* and *B*). Furthermore, the restored activity could be attributed exclusively to the retroviral *hTERT* gene, because the endogenous *hTERT* mRNA was not detectable in the *hTERT* vector-infected cells (Fig. 1*A*).

To ascertain the effects of *hTERT* expression on telomere length and cellular life span, we isolated genomic DNA from the vector control-infected cells and from cells infected with an *hTERT* retrovirus at early passage and after the cultures had grown at least 20 pd after infection. The DNA was analyzed by
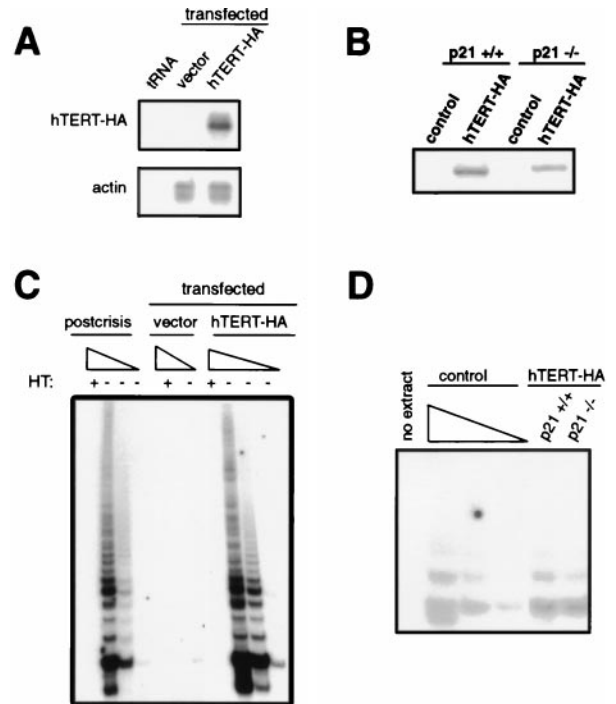

FIG. 3.   *hTERT-HA* expression restores telomerase activity in postsenescent human cells. (*A*) Control or *hTERT-HA* expression vectors were stably introduced into a T-Ag-transformed HEK cell clone, yielding multiple clonal populations. Total RNA (40 μg) was isolated and assayed for *hTERT-HA* expression by an RNase protection assay using an antisense *hTERT* probe that recognizes and distinguishes endogenous hTERT and retroviral *hTERT-HA* transcripts. Results from a representative control and a high *hTERT-HA*-expressing clone are shown. Yeast tRNA and a human β-actin probe demonstrate the specificity of the probe and the presence of equal amounts of RNA, respectively. (*B*) Control or *hTERT-HA* expression vectors were stably introduced into p21$^{+/+}$ and p21$^{-/-}$ fibroblasts, yielding multiple clonal populations. Representative clones from each infection are shown. Total cellular extracts were immunoblotted with an anti-HA antibody to visualize hTERT-HA. (*C*) Cytosolic cellular extracts prepared from the same control (0.2 μg) and *hTERT-HA*-transfected HEK clones (2, 0.2, or 0.02 μg) or from the parental line postcrisis were assayed for telomerase activity. As a negative control, 2 μg of all the extracts tested was heat treated (HT) to inactivate telomerase before telomere repeat amplification protocol assay. (*D*) Four micrograms of cytosolic cellular extracts prepared from p21$^{+/+}$ or p21$^{-/-}$ fibroblast clones was assayed for telomerase activity. Telomerase-positive HeLa cell cytosolic extracts (4, 2, and 1 μg) are included as a positive control.

Southern blots probed with a telomere-specific probe to visualize the terminal restriction fragments (TRF) that include the telomeres. The TRF length of the telomerase-negative control cells was substantially longer at early passage compared with late passage, reflecting the loss of telomeric DNA sustained during extended passage in culture (Fig. 2*A*). Transformed human cells enter crisis once TRF reach a length of ≈4 kb (14–17). Telomeres that have shortened to this degree may no longer protect chromosome ends, which in turn may lead to the genomic instability and cell death associated with crisis. Consistent with these observations, the control vector-infected cells also entered crisis when their telomeres reached a size of ≈4.5 kb (Fig. 2*A*). In contrast, the *hTERT*-expressing cells maintained telomere length over time at a size of ≈9 kb (Fig. 2*A*) and did not enter crisis but instead continued to proliferate (Fig. 2 *B* and *C*). Identical results were obtained in each of five clonal isolates from vector control-infected (open symbols) and *hTERT*-expressing populations (closed symbols) (Fig. 2*C*). Expression of *hTERT* in T-Ag-transformed fibroblasts (LM216) also conferred on these cells the ability to proliferate beyond crisis, confirming these observations in
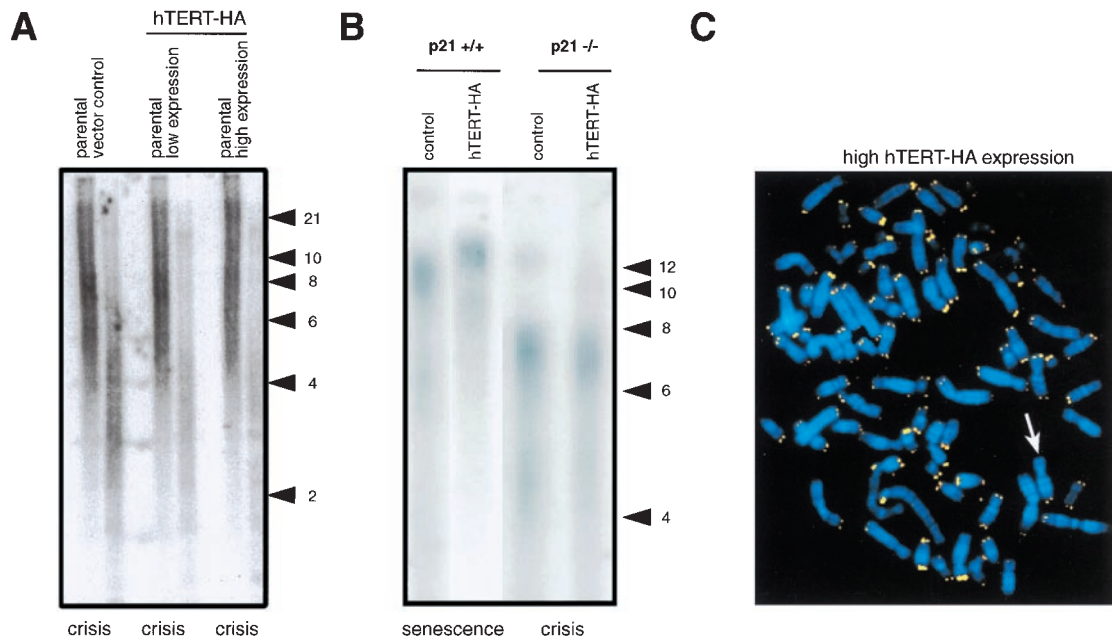
FIG. 4. Expression of *hTERT-HA* does not result in telomere maintenance. (*A*) The TRF of a representative control, low, and high *hTERT-HA*-expressing clones at late passage are shown compared with the parental line at the time of transfection. The relative migration of molecular weight markers is indicated on the right. (*B*) Genomic DNA was isolated from *hTERT-HA*-expressing fibroblast clones one passage before terminal growth arrest and hybridized with a telomeric probe to visualize the TRF. Cultures of uninfected, normal (p21$^{+/+}$) cells in senescence and p21$^{-/-}$ cells in crisis are included to show the telomere length at terminal growth arrest. (*C*) Individual telomeres (yellow) of a metaphase spread of chromosomes (blue) of a high *hTERT-HA*-expressing clone before crisis were identified by fluorescent *in situ* hybridization with a telomeric probe. Arrow denotes a chromosome end that fails to hybridize to the probe.

another cell lineage (data not shown). Thus, ectopic expression of *hTERT* alone was sufficient to allow T-Ag-transformed human cells to bypass crisis.

Alteration of the Carboxyl Terminus of hTERT. A quite different outcome was observed when we expressed a derivative of the hTERT protein (termed hTERT-HA) that had been modified by the attachment of an HA epitope tag to its C terminus. This HA tag had no effect on the ability of the modified hTERT to be expressed (Fig. 3 *A* and *B*) or to reconstitute high levels of measurable telomerase activity (Fig. 3 *C* and *D*) in T-Ag-transformed HEK cells (Fig. 3 *A* and *C*) or human fibroblasts that had circumvented senescence through the deletion of both copies of the p21$^{CIP1/WAF1}$ gene (p21$^{-/-}$), which encodes the widely acting cyclin-dependent kinase inhibitor (26) (Fig. 3 *B* and *D*). However, quite unexpectedly, cells expressing this version of hTERT continued to lose telomeric DNA (Fig. 4 *A* and *B*). Indeed, some chromosomes in *hTERT-HA*-expressing HEK cells lacked sufficient amounts of telomeric DNA to bind to a telomeric probe upon hybridization *in situ* (Fig. 4*C*). Thus, despite high levels of telomerase activity, *hTERT-HA*-expressing cells continued to lose telomeric DNA, even to the point where some chromosome ends had little or no telomeric DNA remaining.

Ectopic expression of hTERT-HA and restoration of telomerase activity did not confer on the transformed HEK cells any substantial proliferative advantage. Both control and *hTERT-HA*-transfected cell clones entered crisis (Fig. 5*A*) when TRF reached an average length of ≈4 kb (Fig. 4*A*). Two clones expressing high levels of *hTERT-HA* did proliferate ≈8–10 pd beyond most of the control clones; however, it is difficult to assess whether this proliferation was caused by *hTERT-HA* expression or interclonal variability (Fig. 5*A*). A polyclonal population of independent *hTERT-HA*-expressing clones, generated by infection of HA-5, a distinct clone of T-Ag-transformed HEK cells used in the aforementioned experiments, with an *hTERT-HA* retrovirus, also underwent crisis with similar kinetics to the vector-control population (data not shown). Rare variants that emerged from crisis (Fig.

5*B*) spontaneously up-regulated their endogenous *hTERT* gene, independent of whether they expressed the *hTERT-HA* gene (Fig. 5*C*). Therefore, *hTERT-HA* expression did not greatly extend the life span, increase the frequency of immortalization, or directly immortalize the transformed HEK cells.

By using human fibroblasts expressing (p21$^{+/+}$) or lacking (p21$^{-/-}$) p21, we were able to analyze the effect of hTERT-HA during both senescence and crisis. Despite readily detectable hTERT-HA expression (Fig. 3*B*) and telomerase activity (Fig. 3*D*), p21$^{+/+}$ fibroblasts entered senescence (Fig. 5*D*), and p21$^{-/-}$ fibroblasts entered crisis (Fig. 5*E*). As with the T-Ag-transformed HEK cells, we noted that both the p21$^{+/+}$ and p21$^{-/-}$ cells infected with the hTERT-HA-expressing retrovirus divided slightly longer (2–10 pd) than the control cells, suggesting that the addition of the HA tag to hTERT may not completely disrupt the *in vivo* function of this protein. Nevertheless, cells expressing this mutant version of hTERT ultimately fail to maintain telomere length and cease to proliferate, irrespective of cell type, culture age, or mechanism by which senescence was bypassed.

CONCLUSION

We conclude that the ectopic expression of *hTERT* and the resulting induction of telomerase activity in T-Ag-transformed HEK cells is sufficient to arrest telomere shortening and to permit cells to overcome crisis. Introduction of an epitope tag to the C terminus of hTERT abolished the ability of this protein to extend the life span of these cells as well as that of human fibroblasts without affecting the assayable *in vitro* catalytic activity of this enzyme. Hence, telomere elongation rather than telomerase enzyme activity is the biochemical attribute most closely tied to extension of the life span of cell lineages. This tight association is illustrated as well in certain human cells that undergo immortalization and maintain telomere length without expressing telomerase (29, 30). Alteration of the hTERT C terminus may perturb its ability to interact with other proteins required for telomere elongation.
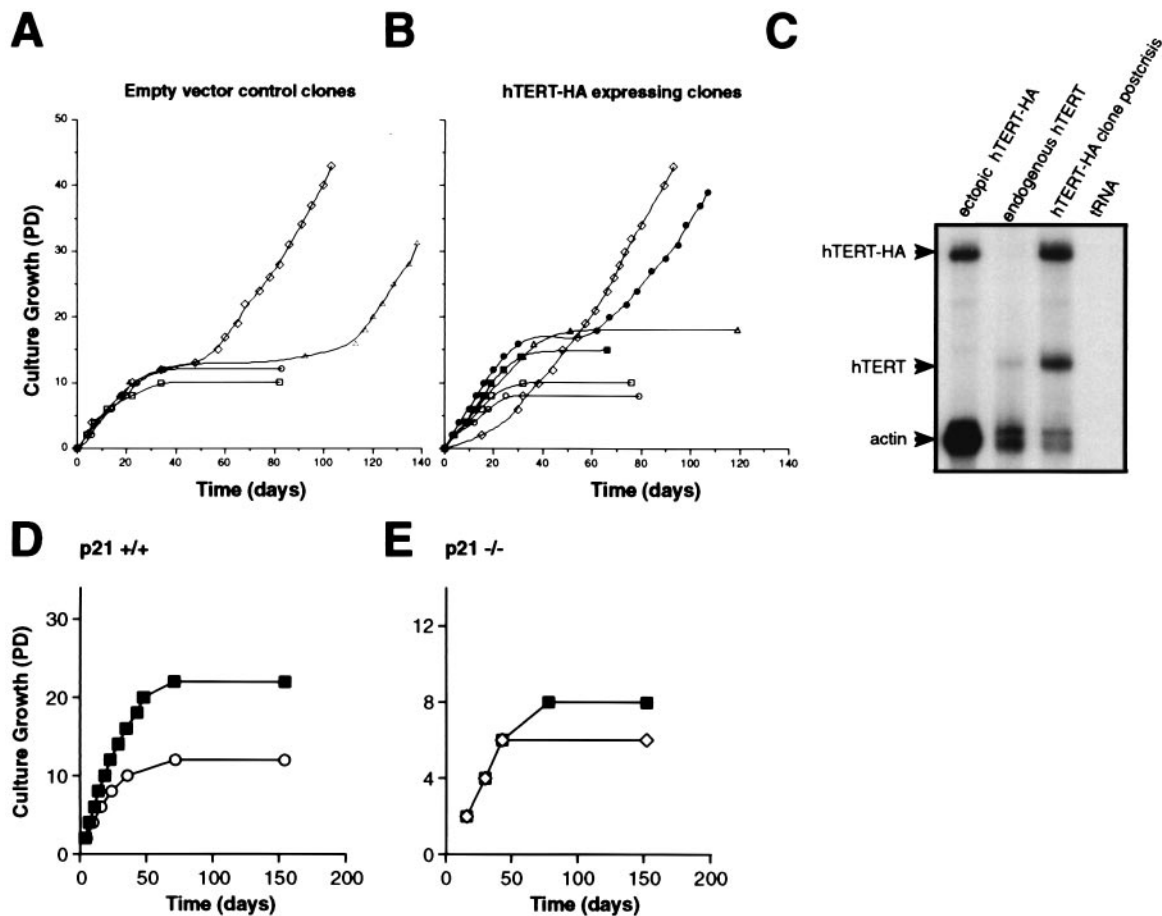
FIG. 5. *hTERT-HA* expression does not immortalize transformed cells. (*A*) Culture growth (pd) versus time (days) is plotted for individual clonal populations of T-Ag-transformed HEK cells transfected with the empty vector clone 1 (○), 2 (□), 3 (△), and 4 (◇) or (*B*) with the *hTERT-HA* expression vector, yielding clones with either low telomerase activity (<50% of the activity detected in immortal T-Ag-transformed HEK cells): clone 1 (○), 2 (□), 3 (△), and 4 (◇) or high levels or telomerase activity (>90% of the telomerase activity detected in immortal T-Ag-transformed HEK cells): clone 5 (●) and 6 (■). (*C*) The level of both the transfected *hTERT-HA* and endogenous *hTERT* mRNAs was measured in a postcrisis, high *hTERT-HA*-expressing clone by an RNase protection assay for *hTERT*. This antisense probe identifies both endogenous and ectopically expressed *hTERT*-HA as shown in total RNA isolated from the telomerase positive human cell line 293 and a murine NIH 3T3 cell line stably expressing *hTERT-HA*. Yeast tRNA and a human β-actin probe demonstrate the specificity of the probe and the presence of equal amounts of RNA, respectively. (*D* and *E*) Culture growth (pd) versus time (days) are shown for normal (p21$^{+/+}$) fibroblasts (*D*) or postsenescent (p21$^{-/-}$) fibroblasts (*E*) that either do (■) or do not (○, ◇) express the *hTERT-HA* protein. The pd values of p21$^{-/-}$ cells were redefined after the second gene targeting event (26) and thus do not correspond to those of p21$^{+/+}$ cells.

Because crisis is the last known proliferative blockade before cellular immortalization (13, 31), we speculate that *hTERT* expression alone may suffice to immortalize T-Ag-transformed cells and possibly other types of precrisis cells. These observations lend further support to the notion that the up-regulation of *hTERT* and telomerase activity observed in human tumors (4–6, 8, 10, 32) may be the direct cause of the ability of cancer cells to divide beyond the replicative capacity of normal somatic cells. Activation of *hTERT* therefore may represent a critical step in tumor progression and correspondingly may predispose cells to become malignant.

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