The hTERTα Splice Variant is a Dominant Negative Inhibitor of Telomerase Activity[1]

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Abstract

The telomerase catalytic subunit (hTERT) is an essential component of the holoenzyme complex that adds telomeric repeats to the ends of human chromosomes. Maintenance of telomeres by telomerase or another mechanism is required for cell immortalization, and loss of telomeric DNA has been proposed as a trigger for cellular senescence. Available evidence suggests that regulation of telomerase activity primarily depends on transcriptional control of hTERT. However, several human tissues as well as some normal cell strains have been shown to express low levels of hTERT mRNA even though they lack telomerase activity. We have previously identified six splice variants of hTERT, including a ''deletion'' variant (hTERTα) that is missing conserved residues from the catalytic core of the protein. Several of the deletion variants have been detected in normal and developing human tissues. We now show that hTERTα inhibits endogenous telomerase activity, which results in telomere shortening and chromosome end-to-end fusions. Telomerase inhibition induced a senescence-like state in HT1080 cells and apoptosis in a jejunal fibroblast cell line. These results suggest a possible role for hTERT splice variants in the regulation of telomerase activity. *Neoplasia* (2000) **2,** 426–432.

Keywords: telomerase, telomeres, hTERT, splicing, dominant negative.

Introduction

Telomeres are composed of repetitive sequences that, along with telomere-binding proteins, act to protect chromosomes from end-to-end fusions (reviewed in Ref. [1]). Telomerase is a ribonucleoprotein enzyme complex that extends telomeres. In most normal human cells telomerase activity is either absent or present at low levels and telomeres gradually shorten due, at least in part, to the DNA ''end-replication'' problem. The telomere hypothesis of cell senescence proposes that it is the critical loss of telomere repeats that signals senescence [2,3]. However, in germ cells and immortalized cells telomerase is sufficiently active to add T_2AG_3 repeats and maintain telomere lengths, allowing these cells to divide indefinitely.

The human telomerase complex includes a catalytic protein, hTERT, and the RNA subunit, hTER, which provides the template for synthesis of T_2AG_3 repeats. hTER RNA is found in most cells whether or not they have telomerase activity [4–6]. Most of the evidence available to date suggests that regulation of telomerase activity primarily depends on transcriptional control of hTERT. There is good correlation between hTERT mRNA expression and telomerase activity in tumors and B lymphocytes [7–11]. Addition of exogenous hTERT is often sufficient to reconstitute telomerase activity in many normal cells and in telomerase-negative immortal lines that express hTER (reviewed in Ref. [1]). However, hTERT expression is not perfectly correlated with telomerase activity. Prostate, heart, brain and other organs as well as some normal cell strains such as WI38 fibroblasts have been shown to express low levels of hTERT mRNA even though they are negative for telomerase activity [8]. This indicates that there may be additional levels of control.

When hTERT was initially identified, six variant cDNAs were found in a colon cancer cell line cDNA library; these were presumed to be the result of alternative splicing [12]. For the studies described here, we focused on the hTERTα deletion variant, which is predicted to remove 12 amino acids from the conserved reverse transcriptase domain A (Figure 1*A*).

hTERTα and other splice variants have been detected in developing human tissues along with undeleted hTERT mRNA [10]. Expression of the undeleted form, in most cases, corresponded with telomerase activity. However, various splice variants, depending on the tissue type, continued to be synthesized even when the undeleted hTERT was not. Thus, alternative splicing may serve as another mechanism for regulation of telomerase activity.

Here we further characterize the hTERTα deletion variant and demonstrate that overexpression of this transcript can inhibit telomerase activity in telomerase positive immortal cell lines. Depending on the cell line, telomerase inhibition resulted either in cell death or in a senescence-like state.

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Methods

Plasmid Vector Construction

hTERT and hTERTα [12] were subcloned into the mammalian expression vector pCIneo (Promega), and the entire sequence was verified by DNA sequencing. For overexpression in telomerase positive cell lines, hTERT and hTERTα were each subcloned from pCIneo vectors into pIRESneo (Clontech). The resultant plasmids are designated pIRES-hTERT, and pIRES-hTERTα. A dominant negative hTERT mutant (D712A, designated 3-1) [13], was also subcloned into pIRESneo.

Cell Culture

SV40-immortalized human skin fibroblast GM847DM ("double mutant," ouabain and 6-thioguanine resistant) cells were obtained from Dr. O. Pereira-Smith, Baylor College of Medicine (Houston, Texas). The following cell lines used for transfections and/or RNA isolation were obtained from ATCC: HT1080, human fibrosarcoma; 293, human embryonic kidney adenovirus transformed; and HeLa, human epithelioid cervical carcinoma line. JFCF-6T/2H cells are human jejunal fibroblasts immortalized following transfection with an SV40 early region expression plasmid. All cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DME) with 10% fetal bovine serum (CSL Biosciences, Parkville, Victoria, Australia) and 50 μg per ml gentamicin (Sigma).

Transfections

Cells were seeded at a density of 10^6 cells per 10-cm dish and transfected with 5 μg plasmid DNA using 30 μl Fugene-6

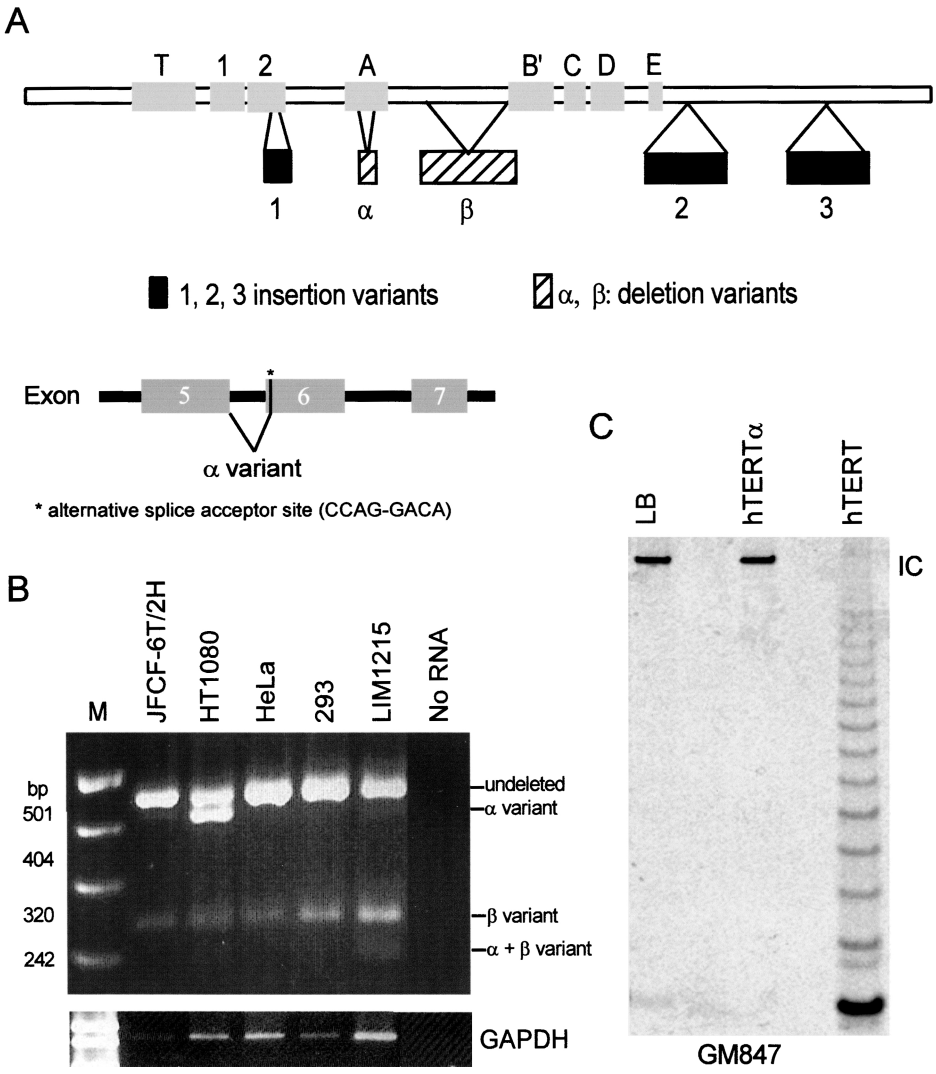

Figure 1. *Deletion variant hTERTα is expressed in some telomerase-positive immortalized cell lines. (A). The sites where the α and β deletion variants occur are shown relative to the conserved reverse transcriptase domains of hTERT [12], and hTERTα employs an alternative splice site within exon 6 (based on genomic sequence [19,20]). (B) GC-rich RT-PCR was performed on total cellular RNA from five telomerase-positive immortal cell lines (indicated above each lane). Primers were used that spanned the 430-bp region encompassing the α and β deletion sites. (M) indicates DNA size markers. Deletion variants are noted, including β variant and α+β variant previously described [12]. RT-PCR for GAPDH, to verify quality of the RNA, is shown in the bottom panel. (C) Telomerase-negative GM847 cells were transiently transfected with plasmid overexpressing hTERT or the deletion variant hTERTα. Telomerase activity was reconstituted by expression of hTERT but not by hTERTα. Protein lysis buffer (LB) was used as a negative control for PCR, and the internal positive control is indicated (IC).*

transfection reagent (Roche) and 1 ml OPTI-MEM reduced serum medium (Gibco) for 2 hours at 37°C, after which time DME medium plus 10% serum was added. Cells were harvested the next day with trypsin–EDTA and seeded at a concentration of 10^4 cells per 10 cm plate in medium containing Geneticin (G418 sulfate, Roche). Geneticin concentrations were (in μg/ml): 300 for GM847DM, 700 for HT1080, 560 for HeLa, 490 for 293, and 245 for JFCF-6T/2H. Colonies were picked after 2 weeks of selection using 4-mm sterile, trypsin-soaked filter paper discs. Colonies were passaged continuously in medium containing Geneticin.

Cell Staining

β-Galactosidase staining was performed as previously described [14]. DAPI staining was performed on cell suspensions in PBS for 5 minutes at room temperature. TUNEL analysis was conducted with the *in situ* cell death detection kit, fluorescein-conjugated (Roche).

Telomere Repeat Amplification Protocol (TRAP) Assay

Cell lysates were prepared using the CHAPS detergent lysis method and 2 μg of total protein was used in each assay. The protein concentration of lysates was measured using the BioRad protein assay kit. The PCR-based TRAP assay for telomerase activity was performed as described [15], using 0.1 fg of myogenin DNA with TRAP primer flanking sequences as an internal control in each reaction [16]. Amplification products were separated on a 10% nondenaturing polyacrylamide gel, stained with SYBR Green I (Molecular Probes) and visualized using a Storm 860 imager (Molecular Dynamics).

Alkaline Phosphatase Assay

For samples that were negative for telomerase activity by the TRAP assay, the quality of protein lysates was verified by assaying for alkaline phosphatase activity. Reactions were performed on a microtiter plate in a total volume of 8 μl, including 2 μl protein lysate, and containing 0.1 M Tris–Cl pH 9.5, 4 mM $MgCl_2$, 6 mM *p*-nitrophenylphosphate. Reactions were incubated for 15 min at 37°C. Fifty microliters 50 mM NaOH was added to stop the reaction. OD_{405} measurements were used to quantitate alkaline phosphatase activity.

RT-PCR Analysis

Primer sets (HT2781R/HT1875F and HT2026F/HT2482R, previously described [12]) flanking nucleotides 2165 through 2620 of the hTERT gene (AF015950) were used to detect α and β deletion variants in RNA samples from cell lines. Total RNA was isolated using RNA Isolation Reagent (Advanced Biotechnologies). For GC Rich PCR, cDNA was synthesized using the Advantage RT for PCR kit (Clontech), and GC Rich amplification was carried out using the manufacturer's recommendations (Roche) with an annealing temperature of 55°C for 30 seconds for both the primary and nested amplification steps. GAPDH control RT-PCR was carried out using primers and conditions supplied in the Advantage RT for PCR kit (Clontech).

Plasmid mRNA was amplified from total RNA of transfected clones using the Titan RT-PCR kit (Roche) and

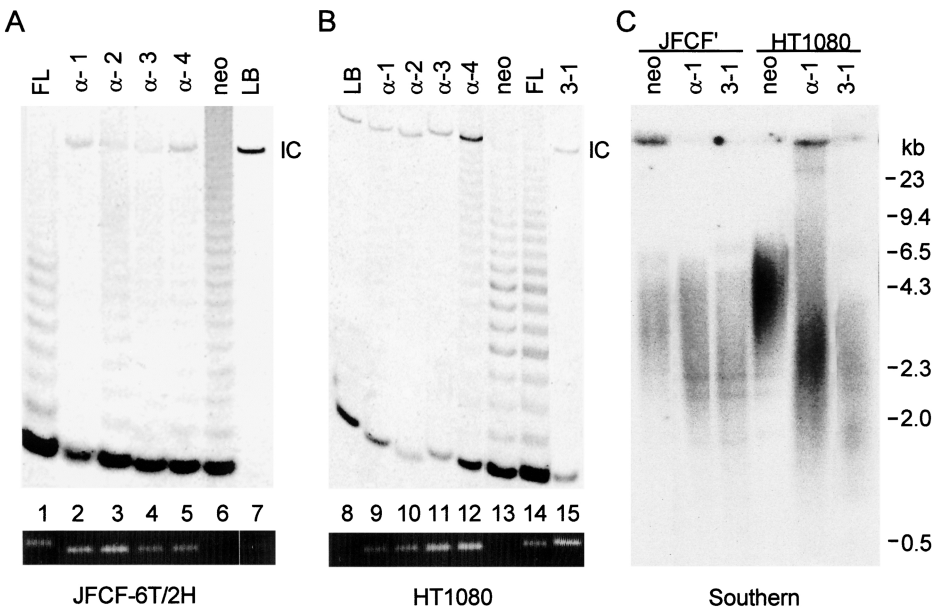

Figure 2. *Overexpression of hTERTα inhibits endogenous telomerase activity in stable clones of JFCF-6T/2H and HT1080. (A–B) The TRAP assay was performed on 2 μg of protein lysate from each of the indicated G418-selected clonal cell lines. Lanes 1 and 14, full length hTERT controls; lanes 2 through 5, JFCF-6T/2H stable clones expressing hTERTα; lanes 6 and 13, empty vector controls; lanes 7 and 8, lysis buffer; lanes 9 through 12, HT1080 stable clones expressing α variant; lane 15, D712A dominant negative control. Results of RT-PCR to check for plasmid transcription are shown below the corresponding clone. IC indicates internal control for the PCR step of the TRAP assay. (C) Example of Southern analysis on stable clones overexpressing dominant negative inhibitors of telomerase. JFCF' indicates JFCF-6T/2H cells. HT1080 or JFCF-6T/2H were transfected with empty vector (neo), pIRES-hTERTα (α) or 3-1 dominant negative control. Telomeric DNA, $(T_2AG_3)_3$, was used as a probe. Positions of size markers are indicated on the right.*

primers HT2026F/HT2482R. Only one round of PCR was used to detect plasmid, unlike the two rounds used for GC-rich PCR, which is why no hTERT products are detectable in vector only controls (neo) (Figure 2).

Terminal Restriction Fragment (TRF) Analysis

High-molecular-weight genomic DNA was isolated from 10^6 cells, and 40 μg was digested with restriction enzymes *Hin*f1 and *Rsa*1. The digested DNA was quantitated by Hoechst dye staining and fluorimetry. One microgram of DNA was loaded onto a 1% agarose gel in 0.5×Tris–borate–EDTA (TBE) buffer. DNA was separated by electrophoresis at 1.2 V/cm for 18 hours. For some experiments, pulsed-field gel electrophoresis was carried out using a CHEF-DR II pulsed-field electrophoresis apparatus (BioRad) in recirculating 0.5×TBE buffer at 14°C with a ramped pulse speed of 1 to 6 seconds at 200 V for 14 hours. The gels were dried, denatured and hybridized to a [γ-^{32}P]dATP, 5′ end-labeled telomeric oligonucleotide probe $(TTAGGG)_3$, washed in 0.1×SSC and finally exposed to Kodak XAR film for 24 hours as described [17].

FISH Analysis of Metaphase and Interphase Nuclei

Chromosome preparations from colcemid arrested metaphases were obtained according to standard cytogenetic methods. Fluorescence *in situ* hybridization (FISH) with a Cy3-conjugated telomere-specific $(C_3TA_2)_3$ PNA (peptide nucleic acid) probe (PE Biosystems, Framingham, MA) was performed according to Lansdorp *et al.* [18]. Slides were evaluated on a Leica DMLB fluorescence microscope with appropriate filter sets for UV and green excitation. Images were captured on a cooled CCD camera (SPOT 2, Diagnostics Instruments), merged using SPOT software and further processed using Adobe Photoshop 5.5.

Results

Comparison of the α transcript (hTERTα) sequence [12] with the genomic structure of hTERT [19,20] indicates that the 36-bp deletion in hTERTα is formed by use of an alternative splice acceptor site within exon 6 (Figure 1*A*). RT-PCR showed that hTERT variant transcripts were detectable in several telomerase-positive immortalized cell lines (Figure 1*B*). Although the PCR is not quantitative, hTERTα appeared to be abundantly expressed in HT1080 cells.

The 12 amino acids missing from hTERTα are from the conserved reverse transcriptase domain A. As expected, hTERTα does not possess telomerase activity. This variant was previously amplified from an LIM1215 cDNA library [12], then subcloned into a mammalian expression vector and transiently transfected into GM847 cells, an immortal cell line that lacks telomerase activity but which expresses the RNA subunit, hTER [21]. Transient expression of hTERT induced telomerase activity, detectable by TRAP assay (Figure 1*C*), whereas hTERTα did not, even though RT-PCR analysis indicated that the variant was expressed from the plasmid (not shown).

It has been shown that single base substitutions artificially introduced into hTERT domain A result in a dominant negative form of the protein [13,22]. Therefore, we tested hTERTα for the ability to inhibit telomerase activity in two telomerase-positive cell lines (HT1080 and JFCF-6T/2H) by generating stable clones overexpressing hTERTα from a CMV promoter. The expression construct contained an internal ribosome entry sequence (IRES) so that the hTERT variant and neomycin phosphotransferase could be expressed as a single transcript. The dominant negative 3-1 construct [13], containing a single amino acid change (D712A), was used as a control. In the majority of stable clones overexpressing 3-1 or hTERTα there was an almost complete inhibition of telomerase activity (Table 1, Figure 2*A* and *B*). Nonquantitative RT-PCR analysis verified that the plasmids were expressed in all clones that showed inhibition of telomerase activity (Figure 2*A* and *B*, lower panels).

Southern analysis of telomere lengths in HT1080 clones overexpressing hTERTα or 3-1 showed that telomerase inhibition resulted in telomere shortening relative to empty vector controls (Figure 2*C*). FISH, using a probe for telomeric DNA also showed shortened telomeres on most chromosomes, and some telomeres were undetectable (Figure 3*A*). In 20/20 hTERTα metaphases examined, there were chromosome ends with no detectable telomere sequence, compared to 1/15 vector control metaphases. Chromosome end-to-end fusions were common in telomerase-inhibited clones (18/20 hTERTα metaphases, versus 0/15 for vector control). In some cases, circularized chromosomes were also apparent (2/20 metaphases, compared to 0/15 in vector control).

Telomeric shortening in JFCF-6T/2H cells was not visible by Southern analysis, except as a small increase in the amount of low-molecular-weight (<2.4 kb) telomeric DNA relative to the pIRES-neo controls (Figure 2*C*). However, the effects of telomerase inhibition were apparent by FISH analysis, as several chromosomes within each cell had undetectable telomeres (20/20 metaphases, versus 0/20 for vector control) (Figure 3*B*). Most telomeres within each cell were still visible as a bright hybridization signal (Figure 3*B*), which may explain why telomere shortening was not

Table 1. Effects of hTERTα on Telomerase Positive Cells.

Plasmid (pIRES-)	HT1080	JFCF-6T/2H
neo	0/5	0/5
hTERT	0/3	0/8
3-1	7/11 (7, PD26-35)	2/3 (2, PD32-39)
hTERT-α	7/7 (6, PD29-35)	4/4 (4, PD23-29)

Telomerase-positive cell lines were transfected with the indicated plasmids and stable G418-resistant clones were analyzed for telomerase activity. The proportion of stable clones showing telomerase inhibition, as determined by semiquantitative TRAP, are shown. The number of telomerase-inhibited clones that underwent senescence or apoptosis are indicated in parenthesis, followed by the range of population doublings (PD) reached by these clones before the onset of senescence or apoptosis.

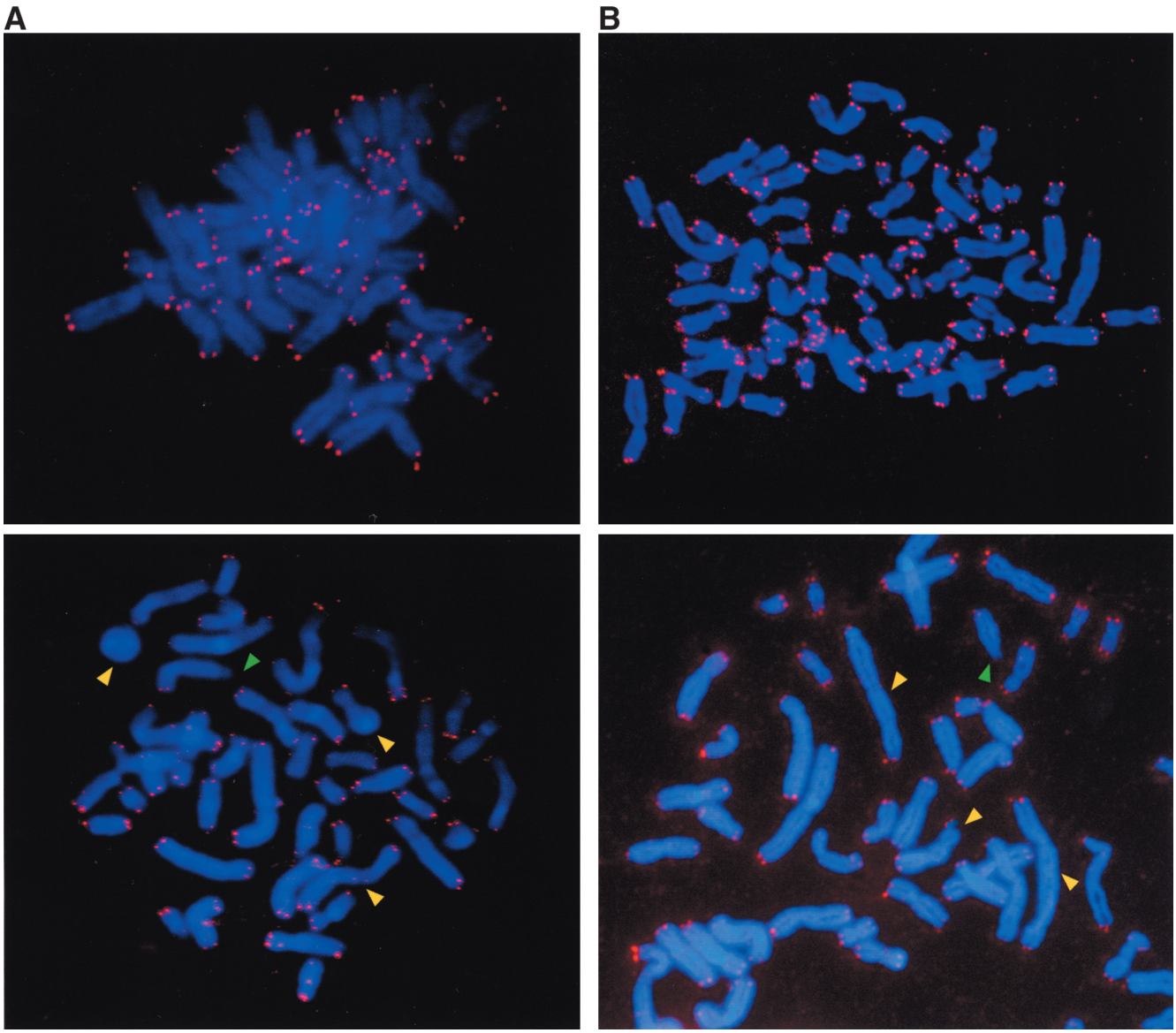

Figure 3. *hTERTα expression causes loss of telomeric DNA and chromosome end-to-end fusions. (A) FISH analysis using Cy3-conjugated telomeric PNA probe on metaphases of HT1080 stable clones overexpressing empty vector (upper panel) or pIRES-hTERTα (lower). DNA was counterstained with DAPI and images merged. Undetectable telomeres (green arrows) and fused or circularized chromosomes (yellow arrows) are indicated. (B) FISH analysis using Cy3-conjugated telomeric PNA probe on metaphases of JFCF-6T/2H stable clones overexpressing empty vector (upper panel), or pIRES-hTERTα (lower). DNA was counterstained with DAPI and images merged. Undetectable telomeres (green arrows) and fused or broken chromosomes (yellow arrows) are indicated.*

detectable by Southern analysis. Every metaphase examined showed chromosome end-to-end fusions (compared to 0/20 controls), although many also had broken chromosomes (5/20 metaphases), or circularized chromosomes (1/20 metaphases) (Figure 3*B*).

In addition to the inhibition of *in vitro* telomerase activity, exogenous expression of hTERTα or 3-1 induced cell death, in the case of JFCF-6T/2H, and a senescence-like state in the case of HT1080. HT1080 cells expressing 3-1 or hTERTα became large and flattened and stained positive for senescence-associated β-galactosidase activity (Figure 4*A*). JFCF-6T/2H clones showed cell loss and debris (Figure 4*B*). DAPI staining (Figure 4*B*) and TUNEL staining (not shown) indicated that cell death was due to apoptosis. This may have been caused by critical shortening of just a few chromosomes within each cell. Although hTERTα was not tested in HeLa cells, these cells, like HT1080, exhibited a senescence-like state after overexpression of the 3-1 dominant negative control (Figure 4*C*).

In most cases, the senescence-like state or cell death occurred within 30 population doublings (PD), 30 to 40 days, after transfection (Table 1). In several clones, revertant colonies arose in the population, which eventually overgrew the culture. RT-PCR indicated that the revertants had stopped expressing the dominant negative mRNAs, and

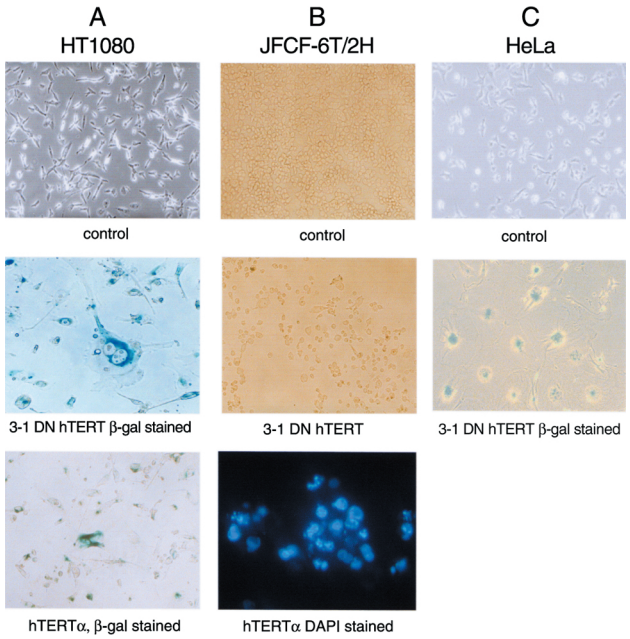

Figure 4. *Dominant negative forms of hTERT induce either apoptosis or a senescence-like state. (A) Photomicrographs of HT1080 stable clones are shown. "control," (upper panel) was transfected with empty vector. Dominant negative (DN) 3-1 control is shown with staining for endogenous β-galactosidase activity (middle). β-Galactosidase staining of an hTERTα transfected stable clone is also shown (lower). (B) JFCF-6T/2H cells are shown: control, empty vector (upper); dominant negative (DN) 3-1 control (middle); α variant transfected stable clone, DAPI stained (lower). (C) Stable clones of HeLa are shown: empty vector (top) and β-galactosidase stained cells expressing DN 3-1 control (bottom).*

TRAP analysis showed that they had reactivated telomerase activity (not shown).

Discussion

The lack of catalytic activity in hTERTα was expected because a critical portion of the catalytic core is deleted. Point mutations introduced into this region have abolished activity, and several dominant negative hTERT mutants have been developed [13,22–24]. However, this is the first demonstration of an endogenous form of hTERT with dominant negative activity.

Although neither hTERTα nor the dominant negative control (3-1) completely abolished telomerase activity, the reduction in activity that was obtained was sufficient to reverse the immortal phenotype. This is encouraging for the development of telomerase inhibitors for tumor treatment.

As would be predicted, HT1080 cells exhibited telomeric shortening after inhibition of telomerase activity. In contrast, in the JFCF-6T/2H cell line telomeric shortening was observed on only a few chromosomes within each cell, rather than an overall loss of telomeric sequences. There were a few chromosomes within each cell with telomeres so short as to be undetectable by FISH, although other chromosomes within the cell had easily detectable telomeres. It is possible that particular chromosome ends in this cell line may be more sensitive to loss of telomerase activity and may have shortened more quickly.

The HT1080 cells entered a senescence-like state after transduction with the dominant negative forms of hTERT, and this is the first report of a senescence-like state occurring after inhibition of telomerase activity. The molecular mechanisms of this effect need to be investigated, but it seems possible that chromosome end-to-end fusions may be involved. Van Steensel and colleagues have shown that expression of dominant negative forms of the telomere-binding protein TRF2 induced end-to-end fusions, without loss of telomeric DNA, and a senescence-like state in an HT1080 derivative [25]. Evidence was presented that the fusions occurred due to loss of telomeric single-stranded G tails. Thus, senescence may be induced in these cells either by loss of telomeric DNA following inhibition of telomerase activity or by disruption of the telomeric architecture. Although we cannot rule out the possibility that the dominant negative hTERT molecules somehow disrupted the telomeric protein cap, it seems more likely that a few critically short telomeres caused the chromosome end-to-end fusions and resultant chromosome breaks that were visible in these cells.

As reported previously [22–24], dominant negative forms of hTERT are able to induce apoptosis. It is of interest that, depending on the cell line, the dominant negative hTERT molecules (3-1 and the hTERTα variant) were able to induce either apoptosis or cellular senescence. SV40-expressing cells at crisis exhibit a similar duality of responses to telomere shortening: cells approaching crisis have telomeres shorter than those seen in senescent cells [26], and at crisis they may become apoptotic or may arrest in a senescence-like state [27]. In this context, it should be noted that DNA double-strand breaks, which are a well-known trigger of apoptosis [28,29], were observed in the telomerase-inhibited JFCF-6T/2H cells.

Identification of the dominant negative properties of the α variant raises the possibility that alternative splicing may represent an additional level of control of telomerase activity. So far, the deletion variants have been detected by RT-PCR in immortalized cells (this paper and [12]), normal tissues [30] and during human development [10]. Expression of hTERTα (and perhaps other variants) may downregulate telomerase activity in a dose-dependent manner, but reliable antibodies need to be developed to investigate this possibility *in vivo*. Progress in understanding control of alternative splicing may eventually permit upregulation of hTERTα being used as an important therapeutic tool in the treatment of human cancers.

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