Amplification of Telomerase Reverse Transcriptase Gene in Human Mammary Epithelial Cells with Limiting Telomerase RNA Expression Levels

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Abstract

Activation of telomerase is a crucial step during cellular immortalization, and in some tumors this results from amplification of the human telomerase reverse transcriptase (hTERT) gene. Immortalization of normal human cells has been achieved by transduction with hTERT cDNA under the control of a strong heterologous enhancer/promoter, but this is sometimes an inefficient process, with periods of poor growth or even crisis occurring before immortalization. Here, we showed that normal human mammary epithelial cells expressing exogenous hTERT amplified the transgene extensively and expressed high levels of hTERT mRNA and protein. Paradoxically, the cells had low levels of telomerase activity and very short telomeres, indicating that telomerase activity did not correlate with hTERT expression. These cells contained only ~20 human telomerase RNA (hTR) molecules/cell (compared with ~120 hTR molecules per 293 cell). Expression of exogenous hTERT caused increased telomerase activity and telomere shortening. These data indicate that some hTERT-transduced normal cells may express high levels of the transgene but fail to up-regulate endogenous hTR expression sufficiently to enable expression of robust levels of telomerase activity. [Cancer Res 2008;68(9):3115–23]

Introduction

Human telomeres are nucleoprotein complexes located at the ends of linear chromosomes (1). They shorten at each cell division, which limits the number of times a cell can divide (2). Telomerase is a ribonucleoprotein enzyme complex that adds telomeric repeat sequences to the ends of chromosomes to counteract this shortening (3). It has recently been found that the active human telomerase enzyme is composed of human telomerase reverse transcriptase (hTERT), human telomerase RNA (hTR), and dyskerin (4). hTERT is the catalytic reverse transcriptase component (5), hTR serves as the RNA template for the addition of telomeric repeats (6), and dyskerin is an RNA-binding protein. Mutations in any of these components may result in dyskeratosis congenita, a human disease syndrome associated with short telomeres (reviewed by Kirwan et al. in ref. 7).

Telomerase is not detectable in most normal human somatic cells, whereas it is expressed in >85% of human tumors (8). How telomerase levels are regulated in these tumors is still poorly understood. A major focus of investigation has been the control of hTERT transcription (reviewed by Horikawa et al. in ref. 9). Amplification of the hTERT gene has been observed in various human cancer cell lines and tumors, and is also thought to be a mechanism of telomerase activation (10–13).

In view of the observations that hTR is ubiquitously expressed (6, 14), whereas hTERT is expressed only in telomerase-positive cells (5), and that expression of exogenous hTERT alone can immortalize normal human cells (15, 16), abundance of hTERT was previously thought to be the sole limiting factor for telomerase activity. Although immortalization of many types of normal human cells has been achieved by transducing them with exogenous hTERT, for some strains of normal human cells, this is an inefficient process, with periods of poor growth or even crisis being experienced before immortalization (17–19). Data are accumulating in support of the notion that hTR levels can also be limiting for telomerase activity. For example, hTR mutations have been found to cause autosomal dominant dyskeratosis congenita (20) and can reduce telomerase activity via haploinsufficiency (21). Furthermore, overexpression of both hTERT and hTR elongates telomeres better than overexpression of hTERT alone in X-linked and autosomal dominant dyskeratosis congenita (22, 23). It has also been shown that concomitant overexpression of hTERT and hTR was necessary to substantially increase telomerase activity in both cancer and primary cells (24). However, none of these studies quantified the number of hTR molecules in the cells. This is important because a previous study has reported that there are 11,000 to 13,500 hTR molecules per cell in the telomerase-negative BJ, IMR90, and SW13 cell strains (25), which would be inconsistent with a limiting role for hTR in light of the 20 to 50 active telomerase molecules per cell in 293 cells (4).

In this study, we explore mechanisms of telomerase activity regulation. Human mammary epithelial cells (HMEC) have been transduced with hTERT in our laboratory, and four independent hTERT-immortalized mass cultures (B80-TERT1, 2, 3a, and 3b) were obtained (26). In the present study, we found that the B80-TERT1 cell line had undergone extensive amplification of the hTERT transgene and was expressing high levels of hTERT mRNA and protein but had low telomerase activity. Furthermore, we showed that hTR levels limit telomerase activity and telomere length in this cell line. More importantly, our quantitation of hTR molecules per cell provides an explanation for why hTR is limiting: the low abundance of hTR (~20 molecules of hTR per cell) is
insufficient for significant telomerase activity and telomere maintenance even in the presence of a vast excess of hTERT. To our knowledge, our study is the first to report the low abundance of hTR in telomerase-negative cells (approximately three hTR molecules in each telomerase-negative B80 cell), and that hTR limits telomerase activity and telomere maintenance in the context of hTERT amplification.

Materials and Methods

Cells and cell culture. HMECs were cultured in MCDB 170 medium (Invitrogen) as described previously (26). PA317 packaging cells were grown in Dulbecco's Modified Eagles Medium with 10% fetal bovine serum in a 5% CO2 incubator at 37°C.

Fluorescence in situ hybridization analysis. Chromosome preparations from colcemid-arrested cells were obtained according to standard cytogenetic methods (27). A 3.4-kb full-length hTERT cDNA excised from pCIneo-hTERT plasmid (28) or a 650-bp NH2-terminal hTERT fragment excised from pJ101-hTERT1-182aa was labeled with bio-16-dUTP using the Biotin-Nick Translation Mix (Roche) according to the manufacturer's instructions. Approximately 30 ng/µL of the full-length hTERT or the 650-bp NH2-terminal hTERT fragment was hybridized onto separately denatured chromosome preparations for 16 to 18 h in a humidified chamber at 37°C. The hybridized probe was detected with Fluorescein Avidin DCS (Vector Laboratories) followed by signal amplification with biotinylated anti-avidin antibody (Vector Laboratories) and another layer of Fluorescein Avidin DCS. Chromosomes were counterstained with diamidino-phenyl-indole-dihydrochloride (DAPI; final concentration, 0.6 µg/mL; Sigma-Aldrich) for chromosome identification, and slides were evaluated on a Leica DMLB fluorescence microscope with appropriate filter sets. Fluorescein and DAPI images were captured separately with a cooled charge-coupled device camera (SPOT2; Diagnostic Instruments), merged using SPOT2 software, and further processed using Adobe Photoshop version 6.0 software.

Southern blot analysis. Genomic DNA was digested with EcoR I and Sal I, separated through a 0.8% agarose gel with Tris-borate-EDTA (TBE) buffer at pH 7.5, and transferred onto Hybond N+ membrane (Amersham Biosciences) by capillary action in 0.4 mol/L NaOH for 4 h. The membrane was blocked in Church buffer [1% bovine serum albumin, 1 mmol/L EDTA, 0.5 mol/L NaPO4 (pH 7.2), and 7% SDS] at 65°C for 1 h, followed by hybridization (in Church buffer at 65°C overnight) with the 3.4-kb full-length hTERT cDNA excised from pCIneo-hTERT plasmid and radiolabeled with α-32P-dCTP. The membrane was washed twice in 2× SSC/0.1% SDS at 65°C for 20 min, followed by another two washes in 0.2× SSC/0.1% SDS at 65°C for 20 min. Washed membrane was exposed to Kodak BioMax MS X-ray film (Sigma-Aldrich).

Reverse transcriptase-PCR. Total RNA was isolated from cell pellets by using TRI-zol reagent (Invitrogen). One microgram of total RNA was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (Promega). Taq polymerase (Roche) was used for the amplification. See Supplementary Data for PCR conditions and primer sequences.

Quantitative real-time reverse transcriptase-PCR. The Qiagen RNeasy Mini kit (Qiagen) was used to extract total RNA from cells that had been pelleted by centrifugation. Total RNA (1 µg) was treated with DNase I (Invitrogen), followed by reverse transcription [SuperScript III First-Strand Synthesis System for reverse transcriptase-PCR (RT-PCR); Invitrogen] according to the manufacturer's instructions. Real-time RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) in a Corbett RotorGene-6000 thermal cycler. Cycle parameters were as follows: incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.
60°C for 1 min. Housekeeping gene gyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Primer pairs were matched for PCR efficiencies. See Supplementary Data for primer sequences.

**Northern blot analysis.** Total RNA from 5 × 10^6 or 1 × 10^7 cells was prepared using an RNaseasy Mini kit (Qiagen), TRI-zol reagent (Invitrogen), or phenol/chloroform extraction from whole cell lysate to compare potential yield differences. Total RNA extracted by any of the above methods was precipitated, resuspended in 10 μL of water and 10 μL of formamide loading buffer, followed by heating at 70°C for 5 min, loaded on a 1.5-mm thick 4% polyacrylamide, 8M urea gel, and electrophoresed at 25 W for 1 h in 1× TBE buffer. RNA was transferred onto Hybond N+ membrane (Amersham Biosciences) by electroblocting at 1.5A for 2 h in 0.5× TBE buffer, with the buffer cooled to 4°C. The membrane was crosslinked with 254 nm UV light (Stratalinker; Stratagene) and prehybridized in Church buffer (see above) at 55°C for 1 h, followed by hybridization in Church buffer at 55°C overnight with 32P 5′-end–labeled hTR probe (5′-CGG TGG AAG GCG GCA GGC CGA GGC-3′). The membrane was washed three times in 0.1× SSC/0.1%SDS at 55°C for 10 min, exposed to a phosphor screen, and scanned with a Storm 860 optical scanner with ImageQuant software (Molecular Dynamics).

**Western blot analysis.** Cell pellets were lysed in freshly made radioimmunoprecipitation assay buffer [10 mmol/L Tris-HCl (pH8.0), 140 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 1× complete protease inhibitors (Roche), 10 mmol/L phenylmethylsulfonflurylhydroxide, and 0.1% sodium deoxycholate]. Lysates equivalent to 50 μg of protein were separated on an 8% polyacrylamide/SDS gel and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose (Amersham Biosciences) by electroblocting. The membrane was blocked with 1× PBS containing 2% skim milk and 0.05% Tween 20 at room temperature for 1 h. The membrane was then incubated with the primary antibody (goat polyclonal anti-hTERT; sc-7215; Santa Cruz) in 1×PBS containing 1% skim milk and 0.05% Tween 20 at 4°C overnight. The membrane was washed in 1×PBS containing 0.05% Tween 20, followed by the incubation with the secondary antibody (rabbit anti-goat immunoglobulin horseradish peroxidase; 1:1,000 dilution; DAKO Cytomation) in 1×PBS containing 1% skim milk and 0.05% Tween 20 at room temperature for 1 h. Amersham ECL plus Western blotting detection system (Amersham Biosciences) and high performance chemiluminescence film (Amersham Biosciences) were used for detection.

**Direct telomerase activity assay.** A direct telomerase activity assay recently developd in our laboratory (29) was used. Cells (1 × 10^7) were lysed and the lysate was incubated with purified anti-hTERT antibody (4). Protein G/Agarose beads were added and the immunoprecipitate was collected into a microspin column. The beads were washed, and antigenic peptide (4) was mixed with the immunoprecipitate to elute telomerase. The activity of immunopurified telomerase was analyzed as described in Supplementary Data.

**Production of retroviruses and infections.** hTR construct pBABE-puro-U3-hTR (kindly provided by Dr. Kathleen Collins, Department of Molecular and Cell Biology, University of California, Berkeley, CA) and vector alone (pBABE-puro) were transfected into PA317 packaging cells separately using siPORT XP-1 transfection reagent (Ambion). After 48 h, medium was harvested and filtered through a 0.45-μm membrane, and polybrene (Sigma-Aldrich) was added to a final concentration of 4 μg/mL. B80-TERT1 cells were grown to 50% confluence and then incubated with retrovirual supernatant at 37°C for 8 h, followed by incubation with fresh medium overnight. Forty-eight hours after infection, cells were passaged and grown in selection medium containing 0.6 μg/mL puromycin (Sigma-Aldrich). Clones were obtained and maintained in medium containing 0.6 μg/mL puromycin.

**Terminal restriction fragment analysis.** HindI- and Bsa I-digested genomic DNA (1.5 μg) was fractionated on a pulsed-field electrophoresis apparatus (Bio-Rad) as previously described (30). The gel was dried, denatured, and hybridized to a [α-32P] dATP 5′-end–labeled telomeric oligonucleotide probe, (TTAGGG)_5. The gel was exposed to a phosphor screen and scanned with a Storm 860 optical scanner with ImageQuant software. Median telomere length was obtained with Telometric software (31).

See Supplementary Data for Preparation of hTR standards and in vitro hTERT translation.

### Results

**Amplification of exogenous hTERT in HMECs.** Four independent hTERT-immortalized mass cultures B80-TERT1, 2, 3a, and 3b were previously obtained in our laboratory by transfecting B80 HMECs with pCIneo-hTERT (26). In the present study, we detected extensive amplification of hTERT DNA in B80-TERT1 cells by flow cytometry in situ hybridization (FISH) analysis of metaphase...
spreads using both a 3.4-kb full-length hTERT cDNA (Fig. 1A) and a
650-bp NH2-terminal hTERT probe (data not shown). Amplification
of hTERT was observed in every metaphase (Fig. 1A) and interphase nucleus (Supplementary Fig. S1), which indicates that
this immortalized cell line has become clonal, although it was
originally established as a mass culture. A similar pattern of
hybridization in B80-TERT1 cells was also observed by FISH
analysis when pCIneo vector alone was used as a probe (data not
shown). These observations indicate that the hTERT transgene and
its accompanying vector sequence were extensively amplified in
B80-TERT1 cells.

Furthermore, hTERT amplification was also detected in B80-
TERT3b cells by FISH analysis (Supplementary Fig. S2), although
to a much lesser extent than in B80-TERT1 cells. No hTERT ampli-
fication was detectable by FISH in B80-TERT2 or 3a cells (data not
shown).

To confirm the hTERT amplification seen by FISH in B80-TERT1
and B80-TERT3b cells, Southern blot analysis was performed using
the full-length hTERT cDNA as a probe. A very high hTERT copy
number was detected in only 1 μg of B80-TERT1 genomic DNA,
whereas only a small amount of hTERT DNA was detected in 10 μg
of genomic DNA from B80-TERT2 and B80-TERT3a. An interme-
diate TERT copy number was observed in B80-TERT3b (Fig. 1B).
The genomic DNA was digested with EcoR I and Sal I restriction
enzymes, which excise the 3.4-kb hTERT cDNA from plasmid
pCIneo-hTERT with which the B80-TERT lines were generated. The
presence of a dominant 3.4-kb band (Fig. 1B, top) showed that it
was the exogenous hTERT that was amplified. This was confirmed
by the observation that a high copy number of the vector backbone
was detected in B80-TERT1 cells when the pCIneo vector alone was
used as a probe in Southern blot analysis (data not shown).

Correlation between hTERT RNA/protein expression and
hTERT gene copy number. Expression of hTERT mRNA was
measured by conventional and real-time RT-PCR. As shown in
Fig. 2A, the highest level of hTERT mRNA was detected in B80-
TERT1 cells, less hTERT mRNA was detected in B80-TERT3b, and
the least amount of hTERT mRNA was detected in B80-TERT2
and B80-TERT3a cells. No hTERT mRNA could be detected in untrans-
fected B80 cells. Similar amounts of GAPDH mRNA were detected
in all five HMEC cultures (Fig. 2A). Quantitation by real-time
RT-PCR is shown in Fig. 2B. No hTERT mRNA was detected in
untransfected B80 cells (data not shown). All hTERT immortalized
lines showed a significantly higher level of hTERT mRNA compared
with telomerase-positive 293 cells, with the highest level of hTERT
mRNA detected in B80-TERT1 (>30-fold greater than in 293 cells).

To determine whether hTERT protein is being expressed and
whether the protein level correlates with the hTERT gene copy
number and hTERT mRNA level, Western blot analysis using an
antibody against hTERT was performed, with in vitro
translated hTERT as a positive control (Fig. 2C). No hTERT could be
detected in untransfected B80 cells (data not shown) and the highest hTERT
protein level was found in B80-TERT1 cells. Therefore, we conclude
that hTERT copy number in this panel of HMEC cell lines
correlates with expression levels of hTERT mRNA and protein.

Lowest telomerase activity and lowest hTR in the highest
hTERT-expressing cells. To determine whether hTERT expression
 correlates with telomerase activity, a direct quantitative telomerase
activity assay was performed in all four hTERT-immortalized lines
(Fig. 3A and B). Surprisingly, B80-TERT1 cells had the lowest
telomerase activity (32% of the activity in B80-TERT3b cells).

hTR levels have previously been shown to limit telomerase
activity in hTERT-overexpressing HeLa, HT1080, and normal

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Figure 3. Telomerase activity measured by a direct
telomerase assay correlates with hTR levels.
Telomerase reaction products were analyzed on a 10% denaturing sequencing gel (A). Note only 2.5 x 10^6
293 cells were assayed, whereas 1 x 10^7 cells were
used for the others. Results from four independent
telomerase assays were quantitated with ImageQuant
software as shown in B. Columns, mean; bars, SE. No telomerase activity was detected in B80
cells. hTR (C) and DKC1 mRNA (D) levels were
measured by real-time RT-PCR. Columns, mean;
bars, SE from three separate PCR runs (C). Columns,
mean; bars, ranges for duplicate PCR reactions from
the same PCR run (D). B80, 1, 2, 3a, and 3b are
defined in Fig. 2 legend.
human lung fibroblasts (24). To determine whether telomerase activity is limited by hTR in hTERT-immortalized B80 cells, relative hTR level was examined using real-time RT-PCR. As shown in Fig. 3C, the hTERT-transduced B80 cells had increased hTR expression (15- to 51-fold) compared with the untransfected controls. The lowest level of hTR was detected in B80-TERT1 (15-fold increase), and the highest level of hTR was detected in B80-TERT3b (51-fold). Different primer sets gave similar real-time RT-PCR results (Supplementary Fig. S3).

To determine the number of hTR molecules in each cell, we performed both Northern blot analysis and real-time RT-PCR using serially diluted *in vitro* transcribed hTR as standards. Both assays showed excellent linearity of the standards over the range used (Fig. 4, *bottom*). Three Northern blot analyses with 293 cells (using three different methods to extract total RNA to compare potential RNA yield difference; see Materials and Methods section) gave very reproducible results, and the number of hTR molecules in each 293 cell was 123 ± 23 (mean ± SE; data not shown). Northern blot analysis showed that the number of hTR molecules per cell was 23, 28, 43, and 90 in B80-TERT1, 2, 3a, and 3b, respectively (Fig. 4A). A repeat Northern gave a 2-fold lower estimate of hTR molecules per cell (data not shown). Three separate real-time RT-PCR analyses with duplicates in each PCR run gave a slightly higher estimate of hTR copy number as follows: 45, 76, 79, and 148 molecules of hTR in B80-TERT1, 2, 3a, and 3b, respectively (Fig. 4B). The ~2-fold difference in hTR molecules per cell quantified by our Northern and real-time RT-PCR could be due to the fact that Northern blot analysis only detects the mature 451-nucleotide hTR, whereas real-time RT-PCR would detect the mature hTR in addition to any form of precursor, incompletely synthesized and even partially degraded hTR, provided the target 150-bp amplicon was intact. There are also some technical reasons that may contribute to the differences between the two methods. For example, the signal to noise ratio may confound quantitation by Northern more than by real-time RT-PCR. In addition, this difference is similar to the 3-fold difference in TLC1 (yeast telomerase RNA) copy number examined by Northern and real-time RT-PCR analysis (32). We consider that the copy number of functional hTR molecules is more likely to correspond to the number obtained by Northern because this technique detects the mature full-length hTR. Regardless of which technique is more accurate, the data indicate that telomerase activity correlates with hTR, rather than with hTERT expression levels in these hTERT-overexpressing HMECs.

As dyskerin (encoded by the *DKC1* gene) is part of the active telomerase enzyme in addition to hTERT and hTR (4), we examined DKC1 mRNA expression to determine whether DKC1 level correlates with levels of hTERT, hTR, or telomerase activity. As shown in Fig. 3D, hTERT overexpression resulted in increased DKC1 expression from 2- to 4-fold in the four B80-TERT lines. However, no consistent correlation was found between expression of DKC1 mRNA and either hTERT or hTR expression, or telomerase activity.

**Increased telomerase activity and telomere lengthening resulted from hTR overexpression.** We next addressed whether overexpression of hTR would increase telomerase activity in B80-TERT1 cells. An hTR-containing retroviral vector, pBABE-U3-hTR, was used to induce hTR expression, with pBABE vector alone as a control. B80-TERT1 cells were infected with the retrovirus and

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**Figure 4.** hTR molecules per cell determined by both quantitative Northern blot analysis and real-time RT-PCR. **A,** a representative Northern blot analysis using *in vitro* transcribed hTR (*i.v. hTR*) as standards (*top*). Total RNA from 5 × 10⁶ 293 cells and 1 × 10⁶ B80-TERT1, 2, 3a, and 3b cells was loaded on the gel. **B,** a recovery control added before RNA precipitation (³²P-labeled 100-mer DNA oligonucleotide). About 40% of the RNA was recovered after RNA precipitation (data not shown), which was taken into account when calculating hTR molecules per cell (given beneath the gel) by interpolation from the standard curve shown at bottom. **B** shows the standard curve (*bottom*). **C** shows the standard curve (*bottom*). **D** shows the standard curve (*bottom*). **E** shows the standard curve (*bottom*). **F** shows the standard curve (*bottom*). **G** shows the standard curve (*bottom*). **H** shows the standard curve (*bottom*). **I** shows the standard curve (*bottom*). **J** shows the standard curve (*bottom*). **K** shows the standard curve (*bottom*). **L** shows the standard curve (*bottom*). **M** shows the standard curve (*bottom*). **N** shows the standard curve (*bottom*). **O** shows the standard curve (*bottom*). **P** shows the standard curve (*bottom*). **Q** shows the standard curve (*bottom*). **R** shows the standard curve (*bottom*). **S** shows the standard curve (*bottom*). **T** shows the standard curve (*bottom*). **U** shows the standard curve (*bottom*). **V** shows the standard curve (*bottom*). **W** shows the standard curve (*bottom*). **X** shows the standard curve (*bottom*). **Y** shows the standard curve (*bottom*). **Z** shows the standard curve (*bottom*).
selected with puromycin. Individual colonies were picked and cultured for further analysis. Seven hTR-overexpressing clones (hTR15, hTR17, and hTR19-23), three pBABE vector-alone clones (BABE2, BABE6, and BABE8), and parental B80-TERT1 cells were examined for hTR, hTERT, and DKC1 expression using real-time RT-PCR.

In the hTR-infected cells, hTR was overexpressed from 35- to 125-fold compared with parental B80-TERT1 cells, whereas in the vector control clones, hTR levels varied from 0.8- to 1.9-fold compared with the parental cells (Fig. 5A). The difference in hTR level between hTR-infected and vector control clones was highly significant (P < 0.0001). hTERT expression varied from 1.2- to 4.3-fold in the three vector control clones and from 1.1- to 6.1-fold in the seven hTR-overexpressing clones compared with parental B80-TERT1 cells (Fig. 5B). DKC1 RNA expression varied from 1.5- to 2.7-fold in the three vector control clones and from 1.7- to 3.0-fold in the seven hTR-overexpressing clones (Fig. 5C). No significant difference was observed in either hTERT level (P = 0.68) or DKC1 level (P = 0.25) between hTR-infected and vector control clones (Fig. 5).

To measure telomerase activity levels, we used a direct telomerase activity assay. Telomerase activity was increased from 33- to 63-fold in hTR-overexpressing clones in comparison with 1.1- to 1.6-fold in vector-alone controls (Fig. 6A). We then determined whether the increased telomerase activity resulting from hTR overexpression in B80-TERT1 cells would cause telomere shortening. As shown in Fig. 6B, B80-TERT1 cells had very short telomeres (median telomere length, 5 kb), whereas B80-TERT3b cells had very long telomeres (median telomere length, 14 kb), correlating with the telomerase activities in those cells (Fig. 3A). As expected, all hTR-overexpressing clones had extensively elongated telomeres (median telomere length varied from 29–36 kb in 7 hTR-overexpressing clones; Fig. 6B), compared with the vector controls that exhibited short telomeres (median telomere length varied from 4.5–5.2 kb in three vector control clones), similar to the parental B80-TERT1 cells. We therefore conclude that hTR overexpression in B80-TERT1 cells resulted in increased telomerase activity and consequent telomere lengthening.

**Discussion**

hTERT amplification has previously been observed in human cancer cell lines (10), neuroblastomas, and tumors of lung, cervix, and breast (10–13). There seems to be an inconsistent relationship between amplification of the hTERT gene and the levels of hTERT mRNA, protein expression, and telomerase activity (10–13). Our study has identified a reason for lack of correlation between hTERT expression levels and telomerase activity, namely inadequate up-regulation of hTR expression.

There was a very low abundance of hTR in telomerase-negative B80 cells before hTERT overexpression, approximately three molecules per cell as measured by real-time RT-PCR (and undetectable by Northern blot; data not shown). Therefore, even a 15-fold up-regulation of hTR in B80-TERT1 (Fig. 3C) was associated with a telomerase activity level that resulted only in maintenance of very short telomeres. That hTR levels were limiting in the B80-TERT1 cells was confirmed by showing that expression of exogenous hTR resulted in high levels of telomerase activity and very long telomeres.

Although until recently it has been assumed that telomerase activity is controlled solely by the availability of hTERT, data are accumulating in support of the notion that hTR and hTERT are both limiting. Notably, overexpression of both hTR and hTERT in HeLa cells and fibroblasts resulted in high levels of telomerase activity and massive telomere elongation (24). Moreover, the important contribution of hTR levels to telomerase activity is indicated by the long-standing observation that hTR levels are substantially elevated in a wide variety of human tumors (33–37) and immortal human cell lines (5). hTR amplification has also been
observed in various tumors (38–40), and it may be one mechanism of increased hTR expression in the tumors. More recently, a viral form of telomerase RNA (vTR) with 88% homology to chicken telomerase RNA was found to be encoded by oncogenic strains of Marek’s disease virus (MDV) but not by nononcogenic MDV strains (41). Furthermore, the wild-type vTR was shown to facilitate avian tumorigenesis (42). More importantly, the decreased hTR levels observed in human dyskeratosis congenita patients due either to hTR or DKC1 mutations are associated with abnormally short telomeres (reviewed by Kirwan et al. in ref. 7), which can be rescued by overexpression of both hTERT and hTR (22, 23). It seems that the hTR mutations associated with dyskeratosis congenita reduce telomerase activity via haploinsufficiency (21). Studies in mice also suggest telomerase RNA can be limiting (43, 44).

However, none of the above mentioned studies quantitated the number of hTR molecules per cell. It has been suggested that there are 20 to 50 active telomerase molecules per cell in 293 cells (4). B80-TERT1 cells are predicted to have many more molecules of hTERT per cell than 20 to 50 because hTERT is readily detectable by our Western conditions in this cell line (Fig. 2C) and not in 293 cells (data not shown). Thus, our quantification of hTR molecules per cell explains the very short telomeres maintained by this very large number of hTERT molecules: ~20 molecules of hTR are insufficient for significant telomere elongation (Fig. 6B), even in the presence of a vast excess of hTERT.

A previous study has reported that there are 11,000 to 13,500 hTR molecules per cell in the telomerase-negative BJ, IMR90, and SW13 cell strains (25). Assuming that other normal human somatic cells have hTR levels within an order of magnitude of the levels in HMECs, our estimate of approximately three molecules of hTR per B80 cell is more consistent than the previous study (25) with the observation that a 2-fold change of hTR abundance (hTR haploinsufficiency) can lead to dyskeratosis congenita (reviewed by Marrone et al. in ref. 45) because a 2-fold decrease in a vast excess of hTR would not be expected to affect telomere length. Our study is also in agreement with the observation of a low TLC1 abundance

![Figure 6](image-url)
in yeast (32). The reason for the discrepancy between our data and those of Yi et al. (25) is likely to be technical rather than an intrinsic difference between B80 cells on the one hand and telomerase-negative BJ, IMR90, and SW13 cells on the other because hTR levels were quantitated in 293 cells in both studies and reported to be 120 to 300 molecules per cell (Northern and real-time RT-PCR; this study) and ~69,000 molecules per cell (competitor RNA RT-PCR method; ref. 25). The number of RNA molecules per cell reported here may be an underestimate due to the yield of RNA during cell extraction being <100%, but this is unlikely to account for a 200- to 600-fold difference; furthermore, three different methods of RNA extraction gave similar results (see Materials and Methods).

The limiting hTERT levels in the B80-TERT1 cells may have been the selection pressure that resulted in a growth advantage for a clone of cells within the original mass culture that had acquired amplification of hTERT. Before amplification of hTERT, it is possible that the cellular or local concentrations of both molecules were below the $K_d$ for the interaction, in which case, a higher hTERT concentration would favor the interaction between the molecules and lead to increased telomerase activity, regardless of stoichiometry. Another possibility is that before amplification, hTERT may have been poorly expressed, as it is in B80-TERT3a (Fig. 2C), such that the number of hTERT molecules was lower than hTR, and this limiting hTERT expression may have selected for amplification of hTERT. Alternatively, cells with increased hTERT may have a growth advantage due to putative telomere length-independent functions of hTERT (46, 47).

Up-regulation of hTERT has previously been observed in cells expressing exogenous hTERT (22, 23, 48). The increased hTERT was suggested to be due to an increased half-life as a result of the association of hTR with hTERT (48). Our results indicate that this cannot be the whole explanation because the cells with the highest hTERT protein levels had the lowest hTR levels.

We found a very consistent correlation between telomere length and telomerase activity (Fig. 6C) as measured by the direct telomerase activity assay. This assay includes a step in which telomerase activity is partially purified by immunoprecipitation from cell extracts with an hTERT antibody, which greatly increases the sensitivity of the assay (e.g., telomerase activity was readily detectable in 2.5 × 10^6 293 cells without overexpression of hTERT or hTERT). In the series of cell lines generated from B80 cells, the rank order according to telomerase activity was the same as that for telomere length, and in the hTERT-transduced B80-TERT1 cells, up-regulation of telomerase activity was accompanied by a corresponding increase in telomere length. In agreement with data from a previous study (24), this indicates that in human cells, telomerase activity level is a major determinant of telomere length.

Our observation that hTERT amplification correlates with hTERT mRNA/protein expression but not telomerase activity in hTERT-immortalized HMECs indicates that hTERT amplification and telomerase activity are not good surrogate markers for telomerase activity levels. Insufficient hTR up-regulation could be one of the explanations for the previously observed nonassociation between hTERT amplification and telomerase activity in some tumors (10–13). Our study is the first to examine the expression of other telomerase components, hTR and dyskerin, in the context of hTERT-immortalized HMECs, and provides further direct evidence that hTR is limiting for telomerase activity and telomere length maintenance. Furthermore, our quantitation of hTR levels (approximately three hTR molecules per cell in telomerase-negative B80 cells) provides an explanation for this observation, which was at odds with the previously reported vast excess of hTR in telomerase-negative cells.

References


Amplification in Cells with Limiting hTR Levels


Amplification of Telomerase Reverse Transcriptase Gene in Human Mammary Epithelial Cells with Limiting Telomerase RNA Expression Levels

Ying Cao, Lily I. Huschtscha, Amanda S. Nouwens, et al.