Clonal Heterogeneity in Telomerase Activity and Telomere Length in Tumor-Derived Cell Lines (44505)

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Abstract. **The ribonucleoprotein, telomerase, is responsible for the maintenance of telomere length in most immortal and cancer cells. Telomerase appears to be a marker of human malignancy with at least 85% of human cancers expressing its activity. In the present study, we examined a series of tumor-derived and *In vitro* immortalized cell lines for telomerase activity levels, telomere lengths, and expression levels of the RNA and catalytic components of telomerase. We found significant variability in both telomere lengths and telomerase activity in clones from tumor cells. In addition, the levels of telomerase components or telomerase activity were not predictive of telomere length. Data from clonally derived cells suggest that critically shortened telomeres in these tumor-derived cell lines may signal activation of telomerase activity through an increase in the expression of the catalytic subunit of telomerase. Although clones with low telomerase shorten their telomeres over time, their subclones all have high levels of telomerase activity with no telomere shortening. In addition, analysis of early clones for telomerase activity indicates substantial variability, which suggests that activity levels fluctuate in individual cells. Our data imply that cell populations exhibit a cyclic expression of telomerase activity, which may be partially regulated by telomere shortening.** [P.S.E.B.M. 2000, Vol 223:379–388]

Telomerase, a cellular reverse transcriptase present in immortal cells, uses its RNA component as the template for the addition of telomeric repeats onto the G-rich strand of human telomeres (1). Telomeres are the specialized structures at the end of chromosomes that in vertebrates consist of tandem hexanucleotide $(TTAGGG)_n$ repeats (2) and specific DNA binding proteins (3). Telomeres cap the chromosome ends and play an essential role in maintaining chromosome stability and integrity (4). In normal somatic cells, there is a progressive loss of telomeric repeats with each round of cell division due to the inability of standard DNA polymerases to replicate the end of a linear DNA molecule, a process known as the end replication problem (5, 6). The progressive shortening of human telomeres has been observed both in cultured cells and as a function of donor age (7–10). These correlations support the hypothesis that telomere length is the counting mechanism that controls the number of divisions that a cell can undergo (7, 11). The cloning of the catalytic subunit of human telomerase (12–15) and its ectopic expression in normal cells (16, 17) prevents cellular senescence by maintaining telomere lengths, showing that telomere length can be a regulatory factor in cellular senescence.

Becoming immortal by maintaining telomere length appears to be a key factor in the progression to cancer. Telomerase activity has been detected in 85% of tumor samples (18) and in almost all tumor-derived human cell lines analyzed (19, 20). Although there are potentially several means to maintain telomere stability, telomerase is the major pathway for maintaining telomere length (19, 21). Telomerase activity is expressed in normal germline and somatic cells

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during embryogenesis (22). After birth, activity is only detected in adult male germline cells and at reduced levels in stem cells and in the hematopoietic system (23–27). However, this reduced activity is not sufficient to maintain telomere length, and telomeres continue to shorten (9, 25, 26, 28). Human cell lines immortalized in culture are generally telomerase positive and have stable but shorter telomeres. However, a subset of immortalized human cells may be telomerase negative with long and heterogeneous telomeres, suggesting an alternate mechanism (ALT pathway) for maintenance of telomere length (29, 30).

Better knowledge of the regulation of telomerase in normal and immortal cells is needed to evaluate the potential of new therapeutic and diagnostic approaches using telomerase as a target. Multiple pathways appear to regulate telomerase activity as a function of cell cycle, growth, and development. In the present study, we analyzed a panel of telomerase-positive tumor-derived human cell lines, *in vitro* immortalized human cells, and clones of these cell lines for telomere length and telomerase activity levels. We determined the expression levels of the RNA component (hTR) and the catalytic subunit (hTERT) of telomerase by semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Our results showed variability in both telomerase activity and telomere lengths in clones from individual cell lines and confirmed that expression of telomerase components or activity levels does not predict telomere length. Clonal analysis of tumor-derived cell lines also suggested that some cell lines may be capable of modulating the regulation of telomerase, and this regulation may be partially controlled by telomere shortening.

Materials and Methods

Cell Culture. The mortal cell strain used was a human fetal lung fibroblast strain (IMR-90) (ATCC number: CCL-186). The immortal human cell lines analyzed were: a dexamethasone (Dex)-inducible T-antigen expressing IMR-90 (IDH4 31), two T-antigens expressing IMR-90 (one with telomerase, SW39, and one without telomerase activity, SW13), a human mammary epithelial cell line (HME) immortalized *in vitro* by introduction of mutant p53 (R-273-H) (HME32(273)-1) (32), a spontaneously immortalized Li-Fraumeni HME cell line (HME50-5) (33), a non-small cell lung carcinoma line (H1299) (ATCC number: CRL-5803), a human fibrosarcoma line (HT1080) (ATCC number: CCL-121), a renal cell carcinoma line (RCC23), and a promyelocytic leukemia cell line (HL60).

All cells except HME50-5 and HME32(273)-1 were grown at 37°C in 5% CO_2 in a 4:1 mixture of Dulbecco's modified Eagle medium and medium 199 (Gibco BRL, Gaithersburg, MD) supplemented with 10% cosmic calf serum (Hyclone, Logan, UT) and 50 μg/ml of gentamicin. IDH4 cells were immortalized by expression of simian virus 40 (SV40) large T antigen under the control of the mouse mammary tumor virus (MMTV) promoter. The MMTV promoter is inducible upon the addition of Dex; therefore, IDH4 cells were maintained in 1 μ*M* Dex (31).

HME50-5 and HME32(273)-1 cell lines were grown in serum-free medium consisting of a modified basal medium MCDB 170 (Gibco BRL) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA), 5 μg/ml insulin (Sigma, St Louis, MO), 0.5 μg/ml hydrocortisone (Sigma), 50 μg/ml gentamicin (Sigma), 5 μg/ml transferrin, and 10 ng/ml epidermal growth factor (Gibco BRL). Medium was changed every 2–3 days.

Control clones of the H1299 cell line (H1299 A, B, and C) were isolated following infection (34) with a parental retroviral vector, pBABE (35) carrying a puromycin resistance gene (pBABE-puro).

Terminal Restriction Fragment (TRF) Length-Measurement. For determining telomere lengths, cells were lyzed, and protein was digested. Samples were incubated in 10 m*M* Tris HCl, pH 8.0, 100 m*M* NaCl, 100 m*M* EDTA, pH 8.0, 0.5% SDS, and 0.25 mg/ml proteinase K overnight at 55°C. DNA was extracted with phenol/chloroform, ethanol precipitated and resuspended in 10 m*M* Tris HCl, pH 7.5, 1 m*M* EDTA. DNA (5–10 μg) was digested overnight to completion at 37°C with *Hin*fI (10 units/μg of DNA) or with a mix of six different restriction endonucleases, *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Msp*I, and *Rsa*I (4 units/μg for each enzyme). The digested DNA was separated on a 0.8% agarose gel run for 16 hr at 74 V in 1X TAE buffer. The gel was dried under vacuum for 45 min at 50°C, then denatured for 30 min in 0.5 *M* NaOH, 1.5 *M* NaCl, and neutralized for 30 min in 1.5 *M* NaCl, 0.5 *M* Tris HCl, pH 7.5.

The gel was hybridized overnight at 42°C in 5X SSC, 5X Denhardt's, 10 m*M* Na_2HPO_4, 1 m*M* $Na_2P_2O_7/10\ H_2O$ with a [$\gamma - {}^{32}P$] ATP-labeled $(TTAGGG)_4$ probe. The gel was washed at room temperature for 10 min in 2X SSC, 0.1% SDS, then three times for 10 min each in 0.1X SSC, 0.1% SDS, exposed to a PhosphorImager screen and analyzed using ImageQuant version 3.3 software (Molecular Dynamics, Sunnyvale, CA). The range of TRF lengths was estimated and in some cases, the median TRF length was estimated at the peak position of the hybridization signal based on electrophoresis of 1 kb and high-molecular weight ladders (Gibco).

Telomerase Assays. Detection of telomerase activity in cultured cells involves the extension of an oligonucleotide, which serves as the substrate for the telomerase enzyme, and PCR amplification of the resultant products with the forward (TS) and reverse (CX) primers. The details for this telomeric repeat amplification protocol (TRAP assay) are discussed elsewhere (19, 20, 36). The TRAP-eze telomerase detection kit was used to detect telomerase activity as recommended by the manufacturer with minor modifications (Intergen, Gaithersburg, MD).

The lysis buffer consisted of 10 m*M* Tris-HCl, pH 8.0, 1 m*M* $MgCl_2$, 1 m*M* EGTA, 1% NP-40, 0.25 m*M* sodium deoxycholate, 10% glycerol, 150 m*M* NaCl, 5 m*M* β-mer-

captoethanol, and 0.1 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) (37). Cells were lyzed, incubated on ice for 30 min, and centrifuged at 14,000*g* for 20 min. The supernatant was flash-frozen and stored at – 80°C. For the PCR reaction, an appropriate amount of extract (2 μl, the amount of extract corresponding to 100 or 1000 cell equivalents) was combined with the 48-μl reaction mixture including two units of Taq DNA polymerase (Gibco).

Detection of telomerase activity on a small number of cells was done by selecting clones in the 100–1000 cell stage and scraping the cells using a 1-mm sterile disposable loop directly into 10–15 μl of lysis buffer. The samples were incubated on ice for 30 min and subjected to a brief spin at 4°C for 10 s. A 5-μl aliquot was used in the TRAP assay as described above; whereas protein concentration (BCA assay, Pierce, Rockville, IL) was tested using the remaining volume (5–10 μl) to determine if clonal isolation was successful.

Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis of hTR and hTERT Expression. Total RNA was isolated by guanidinium thiocyanate and phenol-chloroform extraction as described by Chomczynski and Sacchi (38). After treatment with DNase I (Boehringer Mannheim Corporation/ Roche Molecular Biochemicals, Indianapolis, IN) to remove any genomic DNA contamination, 2.5 μg of total RNA was annealed with short random oligonucleotides (decamers) and retro-transcribed in cDNA using the first-strand synthesis kit RETROscript (Ambion Inc., Austin, TX). PCR reactions were performed using a fraction of the cDNA reaction.

The primers used to amplify hTERT and hTR have been previously reported (12). hTERT was amplified using primers LT5 (5′-CGGAAGAGTGTCTGGAGCAA-3′) and LT6 (5′-GGATGAAGCGGAGTCTGGA-3′) with an initial heating at 94°C for 5 min followed by a total of 34 cycles of 94°C for 45 sec, 62°C for 40 sec and 72°C for 45 sec. hTR (39) was amplified using primers F3b (5′-TCTAACCCTAACTGAGAAGGGCGTAG-3′) and R3c (5′-GTTTGCTCTAGAATGAACGGTGGAAG-3′) with an initial heating at 94°C for 5 min followed by 25 cycles of 94°C for 45 sec, 55°C for 40 sec and 72°C for 45 sec. The control amplification of 18S ribosomal RNA was performed using a 3:7 mixture of 18S rRNA primer pairs and 18S rRNA Competimers (primers modified at their 3′ends to block extension by DNA polymerase) (Ambion Inc.). By mixing primers with increasing amounts of Competimers, the overall amplification efficiency of PCR is reduced without the primers becoming limiting and without loss of relative quantitation. The PCR conditions for 18S were the same as hTERT amplification for 23–25 cycles. All samples were assayed at the same time for a particular pair of primers to minimize experimental variation. Amplified products were resolved on a 6% polyacrylamide gel for hTERT and hTR and on a 2% agarose gel for 18S RNA and visualized by staining with ethidium bromide.

Results

We examined several human cell lines for terminal restriction fragment (TRF) length, levels of telomerase activity, and expression levels of the telomerase template RNA component, hTR, and the mRNA encoding the catalytic protein subunit, hTERT. The cells used were tumor-derived (HT1080, HL60, H1299, and RCC23) and *in vitro* immortalized (IDH4, HME32(273)-1, SW39) lines. As controls, we also included an *in vitro* immortalized cell line (SW13) and a normal human cell strain (IMR-90), both telomerase-negative.

Telomerase Activity Levels, TRF Length, and Expression of hTR and hTERT. We initially analyzed the levels of telomerase activity in each cell line and observed some variability in expression of telomerase activity between the different cell lines (Fig. 1A). Preliminary experiments were performed using the conventional CHAPS lysis buffer, which extracts about 20% of the total telomer-

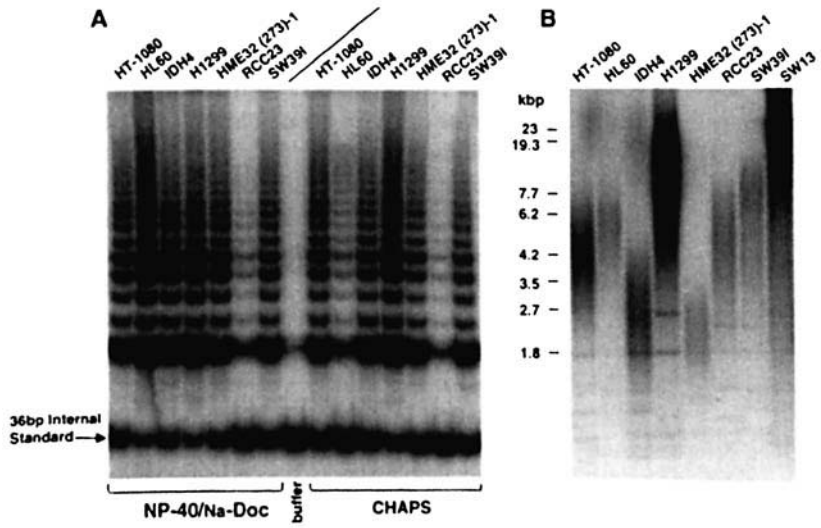

Figure 1. Expression of telomerase activity does not correlate with telomere length in cell lines from different origins. (A) Telomerase activity was analyzed in each cell line (tumor-derived or *in vitro* immortalized) using the TRAP assay. The original CHAPS and the modified NP40 lysis buffers were used. Extracts corresponding to 100-cell equivalents were subjected to PCR, and the products were electrophoresed on 10% polyacrylamide gels. The processivity of the 6-base pair (bp) ladder and the presence of the 36-bp internal control band indicate the specificity of the reaction. NP-40 lysis buffer without cell extract was used as a negative control. (B) Terminal restriction fragment length (TRF) was performed on each cell line to determine telomere length. Total DNA was extracted from each cell line and digested with six different restriction endonucleases (*Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Msp*I, and *Rsa*I). In-gel hybridization with a $(TTAGGG)_4$ radiolabeled probe was used to detect telomeric DNA. The molecular size marker used was Lambda DNA digested with *Sty*I.

ase. This extraction showed significant variability in telomerase activity levels between the different cell lines. By using the modified NP-40 lysis buffer, which extracts about 85% of total telomerase activity (37), we observed an increase in overall extraction of telomerase activity and a decrease in variability (Fig. 1A). HL60 cells with weak telomerase activity using the CHAPS buffer appeared to have a very strong extractable telomerase activity using the NP-40 lysis buffer, with levels similar to H1299. The H1299 and the HL60 cell lines had high levels of telomerase activity such that linearity of the assay was only observed when the amount of extract corresponding to 100 cell equivalents or fewer was analyzed (Fig. 1A, and unpublished data). RCC23 cells had consistently low but detectable telomerase activity. The *in vitro* immortalized cell lines (IDH4, HME32(273)-1 and SW39) exhibit a 4- to 5-fold increase in the level of telomerase activity after extraction with the NP-40 lysis buffer (Fig. 1A). Because we were able to detect significantly higher amounts of telomerase activity using optimized lysis conditions, the *in vitro* immortalized cell line with undetectable telomerase activity, SW13, was tested to determine if telomerase may be expressed in these cells but not efficiently extracted using the conventional CHAPS lysis buffer. However, no telomerase activity was detected for the telomerase-negative, ALT pathway cell line SW13 or for the normal diploid fibroblasts IMR-90 (unpublished data).

Analysis of TRF lengths was also variable for the different cell lines (Fig. 1B). HT1080, HL60, and H1299 that exhibited similarly high telomerase levels had very different telomere lengths, with HT1080 and HL60 having ≈ 3–7-kilobase (kb) telomeres, whereas H1299 had 6–25-kb telomeres. The RCC23 cell line with the lowest telomerase activity had telomeres of similar length (4–7 kb) to HL60 (3–7 kb). The *in vitro* immortalized cells IDH4 and HME32(273)-1 had telomere lengths ranging from 2–3 kb, whereas their telomerase activity was significantly higher than the RCC23 cells. SW39 had similar telomerase activity levels as IDH4 and HME32(273)-1, yet longer telomeres (7–9 kb). The telomerase-negative cell line SW13, originating from the same SV40 T-antigen transfection of IMR-90 fibroblasts as SW39, presented much longer and heterogeneous telomere lengths (15–25 kb) despite undetectable telomerase activity. These data suggest that because telomerase activity and telomere size do not correlate, the levels of telomerase are not predictive of telomere lengths.

Semi-quantitative RT-PCR using specific primers for the RNA component (hTR) and the mRNA for the catalytic subunit (hTERT) of telomerase was carried out on total RNA extracted from the different telomerase-positive and -negative cell lines (Fig. 2). PCR reactions were also performed with 18S ribosomal RNA primers as an internal control for quantitation. The linearity of the RT-PCR amplification was determined, and RT-PCR was performed on purified RNA samples without reverse transcription to confirm the absence of any DNA contamination (unpublished data). When each cell line was analyzed for expression of the RNA component of telomerase, hTR was present in all cell lines, even those lacking detectable telomerase activity (Fig. 2) (39). HL60, IDH4, H1299, and RCC23 had comparably high hTR levels, yet RCC23 had the lowest telomerase activity, suggesting that absolute levels of hTR are not indicative of telomerase activity levels. The mRNA encoding the catalytic subunit of telomerase, hTERT, was expressed at a similar level in all the telomerase-positive cell lines except for RCC23, which had much lower hTERT mRNA levels consistent with its low telomerase activity (Fig. 2). The telomerase-negative cells, IMR-90 and SW13, expressed lower levels of hTR and no detectable hTERT message (Fig. 2). Thus, expression of the hTERT mRNA parallels telomerase activity more closely than the RNA component of telomerase, hTR.

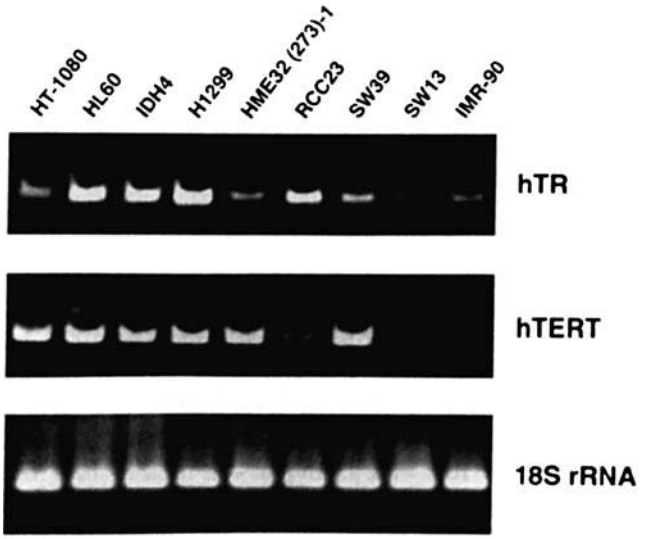

Figure 2. Representative results of semiquantitative RT-PCR analysis for the expression of the RNA template and the catalytic protein subunit of telomerase in telomerase-positive (HT1080, HL60, IDH4, H1299, HME32(273)-1, RCC23, SW39) and telomerase-negative (SW13, IMR-90) cell lines. hTR (human telomerase RNA component) expression was measured by RT-PCR with primers F3b and R3c that amplify a 126-bp fragment of the hTR RNA. Specific primers LT5 and LT6 amplify a 145-bp fragment of hTERT (human telomerase catalytic component). The 18S ribosomal RNA internal control (to normalize for RNA amount) was measured with a 3:7 mix of 18S rRNA primer pairs and 18S rRNA competimer (Ambion), and the RNA internal control amplified a 488-bp fragment. Products were analyzed by electrophoresis on 6% polyacrylamide gels for hTR and hTERT and on 2% agarose gel for 18S rRNA.

The mechanism responsible for the regulation of telomerase activity levels in these immortal cell lines is unclear as some cell types (RCC23) presented with low telomerase activity, whereas others (H1299, for example) expressed very high levels of activity. One formal possibility is that telomerase activity in each cell is just inherently lower for the RCC23 than for the H1299 cell populations. Another plausible explanation is that RCC23 and H1299 express similar levels of telomerase activity on a per-cell basis, but the RCC23 population is more heterogeneous with fewer telomerase-positive cells than the H1299 population. This

would suggest that the level of telomerase activity detected is proportional to the percentage of cells expressing telomerase. With those things in mind, we selected clones from both RCC23 with lower telomerase activity and H1299 with higher activity.

RCC23 Cells Exhibited Variable Expression of Telomerase Activity. RCC23 is a nontumorigenic human renal carcinoma cell line established from a stage III nonpapillary carcinoma (40). When we analyzed this cell line on a clonal basis, we found that some clones, harvested in log phase growth ≈ 25 doublings after cloning, varied in their telomerase activity levels (Fig. 3A). Unfortunately, clone D, which had the lowest telomerase activity, was unable to proliferate beyond the initial harvest and was not chosen for further analysis. Clones B and E (higher telomerase activity relative to the others) and clones H and J (low telomerase activity) were selected for analysis of activity, telomere length, and expression levels of hTR/hTERT RNAs. These clones were followed in culture for about 100 population doublings (PDs). Telomerase activity in each of the clones remained at a constant level over 100 PDs in culture (unpublished data), suggesting possible regulation of telomerase activity within each of the clones as well. Detection of constant levels of activity also suggested that if individual cells within the RCC23 population express varying amounts of telomerase (as measured in the clones), there appears to be no growth advantage for higher (or lower) activity over 100 PDs. Figure 3B shows that initially, there was minimal variability in the telomere length between each of the clones (4–5 kb), which did not differ significantly from the population. However, although clones B, E, and H showed stability of their telomere lengths with increasing PD, clone J with very low telomerase activity progressively shortened its telomeres over time in culture (Fig. 3B). Interestingly, we also found that the growth rates for these clones were not substantially different from each other (unpublished data).

To see if this clonal heterogeneity persisted after the initial cloning, RCC23 clone J was subcloned. Cells from RCC23 J clone at PD 95 were replated at clonal density, and several subclones were isolated and tested for telomerase activity. Figure 4 shows representative results obtained from 3 of the 12 clones isolated. These subclones (J2, J5, and J7) showed dramatically increased levels of telomerase activity when compared with either the RCC23 population or the parental J clone, and these levels did not fluctuate significantly over the next 70 PDs (Fig. 4A). When TRF lengths were measured, subclones RCC23 J2 and J5 initially had short telomere lengths similar to those seen in RCC23 J at PD 95, but after 70 PDs, the telomere lengths appeared to elongate (Fig. 4B). The RCC23 J7 subclone, which initially had a longer TRF length, maintained its telomere lengths over 70 PDs (Fig. 4B). Taken together, these data and the data from Figure 3B, where clone J has shorter telomeres at the time of cloning, suggest two possible mechanisms: (i) only those cells with adequate telomerase can escape a telomere-induced senescence/death phenomenon; or (ii) telomerase is upregulated in clone J in response to telomere shortening. Given that the cloning efficiency is high, and no cellular senescence or death is observed (unpublished data), the latter seems a plausible mechanism.

RCC23 J2 and RCC23 J7 clones were replated for another round of clonal selection at approximately PD 185, and the isolated clones were subjected to the TRAP assay and TRF analysis (Figs. 5A & 5B). Although clones from RCC23 J2 had similar levels of telomerase activity and similar telomere lengths, clones isolated from RCC23 J7 had a more variable expression of telomerase activity and telomere lengths. Therefore, even within a clonal population of cells, there can be significant heterogeneity in the expression of telomerase activity and maintenance of telomere lengths, suggesting that the regulation of telomerase activity differs in individual cells.

Expression levels of the hTR and hTERT RNAs were examined for RCC23 clones B, E, H, J, and subclones J2 and J7 over a period of about 70–100 PDs. RT-PCR analysis

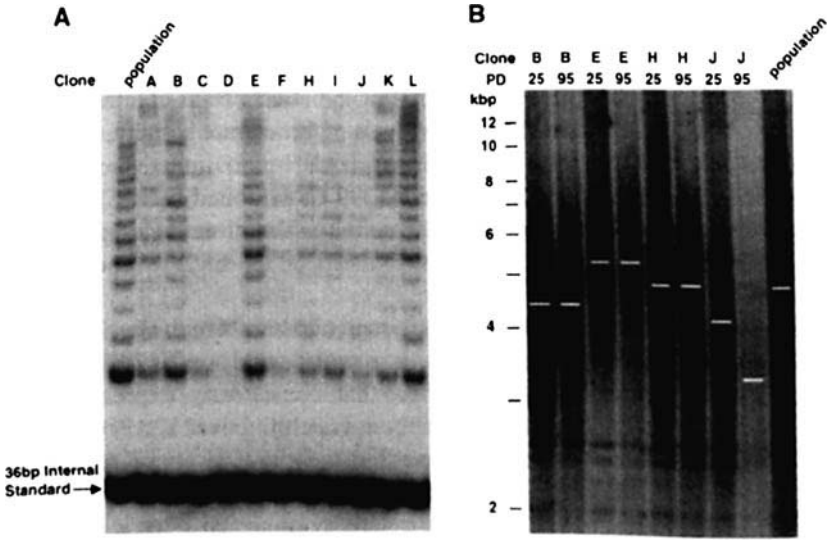

Figure 3. Clones from the RCC23 cell line presented with variable levels of telomerase activity, and one clone exhibited telomere shortening. (A) The RCC23 population was plated at low density with individual clones (A-L) selected, maintained, and collected for use in the TRAP assay. Each clone was tested using 1000 cell equivalents per reaction. (B) TRF analysis was done on genomic DNA digested with *Hinf*I restriction endonuclease. Telomere lengths were estimated based on migration of 1 kb and high-molecular-weight DNA ladders. The white bars indicate peak value of telomere length for visual comparison.

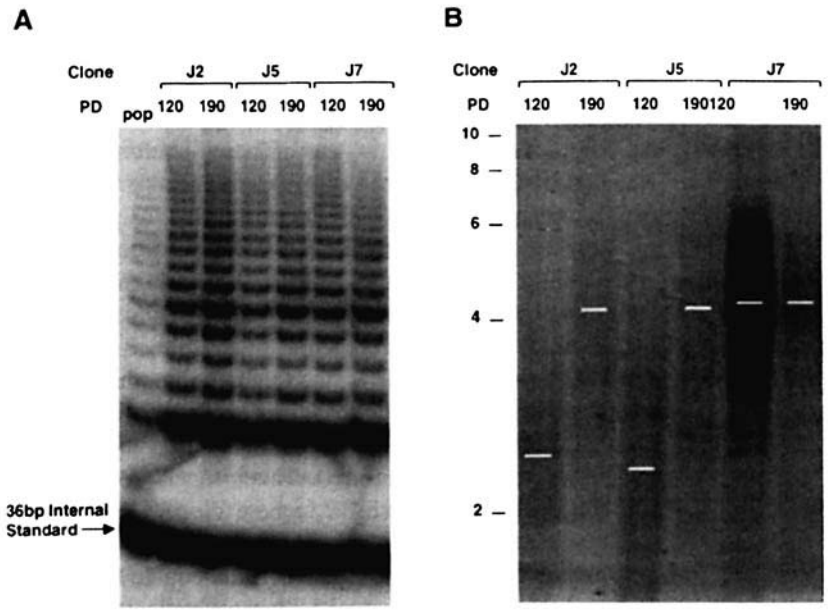

Figure 4. Late passage cloning of RCC23 J yields increased telomerase activity and increasing telomere length. RCC23 J cells at PDs 95 were plated at clonal density, and individual clones were selected for both (A) telomerase activity, using 1000 cell equivalents per reaction, and (B) telomere length. Clones J2, J5, and J7 were chosen for analysis of activity and length over 70 PDs. TRF analysis was done on genomic DNA digested with *Hinf*I restriction endonuclease.

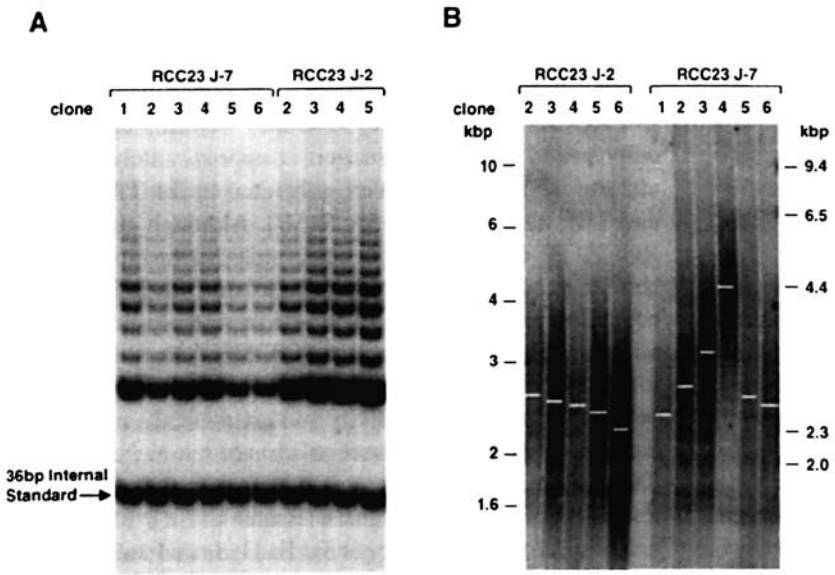

Figure 5. Continued variability in telomerase activity and telomere length in third generation clones of RCC23. RCC23 J2 and RCC23 J7 clones were plated for a second round of clonal selection. Subclones were isolated for (A) telomerase activity using 1000 cell equivalents and (B) TRF analysis using *Hinf*I-digested DNA.

showed that the expression level of hTR between clones with weak or strong telomerase activity did not vary significantly (unpublished data). The expression levels of hTERT mRNA appeared to fluctuate only modestly over 70–90 PDs (unpublished data), even though telomerase activity remained constant (unpublished data). Yet there appeared to be a modest but consistent increase in hTERT mRNA in the subclones of clone J (J2, J5, J7) (unpublished data). Thus, regulated transcriptional expression of the hTR or hTERT message does not appear to be the entire explanation for the variability in the expression of telomerase activity in the RCC23 cell line, suggesting post-transcriptional regulation perhaps *via* association with proteins that modulate telomerase activity (41).

H1299 Cells Show Clonal Variability in Telomerase Activity and Telomere Lengths. Another part of the study consisted of infection of the non-small cell lung carcinoma line, H1299, with the parental retroviral vector, pBABE-puro, followed by clonal isolation. Fourteen H1299 pBABE-puro clones and six clones of uninfected H1299 cells were selected. All of the clones from uninfected cells demonstrated similar levels of telomerase activity, yet of the 14 H1299 clones from pBABE-puro infected cells, two clones, H1299 B and H1299 C, exhibited variability in expression of telomerase activity. Therefore, heterogeneity at the cellular level also existed in the high telomerase cell line, H1299.

Telomerase activity and telomere length were analyzed for these two clones, in parallel with one H1299 pBABE-puro clone, H1299 A, that consistently expressed high telomerase activity without variability over 120 PDs. H1299 B and H1299 C expressed very low levels of telomerase activity at early PD, but this low activity was not maintained (Fig. 6A). Telomerase activity analyzed over a period of

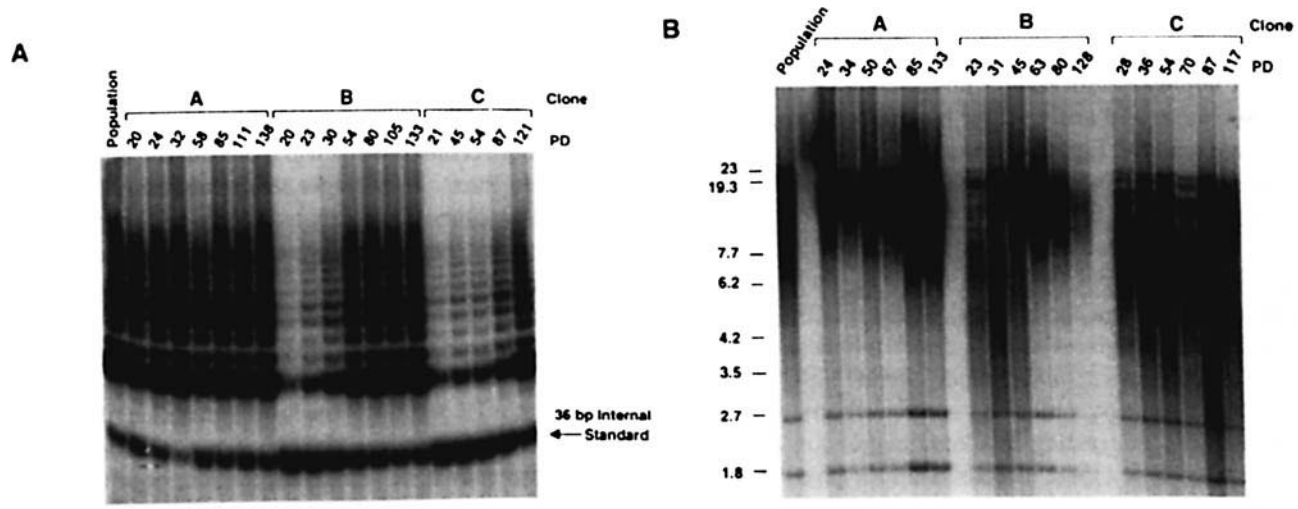

Figure 6. Reactivation of telomerase activity in H1299 clones correlates with shortening of telomeres. H1299 cells infected with the parental retroviral vector pBABE-puro were plated at clonal density and selected with puromycin, and 14 individual clones were collected. Analysis of these clones for telomerase activity showed two clones, H1299 B and H1299 C, with very low telomerase activity. These two clones in parallel with one clone, H1299 A, with telomerase activity similar to the population, were maintained and cultured over 130 PD. (A) Telomerase activity using 1000 cell equivalents was determined over 120–140 PD. (B) TRF lengths were analyzed using genomic DNA digested with six different enzymes over a period of 133, 128, and 117 PDs in culture, respectively, for clones H1299 A, B, and C. Lambda DNA digested with *StyI* served as a molecular size marker.

several PDs increased in clones B and C in conjunction with increased cell division compared with clone H1299 A (Fig. 6A). Both H1299 B and H1299 C clones at high PD had telomerase activity at levels similar to clone H1299 A or the H1299 population, but clone B reactivated telomerase much earlier (around 50 PDs) than clone C (around 120 PDs). For both clone B and C, activity was re-expressed at high levels and appeared to remain stable when analyzed over 80 more PDs (a total of ≈ 200 PDs).

When the telomere dynamics were examined by TRF analysis, clones B and C showed much shorter (6–8 kb) and broad telomere lengths at early PD, compared with clone A or the population (Fig. 6B). Several telomere-specific peak values were observed, representing longer telomeres for clones B and C and demonstrating several populations of distinct telomere length. Clone H1299 A showed stable telomere length over 133 PDs. However, when clones B and C acquired higher telomerase activity, their telomere lengths increased by several kilobases, reaching a length similar to clone A. The timing of telomere elongation for clones B and C corresponded closely to the time course of telomerase upregulation (Fig. 6), and telomere size remained stable after continuous culture to over 200 PDs (unpublished data).

Analysis of expression levels of hTR and hTERT RNAs over a period of 121–138 PDs was done by semiquantitative RT-PCR for the three clones and for the population (Fig. 7). hTR levels appeared to be stable and at a similar level in all three clones over passages in culture. However, variation in the hTERT mRNA expression levels was observed. Although this level appeared stable and high in clone A over 133 PDs, clones B and C with low telomerase activity had a correspondingly low level of hTERT mRNA. The subsequent upregulation in telomerase activity

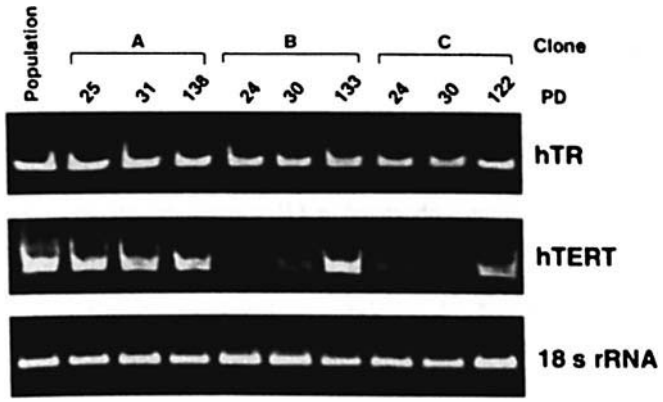

Figure 7. Upregulation of the hTERT mRNA parallels the increase in telomerase activity in H1299 clones B and C. hTR, hTERT, and 18S control RNA levels were determined by semiquantitative RT-PCR from total RNA extracted from the H1299 population and clones H1299 A, B, and C.

coincided with an increase in hTERT mRNA. The level of hTERT mRNA in H1299 B at 133 PD appeared to be similar to that of clone H1299 A and the population. Although elevated at PD 122, clone H1299 C did not reach the same level of hTERT mRNA as the H1299 population, clone H1299 A, or clone H1299 B at PD 133. In both clones B and C, the increase in telomerase activity paralleled the increase in hTERT mRNA, indicating a strong correlation between hTERT mRNA levels and telomerase activity.

Variable Expression of Telomerase Activity in Early Clones. Clones are typically isolated after 20–25 PDs, where a single cell becomes a colony of at least one million cells. Because so many cell divisions have taken place, telomerase may be turned on and off, and shortening of telomeres may be accomplished without a distinguishable change in detectable activity or telomere length in the ma-

ture clone. To overcome this problem, early clones were selected by visually choosing small clones with 100–1000 cells per colony (7–10 PDs). Chosen clones were scraped into lysis buffer and analyzed for protein concentration and telomerase activity. Figure 8 shows the results from 17 early clones from HME50-5, with considerable variability in detectable telomerase activity between clones isolated using this method. Essentially equivalent amounts of protein were used for each sample (within a factor of three). Similar results were obtained for HT1080, IDH4, H1299, RCC23, and SW39. Although we do not detect telomerase heterogeneity in fully grown, mature clones for HT1080, HME50-5, or IDH4 cell lines, variability is often observed in early clonal analysis, suggesting regulated expression of telomerase activity in a subset of cells within a given immortal population.

Discussion

In the present study, we analyzed a panel of tumor-derived and *in vitro* immortalized cell lines for telomerase activity, telomere dynamics, and the expression of the RNA component and the catalytic subunit of telomerase. Whereas all of the immortal cell lines exhibited consistent telomere lengths over many PD in culture, the lengths varied between cell lines and appeared independent of the amount of detectable telomerase activity. Moreover, our results showed that the levels of extracted telomerase activity are not predictive of absolute telomere length. Others have suggested that the differences observed in TRF measurements between cell lines from differing sources may be due to variable lengths of an undefined X-region (3, 22). However, the IMR-90-derived cell lines immortalized using SV40 large T antigen (telomerase-positive SW39 and IDH4 cell lines with TRFs of 7–9 kb and 2–3 kb, respectively) suggest controls on telomere length independent of the X-region factor.

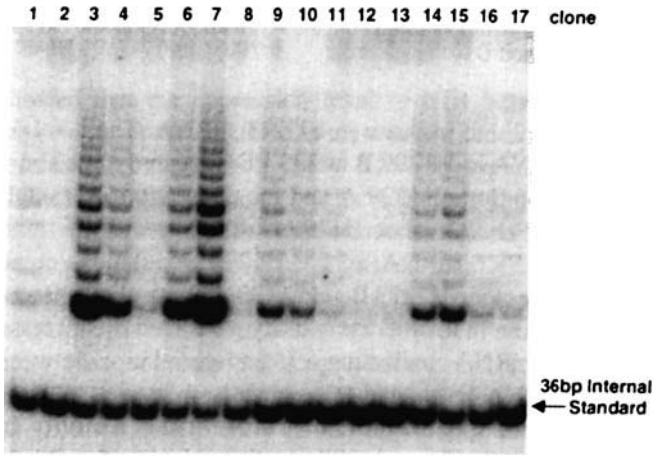

Figure 8. Variability in telomerase activity in early clones. HME50-5 cells were plated at clonal density for detection of telomerase activity early in the clonal selection process (e.g., the 100–200 cell stage). Results from 17 early clones from HME50-5 are presented here. Telomerase activity was measured with the TRAP assay using roughly equal amounts of protein for each sample (within a factor of three).

IDH4 (2–3-kb telomeres) would be expected to have the same X-region as SW39 (7–9 Kb), as these are two immortal cell lines derived from the same parental cell. If X-region were the only thing controlling telomere length, the telomeres would probably be much closer in length. Yet, SW39 has two to three times longer telomeres than IDH4, a difference that cannot be attributed solely to X-region.

When we further analyzed the RCC23 cell line for clonal variability, we observed that initially all the clones tested had similar telomere lengths but differed in their levels of telomerase activity. Late passage cloning yielded subclones with much higher telomere activity and stable telomere lengths. In the H1299 cells, telomere lengthening correlated with telomerase activation. Some cells within these populations may have ceased expression, or at least downregulated telomerase, leading to gradual telomere shortening. In both RCC23 and H1299, the gradual decline to critically short telomeres may signal an upregulation in telomerase activity, preventing senescence and permitting stabilization of telomere lengths. The reappearance of telomerase and telomere lengthening activities in clones with critically shortened telomeres suggests that either short telomeres can produce a signal that reactivates telomerase in tumor cells, or variants within the population in which telomerase is reactivated are able to overgrow the cultures. However, there is no current evidence, either here or elsewhere, indicating that long telomeres provide cells with a selective growth advantage. Thus, it is more likely that for some cells in a population, there is a cyclic event of telomerase repression and activation, and our results suggest that this event is not stochastic but might be controlled or regulated by telomere size. We suggest that the cyclic behavior of telomerase is due to the results obtained in the early clone experiment shown in Figure 8, where choosing clones early (PD 7–10), as near to the single cell stage as possible, showed significant variability in telomerase activity. Mature clones (PD 20–25) expressed relatively uniform levels of telomerase activity, suggesting that by picking clones early, we were able to sample telomerase levels at stages more closely resembling single cells.

Others have also observed a large range of mean telomere sizes and some variability in telomerase activity in three telomerase-positive cell lines: a cervical carcinoma line, HeLa; an adenovirus-immortalized embryonic kidney cell line, 293; and a human thyroid cancer cell line, K1 (42, 43). Our results indicating variable regulation of telomerase activity in cells suggest that there are probably additional controls on telomere length other than telomerase, similar to the Rap1 mechanism for yeast telomeres (44) or the TRF1/TRF2 proteins in mammalian cells (45, 46).

Our telomerase-negative cell line, SW13 (ALT pathway), with 15–25 kb telomeres is consistent with the results of others. Several *in vitro* immortalized cell lines present no detectable telomerase activity but still have very long and heterogeneous telomeres (29, 30). Our data and those of others indicate that in immortal cells, telomere lengths can

be maintained or elongated by telomerase or by another alternative mechanism (ALT pathway). Interestingly, all of the subclones from RCC23 J (low telomerase activity and continually shortening telomeres) activated high levels of telomerase rather than an ALT pathway. We also found that in the H1299 clonal analysis, elongation of telomere lengths required activation of wild-type levels of telomerase activity rather than an immediate lengthening using an ALT pathway. Taken together, our results suggest that immortal and tumor-derived cells that have initially used a telomerase pathway for continual proliferation (to overcome crisis) predominantly reactivate telomerase in response to telomere shortening. The telomerase-positive cell line H1299 has very long and relatively homogeneous telomeres. It is tantalizing to hypothesize that this cell line may use both of these mechanisms to elongate its telomeres, an ALT pathway to yield long telomeres, and a telomerase mechanism to maintain telomeres at a homogeneous length. However, our results do not favor this hypothesis. Our data with the H1299 clones showed that the absence of telomerase leads to shortening of telomeres over time and that stabilization of telomere lengths required upregulation of telomerase activity rather than the ALT pathway.

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1. Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell **59**:521–529, 1989.
2. Moyzis RK, Buckingham JM, Cram S, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu J-R. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci U S A **85**:6622–6626, 1988.
3. de Lange T, Shiue L, Meyers RM, Cox DR, Naylor SL, Killery AM, Varmus HE. Structure and variability of human chromosome ends. Mol Cell Biol **10**:518–527, 1990.
4. Blackburn EH. Structure and function of telomeres. Nature **350**:569–573, 1991.
5. Olovnikov AM. Principle of marginotomy in template synthesis of polynucleotides. Doklady Biochem **201**:394–397, 1971.
6. Watson JD. Origin of concatameric T4 DNA. Nature New Biol **239**:197–201, 1972.
7. Harley CB, Futcher AB, Greider CW. Telomeres shorten with aging. Nature **345**:458–460, 1990.
8. Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC. Telomere reduction in human colorectal carcinoma and with ageing. Nature **346**:866–868, 1990.
9. Lindsey J, McGill N, Lindsey L, Green D, Cooke H. *In vivo* loss of telomeric repeats with age in humans. Mutat Res **256**:45–48, 1991.
10. Allsopp RC, Vaziri H, Patterson C, Goldstein S, Youglai EV, Futcher AB, Greider CW, Harley CB. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci U S A **89**:10114–10118, 1992.
11. Wright WE, Shay JW. The two-stage mechanism controlling cellular senescence and immortalization. Exp Gerontol **27**:383–389, 1992.
12. Nakamura TM, Morin GB, Chapman B, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR. Telomerase catalytic subunit homologs from fission yeast and human. Science **277**:955–959, 1997.
13. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, Ziaugra L, Beijersbergen RL, Davidoff MJ, Liu Q, Bacchetti S, Haber DA, Weinberg RA. hEST2, the putative human telomerase catalytic subunit gene, is upregulated in tumor cells and during immortalization. Cell **90**:785–795, 1997.
14. Harrington L, Zhou W, McPhail T, Oulton R, Yeung DSK, Mar V, Bass MB, Robinson MO. Human telomerase contains evolutionarily conserved catalytic and structural subunits. Genes Dev **11**:3109–3115, 1997.
15. Kilian A, Bowtell DDL, Abud HE, Hime GR, Venter DJ, Keese PK, Duncan EL, Reddel RR, Jefferson RA. Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. Hum Mol Genet **6**:2011–2019, 1997.
16. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chui C-P, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. Extension of lifespan by introduction of telomerase into normal human cells. Science **279**:349–352, 1998.
17. Vaziri H, Benchimol S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol **8**:279–282, 1998.
18. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer **33**:787–791, 1997.
19. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. Science **266**:2011–2015, 1994.
20. Piatyszek MA, Kim NW, Weinrich SL, Keiko H, Hiyama E, Wright WE, Shay JW. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). Methods Cell Sci **17**:1–15, 1995.
21. Counter CM, Avilion AA, Lefeuvre CE, Stewart NG, Greider CW, Harley CB, Bacchetti S. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J **11**:1921–1929, 1992.
22. Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. Telomerase activity in human germline and embryonic tissues and cells. Dev Genet **18**:173–179, 1996.
23. Broccoli D, Young JW, de Lange T. Telomerase activity in normal and malignant hematopoietic cells. Proc Natl Acad Sci U S A **92**:9082–9086, 1995.
24. Counter CM, Gupta J, Harley CB, Leber B, Bacchetti S. Telomerase activity in normal leukocytes and in hematological malignancies. Blood **85**:2315–2320, 1995.
25. Hiyama K, Hirai Y, Kyoizumi S, Akiyamam M, Hiyama E, Piatyszek MA, Shay JW, Ishioka S, Yamakido M. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol **155**:3711–3715, 1995.
26. Hiyama E, Hiyama K, Tatsumoto N, Kodama T, Shay JW, Yokoyama T. Telomerase activity in human intestine. Int J Oncol **9**:453–458, 1996.
27. Taylor RS, Ramirez RD, Ogoshi M, Chaffins M, Piatyszek MA, Shay JW. Detection of telomerase activity in malignant and nonmalignant skin conditions. J Invest Dermatol **106**:759–765, 1996.
28. Bodnar AG, Kim NW, Effros RB, Chui C-P. Mechanisms of telomerase induction during T-cell activation. Exp Cell Res **228**:58–64, 1996.
29. Murnane JP, Sabatier L, Marder BA, Morgan WF. Telomere dynamics in an immortal human cell line. EMBO J **13**:4953–4962, 1994.
30. Bryan TM, Engelzou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal cells without detectable telomerase activity. EMBO J **14**:4240–4248, 1995.
31. Shay JW, West MD, Wright WE. Re-expression of senescent markers in deinduced reversibly immortalized cells. Exp Gerontol **27**:477–492, 1992.
32. Gollahon LS, Shay JW. Immortalization of human mammary epithelial cells transfected with mutant p53 (273his). Oncogene **12**:715–725, 1996.

33. Shay JW, Tomlinson G, Piatyszek MA, Gollahon LS. Spontaneous *in vitro* immortalization of breast epithelial cells from a patient with Li-Fraumeni syndrome. Mol Cell Biol **15**:425–432, 1995.
34. Miller AD, Rosman G. Improved retroviral vectors for gene transfer and expression. Biotechniques **7**:980–990, 1989.
35. Morgenstern JP, Land H. Advanced mammalian gene transfer: High titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res **18**:3587–3596, 1990.
36. Holt SE, Norton JC, Wright WE, Shay JW. Comparison of the telomeric repeat amplification protocol (TRAP) to the new TRAP-eze telomerase detection kit. Methods Cell Sci **18**:237–248, 1996.
37. Norton JC, Holt SE, Wright WE, Shay JW. Enhanced detection of human telomerase activity. DNA Cell Biol **17**:217–219, 1998.
38. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162**:156–159, 1987.
39. Feng J, Funk WD, Wang S-S, Weinrich SL, Avilion AA, Chiu C-P, Adams RR, Chang E, Allsopp RC, Yu J, Le S, West MD, Harley CB, Andrews WH, Greider CW, Villeponteau B. The RNA component of human telomerase. Science **269**:1236–1241, 1995.
40. Ohmura H, Tahara H, Suzuki M, Yoshida MA, Tahara E, Shay JW, Barrett JC, Oshimura M. Restoration of the cellular senescence program and repression of telomerase by human chromosome 3. Jpn J Cancer Res **86**:899–904, 1995.
41. Holt SE, Aisner DL, Baur J, Tesmer VM, Dy M, Ouellette M, Toft DO, Trager JB, Morin GB, Wright WE, Shay JW, White MA. Functional requirement of p23 and hsp90 in telomerase complexes. Genes Dev **13**:817–827, 1999.
42. Bryan TM, Engelzou A, Dunham MA, Reddel RR. Telomere length dynamics in telomerase-positive immortal human cell populations. Exp Cell Res **239**:370–378, 1998.
43. Jones CJ, Soley A, Skinner JW, Gupta J, Haughton MF, Wyllie FS, Schlumberger M, Bacchetti S, Wynford-Thomas D. Dissociation of telomere dynamics from telomerase activity in human thyroid cancer cells. Exp Cell Res **240**:333–339, 1998.
44. Shore D. Telomere length regulation: Getting the measure of chromosome ends. Biol Chem **378**:591–597, 1997.
45. Van Steensel B, de Lange T. Control of telomere length by the human telomeric protein TRF1. Nature **385**:740–743, 1997.
46. Van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. Cell **98**:401–403, 1998.