

Analysis of Mammalian Telomere Position Effect

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Abstract

Methods relating to the positioning of a transgene next to a newly formed telomere in human (HeLa) cells and the subsequent analysis of the resulting clones are described. These include vector design, analysis of integration sites by Southern blotting, pharmacological relief of silencing, and enhancement of silencing by telomere elongation. Several potential pitfalls of applying these techniques to other cell lines are discussed. In addition, detailed instructions are provided for several more general methods related to human telomeres including terminal restriction fragment (TRF) analysis and purification of telomeres from digested genomic DNA. These procedures summarize the techniques currently in use that relate to human telomere position effect.

Key Words

Chromosome
Effect
Healing
Human
Position
Repression
Seeding
Silencing
Subtelomeric
Telomerase
Telomere
Truncation
Variegation

1. Introduction

Reversible silencing of genes near telomeres, termed telomere position effect (TPE), has been studied in lower organisms for over a decade (1,2). Although the mechanism of silencing, particularly in *S. cerevisiae*, is becoming clearer, its biological significance remains a mystery (3). This phenomenon has recently been described in human cells (4,5). Since the strength of silencing is proportional to telomere length and human telomeres shorten with each cell division (6), loss of TPE has the potential to play a role in human aging. Positioning a reporter gene next to a human telomere and subsequent analysis of the resulting clones require utilization of several non-standard molecular techniques. The generation and analysis of clones of human (HeLa) cells

bearing a subtelomeric reporter gene (**Fig. 1**) will be described here to illustrate these methods.

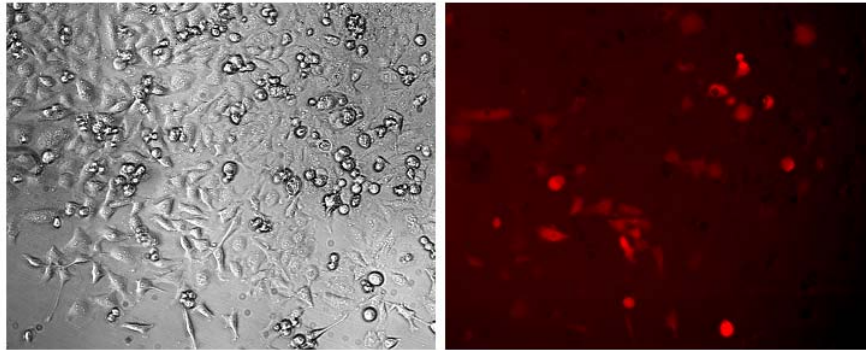


Fig. 1. Expression of a telomeric DsRed2 reporter in HeLa cells. A HeLa clone bearing the DsRed2 reporter at a telomeric site was examined (unfixed) on a Zeiss Axiovert 100M inverted fluorescent microscope. The mosaic pattern of expression is characteristic of transgenes in mammalian cells (20-24) and is particularly prominent for those at telomeric locations (5).

2. Materials

1. Plasmid containing 1.6 kb human telomere repeats $(T_2AG_3)_n$ (7)
2. Expression cassette for the reporter gene of interest (DsRed2-N1, Clontech, Palo Alto, CA, in this example)
3. Appropriate drugs for selection based on the final plasmids and retroviruses used
4. Restriction enzymes, T4 polymerase (for blunting ends if necessary), T4 ligase
5. Bacteria and related materials for plasmid transformation and amplification
6. HeLa cells and a culture facility
7. Materials for Southern blotting
8. Random-primer labeling kit
9. Phosphate-buffered saline (PBS)
10. Digestion buffer: 100mM NaCl, 10 mM Tris pH 8, 25 mM EDTA pH 8, 0.5% SDS and 0.1 mg/mL proteinase K
11. Phenol/chloroform/isoamyl alcohol
12. Large volume of TE, membrane and equipment for dialysis
13. Trichostatin A (TSA) and/or 5-bromodeoxyuridine (BrdU), stored at -20°C at stock concentrations of 1 mg/mL in DMSO and 1 M in H_2O , respectively
14. Retroviral vector encoding hTERT and empty vector control
15. Ecotrophic retroviral packaging cell line such as PE501 (8)
16. Amphitrophic retroviral packaging cell line such as PA317 (8)
17. Resuspension buffer: 100 mM NaCl, 100 mM EDTA, and 10 mM Tris (pH 8)
18. Proteinase K
19. Triton X-100

20. Denaturing solution: 0.5 M NaOH and 1.5 M NaCl.
 21. Neutralization buffer: 1.5 M NaCl and 0.5 M Tris (pH 8)
 22. ³²P-labeled (T₂AG₃)₄ oligonucleotide
 23. Sodium dodecyl sulfate (SDS)
 24. Sodium chloride/sodium citrate buffer (SSC)
 25. Phosphor screen or film and apparatus for detection/quantification (Amersham, Piscataway, NJ)
 26. Fluorescence microscope, fluorescence activated cell sorter or other appropriate apparatus for detection of the chosen reporter gene
- For optional telomere purification (3.2.4):*
27. Biotinylated (CCCTAA)₆ oligonucleotide
 28. Streptavidin-coated magnetic beads such as Dynabeads (Dyna, Oslo, Norway)
 29. 5X Denhardt's solution
 30. Samarium-cobalt or other strong magnet (Edmund Scientifics, Tonawanda, NY)

3. Methods

The methods described below outline (1) construction of the chromosome truncation vector, (2) analysis of the resulting clones by telomere purification and Southern blotting, (3) relief of silencing by trichostatin A or BrdU, (4) elongation of telomeres by hTERT overexpression, and (5) representative results.

3.1 Truncation Vector

The construction of a vector designed to place a gene of interest next to a newly formed telomere by chromosome truncation is described here. A tract of telomere repeats contained within the vector “seeds” the formation of a new telomere at the site of integration when this construct is transfected into cells (7,9-11), resulting in the truncation of a chromosome (*see Note 1*).

3.1.1 T₂AG₃-Containing Plasmid

A plasmid (pSXneo-1.6-T₂AG₃) containing a 1.6 kb tract of telomere repeats (7) was kindly provided by the laboratory of T. de Lange (*see Note 2*). The backbone containing telomere repeats, the origin of replication, and the β-lactamase (ampicillin resistance) gene, was obtained by digestion with Sma I and Hpa I using standard molecular biology techniques (12).

3.1.2 Expression Vector

The plasmid DsRed2-N1 (Clontech, Palo Alto, CA) contains a mammalian expression cassette for the fluorescent protein DsRed2 consisting of the cytomegalovirus (CMV) promoter, the coding region, and the SV40 poly-adenylation signal. A fragment

containing an internal ribosome entry site (IRES) in front of the blasticidin-resistance gene was excised from the vector pWZL-Blast (gift of J. Morgenstern, Millennium Pharmaceuticals, Cambridge, MA) and inserted into the Hpa I site between the DsRed2 coding region and the SV40 poly-adenylation signal by standard molecular biology techniques (*12*), to allow translation of two proteins from only one mRNA (*13*), thus ensuring that blasticidin-resistant cells also expressed mRNA for DsRed2 (*see Note 3*).

3.1.3 Cloning

DNA manipulations were performed by standard methods and are not described here in detail (*12*). Briefly, the DsRed2/IRES-Blast expression cassette was removed from the modified DsRed2-N1 by digestion with Afl III and Bfr I and the ends blunted with T4 DNA polymerase. This fragment was ligated into the backbone derived from pSXneo-1.6-T₂AG₃ such that the CMV promoter was placed adjacent to the base of the T₂AG₃ repeats (**Fig. 2**). A control vector lacking telomere repeats was generated by excision of the telomere tract using Cla I and Sac II, followed by blunting with T4 DNA polymerase and re-ligation (*see Note 4*). After ligation, the DNA was transformed into DH5 α (Invitrogen, Carlsbad, CA) by standard methods, plated on media containing 75 μ g/mL ampicillin, and grown overnight at 37°C. Single colonies were then isolated and grown overnight in liquid media containing 75 μ g/mL ampicillin. Plasmid DNA was isolated and checked for the presence and correct orientation of the insert by restriction enzyme digest.

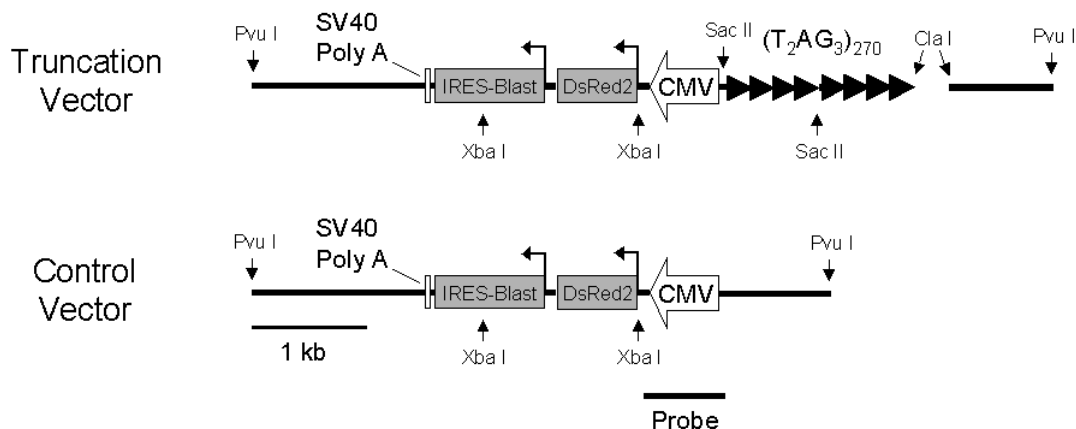


Fig. 2. Structure of the truncation and control vectors. Expression of a single transcript encoding both the DsRed2 and the blasticidin-resistance proteins is driven by the CMV promoter. The linearized forms (as transfected) are shown. Gel purification was used to remove the smaller Cla I/Pvu I fragment from the truncation vector prior to transfection. The restriction sites (Xba I) and probe region used during Southern blotting are indicated below.

3.2 Generation and analysis of clones

3.2.1 Generation of clones

The vectors were linearized with Cla I and Pvu I (truncation vector) or Pvu I alone (control vector) as shown (**Fig. 2**) and gel purified. HeLa cells at 30-50% confluence on 10 cm dishes were transfected with 10 µg of plasmid using FuGENE 6 transfection reagent (Roche, Basel, Switzerland) according the manufacturer's instructions. Cells were grown in 4:1 DMEM:Medium 199 (Invitrogen, Carlsbad, CA, *see Note 5*) containing 10% calf serum and 1 µg/mL blasticidin until individual clones could be easily distinguished (approximately 2 weeks). Clones were then transferred to separate dishes by ring-cloning (*see Note 6*). A glass ring, sealed with sterile silicone vacuum grease, was placed over each clone and the cells were then released by standard trypsinization methods. Clones were then cultured until a sufficient number of cells were obtained from which to extract genomic DNA (10-20 million). DNA was extracted by standard methods and used in the subsequent analysis.

3.2.2 Extraction of genomic DNA

DNA was extracted using a standard dialysis procedure (**14**) instead of precipitation both to preserve the integrity of large DNA fragments and because genomic DNA from our HeLa cells was extremely difficult to re-dissolve after pelleting.

1. Trypsinize cells, wash in PBS, and pellet.
2. Resuspend cells at 10^8 /mL in digestion buffer (100mM NaCl, 10 mM Tris pH 8, 25 mM EDTA pH 8, 0.5% SDS and 0.1 mg/mL proteinase K).
3. Incubate at least 12 hours at 50°C with shaking.
4. Extract with phenol/chloroform/isoamyl alcohol, sacrificing volume if necessary to avoid any white precipitate when transferring the aqueous layer to a new tube. Repeat if necessary.
5. Dialyze the aqueous layer twice against 100 volumes of Tris/EDTA (TE) buffer for a total of at least 24 hours.

3.2.3 Digestion of DNA

Genomic DNA (60-80 µg if performing telomere purification, 15-20 µg if not) was digested with an enzyme (Xba I) that cut within the vector sequence, leaving a region corresponding to the Southern blot probe attached to the telomere repeats (**Fig. 2**). If the vector had integrated at an internal site, this digest produced a discreet band on a Southern blot since a second restriction site was present in the genomic DNA. If the vector sequences had seeded the formation of a new telomere, however, this digest produced a smear on a Southern blot because the heterogeneous repetitive telomere fragments (which do not contain an Xba I site) remained attached to the probed vector sequences (**Fig. 3**).

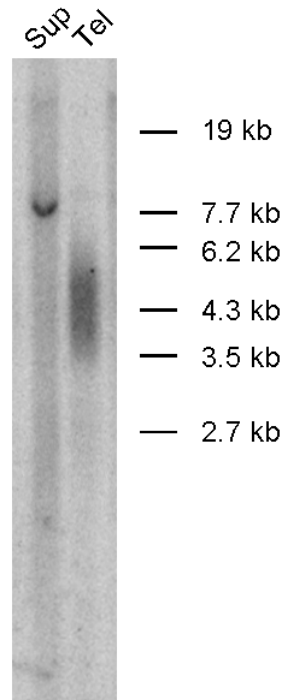


Fig. 3. Southern blot showing telomeric and internal integration sites. Genomic DNA was extracted from a clone in which both a telomeric and an internal integration have taken place. After digestion with Xba I, telomeres were purified by the optional procedure described in **section 3.2.4**. Both the supernatant (Sup, containing bulk genomic DNA) and the purified telomeres (Tel) were analyzed by Southern blot using the CMV promoter region as a probe. Because the heterogeneous telomere fragments remain attached to the CMV promoter after digestion, the telomeric integration site is indicated by a smear in the telomere fraction while the internal integration site is indicated by a single band in the supernatant fraction. Size markers (λ Sty I) are indicated in kilobases.

3.2.4 (Optional) Telomere Purification

This protocol allows purification of telomere-containing DNA fragments after digestion based on the 3' overhang that is present at each chromosome end. This eliminates most of the background signal on a Southern blot, allowing a smear indicating a telomeric integration site (*see section 3.2.5*) to be more clearly distinguished. This method also provides an additional confirmation of a telomeric or internal integration site since telomeric fragments will be present in the telomere fraction while internal fragments will be present only in the supernatant.

1. To the digested DNA (it is not necessary to remove the digestion buffer), add 12.5 μ L 20X SSC, 1.5 μ L 25% Triton X-100, 4.5 pmol biotinylated (CCCTAA)₆ oligonucleotide, and water up to 250 μ L.

2. Anneal the biotinylated oligonucleotide to the telomeric 3' overhangs by heating to 80°C for 20 minutes, 65°C for 30 minutes, 55°C for 20 minutes, 45°C for 15 minutes and 35°C for 15 minutes.
3. Add 20 µL of a 10 mg/mL stock of streptavidin-coated magnetic beads (Dynabeads) that have been washed in 1X SSC, coated with 5X Denhardt's solution for 30 minutes, and resuspended in 1X SSC.
4. Incubate at 4°C overnight, rotating the sample end-over-end at approximately 3 rpm to keep the beads suspended.
5. On day 2, pre-chill (on ice) a magnet (Edmund Scientifics, Tonawanda, NY), 1X SSC with 1% Triton X-100, 0.2X SSC with 1% Triton X-100, and TE, pH 8. Perform the remaining steps keeping all materials (including samples) on ice.
6. Spin tubes briefly to collect samples at the bottom, then "pellet" beads by holding the samples against the magnet. While holding the sample tube against the magnet, carefully remove the supernatant with a pipetor and save (this contains genomic DNA excluding telomere fragments).
7. Resuspend using a wide-bore pipet tip in 150 µL of the 1X SSC buffer, "pellet" the beads with the magnet and discard the supernatant (remove either by pipeting or with a vacuum).
8. Resuspend in 150 µL 1X SSC, this time transferring the slurry to a new tube to avoid recovering DNA non-specifically bound to the walls of the tube. "Pellet" beads and discard supernatant as in the previous step.
9. Resuspend in 150 µL 0.2X SSC, "pellet" beads and discard supernatant.
10. Without disturbing the pellet, hold tube against the magnet and gently add and remove 50 µL of TE with a pipetor. (This step is to remove residual SSC that could interfere with elution in the next step.)
11. Resuspend in 20 µL of TE and elute beads by heating to 65°C for 10 minutes.
12. Spin tubes briefly, "pellet" beads on a warmed magnet and this time recover the supernatant containing telomere fragments.

3.2.5 Southern blotting

Southern blotting was performed by standard methods according to the instructions provided with the Zeta Probe GT (Bio-Rad, Hercules, CA) nylon membrane. Samples were run on a 0.7% agarose gel and capillary transferred for at least 12 hours in 10X SSX. The membrane was then crosslinked twice in a Stratalinker UV crosslinker (Stratagene, La Jolla, CA) at the "autocrosslink" setting. An α -P³²-dCTP labeled probe was generated from a 600 bp fragment of the truncation vector containing primarily the CMV promoter by random priming. Blocking and hybridization were performed in sodium phosphate-buffered (0.25 M, pH 7.2) 7% SDS at 65°C. Washing steps were performed according to the membrane manufacturer's instructions and the blot was exposed to a Phosphor screen and visualized using a STORM 860 Phosphorimager (Amersham, Piscataway, NJ). Telomeric insertions were indicated by a smear while internal insertions gave discreet bands (of characteristic size, **Fig. 3**). If telomeres were purified in the previous step then telomeric clones also gave a positive signal in the telomere fraction while internal clones did not.

3.3 Relief of silencing

Silencing can be relieved in telomeric clones (and internal controls) by treatment with either trichostatin A (TSA) (**4**) or 5-bromodeoxyuridine (BrdU) (**15**). TSA has the advantage that the mechanism is understood, since it is a known histone deacetylase inhibitor, however this drug is highly toxic to HeLa cells, killing greater than 50% during the treatment required for the assay. BrdU on the other hand is less toxic, but its mechanism of action is unknown.

3.3.1 Trichostatin A

Cells were treated with 200 ng/mL trichostatin A in regular medium for 24 hours. The TSA-containing medium was then replaced with fresh medium and the cells were incubated an additional 24 hours before assaying (*see Note 7*).

3.3.2 BrdU

Cells were treated for 72 hours with 50 μ M 5-bromodeoxyuridine in regular medium. Effects were visible by about 48 hours and persisted for several days.

3.4 Enhancement of silencing by telomere elongation

Telomerase is the enzyme that maintains telomeres in germ line and most tumor cells. It consists of an integral RNA component and a catalytic protein component. Although HeLa cells are telomerase positive, additional exogenous telomerase protein (hTERT) can dramatically increase telomere length (*see Note 8*).

3.4.1 Retroviral vectors

The telomerase catalytic component, kindly provided by the Geron Corporation (Menlo Park, CA), was subcloned into the EcoRI site of the retroviral vector pBabe-puro (**16**). Retroviral supernatant was obtained from this and a control (empty) vector by standard methods (**16**). The vector was transiently transfected in to the ecotropic packaging cell line PE501 (**8**) and supernatant from these cells was used to stably infect the amphotrophic cell line PA317 (**8**). Supernatant from PA317 was then used to infect HeLa cells.

3.4.2 Changes in telomere length

Telomere length in HeLa cells increased from approximately 5 kb to approximately 15 kb within one month after hTERT infection. Changes in telomere length were monitored by Southern blot (as described above) for telomeric clones, since the size of the plasmid sequences attached to the telomeric smear was known and could

be subtracted, or alternatively by terminal restriction fragment (TRF) analysis as described in the next section.

3.4.3 Terminal restriction fragment analysis

A generally applicable method for determining telomere length is terminal restriction fragment (TRF) analysis, first described in **ref. (17)** and further developed in **ref. (18)**. This method saves a few steps relative to the Southern blotting technique described previously and can be used to determine telomere length in both telomeric and internal clones. Genomic DNA is digested with a mixture of restriction enzymes so that only repetitive sequences (such as telomeres) that contain no restriction sites remain intact. Samples are run on an agarose gel, which is then probed for telomere sequences (**Fig. 4**). A more detailed description of the TRF procedure is provided in **ref. (19)**.

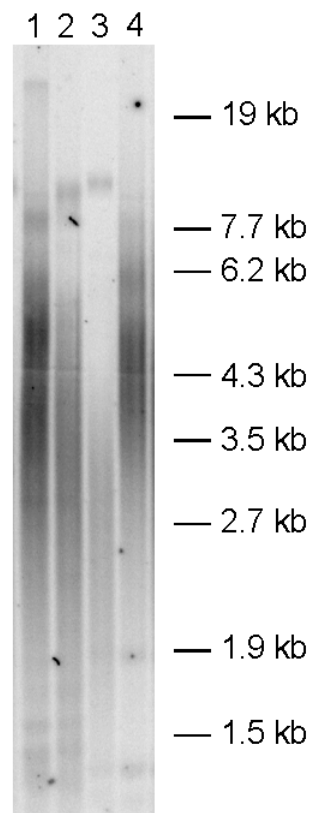


Fig. 4. Terminal restriction fragments from human cells. Genomic DNA from 4 human cell lines was digested with a mixture of restriction enzymes with 4-base recognition sites in order to degrade non-repetitive DNA. Samples were then run on an agarose gel, which was subsequently dried and probed with a $(T_2AG_3)_4$ oligonucleotide (corresponding to the telomere repeat sequence). Care must be taken when determining average size (*see Note 9*) due to the extensive heterogeneity. Lane 2, in particular, is a good example of this. Size markers (λ Sty I) are indicated in kilobases.

1. Resuspend cells in 100 mM NaCl, 100 mM EDTA, and 10 mM Tris (pH 8) at 20 000 cells/ μ L.
2. Extract genomic DNA by bringing the final concentrations of Triton X-100 and proteinase K up to 1% and 2 mg/mL respectively and incubating for 2-16 hours at 55°C.
3. Inactivate proteinase K at 70°C for 30 minutes.
4. (Optional) Extract with an equal volume of phenol/chloroform/isoamyl alcohol. This step is not necessary when using the restriction enzymes specified in step 6, however some enzymes may cut less efficiently if this extraction has not been carried out.
5. Dialyze samples overnight against TE (pH 8).
6. After dialysis, digest 1 μ g DNA with a mixture of six restriction enzymes (1-2 units each of Alu I, Cfo I, Hae I, Hinf I, Msp I, and Rsa I) with 4 bp target sites.
7. Run on a 0.7% agarose gel overnight at approximately 2.5 V/cm.
8. Denature the gel for 20 minutes in 0.5 M NaOH and 1.5 M NaCl.
9. Rinse 10 minutes in water.
10. Dry 1 hour at 55°C. The gel is delicate at this stage and can be transported by rolling loosely around a 10 or 25 mL pipet.
11. Neutralize for 15 minutes in 1.5 M NaCl and 0.5 M Tris (pH 8).
12. Probe with 32 P-labeled (T₂AG₃)₄ oligonucleotide (end-labeled using T4 polynucleotide kinase).
13. Wash in 2X SSC for 15 minutes and 0.1X SSC with 0.1% SDS twice for 10 minutes.
14. Expose the gel to a Phosphor screen or film and analyze, keeping in mind that signal strength will be proportional to both the number of telomeres and the length of each telomere (*see Note 9*).

3.5 Detection of reporter expression

DsRed2 expression was detected by a combination of fluorescence microscopy and fluorescence activated cell sorting (FACS). Expression was highly variable in internal clones, in terms of both the fraction of cells positive and the intensity within each positive cell. Expression in telomeric clones was always limited to a few percent of the cells (**Fig. 5**, *see Note 10*).

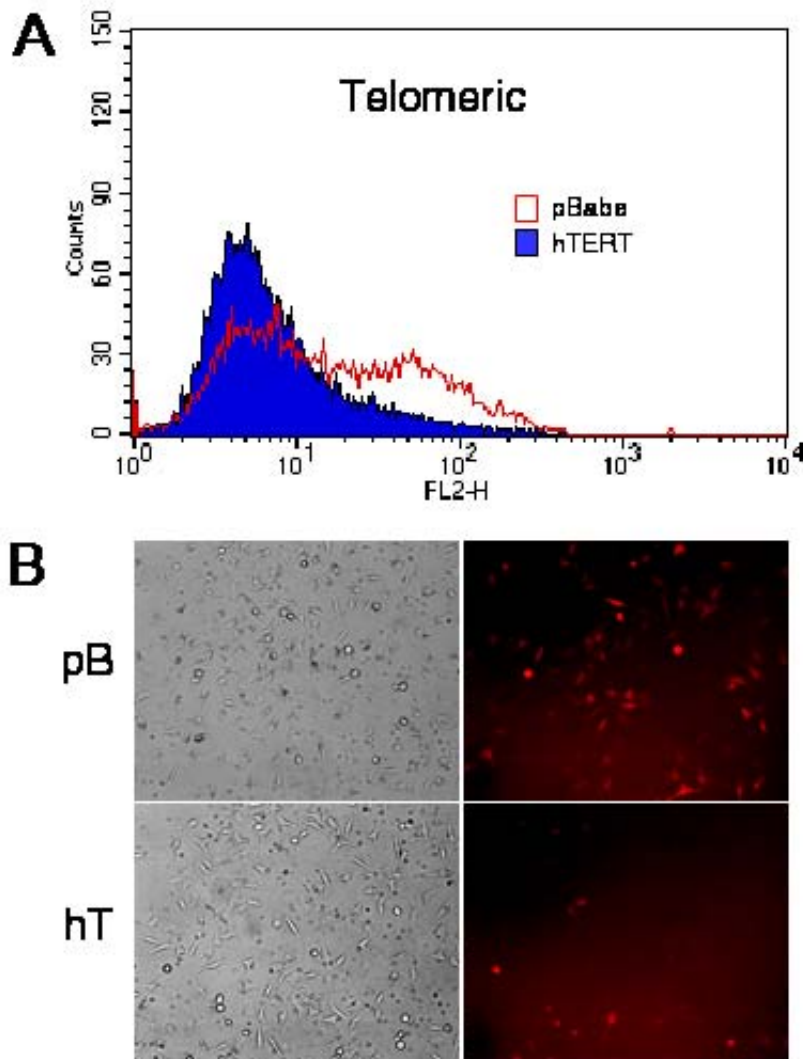


Fig. 5. Elongation of telomeres by hTERT overexpression decreases expression of DsRed2 in telomeric clones. Clones were infected with an empty vector (pBabe) or a retrovirus encoding the telomerase catalytic component (hTERT). (A) FACS analysis of a clone bearing a telomeric DsRed2 reporter with short telomeres (red outline) or long telomeres (solid blue histogram). (B) Bright field and fluorescent images for the cells analyzed in panel A.

4. Notes

1. This procedure was originally used in mammalian cells that were telomerase-positive and aneuploid (7,9-11), such as the HeLa cells described here. Telomerase was thought to be necessary for the “healing” of a new telomere by extension of the plasmid-based telomere repeats and aneuploidy was thought to indicate that cells might tolerate the loss of a chromosome arm after a truncation event. More recently, it was shown that telomere-healing

events can be detected in some normal (telomerase-negative) human fibroblast strains using a 2 kb tract of telomere repeats and that overexpression of TRF1 can enhance this process in telomerase-positive cells (25). In another report telomere-healing was demonstrated in SV40-transformed human embryonic kidney (HEK) cells in the presence of wild type or catalytically inactive telomerase, but not in the absence of telomerase (26). In this report, no telomere-healing events were detected in human diploid fibroblasts transfected with a 1.6 kb tract of telomere repeats or in HEK cells transfected with a 3.2 kb tract of telomere repeats (both telomerase-negative), even in the presence of SV40 T antigen. It is therefore necessary to carefully consider the cell type that will be used (*also see Note 8*) and the strategy for generating telomere-healing events.

2. Telomere (T2AG3) repeats are unstable in certain positions and/or orientations within a plasmid for reasons that are not well understood (27). Rearrangements can be minimized by harvesting bacteria containing the plasmid before the end of log phase growth (usually 10-12 hours for a 250 mL culture). It is necessary to check the integrity of the telomere repeat array by restriction digest at each step in the cloning process. Smaller, sub-stoichiometric bands below the band containing telomere repeats are indicative of deletion products. In some cases, it may be necessary to adopt an alternative strategy if the desired product cannot be obtained intact.
3. An important caveat to keep in mind is that, regardless of the way the vector is organized, this type of strategy will produce only clones in which there is sufficient expression of the marker gene to get drug resistance. It is possible that many telomeric clones exhibiting complete silencing are lost during the selection process. Linking the reporter to the resistance marker, as is done here simply assures that the reporter will be intact in the isolated clones (i.e. prevents the isolation of clones in which the reporter gene has become damaged or lost during integration but that still contain the resistance marker).
4. A linearized control vector could be generated simply by cutting the truncation vector on both sides of the telomere repeats. However, inclusion of extraneous sequences at the end of the linearized control vector (as shown in Fig. 1) increases the number of expressing clones that are obtained in some systems (27). It is not clear whether this is due to degradation of the transfected material, loss of some sequences during integration, or some other mechanism.
5. Our lab uses this particular mix of cell culture media for historical reasons. Is it not expected to differ significantly from 100% DMEM.
6. Transfection of a repeat-containing vector typically produces 1-3 times as many clones as the corresponding vector lacking repeats (27). Within these clones, it is not unusual for >50% to be telomeric integration events (7) although as few as 10% may be obtained, depending on the construct and/or cell line (27).
7. TSA induces either apoptosis, differentiation, or senescence, depending on the cell line (28). HeLa cells treated with TSA undergo apoptosis and the effective doses for killing cells and relieving transgene silencing are very

similar. The procedure described here results in the apoptosis of over half of the treated cells, however lower doses are much less effective at relieving silencing. The effects of TSA (and BrdU) are not specific to telomeric clones (4). Both of these agents appear to generally relieve silencing of transgenes, but have more dramatic effects on telomeric clones due to the stronger initial repression.

8. The behavior of telomeres in the presence of exogenous telomerase is characteristic of each cell line (29). Some, like the HeLa cells described here, will elongate their telomeres rapidly and almost indefinitely in the presence of excess hTERT while others elongate their telomeres more slowly and can reach a stable length that may be very close to the starting length. This should be determined beforehand for the cell line in question if telomere elongation will form an important part of the experiment.
9. Since the probe can bind along the length of the entire telomere, long telomeres will not only run at a higher molecular weight, they will also give a more intense signal per end. This becomes important when attempting to determine the average telomere length. Mean size can be estimated by subdividing the telomere smear into discreet regions and then dividing the sum of volume (phosphorimager) or optical density (film) minus background for all regions by the sum of volume minus background divided by length (of the fragments in that region) for all regions (6). The formula for estimating average telomere length by this method is this method is $\Sigma(\text{volume}_i - \text{background}) / \Sigma[(\text{volume}_i - \text{background})/L_i]$, where volume_i is the signal intensity and L_i is the length of telomere fragments in region i . The program Telorun for this analysis is available at URL: http://www.swmed.edu/home_pages/cellbio/shay-wright/research/sw_lab_methods.htm.
10. In contrast to the stable expression typically observed for transgenes in yeast and other model systems, transgenes in mammalian cells are typically repressed to some degree and are frequently expressed in a mosaic pattern even within clones (20-24). Repression at telomeres appears to be particularly strong since there are no reported cases of telomeric transgenes being expressed in a high fraction of cells and the average expression at telomeres is ten-fold lower than at internal loci (4). However, any experiment involving telomere position effect in human cells should take into account the variability inherent in the expression of mammalian transgenes.

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References

1. Hazelrigg, T., Levis, R., and Rubin, G. M. (1984) *Cell*, **36**, 469-481.

2. Gottschling, D. E., Aparicio, O. M., Billington, B. L., and Zakian, V. A. (1990) *Cell*, **63**, 751-762.
3. Tham, W. H., and Zakian, V. A. (2002) *Oncogene*, **21**, 512-521.
4. Baur, J. A., Zou, Y., Shay, J. W., and Wright, W. E. (2001) *Science*, **292**, 2075-2077.
5. Koering, C. E., Pollice, A., Zibella, M. P., Bauwens, S., Puisieux, A., Brunori, M., Brun, C., Martins, L., Sabatier, L., Pulitzer, J. F., and Gilson, E. (2002) *EMBO Rep*, **3**, 1055-1061.
6. Harley, C. B., Futcher, A. B., and Greider, C. W. (1990) *Nature*, **345**, 458-460.
7. Hanish, J. P., Yanowitz, J. L., and de Lange, T. (1994) *Proc Natl Acad Sci U S A*, **91**, 8861-8865.
8. Miller, A. D., and Rosman, G. J. (1989) *Biotechniques*, **7**, 980-982, 984-986, 989-990.
9. Farr, C., Fantes, J., Goodfellow, P., and Cooke, H. (1991) *Proc Natl Acad Sci U S A*, **88**, 7006-7010.
10. Farr, C. J., Stevanovic, M., Thomson, E. J., Goodfellow, P. N., and Cooke, H. J. (1992) *Nat Genet*, **2**, 275-282.
11. Barnett, M. A., Buckle, V. J., Evans, E. P., Porter, A. C., Rout, D., Smith, A. G., and Brown, W. R. (1993) *Nucleic Acids Res*, **21**, 27-36.
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning : a laboratory manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. Jang, S. K., Pestova, T. V., Hellen, C. U., Witherell, G. W., and Wimmer, E. (1990) *Enzyme*, **44**, 292-309.
14. Ausubel, F. M. (1993) *Current protocols in molecular biology*, Greene Pub. Associates and Wiley-Interscience : J. Wiley, New York.
15. Suzuki, T., Yaginuma, M., Oishi, T., Michishita, E., Ogino, H., Fujii, M., and Ayusawa, D. (2001) *Exp Cell Res*, **266**, 53-63.
16. Morgenstern, J. P., and Land, H. (1990) *Nucleic Acids Res*, **18**, 3587-3596.
17. de Lange, T., Shiue, L., Myers, R. M., Cox, D. R., Naylor, S. L., Killery, A. M., and Varmus, H. E. (1990) *Mol Cell Biol*, **10**, 518-527.
18. Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992) *Proc Natl Acad Sci U S A*, **89**, 10114-10118.
19. Herbert, B.-S., Shay, J. W., and Wright, W. E. (2003) in *Current protocols in cell biology* (Dasso, M., and Morgan, K., eds), pp. in press.
20. Kalos, M., and Fournier, R. E. (1995) *Mol Cell Biol*, **15**, 198-207.
21. Walters, M. C., Fiering, S., Eidemiller, J., Magis, W., Groudine, M., and Martin, D. I. (1995) *Proc Natl Acad Sci U S A*, **92**, 7125-7129.
22. Walters, M. C., Magis, W., Fiering, S., Eidemiller, J., Scalzo, D., Groudine, M., and Martin, D. I. (1996) *Genes Dev*, **10**, 185-195.
23. Martin, D. I., and Whitelaw, E. (1996) *Bioessays*, **18**, 919-923.
24. Dorer, D. R. (1997) *Transgenic Res*, **6**, 3-10.
25. Okabe, J., Eguchi, A., Masago, A., Hayakawa, T., and Nakanishi, M. (2000) *Hum Mol Genet*, **9**, 2639-2650.

26. Guiducci, C., Anglana, M., Wang, A., and Bacchetti, S. (2001) *Exp Cell Res*, **265**, 304-311.
27. Baur, J. A., Shay, J. W., and Wright, W. E., unpublished results.
28. Marks, P. A., Richon, V. M., and Rifkind, R. A. (2000) *J Natl Cancer Inst*, **92**, 1210-1216.
29. McChesney, P. A., Aisner, D. L., Frank, B. C., Wright, W. E., and Shay, J. W. (2000) *Mol Cell Biol Res Commun*, **3**, 312-318.