Telomere Amount and Length Assay

Yuebo Gan,[1] Kory J. Engelke,[1] Christopher A. Brown,[1] and Jessie L.-S. Au[1–3]

Received July 25, 2001; accepted August 30, 2001

Purpose. Telomeres are specific DNA structure at the ends of chromosomes to protect chromosomes from fusion, recombination, and degradation. Telomere length changes are implicated in cell senescence, aging, tumorigenesis, and DNA repair. The standard method for measuring telomere length is Southern blot analysis. This method has several disadvantages, i.e., loss of DNA during membrane blotting, high background due to nonspecific binding of telomere probe to membrane, and loss of telomeric signal due to extensive washing. These limitations resulted in a low signal-to-noise ratio and, therefore, reduced sensitivity and reproducibility. The multi-step Southern blot is also highly labor-intensive. The present study was to develop a more quantitative assay of telomeric amount and length (TALA).
Methods. TALA was based on solution hybridization and did not require blotting, prehybridization, and washing. The major steps were (a) DNA preparation and digestion with restriction endonucleases, (b) hybridization between DNA and telomeric probe, (c) agarose gel electrophoresis, and (d) autoradiography and data analysis.
Results. The telomere amount measured by TALA was linearly correlated with the amount of DNA analyzed ($r^2 = 0.985$, $P < 0.01$). The telomere length measured by TALA also correlated with the telomere length determined by fluorescence in situ hybridization ($r^2 = 0.99$, $P < 0.01$). Compared to the Southern blot analysis, TALA showed a 4-fold greater sensitivity, 4.6-fold higher signal-to-noise ratio, >2 fold-higher reproducibility, and 4-fold less time requirement.
Conclusion. We report here a rapid, sensitive, and quantitative assay for measuring telomere length and amount.

KEY WORDS: telomere; TRF; FISH.

INTRODUCTION

Telomeres are specific DNA structure at the ends of chromosomes consisting of repeat-unit TTAGGG in vertebrates. Telomere protects the chromosome from end-to-end fusion and helps to maintain chromosomal integrity. In somatic cells, telomeres are shortened by 50 to 200 nucleotides per cell division. Shortening of the telomere length to below a threshold value is believed to induce senescence. Recent findings indicate the telomere length is also correlated with aging, tumorigenesis, and DNA repair (1,2). Hence, quantitation of telomere amount and length may have broad implications.

Telomere length is usually reported as a mean length of the terminal restriction fragments (TRF) which include the telomeres and subtelomeric regions. The standard method for measuring telomere length is Southern blot analysis (3,4). This is a multi-step method which entails (a) cleaving purified DNA with restriction enzymes, (b) separating the DNA fragments by size on an agarose gel, (c) denaturing and transferring the DNA fragments to a membrane, (d) hybridizing the telomere with a radioactive telomere probe, (e) removing the unhybridized probe by washing the membrane, and (f) analyzing the data by autoradiography and image analysis (5). There are several disadvantages of this method, including (a) potential loss of DNA molecules due to their inability to be immobilized on the membrane (which is more serious for short DNA molecules), (b) incomplete availability of the immobilized DNA molecules for hybridization with the telomere probe, (c) probe binding to unintended molecules leading to background noise, and (d) loss of signal due to the extensive washing required to reduce the background. These limitations result in a low signal-to-noise ratio. In addition, although the Southern blot is in theory useful for both qualitative and quantitative measurements of the relative telomere amount and the telomere length, it is usually only adequate for analysis of the relative telomere length (e.g., changes with time or due to drug treatment) because of the problems mentioned above, and is seldom used to quantify the changes in telomere amount.

This report describes a solution hybridization-based method to measure the amount and the average length of telomeres. In this telomere amount and length assay (TALA), the probe is added to a solution containing restriction endonuclease-digested DNA fragments and allowed to hybridize with the telomeres. These hybridized telomeres are then separated from the unhybridized probes using gel electrophoresis and quantified using autoradiography. As shown below, TALA is more rapid and has a higher sensitivity and reproducibility when compared to the Southern blot analysis.

METHODS AND MATERIALS

Cell Culture

Human breast MCF-7, pharynx FaDu, prostate PC-3 and ovarian SKOV-3 cancer cells were purchased from American Type Culture Collection (Rockville, MD). Cefotaxime sodium was purchased from Hoechst-Roussel Pharmaceuticals Inc. (Somerville, NJ), and all other culture supplies from Gibco BRL (Grand Island, NY). MCF-7 and PC-3 cells were maintained in RPMI 1640 medium, SKOV-3 cells in McCoy's 5A medium, and FaDu cells in MEM medium. All media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 90 μg/ml gentamicin, and 90 μg/ml cefotaxime sodium. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Extraction of DNA

Exponentially growing monolayer cells were collected by scraping with a rubber policeman, collected and washed two times with phosphate buffered saline (PBS). Genomic DNA was extracted as described by Sambrook *et al.* (5). In brief, cells (2.5×10^6) were lysed by incubating at 37°C for 30 min

[1] College of Pharmacy, The Ohio State University, Columbus, Ohio 43210.
[2] Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210.
[3] To whom correspondence should be addressed. (e-mail: Au.1@osu.edu)

ABBREVIATIONS: FISH, fluorescence in situ hybridization; PBS, phosphate buffered saline; PNA, peptide nucleic acid. SSC, sodium chloride, sodium citrate solution; TALA, telomere amount and length assay; TRF, terminal restriction fragment.

in 10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% SDS, 20 μg/ml DNase-free RNase. Proteinase K (100 μg/ml) (Sigma, St. Louis, MO) was then added and the mixture was incubated at 50°C overnight. The mixture was extracted with 1 volumes of phenol, chloroform/isoamyl alcohol (24:1)/phenol (50:50), and chloroform/isoamyl alcohol. The genomic DNA in the supernatant was precipitated with 2 volumes 100% ethanol/0.1 volume of 3 M sodium acetate (pH 5.2), washed with 70% ethanol, allowed to air dry for 30 min, and dissolved in double distilled water overnight at 4°C. The DNA concentration was determined by A_{260} using a spectrophotometer (Beckman Model DU 640).

Preparation of ^{32}P-Labeled Telomere Probe

The telomere probe $(TTAGGG)_4$ was end-labeled with ^{32}P. Briefly, 100 ng of $(TTAGGG)_4$ oligonucleotide was incubated with the forward reaction buffer (Gibco BRL), 50 μCi γ-^{32}P-ATP (ICN, Costa Mesa, CA) and 10 units Polynucleotide T4 Kinase (Gibco BRL) for 30 min at 37°C. The labeled probe was purified using the QIAquick Nucleotide Removal Kit (QIAGEN Inc, Valencia, CA).

Telomere Amount and Length Assay (TALA)

The hybridization of telomere DNA fragments with the telomere probe was performed using solution hybridization (6), with the following modifications. Genomic DNA (2.5 μg) was digested with restriction enzymes, i.e., 10 units each of Hinf 1, Hae III, and Hha I in ReACT 2 buffer (all from Gibco BRL), for 2 h at 37°C. The digested DNA was mixed with 1.5 ng ^{32}P-labeled probe in the ReACT 2 buffer (Gibco BRL). The mixture was denatured at 98°C for 6 min, hybridized at 55°C for various times ranging from 0.5 to 20 h, and further cooled to 4°C for at least 5 min. The denaturation, hybridization and cooling were performed in a thermocycler (Perkin Elmer model 2400 PCR machine). The unhybridized excess probe and digested non-telomeric DNA fragments were separated from the hybridized telomeres by gel electrophoresis on a 0.7% agarose (Gibco BRL) gel for 6 h at 90 volts in 1X TBE (0.045 M Tris-borate, 0.001 M EDTA). The gel was then dried under vacuum for 1.5 h at room temperature. The dried gel was transferred on a new filter, covered with a plastic wrap film and exposed to a phosphorimager screen for at least 6 h. The resulting autoradiograph was captured using a phosphorimager, and the intensity of the signal was determined using ImageQuaNT software (all from Molecular Dynamics, Sunnyvale, CA). The background signal was calculated as the average of the signals found in the background areas. The telomere amount was calculated as total intensity of the telomeric signal using the area under curve method minus the background signal. The mean telomere length was defined as the length corresponding to the position where 50% of the total signal occurred. Data calculation and statistical analysis (ANOVA) were performed using the Microsoft Excel program.

Southern Blot Hybridization

Southern blot hybridization was performed as described (3,4). Briefly, genomic DNA (10–15 μg) was digested with restriction enzymes as described above. The digested DNA was separated by gel electrophoresis on a 0.7% agarose gel for 6 h at 90 volts in 1 × TBE, and then transferred to a nylon membrane (MagnaCharge, MSI, Westboro, MA). After heating at 80°C for 2 h, the membrane was incubated for 2 h at 50°C with a prehybridization buffer, consisted of 5X Denhardts, 6X SSC, 0.5% SDS, and 100 μg/ml sonicated salmon sperm DNA (Gibco BRL). The membrane was then hybridized with the ^{32}P-labeled telomere probe for 20 h at 50°C, washed twice at 50°C with 0.1% SDS, 1.0 × SSC and twice with 0.1% SDS, 0.1 × SSC. The telomere signal was detected as described above.

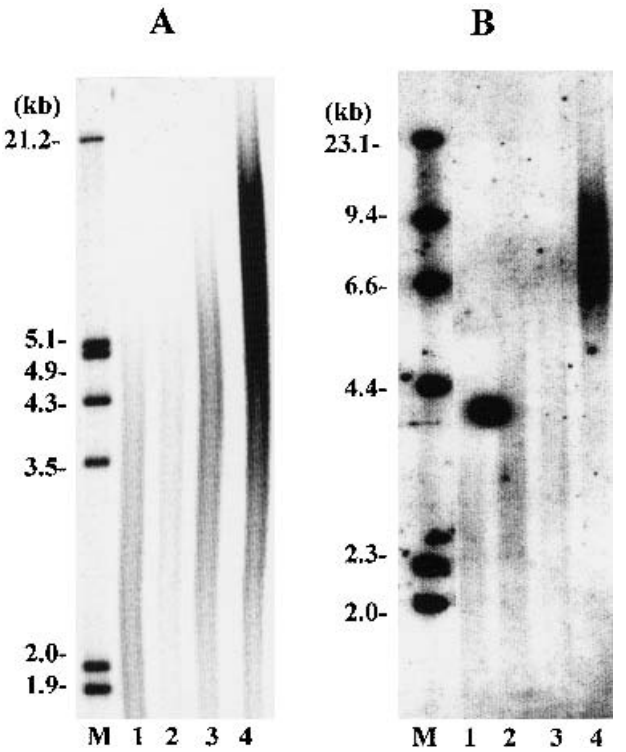

Fig. 1. Telomere length measured by TALA and Southern blot. DNA was isolated from four human cancer cell lines and analyzed for telomere length, as described in the Methods. (A) TALA results obtained with 2.5 μg DNA. (B) Southern blot results obtained with 10 μg DNA. M: Molecular weight standards, lane 1 to 4: MCF-7, PC-3, FaDu, and SKOV-3, respectively.

Table I. Determination of Telomere Length by TALA and Southern Blot Analysis

Cells	TALA telomere length mean ± SD (CV)	Southern blot telomere length mean ± SD (CV)
FaDu	3780 ± 390 (10.3%)	3596*
MCF-7	2670 ± 106 (4.0%)	2720*
PC-3	2878 ± 397 (13.8%)	2844*
SKOV-3	7495 ± 563 (7.5%)	8342 ± 1418 (17.0%)

The mean telomere lengths (length of the terminal restriction fragment) were determined using TALA or Southern blot analysis. Data are reported as mean ± standard deviation (n = 4), with coefficients of variation (CV) in parenthesis, except the data labeled with asterisks were obtained from one experiment.

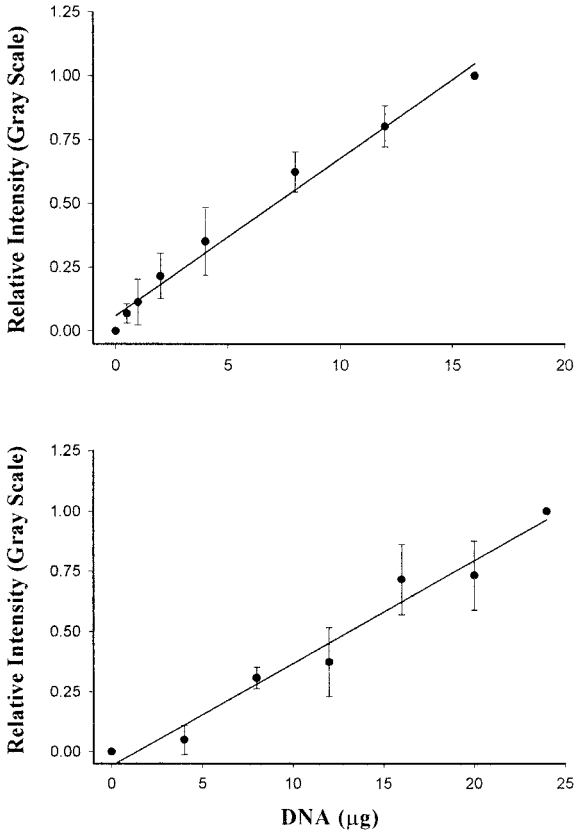

Fig. 2. Telomere amount measured by TALA and Southern blot. Various amounts of DNA were digested and analyzed by either TALA (top panel) or Southern blot (bottom panel). Intensity of telomere signal for each DNA concentration was analyzed by ImageQuaNT. Relative intensity was calculated as the ratio of intensity of telomeric signal of sample to intensity of telomeric signal of the highest amount of DNA used for analysis, i.e., 16 μg for TALA and 24 μg for Southern blot. The coefficient of determination (r^2) for TALA and the Southern blot was 0.985 and 0.923, respectively.

Fluorescence in Situ Hybridization (FISH) to Detect Telomere Length

To verify the validity of the TALA results, telomere signals in intact cells were determined by FISH using fluorescein-labeled peptide nucleic acid (PNA) probe $(CCCTAA)_3$ (PerSeptive Biosystems, Framingham, MA). FaDu, PC-3, and SKOV-3 cells were exposed to 0.1 μg/ml Colcemid for 4 h before harvesting. To spread the chromosomes, the cell pellet was incubated in a hypotonic solution (75 mM KCl) at 37°C for 20 min, washed and fixed thrice, each for 10 min, with acetic acid:methanol (1:3). The fixed cells were dropped onto slides, air dried and kept at –20°C. The slides were sequentially processed in the following solutions: PBS for 5 min, 4% formaldehyde in PBS for 2 min, PBS for 5 min, twice, and dehydrated through 70%, 80%, 95%, and 100% ethanol. Each slide was coated with 10 to 15 μl of PNA probe (1.0 μg/ml), covered with a coverslip, heated at 80°C for 3 min, and then kept at room temperature for 2 h. The slides were washed at room temperature once with PBS for 2 min, twice with the 70% formamide/10 mM Tris-HCl (pH 7.2), and then three times with PBS. The chromosomes were counterstained with 0.1 μg/ml propidium iodide in PBS for 2 min, washed in PBS for 5 min, and mounted in antifade solution (glycerol:PBS (9:1) containing 0.1% p-phenylenediamine, pH 8.0). Slides were examined under a fluorescence microscopy and the images were captured using a digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). The telomeric length was analyzed by ScnImage software (Scion Corporation, Frederick, MD) and calculated as the ratio of area corresponding to telomere to area corresponding to entire chromosome.

RESULTS

Telomere Amount and Length in Human Cancer Cells Analyzed by TALA

Figure 1A shows the TALA results for four human cancer cell lines. The mean telomere length was SKOV-3 > FaDu > PC-3 ~ MCF-7 (Table I). These values are comparable to the values reported in the literature (7). Figure 2 shows the linear correlation between the amount of telomere as measured by TALA and the amount of DNA used for TALA analysis.

Effect of Hybridization Time on TALA Results

To determine the time required for efficient hybridization of telomere probes with the telomeres, the telomere signal in the SKOV-3 cells was compared after hybridization for 0.5 to 20 h. The SKOV-3 cells were used because they showed the longest telomere length and highest signal intensity among the four cell lines tested. The results showed a relatively constant amount of telomere at these hybridization times with slightly lower signal at 20 h (Fig. 3). It was concluded that 0.5 h was sufficient to achieve maximum hybridization between telomeres and telomere probe.

Comparison of Telomere Length Measured by TALA and FISH

Figure 4 shows the detection of telomere in SKOV-3, FaDu, and PC-3 cells using FISH. A comparison of the telo-

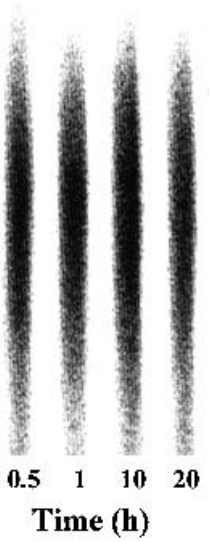

Fig. 3. Effect of hybridization time on TALA results. DNA extracted from SKOV-3 cells (2.5 μg DNA each) was analyzed by TALA. The hybridization time was varied between 0.5, 1, 10, and 20 h.

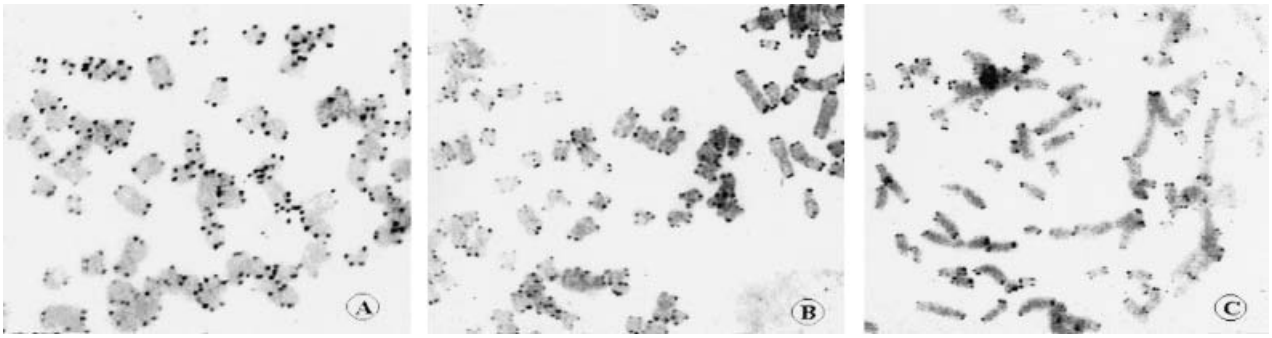

Fig. 4. Telomere detection by FISH. Telomeres in SKOV-3 (A), FaDu (B), and PC-3 (C) cells were detected by the fluorescein-labeled PNA $(CCCTAA)_3$ probe. The black dots are telomere signal at the ends of chromosomes (grayish rods). 1000× magnification.

mere length measured by TALA and FISH shows a highly significant correlation (Fig. 5).

Comparison of TALA and Southern Blot Results

Table I and Fig.1 summarize the results of telomere length measured by TALA and Southern blot. The two methods show comparable results. Both methods required DNA digestion and quantification of the ^{32}P signal. The major difference between the two methods is that the TALA does not require the membrane blotting, prehybridization and washing steps. The fewer sample manipulations in TALA minimized the loss of sample and significantly shortened the experimental time (excluding the time required for autoradiography) from ~42 h required for the Southern blot to ~11 h for TALA. Compared to the TALA assay, the results of the Southern blot analysis (Fig. 1) showed a higher background noise, presumably due to non-specific binding of the telomere probe with non-telomeric molecules on the membrane. The higher background noise and the lower signal intensity contributed to 4.6-fold (range, 2.7 to 5.5) lower signal-to-noise ratio in the Southern blot results, compared with the TALA results. The higher signal-to-noise ratio in TALA further shortened the time required for autoradiography, i.e., 24 h for Southern blot *vs* 6 h for the TALA.

A plot of the telomere amount determined using Southern blot as a function of the amount of DNA analyzed showed a linear relationship (Fig. 2), as observed for TALA. However, the TALA results showed a better correlation and a wider range of the linear relationship (i.e., r^2 of 0.985 over 0.5 μg – 16 μg DNA for TALA vs. r^2 of 0.923 over 2 μg – 24 μg DNA for Southern blot), and a 4-fold greater sensitivity (i.e., a lower detection limit of 0.5 μg for TALA vs. 2 μg for Southern blot).

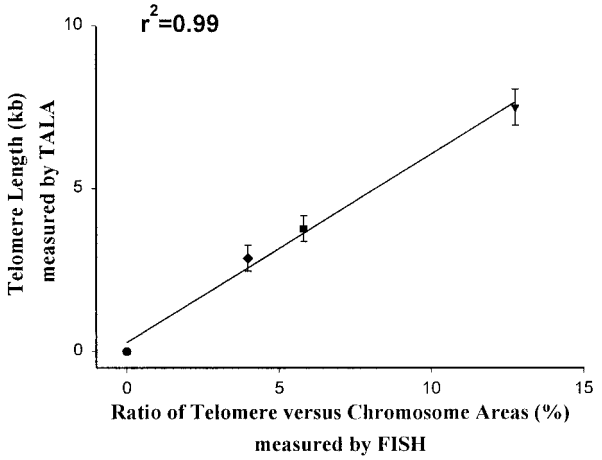

Fig. 5. Comparison of telomere length measured by TALA and FISH. Telomere length detected by TALA was compared with the telomeric signals in FISH in SKOV-3 (▼), FaDu (■), and PC-3 (♦) cells ($r^2 = 0.99$, $P < 0.01$).

The reproducibility of the telomere length determination by TALA and Southern blot analysis was compared in 4 different experiments. Data in Table I show smaller coefficients of variation for TALA compared to Souther blot (i.e., 7.5% vs 17%).

The reproducibility of the telomere amount determination by TALA and Southern blot analysis was also compared. For TALA, the amount of telomeres in three cell lines were measured in 3 experiments with 5 replicates per experiment. The results show average coefficients of variation of 16% for PC-3 cells, 14% for MCF-7 cells, and 9% for SKOV-3 cells. For Southern blot using SKOV-3 cells, the average coefficient of variation from 3 experiments with 4 replicates per experiment was 23%.

DISCUSSION

This report describes a method for measuring telomere amount and length. Compared to the commonly used Southern blot analysis, TALA is 4 times more rapid with less labor intensive, 4 times more sensitive with higher signal to noise ratio, and produces more reproducible results. These advantages are in part due to the fact that in TALA, the hybridization of telomeres with the telomere probe occurs in solution, thus eliminating the problems associated with the need of immobilizing the DNA on a solid support and the high background due to the nonspecific binding of probes to the membrane as in the Southern blot method. The significant correlation between the mean telomere length by TALA and the mean telomeric signal in FISH further validates TALA as a useful method to measure telomere length and amount in cells.

Telomere length, as a "biological clock", is closely related to ageing process (8). The telomere is also implicated in multiple cell functions, including cell senescence, apoptosis, proliferation, DNA repair, and carcinogenesis (9,10). Telomere is a potential therapeutic target for the development of anticancer drugs as well as anti-aging agents (11). However, the existing telomere assays such as Southern blotting are

mainly qualitative and semi-quantitative. Measurement of telomere length with accuracy and precision is important to the evaluation of drug activity on this target. Owing to the inherent complexity, biological assays are often less quantitative than chemical methods such as high pressure liquid chromatography. The present study exemplifies an approach to eliminate and/or better control the steps in biological assays that can cause the assay variability (e.g., membrane transfer, incomplete and background hybridization, and washing steps used in Southern hybridization) and thereby improve the quantitation of a potentially important molecular target.

ACKNOWLEDGMENT

This work was supported in part by a research grant R01CA77091 from the National Cancer Institute, NIH, DHHS.

REFERENCES

1. V. Urquidi, D. Tarin, and S. Goodison. Role of telomerase in cell senescence and oncogenesis. *Ann. Rev. Med.* **51**:65–79 (2000).
2. E. H. Blackburn. Telomere states and cell fates. *Nature* **408**:53–56 (2000).
3. R. C. Allshire, J. R. Gosden, S. H. Cross, G. Cranston, D. Rout, N. Sugawara, J. W. Szostak, P. A. Fantes, and N. D. Hastie. Telomeric repeat from T. thermophila cross-hybridizes with human telomerase. *Nature* **332**:656–659 (1988).
4. T. de Lange, L. Shiue, R. Myers, D. R. Cox, S. L. Naylor, A. M. Killery, and H. E. Varmus. Structure and variability of human chromosome ends. *Mol. Cell Biol.* **10**:518–527 (1990).
5. J. Sambrook, E. F. Fritsch, and T. Maniatis. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989.
6. P. A. Bertram, C. S. Buller, G. C. Stewart, and J.M. Akagi. Isolation and characterization of a Bacillus strain capable of degrading the extracellular glucan from Cellulomonas flavigrna strain KU. *J. Appl. Bact.* **74**:460–466 (1993).
7. C. T. Chu, M. A. Piatyszek, S. S.Y. Wong, C. Honchell, L. A. Holeman, E. W. Wunder, N. Trees, M. A. Palencia, S. Li, and A. C. Chin. Telomerase activity and telomere lengths in the NCI panel of human cancer cell lines. *Proc. Am. Assoc. Cancer Res.* **41**:3405 (2000).
8. C. B. Harley, A. B. Futcher, and C. W. Greider. Telomeres shortening during ageing of human fibroblasts. *Nature* **345**:458–460 (1990).
9. V. Lunblad. DNA ends: Maintenance of chromosome termini vs. repair of double strand breaks. *Mut. Res.* **451**:227–240 (2000).
10. J. W. Shay and W. E. Wright. Telomeres and telomerase: implications for cancer and aging. *Radiat. Res.* **155**:188–193 (2001).
11. E. Raymond, J. C. Soria, E. Izbicka, F. Boussin, L. Hurley, and D. D. Von Hoff. DNA G-quadruplexes, telomere-specific proteins and telomere-associated enzymes as potential targets for new anticancer drugs. *Invest. New Drugs* **18**:123–137 (2000).