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Sensitive Method for Measuring Telomere Lengths by Quantifying Telomeric DNA Content of Whole Cells

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ABSTRACT

Recently, a new method for measuring telomere lengths based on telomere DNA content was developed. The method, which is based on the ratio of telomere to centromere DNA content (TC ratio), is highly sensitive, allowing the analysis of small quantities of DNA. However, the method required the isolation of DNA, which can be difficult or impossible for small numbers of cells. Here, we suggest an improvement of this method that can directly estimate telomere lengths from whole cells. We optimized the method for whole cells and purified DNA and found that accurate TC ratios can be obtained from as little as 9 ng of DNA or 800 whole cells. There was no statistically significant difference between the ratios obtained with purified DNA or with whole cells, indicating that the isolation of DNA is not necessary for small samples.

INTRODUCTION

Telomeres are highly conserved complexes of DNA and protein that exist at the ends of eukaryotic chromosomes (8). Telomeres prevent nuclease degradation and chromosome fusions, making them critical structures for maintaining the fidelity of eukaryotic chromosomes (8). The human telomeric DNA consists of an array of TTA-GGG repeats, which can vary widely in length; 2 kbp in some rapidly dividing cancer cells and 6–11 kbp in human somatic cells (1,4–7). We recently found that the telomere restriction fragments (TRFs) are even longer in chimpanzees and up to 16 kbp in macaque monkeys (Reference 3 and unpublished data). Telomeres in some cells in humans, including peripheral blood mononuclear cells (PBMCs), shorten after each cell division and with age (1,4–7). Based on this observation, we and others hypothesized that telomere lengths could be used for evaluating cell turnover rates in human immunodeficiency virus (HIV) infections. We measured telomere lengths using an improved method (accuracy ± 0.3 kbp) based on two-dimensional calibration of DNA sizes, pulsed-field electrophoresis and high-resolution Southern blot images. However, because Southern blots require at least 1 μ g of DNA to measure telomere lengths, our analysis was not possible when sample cell numbers were extremely low, as is observed for CD4 cells in HIV-infected individuals progressing to AIDS.

A new method of measuring telomere lengths based on the ratio of telomere to centromere DNA content (TC ratio) was recently published (2). This method is based on the ratio of telo-

mere to centromere DNA that can be quantified by a slot-blot procedure. Slot blots are extremely sensitive, allowing detection and analysis of small quantities of DNA (2). However, when only small numbers of cells are available, DNA isolation can be difficult or infeasible. Therefore, we eliminated DNA isolation and performed slot blots for telomeric DNA with whole cells. We measured telomere DNA content of purified DNA and whole cells from cells of known telomere lengths obtained from Southern analysis. In this study, we compared TC ratios obtained from purified DNA and whole cells, and we evaluated the ability of telomere DNA content to predict telomere lengths.

MATERIALS AND METHODS

Samples

The following immortal cell lines or PBMC samples were used for calibrations because their telomere lengths were recently examined in our laboratory by Southern analysis. TC ratios and Southern blots were performed on CEM cells (3.5 kb), TF228 cells (6 kb) and chimpanzee PBMCs (11 kb).

DNA Isolation and Southern Blots

DNA was prepared with a commercially available kit (Puregene®; Gentra Systems, Minneapolis, MN, USA), and equal amounts of DNA (1 μ g) were digested by *AluI*. Samples and two different markers (*HindIII* digest of λ DNA: 4–23 kb and a DNA marker of larger size: 10–50 kb) were then loaded on a 1% 20- $\times$ 25-cm agarose gel. The markers were placed at different positions in

Table 1. A Summary of the TC Ratio Data Including Observed and Fitted TC Ratio Averages and Standard Deviations (SD)

Sample ^a	Observed $\bar{x}$	SD	Fitted $\bar{x}$	Fitted SD	TRF Size	Sample ^a	Observed $\bar{x}$	SD	Fitted $\bar{x}$	Fitted SD	TRF Size
CEM (cells)	0.22	0.09	0.22	0.08	3.5	CEM (DNA)	0.38	0.14	0.37	0.04	3.5
CEM (cells)	0.26	0.10	0.26	0.07	3.5	CEM (DNA)	0.21	0.15	0.20	0.02	3.5
CEM (cells)	0.22	0.08	0.21	0.07	3.5	CEM (DNA)	0.18	0.09	0.18	0.08	3.5
CEM (cells)	0.16	0.02	0.16	0.01	3.5	CEM (DNA)	0.18	0.19	0.18	0.12	3.5
CEM (cells)	0.19	0.09	0.20	0.07	3.5						
CEM (cells)	0.15	0.07	0.16	0.05	3.5						
Average	0.20	0.04	0.20	0.04	3.5	Average	0.24	0.09	0.23	0.09	3.5
TF228 (cells)	0.25	0.10	0.25	0.10	6	TF228 (DNA)	0.31	0.08	0.31	0.08	6
TF228 (cells)	0.34	0.14	0.34	0.13	6	TF228 (DNA)	0.40	0.19	0.40	0.18	6
TF228 (cells)	0.27	0.17	0.28	0.14	6	TF228 (DNA)	0.43	0.18	0.43	0.13	6
TF228 (cells)	0.42	0.17	0.43	0.14	6	TF228 (DNA)	0.32	0.06	0.32	0.06	6
TF228 (cells)	0.34	0.17	0.34	0.16	6						
TF228 (cells)	0.39	0.20	0.41	0.19	6						
Average	0.34	0.07	0.34	0.07	3.5	Average	0.36	0.06	0.37	0.06	6
CHIMP (cells)	0.67	0.06	0.67	0.03	11	CHIMP (DNA)	0.65	0.38	0.65	0.32	11
CHIMP (cells)	0.61	0.16	0.61	0.14	11	CHIMP (DNA)	0.62	0.44	0.62	0.42	11
CHIMP (cells)	0.69	0.29	0.69	0.24	11	CHIMP (DNA)	0.70	0.29	0.70	0.24	11
CHIMP (cells)	0.65	0.18	0.66	0.09	11	CHIMP (DNA)	0.59	0.10	0.59	0.08	11
CHIMP (cells)	0.58	0.26	0.60	0.19	11						
CHIMP (cells)	0.48	0.21	0.50	0.11	11						
Average	0.61	0.08	0.62	0.07	11	Average	0.64	0.05	0.64	0.05	11
<p>Regression Lines</p> <p>cells (observed): TC ratio = telomere length $\times$ (0.0575) $R^2 = 0.935$</p> <p>DNA (observed): TC ratio = telomere length $\times$ (0.0586) $R^2 = 0.951$</p> <p>cells (corrected): TC ratio = telomere length $\times$ (0.0585) $R^2 = 0.949$</p> <p>DNA (corrected): TC ratio = telomere length $\times$ (0.0583) $R^2 = 0.936$</p> <p>^aEach individual sample represents duplicate sample application of four DNA or cell concentrations on an individual slot blot. Therefore, all individual sample averages and SD are the result of 8 independent TC ratio calculations from the same blot. Increased TC ratios with larger telomere lengths are readily observed for whole cells and purified DNA. Formulas and R^2 values for the regression lines describing the relationship between TC ratios and telomere lengths are presented.</p> <p>$\bar{x}$ = average.</p>											

the gel to control for nonuniformities from lane to lane, which might lead to critical errors when comparing the telomere lengths. The gels were run by pulsed-field electrophoresis at 15°C for 20 h and 6 V/cm by using a pulse sequence designed to ensure good separation for sizes between 1 and 37 kb (PPI-200 Programmable Power Inverter; MJ

Research, Watertown, MA, USA). Images of the gels stained with ethidium bromide were acquired by a quantitative imaging system constructed in our laboratory. The DNA was then transferred by a standard Southern blotting procedure for 16 h. The blotted membranes were hybridized with an alkaline phosphatase (AP)-linked telomere

probe and a probe for DNA length calibrating standards (Quick-Light Hybridization KitTM; Lifecodes Corporation, Stamford, CT, USA). The chemiluminescence signal was acquired by a Model GS-505 Molecular Imager[®] System (Bio-Rad, Hercules, CA, USA) at the highest resolution of 0.1 mm. The acquired 16-bit images

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were then processed by using the Molecular Analyst® software (Bio-Rad), and the numbers providing the intensity-distance relationships were transferred to the Scientist® Version 2.01 program (MicroMath Scientific Software, Salt Lake City, UT, USA) for mathematical analysis.

Telomere Length Calculations

The first step in the analysis of the telomere length was to calibrate the distance in the gel vs. the DNA size by using two methods: (i) the actual distance in the gel for each of the markers was measured and fitted to an exponential dependence in the formula:

$$[l = a(\exp)(-bd + cd^2)] \quad [\text{Eq. 1}]$$

where l is the DNA size (kbp), d is the distance in the gel from the wells to the actual marker (mm), and a , b and c are constants determined for each particular run from the fitting procedure; and (ii) the distance from the wells ($d = 0$) to the

actual marker in the image was measured and fitted to the same exponential function. In cases in which the DNA ran nonuniformly, we used a two-dimensional calibration analysis such that the distance was represented as a sum of the distance (d_i) for a particular lane i , commonly lane $i = 1$ and a correction:

$$[d_c = b_c n + c_c n^2] \quad [\text{Eq. 2}]$$

in which b_c and c_c are constants characterizing the deviation, and n is the lane number ($n = 1-42$). In most cases, the nonuniformities were small and could be easily corrected but nevertheless produced large (up to 2–3 kbp) changes if not taken into account. Once the distance calibration was obtained, all distances were converted to telomere lengths, and the dependence of the signal intensity on the telomere length was analyzed. The telomere length was calculated by using a formula for the statistical mean:

$$m = \sum SI / \sum S \quad [\text{Eq. 3}]$$

where S is signal intensity, and l is the telomere length at each point (.01 mm) within the lane. We also evaluated two

other methods based on the point of maximal intensity and fitting data to normal distributions; however, they produced larger fluctuations in the calculated telomere lengths and therefore were not used in this study.

Slot Blots

Appropriate dilutions of DNA were prepared in TE buffer (10 mM Tris-base, 1 mM EDTA, pH 7.4), typically 100, 50, 25 and 12.5 ng per sample were prepared. Cells were washed once in phosphate-buffered saline (PBS), then diluted in PBS, typically 10000, 5000, 2500 and 1250 cells per sample were prepared. The slot-blot apparatus (Bio-Dot® SF System; Bio-Rad) was assembled according to the manufacturers instructions, using 9- × 12-cm Zeta-Probe® Nylon Membranes (Bio-Rad). Slots were washed with 200 µL of neutralization solution (1.5 M NaCl, 0.5 M Tris-base, pH 7.5), and samples were subsequently applied. Two hundred microliters of 1.5 M NaCl, 0.5 M NaOH were applied to the slots to

Figure 1. A Southern blot illustrating the telomere lengths of a subset of samples analyzed in our laboratory, including the CEM, TF228 and chimpanzee cells used in this study. Mean telomere lengths were derived from the integration of signal intensity of the TRFs.

Figure 2. Slot blots of whole cells and purified DNA from CEM, TF228 and chimpanzee cells hybridized with centromere (cen.) or telomere (tel.) probes are shown. Cell and DNA concentrations blotted are indicated.

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denature the DNA, and after 7 min the slots were drained. Neutralization solution (200 μ L) was applied to the slots, and after 7 min the slots were drained. Membranes were removed, rinsed in 2 $\times$ standard saline citrate (SSC) and air-dried for 1 h. DNA was cross-linked to the membrane with 1200 μ J UV light. Hybridization was performed using the Quick-Light Hybridization Kit. Briefly, membranes were prehybridized in 20 mL high-stringency Wash Solution I (Lifecodes) for 15 min at 42°C. Membranes were hybridized in 12.5 mL of hybridization buffer (Lifecodes) containing 4 μ L of telomere probe, 5 tandem repeats of the telomeric repeat unit TTAGGG (Telom/Sli935; Lifecodes) for 3 h at 42°C. Membranes were washed twice in 30 mL Wash Solution I for 10 min at 42°C. Membranes were washed twice in 30 mL Wash Solution II (low stringency) for 10 min at 42°C. Membranes were rinsed 4 times in 20 mL 1 $\times$ Quick Light Buffer, placed in open development folders and sprayed evenly with Lumi-Phos 480TM dioxetane (Lifecodes). Folders were heat-sealed and exposed to a chemiluminescent capture screen overnight. Chemiluminescence was captured with the Molecular Imager System, and signal intensities were quantified with the Molecular Analyst image analysis program. Once telomere signals were acquired, membranes were stripped in 20 mL Wash Solution I for 1 h at 65°C. Membranes were then hybridized and developed as previously described with 4 μ L centromere probe, a consensus alphoid repeat sequence (Alphoid/Sli928; Lifecodes).

TC Ratio Quantification

Telomere and centromere signal intensities were quantified with the Molecular Analyst software by using the volume-integration function corrected for local background. Telomere and centromere signal intensities were plotted as a function of concentration (nanograms DNA or number of cells) to ensure that data were obtained within the linear range of detection. Intensities were fit to a standard linear regression ($i = mc + b$), where i is the signal intensity, and c is the known concentration of cells or DNA. The slope and y-

intercepts were determined for the observed data, and corrected intensities were obtained from the known concentrations. Corrected intensities were almost always in close agreement with

observed values. In those rare instances in which intensities were not linear with respect to concentration, TC ratios were disregarded. TC ratios were calculated by dividing the telomere inten-

Figure 3. The linear relationships between signal intensity and DNA concentration or cell number are depicted for CEM, TF228 and chimpanzee cells. Similar intensity values for whole cells and purified DNA are observed.

sity by the centromere intensity for both the observed and the corrected intensity values at each concentration of DNA or whole cells. The mean values for four concentrations of the same sample done in duplicate on the same blot were then calculated, and these values are reported here.

RESULTS AND DISCUSSION

Slot blots were performed on whole cells and purified DNA from two cell lines, CEM and TF228, and on several chimpanzee samples that were available in our laboratory. Telomere lengths were derived by an improved method of calibrating and integrating signal intensities of the telomere restriction fragments. Telomere lengths of CEM, TF228 and chimpanzee cells were 3.5, 6 and 11 kbp, respectively (Figure 1). Blots were performed within the detection method's linear range for both centromere and telomere probes. Figure 2 depicts the results of a typical slot blot. For whole cells, we found the linear range to be between 1250 and 10000 cells. For purified DNA, we found the linear range to be between 12.5 and 100 ng. Concentrations above these ranges began to saturate the centromere signal, while concentrations below these ranges became undetectable with the telomere probe. We found that a minimum of 800 cells, or 9 ng of DNA, were needed to generate a quantifiable signal.

To ensure that signal intensity was dependent on DNA concentration, we fit all slot-blot data to a linear model. The data consistently fit the model, indicating that the blots were performed within the linear range of the assay. Figure 3 shows the linear relationships used to generate fitted values of signal intensities. The TC ratio was calculated with the observed and fitted values for all concentrations of each sample, and these values were averaged to obtain the TC ratio for each sample. TC ratios were consistent for the observed and fitted averages, generally within 0.01–0.03 U, although greater variability existed among individual concentrations.

To determine the ability of TC ratios to predict telomere length, we compared TC ratios and telomere lengths

for both whole cells and purified DNA (Table 1). The data indicated a statistically significant linear relationship between telomere DNA content and telomere length. Average TC ratios for purified DNA from CEM, TF228 and chimpanzee PBMCs were 0.24, 0.36 and 0.64, respectively. The corresponding TC ratios for whole cells of CEM, TF228 and chimpanzee PBMCs were 0.20, 0.34 and 0.61, respectively. There was virtually no difference between the regression lines for whole cells (slope = 0.0586, $R^2 = 0.95$) or purified DNA (slope = 0.0575, $R^2 = 0.94$). However, the variability among TC ratios obtained for repeated trials of individual samples resulted in telomere length variations of up to ± 1 kbp, which is larger than the variations obtained from Southern analysis (± 0.3 kbp).

In summary, we improved a method for measuring telomere length based on TC ratios by using whole cells in place of purified DNA. TC ratios were virtually identical for both whole cells and purified DNA, indicating that DNA isolation is not necessary when the sample size is too small. Telomere DNA content appears to be very useful for estimating telomere lengths when small amounts of sample are available, such as CD4 cell samples of patients progressing to AIDS. Unfortunately, the method might not reveal the small changes in telomere lengths that can accompany increased cell division and proliferation in these samples. Telomere DNA content might prove most valuable in samples from pediatric patients when the amount of blood is small. Likewise, TC ratios might be capable of determining whether small subsets of cells turn over at a very high rate, which is not reflected in the average rate of cell turnover. Subpopulations of rapidly proliferating cells with telomeres much smaller (ca. 1 kbp) than the rest of the population can be detected through telomere DNA content measurements. TC ratios will probably not replace Southern analysis as the method of choice for monitoring small changes in telomere lengths, but the method can provide an opportunity to estimate telomere lengths in cell populations too small to be analyzed by conventional methods and does not require sophisticated and costly techniques.

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