Measurement of Telomeric DNA Content in Human Tissues

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ABSTRACT

Telomeres, nucleoprotein complexes at the ends of eukaryotic chromosomes, are 10–12 kbp in length in somatic cells, but as small as 1–2 kbp in rapidly growing cancer cells. Southern blot analysis is currently the standard method for the measurement of telomere length. However, accurate determinations are not possible when DNA is broken or scant. To avoid these problems, a slot blot assay that quantitates the relative content, instead of length, of telomere DNA was developed. The relative contents of telomere DNA determined by this slot blot assay were directly proportional to the relative contents of telomere DNA in samples containing as little as 15 mg of total DNA. Relative telomere DNA content, but not length, also was unaffected by breakage of DNA into fragments 1 kbp or less in length.

INTRODUCTION

Telomeres, highly conserved nucleo-protein complexes at the ends of eukaryotic chromosomes, consist of tandem repetitive arrays of a TTAGGG motif (10), ranging from 1–12 kb in length (1,2,4–8,10–14). Although the prognostic value of telomere length remains to be defined, several reports have correlated genomic variability, progression and the aggressiveness of tumors with telomere length (4,8,11,13,14), implying that telomere length could have potential prognostic importance.

The standard method for the measurement of the terminal restriction fragment (TRF) is Southern blot analysis using a telomere-specific DNA probe. However, there are three significant limitations to this method. First, Southern blot analysis of telomere DNA typically requires 1–10 µg of DNA per sample (1,2,4–8,10–14). Second, DNA breakage reduces the observed telomere length. Finally, the TRF includes telomere-associated DNA other than the TTAGGG sequence (3). In this study, the sensitivity, specificity and dependence on DNA integrity of a new slot blot-based assay of telomere content are evaluated.

MATERIALS AND METHODS

DNA Isolation

Tissue samples were obtained from the Cooperative Human Tissue Network (Columbus, OH, USA) and The University of New Mexico Cancer Research and Treatment Center, Albuquerque. HeLa cells (ATCC CCL2 HeLa) were obtained, subcloned and cultured as specified by ATCC (Rockville, MD, USA). Frozen, finely powdered tissue or suspensions of cultured cells were mixed with 5 vol of lysis buffer (0.1 M EDTA, 0.5% Sarkosyl, pH 8.0) and 20 µg/mL boiled RNase at 55°C in a shaking water bath for 30 min. Proteinase K (USB/Amer sham, Cleveland, OH, USA) was then added to 200 µg/mL, and after 4 h, the mixture was extracted twice with 2.5 vol of a 1:1 mixture of phenol and chloroform and twice with 2.5 vol of chloroform alone. The solutions containing DNA were then exhaustively dialyzed against TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.8), precipitated with 2.5 vol of ethanol, resuspended in TE and stored at 4°C.

Preparation of Slot Blots

DNA samples (15–500 ng) were digested with 2 vol of 0.5 M NaOH, 1.5 M NaCl and denatured at 55°C for 30 min. The slot blot apparatus (Minifold® Slot-Blot System; Schleicher & Schuell, Keene, NH, USA) was assembled according to the manufacturer’s instructions, using Hybond®-N+ Nylon Membrane. (Amersham, Arlington Heights, IL, USA). Slot blot wells were washed twice under vacuum with neutralization solution (0.5 M Tris-HCl, 1.5 M NaCl), and the DNA samples, neutralized with 3.3 vol of neutralizing solution, were then loaded. Following a wash with neutralizing solution, the membrane was fixed with NaOH as described by the supplier, sealed in plastic wrap and stored at 4°C.

Preparation of Southern Blots

Approximately 5 µg DNA were digested with 10–20 U (final concentration 1 U/µL) each of restriction enzymes RsaI and Hinfl (New England Biolabs, Beverly, MA, USA) for at least 1 h at 37°C, resolved by electrophoresis at 30 V on 0.8% agarose gels and then transferred to nylon Hy bond-N+ membrane essentially as described by the supplier. Molecular weight standards (HindIII-digested λ phage DNA and 1 Kb DNA Ladders; Life Technologies, Gaithersburg, MD, USA) were run in parallel.

Preparation of Probes

The telomere-specific oligonucleotide [TTAGGG]₄ (10) and centromere-specific oligonucleotide [GTTTTGAAACCTCTTTTGTAGAATCTGTC] (9) were end-labeled with 100 µCi[γ³₂P]ATP (3000 Ci/mmole; NEN Life Science Products, Boston, MA, USA) at 37°C for 40 min using T4 Polynucleotide Kinase (New England Biolabs) with the buffer provided by the supplier. Unincorporated nucleotides were removed by chromatography on Sephadex® G-50 NICK Columns® as described by the supplier (Pharmacia Biotech, Piscataway, NJ, USA).

Hybridization

Membranes were incubated in separate 100-mL Autoblot™ Hybridization Bottles for 2 h at 60°C in an Autoblot Hybridization Oven (both from Belco Glass, Vineland, NJ, USA) with 20 mL of hybridization solution, containing 5× sodium chloride sodium phosphate EDTA (SSPE: 0.9 M NaCl, 0.05 M Na₂HPO₄, 0.028 M NaOH, 0.005 M Na₂EDTA), 0.1% sodium dodecyl sulfate and 20 µg/mL tRNA (Type X; Sigma Chemical, St. Louis, MO, USA). The specified probes were added, and hybridization was performed for 16–24 h (stringency 83%–90%). The post-hybridization washes consisted of two rinses and two subsequent 30-min washes with agitation using 100 mL of
60°C 5× SSPE (stringency 83%–90%) and three 7–10-min washes with agitation using 200 mL of room temperature 0.1× SSPE (stringency 76%–85%).

**Quantification of Telomere Content**

Membranes were air-dried for 30 min and exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA, USA). The position and density of the telomeric hybridization signals of both the Southern blots and slot blots were determined with a PhosphorImager™ (Molecular Dynamics). Telomere content was determined using the volume-integration function of the ImageQuant™ software (Molecular Dynamics). A ratio of telomeric DNA content-to-centromeric DNA content (T/C ratio) was calculated for each sample to prevent errors that could arise from inaccuracies in DNA quantification or incomplete retention of DNA on the membrane. Assuming that the content of centromere DNA is relatively constant between chromosomes, the T/C ratio also normalizes for changes in DNA content resulting from chromosome rearrangements or differences in ploidy. The T/C ratios for each dilution of the sample were then averaged and expressed as a percentage of the placenta DNA. This permitted telomere DNA contents to be compared between independent experiments, even when probes varied in specific activity.

**Quantification of Telomere Length**

Modal TRF length was determined by comparing the position of the greatest hybridization signal in each lane of the Southern blot to a standard curve derived from the migration of DNA standards of known molecular weight. Twenty-eight DNA samples with modal TRF lengths ranging from 2.2–11.5 kbp were selected for the analysis in Figure 2.

**RESULTS**

**Sensitivity and Specificity**

When blots containing serial dilutions of placenta or HeLa cell DNA were analyzed by PhosphorImager, the intensities of the telomere and centromere probe hybridization signals were directly proportional to the mass of DNA in each “slot” over the entire range of DNA masses tested (Figure 1, A–C). In contrast, neither the telomere- nor centromere-specific probes produced detectable signals with 1 µg of DNA from the bacterium *Micrococcus luteus* (not shown), verifying the specificity of hybridization.

**Telomere Content in HeLa and Placenta DNA**

Slot blot analysis demonstrated that the mean T/C ratio for the HeLa cell DNA, a model for immortalized cells,
was 51.9% (standard deviation [SD] ± 3.5%, n = 11) of the T/C ratio for the placenta DNA, a model for somatic cells. Virtually identical results were obtained with Southern blot analysis. The modal TRF length in the HeLa DNA was 53.1% (SD ± 8.1%, n = 5) of the length in the placenta DNA.

**Telomere Contents and TRF Lengths in Human Cell Lines and Tissue Samples**

To further validate this measurement of telomere DNA content, T/C ratios were measured in human DNA samples with known differences in modal TRF length. As shown in Figure 2, the relative content of telomere DNA was directly proportional to telomere length ($r = 0.904$). At a T/C ratio of zero, the TRF length was estimated to be 0.7 kbp. Consistent with this result, non-TTAGGG, telomere-associated DNA sequences, strings of distinct repeated sequence elements collectively com-

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**Figure 2. Length vs. content of telomere DNA.** T/C ratios were measured in 28 DNA samples with known differences in TRF length. Samples included normal human tissue (circles), cancerous human tissue (squares) and HeLa cell subclones (diamonds). See text and Materials and Methods for other details.
prising 0.5–1.0 kbp, are associated with approximately 22 human telomeres (3). The TTAGGG sequence also exists outside of the telomere (15,16). However, because the telomere DNA content and telomere-associated DNA together account for the observed TRF length, it is unlikely that interstitial TTAGGG sequences significantly affect the background of the assay.

**Effect of DNA Breakage**

DNA was progressively fragmented by sonication for 1, 5, 10, and 15 s (Figure 3), and the effects on telomere content and TRF length measurements were analyzed using slot blot and Southern blot assays, respectively. When analyzed by Southern blot, there was a progressive shortening of TRF that mirrored the extent of fragmentation of the bulk DNA. In contrast, the content of telomeric DNA, as defined by the slot blot, was essentially identical for each sample regardless of fragment length.

**DISCUSSION**

Although several reports have correlated telomere length with genetic variability, progression and aggressiveness of tumors (4,8,11,13,14), the value of telomere length as a prognostic tool has not been proven. A definitive answer to this question could be obtained by retrospective measurements of telomere length in archival (paraffin-embedded) biopsy or tumor specimens from patients where the course of disease and outcome are known. Quantification of the content of telomere DNA is independent of DNA breakage, DNA ploidy, DNA sample size and can be performed on as little as 10–20 ng of total DNA, making it particularly well suited for such retrospective investigations.

The principle limitation of the slot blot analysis is that it does not reveal the variability in the TRF length that may exist in the telomere population; for example, when a tumor is not clonal or contains normal cells. However, in archival material, contamination by normal cells can be significantly reduced by well-established methods of microdissection (17,18).

**REFERENCES**

Controlled Gene Gun Delivery and Expression of DNA Within the Cornea

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ABSTRACT

Selective delivery of genes to ocular tissues in vivo has been a long sought after goal for potential gene therapy of ocular disease. The gene gun was considered for this purpose because of its ability to focally transfer DNA to cells through gold micro-particles coated with DNA. Through experimentation, we optimized a technique that allows focal delivery and expression of a plasmid encoding green fluorescent protein in the corneal epithelium 100% of the time. Though the corneal epithelium has a delicate structure, this introduction was not associated with any corneal or ocular damage and did not produce any apparent ocular irritation. These findings demonstrate the utility of gene gun delivery of DNA to selected ocular tissues for potential experimental and therapeutic purposes.

INTRODUCTION

Gene transfer offers a way to potentially correct inherited and acquired ocular disease. Currently, to our knowledge, there have been no successful demonstrations of focal gene delivery to specific ocular structures. Studies have been done that demonstrate non-specific gene transfer to many ocular structures using adenovirus and liposomes (1,2,6,8). In addition to their lack of focal gene expression, these techniques are limited by immunogenicity and low efficiency, respectively.

Gene gun transfer of DNA has been successfully used in plant, microbial and mammalian cells (4,5). It has the potential advantages of lack of immunogenicity, good in vivo gene transfer efficiency and focal gene delivery. Given that gene gun transfection has been successful in many different cell types, we tested whether this method of gene transfer could be used in the eye. To demonstrate gene transfer in vivo, we used a plasmid, mGFP4ST3, encoding green fluorescent protein (GFP), which is nontoxic and can be easily and repeatedly visualized in cornea of live rabbits and in excised tissue.

MATERIALS AND METHODS

Materials

Nine New Zealand white rabbits (2–3 kg) were obtained from Myrtle (Thompson Station, TN, USA). Gold microparticles (2.6-µm diameter) were generously provided by Aurogen (Middleton, WI, USA). Gene gun consumables were purchased from Rumsey-Loomis (Ithaca, NY, USA). All other reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

Plasmid Expression Vector

A GFP gene with the cryptic intron sequences removed was supplied by J. Hasselhoff (MRC Laboratory of Molecular Biology, Cambridge, England, UK) in the plasmid pBIN35S-mGFP4. The GFP sequence was cloned into SacI-XbaI of pUC118 as a SacI-XbaI fragment. This was subsequently cloned into the NheI-EcoRI sites of pCI (Promega, Madison, WI, USA), as an XbaI-EcoRI fragment to drive GFP expression in mammalian cells using the cytomegalovirus promoter. The GFP S65T mutation (3) was subsequently introduced by site-directed mutagenesis yielding the plasmid pCI-mGFP4ST.

Gene Delivery into the Eye Using a Biolistic Gene Gun

For each transfection, 1 µg of DNA coated on 0.2–0.5 mg of gold microparticles was loaded onto a kapton microprojectile as previously detailed (12). This DNA was delivered into the target site using a handheld, helium-driven biolistic gene gun (9). For transfection of the cornea, a 5-mm exit port was used with a Parafilm® gasket affixed to the exit port with vacuum grease. The Parafilm surface was essential for preventing corneal epithelial damage and also served to form a vacuum seal.

Rabbits were anesthetized with a combination of ketamine (20 mg/kg) and xylazine (8 mg/kg) i.m. The ocular