Cell, Vol. 88, 657–666, March 7, 1997, Copyright ©1997 by Cell Press

Long G Tails at Both Ends of Human Chromosomes Suggest a C Strand Degradation Mechanism for Telomere Shortening

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Summary

The chromosomes of lower eukaryotes have short telomeric 3′ extensions. Using a primer-extension/nick-translation technique and nondenaturing hybridization, we find long 3′ G-rich tails at human chromosome ends in mortal primary fibroblasts, umbilical vein endothelial cells, and leukocytes, as well as in immortalized fibroblasts. For all cells tested, >80% of the telomeres have long G-rich overhangs, averaging 130–210 bases in length, in disagreement with the conventional model for incomplete lagging-strand replication, which predicts overhangs on 50% of the chromosome ends. The observed G tails must exist during most of the cell cycle and probably result from degradation of both chromosome ends. The average lengths of the G tails are quantitatively consistent with the observed rates of human chromosome shortening.

Introduction

Telomeres are essential nucleoprotein structures at the ends of all eukaryotic chromosomes (see Zakian, 1989, 1995; Blackburn, 1991). They protect chromosome ends from fusion and degradation (McClintock, 1939; Sandell and Zakian, 1993). In most eukaryotes, telomeric DNA consists of simple repetitive sequences with G-rich 3′ termini. In human somatic cells, telomeres have 500–3000 repeats of TTAGGG, which gradually shorten with age in vivo and in vitro (see de Lange, 1994; Harley, 1995; Harley and Villeponteau, 1995). In contrast, the telomeres of germ line and cancer cells do not shorten, consistent with the behavior of immortal, unicellular organisms. The telomeres of immortal cells can be maintained by the enzyme telomerase, which is able to extend 3′ telomere overhangs, or by recombination (Blackburn, 1992; Lingner et al., 1995; Lingner and Cech, 1996; Greider, 1995). According to the telomere hypothesis of aging (Harley, 1991), progressive telomere shortening in somatic cells is a mitotic clock that limits the proliferative capacity of most human cells, thereby suppressing indefinite growth of abnormal cells while contributing to genome instability and senescence.

Lower eukaryotes have telomeres with short 3′ overhangs. The macronuclear minichromosomes of ciliated protozoans have 12- to 16-base (b) G-rich tails throughout the cell cycle (Klobutcher et al., 1981; Pluta et al., 1982; Henderson and Blackburn, 1989). Saccharomyces cerevisiae has G tails >30 b long late in S phase (Wellinger et al., 1993a, 1993b), which apparently lead to circularization of linear plasmids even in the absence of telomerase activity (Wellinger et al., 1996). Specific proteins bind to the G tails of Oxytricha and Euplotes (Gottschling and Zakian, 1986; Price, 1990). In S. cerevisiae Cdc13p binds to 3′ G-rich single-stranded telomeric DNA and protects the 5′ telomeric end from degradation (Garvik et al., 1995; Nugent et al., 1996). In vitro, single-stranded G-rich telomere DNA can form a variety of noncanonical structures including G quartets, triple helices and G·G base pairing (see Henderson, 1995).

In higher eukaryotes, G tails might be important to control telomerase activity, to protect the chromosome ends from fusion and degradation, to promote telomere–telomere interactions during meiosis, attach chromosomes to specific nuclear sites, or influence telomere shortening. However, there are no data on the existence or lengths of 3′ overhangs in multicellular organisms. Xenopus eggs contain a protein that binds to G-rich single strands in vitro (Cardenas et al., 1993). On the other hand, blunt-ended telomeric DNA constructs are protected from degradation and fusion in Xenopus eggs and egg extracts (L. Li et al., submitted), and mammalian telomerase is also capable of removing nucleotides from the 3′ end of telomeres (Collins and Greider, 1993). Therefore, it is important to determine whether vertebrate telomeres have G tails and, if so, to quantitate the fraction of ends with overhangs and the length of the overhangs. The presence of long G tails in mortal human cells would suggest a relationship between the overhangs and telomere shortening and raise questions about the role of unusual DNA secondary structure in telomere function.

We developed a method to detect and analyze 3′ overhangs, and applied it to human telomeres. $(CCCTAA)_4$ oligonucleotides were hybridized under nondenaturing conditions to those telomeres having G-rich overhangs and extended using Taq polymerase. The resulting-primer/extension-nick translation (PENT) reaction products were analyzed by 1-D alkaline or 2-D neutral/alkaline agarose electrophoresis to determine the fraction of chromosome ends having long G-rich overhangs. Quantitative nondenaturing hybridization to these tails determined the average length of the tails. The abundance and length of the G tails suggest an important function for the overhangs and an explanation for rapid telomere shortening in human cells.

Results

Primer-Extension/Nick-Translation Reaction and Alkaline Agarose Gel Electrophoresis Can Detect 3′ Overhangs

Figure 1 shows the principle of PENT as applied to the detection and quantitation of G tails in human chromosomes. The oligonucleotide $(CCCTAA)_4$ (TelC) is hybridized to available G-rich tails and extended using Taq polymerase, which has 5′-to-3′ exonuclease activity (Longley et al., 1990; Holland et al., 1991). The polymerase fills the gap between the primer and 5′-end of the C strand and then propagates the nick in the 3′ direction.

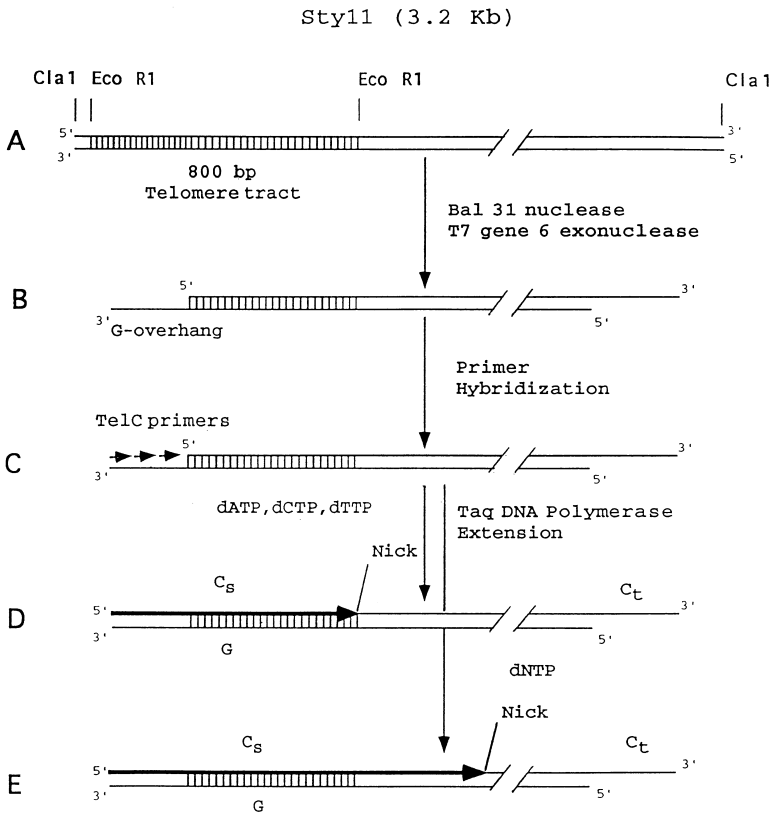

Figure 1. Model Telomere Construct and Description of the PENT Reaction

(A) Structure of the ClaI-linearized plasmid Sty11. Hatched block represents the 800 bp tract of human telomere DNA. The tract is oriented as the telomere end of a chromosome: 5′ end, (CCCTAA).

(B) Procedures to expose telomere ends and produce G tails on DNA constructs. The Sty11/ClaI plasmid fragment was trimmed briefly with Bal31 nuclease and then incubated with T7 gene *6* exonuclease to produce a 3′ overhang.

(C) Hybridization of TelC oligonucleotides to the G-rich overhang.

(D) Extension of the TelC oligonucleotides by Taq DNA polymerase using three triphosphates. The reaction stops when the nick reaches the end of the telomere tract.

(E) The extension reaction in the presence of all four dNTPs. The PENT reaction proceeds beyond the end of the telomere tract.

If several molecules of TelC bind to the overhang, all but the last one will be degraded during the reaction. When electrophoresed on a denaturing alkaline agarose gel and probed with both the G-rich and C-rich telomeric sequences, the reaction products should appear as three bands: C_s corresponds to the newly-synthesized extension products; C_t corresponds to the trimmed original C-rich strands; and C_o corresponds to the original G-rich strands and untrimmed C-rich strands from any telomeric ends without overhangs or with such short overhangs that they cannot bind the primer.

PENT was experimentally tested using a 3.2 kb model telomeric construct with a 100 b G tail and a 650 bp terminal tract of double-stranded telomeric DNA, prepared from Sty11 (Figure 1A). Figures 2A–2C show the results of PENT. The size of the C_s strand increased at the same rate as the size of the C_t strand decreased, ruling out strand displacement (Henderson et al., 1988). In the presence of four dNTPs, the nick-translation reaction proceeded to the opposite end of the linear construct (Figure 2A, lanes 2–9). In the presence of only dATP, dTTP, and dCTP, the reaction proceeded only to the end of the telomeric tract, producing a discrete 750 b C-rich strand (Figures 2B and 2C). Substitution of dTTP with dUTP and incubation of the reaction products with dU-glycosylase followed by alkaline treatment led to complete elimination of the C_s strand (Figure 2B), supporting our interpretation of the PENT reaction products. After long reactions, the C_t strand hybridized with the random-primed plasmid (Figures 2A and 2B), but not $(TTAGGG)_4$ (TelG) (Figure 2C). A 100 b overhang is long enough to initiate multiple nick-translation reactions, however the terminal C_s strand should destroy and replace internally located primers and products. Thus, the C_s product made without dGTP (Figure 2C, lane 28) had the same size as the C-rich fragment without T7 gene *6* treatment (Figure 2D, lane 29). No PENT products were found (i) without primers (Figure 2A, lane 10), (ii) with TelG primers (not shown), (iii) with nontelomeric primers (not shown), or (iv) on constructs without G tails (Figure 2A, lane 11).

Human Telomeres Have 3′ Overhangs as Detected by the PENT/Alkaline Gel Method

We used PENT to detect G tails in IMR-90 normal primary human fibroblasts, which have only weak telomerase activity (Shay et al., 1994; Kim et al., 1994; this study [Experimental Procedures]) and undergo telomere shortening (not shown). High molecular weight (>100 kb) IMR-90 DNA was subjected to PENT, and the products analyzed by 1-D alkaline gel electrophoresis (Figure 3). Lanes 1–3 show the time course of the reactions with TelC primer and four dNTPs. As for the plasmid construct, the rate of C_s synthesis was ~250 b/min. DNA fragments of similar size were synthesized when dGTP was omitted (lanes 7–9), indicating the telomeric origin of the products and the absence of guanine blocks in the terminal 4 kb of the human telomere C strands. Incorporation of dUTP followed by incubation with dU-glycosylase and alkaline treatment caused loss of the C_s products (not shown). Reactions with equal numbers of human and rat telomeres gave nearly identical amounts of C_s product, even though the rat telomeres are 10 times longer (Makarov et al., 1993), consistent with priming only at termini (not shown).

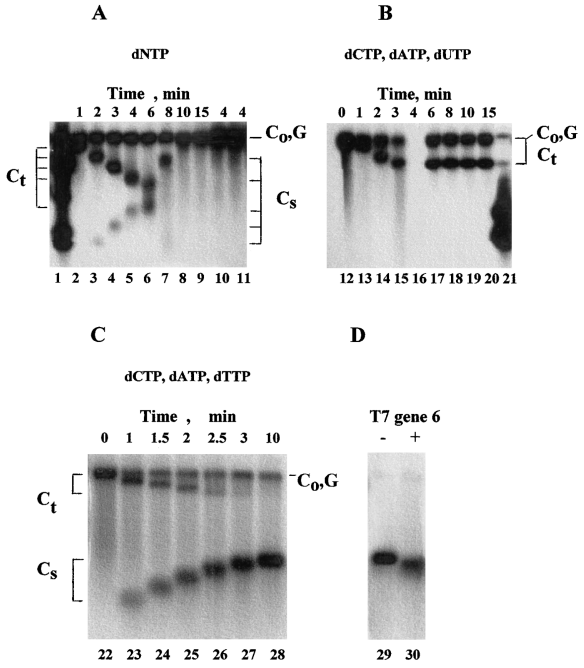

Figure 2. Detection of G-Rich Tails on Model Telomeric Constructs Using PENT and 1-D Electrophoresis

(A) PENT reactions with the Sty11 construct with 100 b G tails in the presence of TelC primer, Taq DNA polymerase, and 4 dNTPs. DNA was electrophoresed in alkaline 1% agarose, transferred to a filter, and probed with Sty11. C_s, primer-extended synthesized C strands; C_t, trimmed C strands; C_o, untrimmed C strands from constructs with blunt ends or very short overhangs (and all full-length G-rich strands). Lane 1, Sty11/AvaII/EcoRI marker; lanes 2–9, PENT time series; lane 10, control 4 min PENT reaction with Sty11 100 b G tail construct in the absence of TelC primer; lane 11, control 4 min PENT reaction with a Sty11 construct without G-rich tail.

(B) PENT reactions, as above, with only dCTP, dATP, and dUTP. DNA was incubated with dU-glycosylase and treated with NaOH to degrade the C_s strands and probed with random-primed Sty11. Lanes 12–20, PENT time series (4 min DNA sample, lane 16, was lost during purification); lane 21, Sty11/EcoRI digest.

(C) PENT reactions, as above, with dCTP, dATP, and dTTP, probed with radioactive TelG. Lanes 22–28, reaction time series.

(D) The size of C strand of the model telomere construct before (−) and after (+) T7 gene *6* exonuclease treatment to create the G tail. DNA was digested with EcoRI before loading and probed with radioactive TelG.

Several alternative explanations for the positive PENT reaction on human chromosomes were ruled out. We found no PENT products in the absence of TelC primer (Figure 3, lanes 4–6), showing that there are not significant numbers of gaps or nicks in the C-rich strands, as found in Tetrahymena (Blackburn and Gall, 1978). Discontinuities in the G-rich strands are ruled out by the fact that the PENT products were of high molecular weight. T4 DNA polymerase trimming reduced the amount of PENT product by more than 10-fold in reactions with (i) the plasmid construct (Figure 4, lanes 2 and 3), (ii) IMR-90 DNA (lanes 4 and 5), or (iii) a mixture of IMR-90 DNA and construct (lanes 6 and 7). Treatment of IMR-90 DNA with S1 nuclease (lane 8) or with Bal-31 nuclease (lane 9) completely eliminated the PENT reaction. These data strongly indicate that PENT requires a 3′ G-rich terminus.

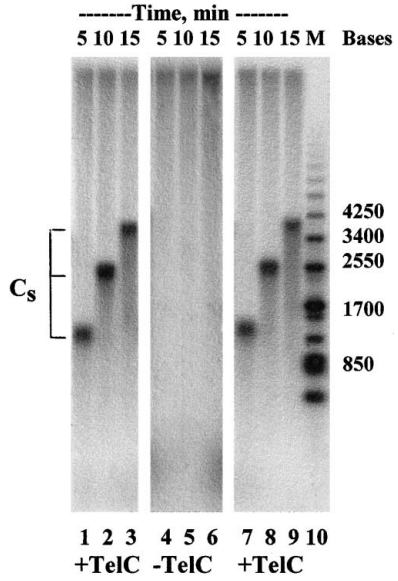

Figure 3. Detection of G Tails on Human IMR-90 DNA Using PENT and 1-D Electrophoresis

High molecular weight primary IMR-90 cell DNA was subjected to PENT for 5, 10, and 15 min, electrophoresed under standard denaturing conditions, transferred to membrane and probed with radioactive TelG. Lanes 1–3, reaction with 4 dNTPs with TelC primer; lanes 4–6, reaction with 4 dNTPs without TelC primer; lanes 7–9, reaction with 3 dNTPs with TelC primer; lane 10, DNA marker 2 (additional bands are circular ligation products).

G tails do not seem to be generated or lost during DNA isolation. Blunt-ended telomeric constructs could be coisolated with human DNA without acquiring G tails (Figure 4, lane 11). G-tailed constructs could be coisolated with DNA without losing the 3′ overhangs (Figure 4, lane 12).

Most Telomeres in Normal Human Fibroblasts Have 3′ Overhangs

PENT can be used to quantitate the fraction of telomere ends with G-rich overhangs. Electrophoretic analysis of the Sty11 constructs with 100 b overhangs indicated that about 7% of the overhangs were < 18 b long (not shown). The fraction of molecules not producing PENT products in Figure 2C was calculated from the ratio $I_o / (I_t + I_s + I_o)$, where I_t, I_s and I_o are integrated intensities of C_t, C_s, and C_o electrophoretic bands probed with TelG, reflecting the amount of trimmed, primer-extended, and unreacted C strand DNA, respectively. Only 15% of the TelG hybridization in Figure 2C (lanes 23–28) is at the position of untrimmed C strands. The residual signal at the position of C_o could be due to a combination of inefficiency of priming and cross-hybridization of TelG with the intact G-rich strands. This assay shows that PENT detects 80%–90% of G tails.

Due to the broad size distribution of telomeres (Allshire et al., 1989), it was necessary to fractionate the human telomere restriction fragments according to molecular weight before analysis on denaturing gels. To minimize the risk of nicking the DNA we first performed PENT on high molecular weight IMR-90 mortal-cell DNA, then digested with Hinf I, electrophoretically separated

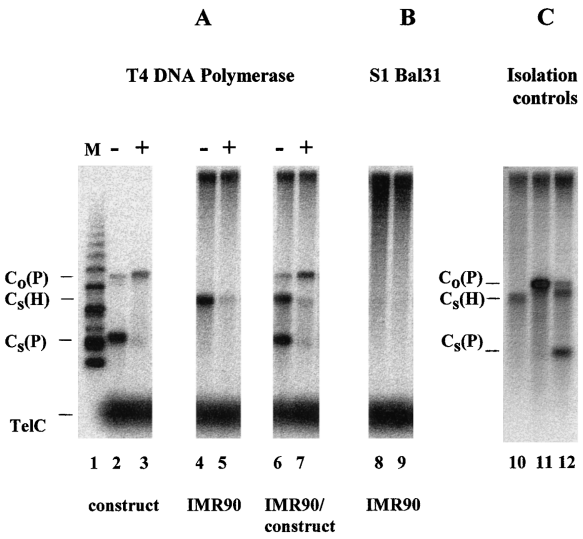

Figure 4. The PENT Reaction Depends upon Presence of 3′ Overhangs

The products of an 8 min PENT reaction were analyzed by alkaline agarose electrophoresis and detected by filter hybridization with TelG.

(A) Removal of G tails by treatment with T4 DNA polymerase; (−) without treatment, (+) after 10 min incubation with T4 DNA polymerase. Lane 1, marker 2; lanes 2 and 3, Sty11 model construct originally having 100 b G tails; lanes 4 and 5, intact IMR-90 DNA; lanes 6 and 7, mixture of the model construct and IMR-90 DNA. C_s(P) and C_s(H) are the PENT products of plasmid and human DNA, respectively. The PENT reaction was carried out for 8 min in the presence of dCTP, dATP, and dTTP, which limits the size of C_s(P) to 750 b and generates a C_s(H) product of 2 kb.

(B) The PENT reaction was prevented by removal of G tails from intact IMR-90 DNA by S1 or Bal31 treatment. Lane 8, S1 digestion; lane 9, Bal31 digestion.

(C) G tails are neither created nor destroyed during isolation of DNA from nuclei. Lane 10, control PENT reaction with intact IMR-90 DNA. Lane 11, reaction of coisolated IMR-90 and blunt-ended construct DNA. The absence of significant PENT products from the plasmid construct indicates that isolation does not generate G tails. Lane 12, reaction of coisolated IMR-90 DNA and Sty11 construct with 100 b overhangs. The presence of PENT plasmid products indicates that isolation does not remove G tails.

the double-stranded products, cut out DNA of different size, ran the products on an alkaline agarose gel, and probed with TelG (Figure 5A). All three C strand products were resolved. The size of C_s progressively increased (but was independent of the size of the restriction fragment) and the size of C_t decreased during the reaction. Integration of the intensities under the peaks (as shown in Figure 5B) from a number of lanes and different reactions show an average of 86% ± 3% of the IMR-90 telomeres were extended by PENT, approaching the efficiency of extension of the plasmid control molecules (Table 1) and indicating that 80%–90% of the human telomeres have long G tails, even in cells with minimal telomerase activity.

Telomeric G Tails Are Conserved in Human Tissue and Mortal and Immortal Tissue Cultures as Shown by 2-D Electrophoretic Analysis of PENT Products

The PENT products can be analyzed in a single 2-D gel by first separating the double-stranded restriction

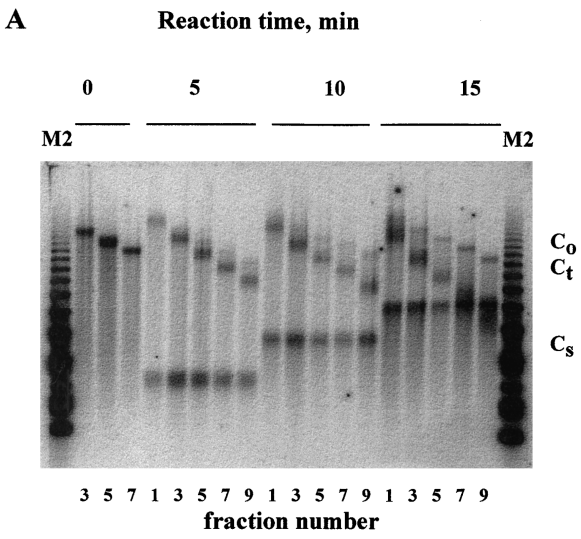

Figure 5. Chromosomes of Normal IMR-90 Cells Have Single-Stranded $(TTAGGG)_n$ Tails at Both Ends

(A) Alkaline electrophoretic patterns of PENT reacted, HinfI digested, size-fractionated IMR-90 DNA demonstrate that most telomere ends undergo PENT reactions and thus have 3′ overhangs. After PENT, the double-stranded products were separated according to molecular weight by slicing bands from the agarose gel and extracting the DNA. The three unreacted lanes (0 min, no Taq) show the molecular weight distributions of gel slices 3, 5, and 7. DNA from gel slices 1, 3, 5, 7, and 9 from the Taq polymerase reactions for 5, 10, and 15 min show strong PENT products, C_t and C_s, and very weak C_o bands which originate from telomeres with blunt or short overhanging ends. M2, 850 b telomere ladder.

(B) Electrophoretic profiles of slice 7 after 0 min PENT reaction (top), and of slice 9 after the 10 min PENT reaction, having comparable lengths of unreacted strand C_o. The observed distribution of molecular weights of the unreacted band was determined from the top profile. Only 21.3% of the hybridization signal was present in the first half of the C_o peak (shaded area). The hybridization signal in the first half of the C_o peak was also measured in the profile of the DNA after the 10 min PENT reaction, and multiplied by 4.7 in order to estimate the total contribution from the unreacted C strands, I_o. The fraction of unreacted strands was calculated by dividing I_o by the total hybridization in all three bands, in this specific case 13%.

fragments in TAE followed by denaturation in NaOH, electrophoresis in the second direction, transfer to filter, and hybridization to TelG. Figure 6A schematically represents the resulting autoradiograms, showing each of the C strand species: C_s, as a band parallel to the first direction of electrophoresis; C_t, as a diagonal band; and C_o, as a slower migrating diagonal band. Figures 6B and

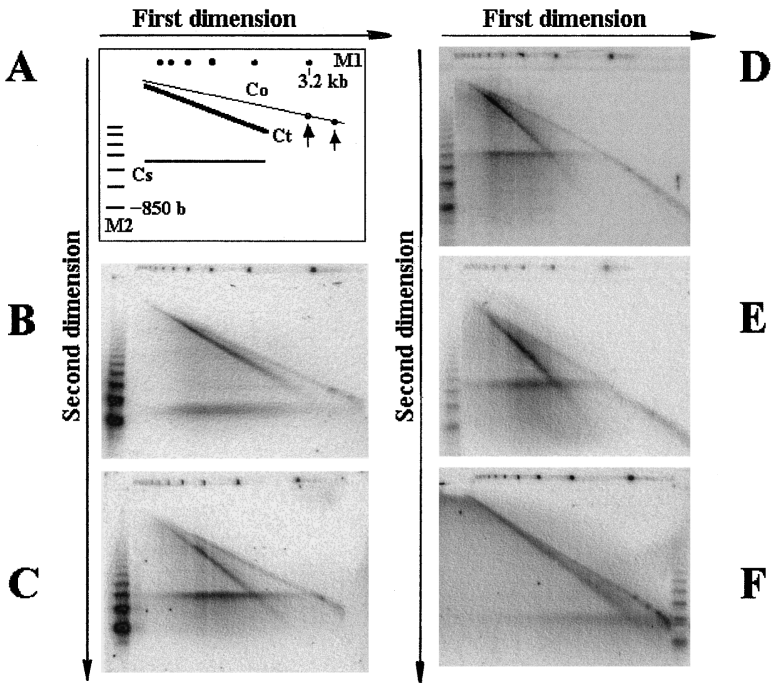

Figure 6. Two-Dimensional PENT Analysis of Terminal and Interstitial Telomeric Restriction Fragments

After the PENT reaction with intact DNA, the samples were restricted with HinfI and subjected to electrophoresis in 0.6% agarose/TAE in the first direction (M1 marker, 3.2 kb ladder) and in 0.8% agarose/NaOH in the second direction (M2 marker, 850 b ladder). The Southern blots were probed with TelG.

(A) A schematic representation of the autoradiograms, as described in the text.

(B and C) PENT products of primary IMR-90 cell DNA after 5 min and 10 min reactions, respectively. The arrows in (A) show the position of interstitial telomere restriction fragments, present as unreacted molecules, C_o, but not as PENT products, C_s.

(D) PENT analysis of cultured primary human umbilical vein endothelial cells.

(E) PENT analysis of human leukocytes.

(F) PENT analysis of postcrisis SV40-transformed IMR-90 cells.

6C show the results of 5 and 10 min PENT reactions of IMR-90 DNA primary cells, showing all of the expected bands when probed with TelG. When the filters were probed with TelC, only the top diagonals were detected, confirming that the G-rich strands had not participated in the PENT reaction (not shown). Figures 6B and 6C also show spots due to interstitial telomere restriction fragments, which have discrete lengths and do not participate in PENT. Two other mortal cells, primary human umbilical vein endothelial (HUVE) cells (Figure 6D) and human leukocytes (Figure 6E) gave very similar PENT results, as did the immortal, SV40-transformed IMR-90 cells (Figure 6F). The fractions of telomere ends that participated in the 10 min reactions were determined on each 2-D gel at 3 different molecular weights. In every case, >80% of the telomeres gave PENT products (Table 1). Thus, long G tails are conserved features at most, if not all, chromosome ends of cells with strong telomerase activity (immortal IMR-90) and weak telomerase activity (primary IMR-90, HUVE, and leukocytes).

Humans Have 130- to 210-Base Telomeric 3′ Overhangs as Determined by Nondenaturing Hybridization and PENT

Nondenaturing hybridization has been used nonquantitatively to detect 3′ overhangs in yeast (Wellinger et al., 1993a, 1993b). We combined nondenaturing hybridization with PENT to determine the average lengths of 3′ tails in humans. ^{32}P-labeled TelC was hybridized under native conditions to the same numbers of human telomeres and control DNA constructs with known lengths of 3′ overhangs. The telomeres and constructs were electrophoresed to remove unbound TelC, and the average length of G tails was determined by two independent methods. Figure 7A (lanes 1–3) shows the autoradiogram of DNA samples from blood, HUVE, and primary IMR-90 cells, showing broad bands of radioactivity at 10–12 kb, coinciding with the telomere terminal restriction fragments found by denaturing hybridization (lanes 4–6), except for the absence of the sharp bands due to the interstitial $(TTAGGG)_n$ tracts. Figure 7A (lanes 7–10)

Table 1. Measured Fractions and Lengths of G-Rich Tails in Human and Control DNA

DNA Sample	Control Plasmid	IMR-90/P04	IMR-90/P10	IMR-90 Immortal	HUVE	Leukocytes
Fraction of strands with detectable G tails[a]	0.85 (n = 1)	0.86 ± 0.03 (n = 17)	0.89 ± 0.03 (n = 4)	0.88 ± 0.03 (n = 3)	0.87 ± 0.03 (n = 3)	0.82 ± 0.05 (n = 3)
Average length of G tails (bases)[b]	100[c] (108)[d]	154 (149)[d]	210	130	150	200

[a] Calculated from the ratio of the amount of unreacted C strand DNA to the total amount of C strand DNA (see text). Averages and standard deviations were determined from N different telomere molecular weights or reactions analyzed by the 1-D or 2-D methods. Approximately 7% of the plasmid control molecules were estimated to have G tails shorter than 18 b. No corrections were applied for any TelG cross-hybridization with the G strands which comigrate with the C_o DNA, which tends to overestimate the amount of unreacted DNA.

[b] Calculated from the relative amount of nondenaturing hybridization of TelC to known amounts of human ends and construct ends with 100 b overhangs. The uncertainties in our determination of the overhang lengths are about 15%.

[c] Determined directly by denaturing gel electrophoresis.

[d] Calculated from the ratio of nondenaturing hybridization of TelC before and after PENT, assuming that TelC fully occupies all sites on the G tails before PENT and occupies only the terminal site after PENT.

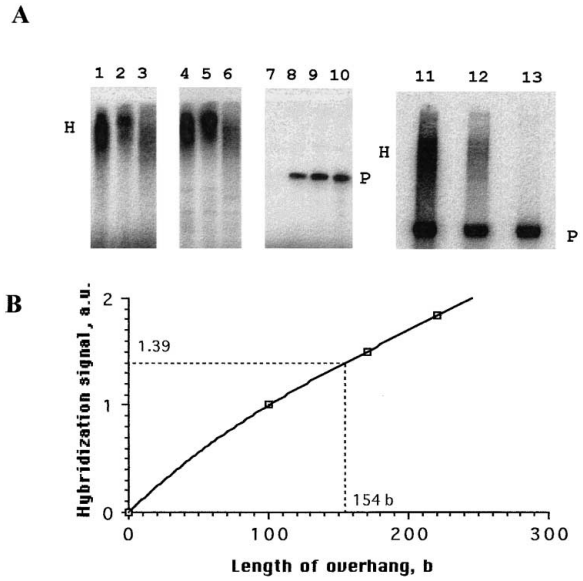

Figure 7. Nondenaturing Solution Hybridization Analysis of the Lengths of $(TTAGGG)_n$ Tails in Human Cells

All samples were hybridized to ^{32}P-labeled TelC, separated by electrophoresis in 1% agarose, and detected by autoradiography.

(A) Lanes 1–3, nondenaturing hybridization of 10 μg HinfI restriction fragments from leukocytes, HUVE cells, and IMR-90/P10 cells, respectively. Lanes 4–6, denaturing hybridization of the same human DNA samples, after denaturing the filter with 0.4 M NaOH and rehybridization with TelC. Lane 7–10, nondenaturing hybridization to 0.1 ng of plasmid constructs with 0, 100, 170, and 220 b G tails. Lane 11, nondenaturing hybridization to a mixture of 10 μg IMR-90/P04 DNA and 0.4 ng 100 b G tail construct. Lane 12, nondenaturing hybridization to 20 μg IMR-90/P04 DNA and 0.8 ng 100 b G tail construct after PENT. Lane 13, nondenaturing hybridization to 0.8 ng 100 b G tail construct alone after PENT. (H) and (P), human and plasmid DNA, respectively.

(B) Magnitude of nondenaturing hybridization signal for constructs with 100 b, 170 b, and 220 b G tails (see details in the text).

shows how TelC hybridizes to the constructs with weight-average G tail lengths of 0, 100, 170, and 220 b. The TelC hybridization signals were nearly proportional to the average lengths of the G overhangs (Figure 7B).

Treatment of the human and construct DNA with S1, mung bean, or Bal-31 nucleases, or with T4 DNA polymerase led to elimination or significant reduction (after T4 polymerase) of the nondenaturing hybridization signal without affecting the size or intensity of the denaturing hybridization signal (not shown). The strength of the TelC hybridization was the same for DNA isolated from both cells and nuclei, prepared by phenol extraction or by only proteinase K/SDS digestion. The low efficiency of nondenaturing hybridization with TelG (data not shown) suggests that the G tails are not formed by slippage of telomere strands to create C loops.

The lengths of the G tails were measured by comparing the hybridization signal of TelC to genomic DNA with that of TelC to DNA constructs having G tails of known lengths. Figure 7A (lane 11) shows nondenaturing hybridization of HinfI-digested IMR-90/P04 DNA mixed with an approximately equimolar amount (according to optical density measurements of DNA stock solutions) of the construct with a 100 b G tail. To accurately determine the relative molarity of the human and plasmid overhangs, the same samples were subjected to a 10 min PENT reaction (Figure 7A, lane 12), which should destroy all but the terminal TelC. The relative hybridization signals for the human and plasmid DNA after such treatment were easily measured, because of the low background in the plasmid-only control (Figure 7A, lane 13). Thus, we found that the molarity of the plasmid ends was 11% greater than that of the human DNA. With this correction, the measurement of hybridization signals before PENT (Figure 7A, lane 11) allowed us to conclude that the nondenaturing hybridization signal for the human DNA was 1.39 times greater than to the same number of moles of plasmid with 100 b overhang. Using the experimental dependence of hybridization upon G tail length, we calculate that the IMR-90/P04 overhangs were 154 b long (Figure 7B). In a separate experiment, Tel C was hybridized under nondenaturing conditions to IMR-90/P04, IMR-90/P10, immortal IMR-90, leukocyte, and HUVE cells. The relative amounts of DNA were determined from ethidium bromide fluorescence, and the relative amounts of hybridization by autoradiography. The lengths of the G tails were between 130 and 210 b long, assuming that the IMR-90/P04 overhangs were 154 b long (Table 1). These results indicate that long G-rich protruding termini are a general feature of human chromosome ends.

The lengths of the IMR90-P04 G tails were also estimated from the fraction of hybridized TelC that is removed by the PENT reaction shown in Figure 7, lanes 11 and 12. PENT decreased the radioactivity of the human and plasmid DNA by factors of 6.2 and 4.5, respectively, leading us to conclude that the human and plasmid ends bound an average of 6.2 and 4.5 oligonucleotides. Assuming that TELC saturated the G tails, we estimate the size of the overhangs to be 149 in human and 108 bases in the construct. The consistency of these numbers with our earlier results increases our confidence in our estimates of the length and abundance of telomere G tails.

Discussion

G Tails Are Covalent Structures at the Ends of Human Chromosomes

We detect long 3′ overhangs in cultured primary lung fibroblasts and umbilical vein endothelial cells, in leukocytes, and in immortal lung fibroblasts using PENT and nondenaturing hybridization. The PENT signal is dependent upon the presence of the TelC primer showing that products are not formed from internal nicks or gaps. The PENT and nondenaturing hybridization signals disappear when the DNA is digested with S1 or Bal-31 and decrease about 10-fold when trimmed with T4 polymerase, showing that both reactions depend upon the presence of the 3′ overhang. Because the dissociation temperature should be about 60°C for a 24 b primer and about 42°C for an 18 b primer, it is likely that overhangs shorter than 18 b are not efficiently detected. The fraction of molecules with G tails detected by PENT and the nondenaturing hybridization signals are insensitive to the method of DNA preparation, and the procedures used for DNA isolation from nuclei do not create or

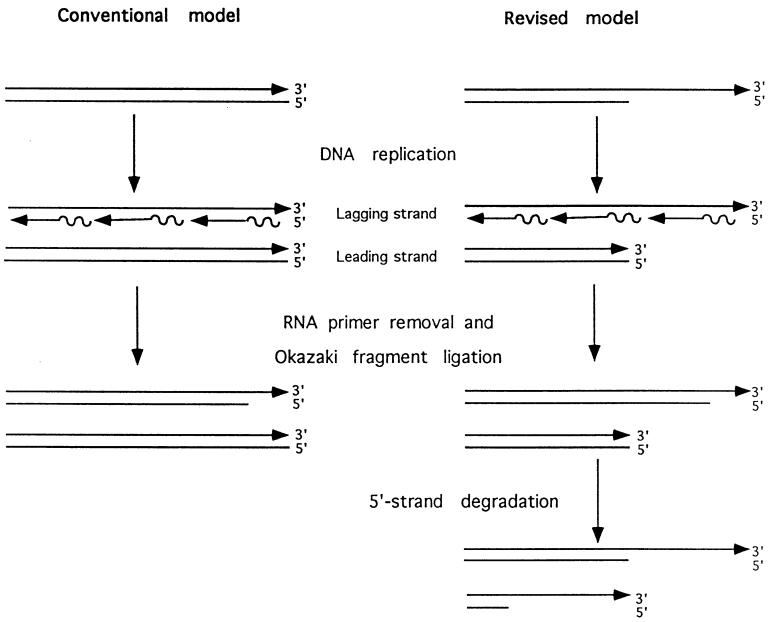

Figure 8. Schematic Drawing of the Conventional Incomplete Lagging-Strand Synthesis Model and Revised 5′ Degradation Model for Telomere Shortening

In both models, lagging-strand DNA synthesis produces a series of Okazaki fragments covalently attached to the short RNA primers at their 5′ ends. Subsequent degradation of the primers, gap repair, and ligation replaces all but the last primer with DNA, resulting in an unfillable gap at half of the 5′ termini. The strand degradation model for telomere shortening states that DNA is lost from both ends of the chromosome subsequent to replication due to degradation of 5′ strands so as to leave a long 3′ overhang at both ends.

destroy G tails. Our data strongly indicate the existence of G-rich extensions at >80% of the ends of human chromosomes. These ubiquitous G tails are probably important for many telomere functions such as protection against degradation and fusion, and nuclear positioning. In immortal cells, the 3′ overhangs might be critical for maintaining telomere length by allowing extension by telomerase, which requires an exposed 3′ terminus (Lingner and Cech, 1996), or by nonreciprical recombination, which should be facilitated by the single-stranded tails (Wang and Zakian, 1990; Murnane et al., 1994; Bryan et al., 1995; Strahl and Blackburn, 1996; Li and Lustig, 1996).

The observed overhangs are unlikely to be due to telomerase activity, because primary IMR-90 cells and leukocytes have very low telomerase activities (Kim et al., 1994; Shay et al., 1994; Broccoli et al., 1995; this study [Experimental Procedures]). Telomeres from post-crisis SV40-transformed IMR-90 cells have the shortest G tails, despite the presence of high telomerase activity. Although even weak telomerase activities might be capable of increasing the length of existing G tails, recent studies of the Euplotes enzyme suggest that telomerase is incapable of creating 3′ overhangs (Lingner and Cech, 1996). Therefore, our results suggest the existence of a telomerase-independent mechanism for generating the 3′ overhangs.

Most Human Chromosome Ends Have G Tails, Inconsistent with the Conventional Model for Telomere Shortening

Replication of chromosome termini should result in loss of DNA from the 5′ strand of half the telomeres due to incomplete lagging-strand replication concomitant with degradation of the terminal RNA primer (Watson, 1972; Olovnikov, 1973). The conventional model for telomere shortening is that this inherent feature of end replication is solely responsible for telomere shortening, as shown in Figure 8. Although the rate of shortening would depend upon the size and position of the last primer, only half of the ends would lose DNA. In contrast, our results on human cells show that 80%–100% of the telomeres have G-rich overhangs. To account for these data, we propose a revised model for telomere shortening. The strand degradation hypothesis for telomere shortening states that DNA is lost from both ends of the chromosome due to degradation of both 5′ strands of the DNA at the time of or soon after replication. Because a 3′ overhang might serve as a substrate for telomerase, the rate of telomere shortening predicted by the strand degradation model would depend upon the extent of 5′ strand degradation as well as the extent of 3′ strand extension or degradation by telomerase. Zakian and coworkers recently suggested that yeast has a cell cycle-controlled degradation of the telomere 5′ end (Wellinger et al., 1996). However, unlike human chromosomes, which have G-rich overhangs during most of the cell cycle, the yeast overhangs disappear soon after DNA synthesis (Wellinger et al., 1996), apparently by repairing the 5′ gap, as evidenced by a very slow loss of telomere DNA in telomerase null mutants (Lundblad and Szostak, 1989).

There is a mammalian candidate for the proposed degradative activity, which could be coupled with DNA replication. Recent discovery, purification, and in vitro studies of the 5′-to-3′ exonuclease from calf thymus (Siegal et al., 1992), mouse (Goulian et al., 1990), and human (Ishimi et al., 1988) have suggested an important role for this exonuclease in the final degradation of RNA primers from the lagging strand. This nuclease can degrade DNA as well as RNA, and is active by itself or as a component of DNA polymerase (Siegal et al., 1992).

There are a number of possible mechanisms for regulating the length of G tails. During evolution, the sequences of telomeres have been selected to satisfy several important functions, including protection against degradation. These sequences have in common G-rich strands capable of forming unusual secondary structures in vitro. Unlike the situation in ciliates, human G tails are of sufficient length to form stable intramolecular

G·G hydrogen-bonded structures, renewing interest in the role of G quartets, triplexes, and duplexes in telomere function. One possibility is that generation of a G-rich tail of some critical length could allow formation of secondary DNA structure that is resistant to further degradation and/or is bound by specific proteins. Alternatively, after replication there could be competition between the putative exonuclease and telomere binding factors, or active regulation of the G tail length by the degradative or synthetic activities of telomerase in conjunction with telomere binding factors.

Long G Tails Might Account for Rapid Human Chromosome Shortening

Shortening of human telomeres is a well-documented phenomenon, which has led to much speculation about the role of telomeres in cellular senescence of normal cells and immortality of cancer cells (Harley, 1991, 1995). The conventional model for telomere shortening due to incomplete lagging-strand replication predicts that, if the last RNA primer is terminally located, the average number of base pairs lost from each telomere per cell duplication should be 0.25 times the length of the RNA primer (estimated to be 8–12 b long; Tseng et al., 1979). Such low rates of chromosome shortening have been detected in telomerase null mutants of yeast (Lundblad and Szostak, 1989) and in Drosophila (Biessmann and Mason, 1988; Levis, 1989), and are consistent with primer removal being important for telomere shortening in those organisms. In contrast, the chromosomes of telomerase-deficient human fibroblasts shorten by 31–85 bp per cell doubling (Harley et al., 1990). Our evidence that there is an average of 154 b of G tail per chromosome end in human IMR-90 fibroblasts suggests a mechanism for human telomere shortening that is consistent with the observed rates of telomere loss. If the C strand gaps are not filled in before replication or augmented by telomerase, the telomeres should shorten an average of 77 bp per cell cycle, consistent with the observed losses in fibroblasts. To directly test the hypothesis that the 3′ tails (or more descriptively, the 5′ recessed ends) are the key to telomere shortening, the observed rates of shortening in different cell types should be directly compared with the lengths of the G tails. If the strand degradation hypothesis is correct, there would be at least three mechanisms by which telomere length could be regulated in proliferating cells: (i) elongation of the 3′ ends by telomerase, (ii) degradation of the 5′ ends, probably by an exonuclease, and (iii) recombination. In the context of the telomere hypothesis for cellular senescence and immortality, all three factors might be involved in control of the proliferative lifespan of human cells.

Experimental Procedures

Cell Culture, Nuclear Isolation, and Telomerase Assay

Three derivative cultures of human fetal lung fibroblasts were purchased and grown strictly according to instructions from the NIA Aging Cell Repository (Coriell Institute for Medical Research, Camden, NJ). Normal IMR-90 primary cells (catalog #190 P04 and #190 P10, after 4 and 10 laboratory passages) and postcrisis immortal SV40 virus-transformed IMR-90 (#AG02804C) were harvested at about 80% confluence. The IMR-90/P04 and IMR-90/P10 cells were harvested after ~33 and ~63 postfetal population doublings, respectively. Human umbilical vein endothelial (HUVE) cells (gift from R. Marks) were grown as described (Dixit et al., 1989) and harvested after 11 passages. Human leukocytes were separated from fresh blood by isotonic lysis (Birren and Lai, 1993). Cells ($1\text{-}2 \times 10^8$) were harvested by centrifuging 3× for 10 min at 800 × g in 15 ml cold PBS followed by resuspension in PBS ($\sim 2 \times 10^8$/ml). Nuclei were isolated as described elsewhere (Lejnine et al., 1995).

Telomerase activity was assayed in IMR-90/P04, HUVE, and immortal IMR-90 cells using the TRAPeze Telomerase Detection Kit (catalog # S7700-KIT) and PCR protocol, which included all appropriate positive and negative controls (Oncor Inc., Gaithersburg, MD). The mortal IMR-90 cells had weak telomerase activities that were 10–20 times lower than in the immortal IMR-90 cells, while HUVE cells had intermediate activities. Telomerase activity has previously been undetectable in IMR-90 cells by conventional or PCR assays (Kim et al., 1994; Shay et al., 1994).

Oligonucleotides

Oligonucleotides were synthesized at the University of Michigan Biomedical Research Core Facility. Oligonucleotide $(CCCUAA)_4$ (TelC) was used as primer for PENT reactions. Oligonucleotides $(CCCTAA)_3CCC$, $(UUAGGG)_4$ (TelG), CCCTCCAGCGGCCGG(TTAGGG)$_3$, and $(CCCUAA)_4$ were used for probe preparation.

DNA Purification

We usually used a protocol for isolation of high molecular weight DNA in solution (Birren and Lai, 1993). Washed tissue culture or fresh blood cells or nuclei were incubated with Proteinase K/lysis solution (50 mM EDTA [pH 8.0], 1% SDS, 1 mg/ml proteinase K) for 24–48 hr, extracted with phenol, then with phenol/chloroform, and dialyzed against TE. For certain critical experiments (e.g., for G overhang length analysis), the DNA was digested with RNase. DNA concentrations were determined by spectrophotometry (usually 100–200 μg/ml). Telomere molarity was calculated assuming 75×10^6 bp per telomere (or 3.4×10^9 bp per haploid genome).

Preparation of Model Telomere Constructs

Linear constructs with 520–700 bp of double-stranded human telomere DNA and 100–220 b of G-rich overhang were constructed from plasmid Sty11 (gift from Dr. T. de Lange). Sty11 was cut with ClaI which leaves 10 bp of polylinker DNA at the end of an 800 bp telomere tract (Figure 1). To expose the telomeric repeat, the linearized plasmid was digested with Bal-31 for 30 s at 30°C using 2 units of enzyme with 10 μg DNA (Lejnine et al., 1995). To produce a 3′ overhang, 5 μg of linearized or linearized/Bal-31-treated DNA was incubated with 100 units of T7 gene *6* exonuclease in 50 μl of 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl at 20°C for different times, extracted, and resuspended in TE. The average G tail length and length distribution were determined by digestion with EcoRI, electrophoresis in 1.5% agarose/40 mM NaOH, and analysis of the length of the C strand.

Primer-Extension/Nick-Translation Reaction

The optimized reaction was performed in 50 μl PENT buffer (the standard Taq polymerase buffer composed of 20 mM Tris-HCl [pH 8.3], 50 mM KCl, and 2 mM $MgCl_2$) containing 50 μM dNTPs, 5–10 nM TelC primer, 0.1–1 fmol of DNA telomere ends (5–50 μg of human DNA or 0.1–1 ng of Sty11 telomere construct) and 40 units/ml of Taq polymerase at 55°C. To insure the hybridization of the TelC primers to all single stranded telomere ends, the ingredients of the reaction (except Taq polymerase) were placed into 0.5 ml thin-wall PCR tubes, mixed, covered with mineral oil, and incubated at 45°C for 1 hr in a DNA Thermal Cycler 480 (Perkin-Elmer, Cetus). The temperature was increased to 55°C for 5 min, and Taq polymerase was added. Aliquots were removed at the desired times and quenched on ice with 10 mM EDTA. All DNA samples were incubated with dU-glycosylase (1 μl enzyme per 50 μl reaction) at 37°C for 1–2 hr, ethanol precipitated, washed, and dried. The dU-glycosylase promoted primer degradation during alkaline electrophoresis, greatly reducing the background on Southern blots. Similar treatment was used to destroy PENT reaction synthesis products in those cases when dUTP was incorporated.

Other Enzymatic Treatments

To remove G tails, 10 mg of IMR-90 DNA was incubated with 300 units/ml S1 nuclease for 15 min at 37°C in 50 mM NaAc (pH 4.5), 1 mM $ZnCl_2$, and 200 mM NaCl, or with 20 units/ml Bal31 nuclease for 5 min at 30°C in Bal31 buffer. For the same purpose, 2 ng of plasmid construct, 10 mg of IMR-90 DNA, or a mixture of the two was incubated with 10 units of T4 DNA polymerase for 10 min at 37°C in 50 mM Tris-HCl (pH 8.8), 15 mM $(NH_4)_2SO_4$, 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 100 μg/ml bovine serum albumin DNA was extracted and resuspended in PENT buffer.

One- and Two-Dimensional Alkaline Gel Analysis

Alkaline electrophoresis was performed in 0.8–1% agarose as described elsewhere (Maniatis et al., 1982) with 40 mM NaOH. The gels were run at 1 V/cm (250–300 mA) for 12–16 hr at room temperature with buffer circulation. Double-stranded, PENT-treated, HinfI-digested human DNA and DNA size markers were run on 0.5% SeaKem Gold agarose in TAE for 36 hr at 0.8 V/cm. When the positions of the DNA markers were established, the lane corresponding to human DNA/HinfI restriction fragments was excised and either cut into narrow gel slices for size fraction analysis or used for 2-D electrophoresis. In the latter case, the agarose inserts with human DNA and DNA marker 2 (0.1 ng in 100 μl of 0.6% agarose) were placed in the same gap made in 0.8% agarose gel, and the gap was filled with melted 0.5% SeaKem Gold agarose. After solidifying, a second gap of the same length but 2.0 mm wide was cut adjacent and parallel to the first one and filled with 0.1% SeaKem Gold agarose to provide the zone where large DNA strands could denature. The gels were soaked in 40 mM NaOH and 1 mM EDTA and electrophoresed as above. After electrophoresis, gels were neutralized and vacuum blotted. In the case of 2-D analysis, before blotting, the agarose lane with the 3.2 kb DNA ladder (cut from the first direction gel and stored at 4°C) was embedded in the gel adjacent to the human DNA to provide a marker for the first direction.

Length Analysis of G-Rich Tails by Nondenaturing Hybridization and PENT

HinfI-digested human DNA, plasmid constructs with 100 b, 170 b, and 220 b overhangs, or a nearly equimolar (in terms of telomere ends) mixture of human and plasmid DNA were hybridized at 50°C with 1 nM [^{32}P]TelC in 20–30 μl of hybridization buffer (50 mM NaCl, 1 mM EDTA, and 50 mM Tris-HCl [pH 8.0]) or PENT buffer for 12–16 hr. Some of the samples were subjected to PENT (100 mM dNTP, 5U Taq DNA polymerase; 10 min at 55°C), then all samples were electrophoresed on a 1% agarose/TAE gel, electroblotted onto a nylon membrane for 16 hr, and quantitated. The absolute telomere molarity of the IMR90/P04 DNA solution was approximated by spectrophotometry. The molarities of plasmid constructs and telomeres from different human cells were determined by CCD analysis of fluorescence of ethidium bromide stained gels; the signal intensities of plasmids and telomeres were normalized to the signal intensities of a DNA Mass Ladder (GIBCO–BRL) and IMR90/P04 DNA, respectively.

Probes, Hybridization, and Quantitation of Gels

To prepare radioactive TelC probe, $(CCCTAA)_3CCC$ was hybridized to $(UUAGGG)_4$, and the extension reaction was performed in 20 μl of solution containing 5 pmol of the hybrid, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 μg/ml bovine serum albumin, 50 μM dTTP, 50 μCi [^{32}P]dATP, and 4 units of Klenow fragment (exo$^-$) at 25°C for 30 min. The reaction was terminated with 20 mM EDTA. After adding 1 μl of dU-glycosylase, the reaction mixture was incubated at 37°C for 1–2 hr, heated at 95°C for 30 min to introduce breaks at abasic sites, and used for hybridization. TelG probe was prepared by a similar primer-extension and degradation reaction using $CCCTCCAGCGGCCGG(TTAGGG)_3$ and $(CCCUAA)_4$. Radioactive Sty11 plasmid probe was obtained by random priming labeling with [^{32}P]dATP. Hybridization with oligonucleotide probes and filter washing were performed as described before (Makarov et al., 1993). Hybridization was quantitated with a Molecular Dynamics 400A PhosphorImager and ImageQuant software.

DNA Markers

Marker 1 (3.2 kb ladder) was prepared by ligation of linear DNA fragments produced by HindIII digestion of Sty11. Marker 2 (850 b ladder) was synthesized using 850 bp BglII/BamHI Sty11 fragments. The ligation reaction was performed at 37°C in the presence of T4 DNA ligase and both restriction enzymes. After ligation, the products were additionally digested with BglII and BamHI to cut completely all BglII–BglII and BamHI–BamHI junctions. Only BglII–BamHI linkages survived after this treatment, which gave rise to the 850 b ladder with G-rich and C-rich DNA sequences located on opposite strands.

Acknowledgments

We thank David Engelke for Taq DNA polymerase, R. Marks for HUVE cells, and Titia de Lange for Sty11. We thank Kuenok Lee and Roy Bellville for culturing IMR-90 cells and isolation of leukocytes from human blood, and Graeme Tank for critical reading of the manuscript. This project was supported by an award from the Claude Pepper Older Americans Independence Center to V. L. M., a fellowship from the Horace Rackham School of Graduate Studies to Y. H., the Office of the Vice-President for Research, and NSF grant MCB 9514196.

Received October 2, 1996; revised January 23, 1997.

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