Long Telomeric C-rich 5’-Tails in Human Replicating Cells*

Received for publication, September 3, 2002, and in revised form, October 24, 2002
Published, JBC Papers in Press, November 14, 2002, DOI 10.1074/jbc.M208939200

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Telomeres protect the ends of linear chromosomes from abnormal recombination events and buffer them against terminal DNA loss. Models of telomere replication predict that two daughter molecules have one end that is blunt, the product of leading-strand synthesis, and one end with a short G-rich 3’-overhang. However, experimental data from proliferating cells are not completely consistent with this model. For example, telomeres of human chromosomes have long G-rich 3’-overhangs, and the persistence of blunt ends is uncertain. Here we show that the product of leading-strand synthesis is not always blunt but can contain a long C-rich 5’-tail, the incompletely replicated template of the leading strand. We examined the presence of G-rich and C-rich single-strand DNA in fibroblasts and HeLa cells. Although there were no significant changes in the length distribution of the 3’-overhang, the 5’-overhangs were mostly present in S phase. Similar results were obtained using telomerase-negative fibroblasts. The amount and the length distribution of the 5’ C-rich tails strongly correlate with the proliferative rate of the cell cultures. Our results suggest that, contrary to what has commonly been supposed, completion of leading-strand synthesis is inefficient and could well drive telomere shortening.

Telomeres are complex specialized structures that seal the termini of eukaryotic chromosomes protecting them from DNA loss, end-to-end fusion, and other potential genetic rearrangements. Telomeric DNA of most eukaryotic organisms is made of short tandem repeated sequences (1–4). The nature and the organization of the repeats is such that one strand is usually rich in guanines (G), and this strand always runs 5’ to 3’ toward the end of the chromosome. Consequently, its complementary cytosine (C)-rich strand always runs 5’ to 3’ toward the centromere. When a telomere is replicated, leading-strand synthesis produces the G-rich copy of the C-rich template minus, and lagging-strand synthesis produces the C-rich copy of the G-rich template minus. As a consequence, a terminal G-rich 3’-overhang at least as long as the removed RNA primer from the parental strand is expected to be present in the lagging-strand telomere, whereas the leading-strand telomere is expected to be blunt (5, 6). Upon successive rounds of DNA replication, telomeres will progressively shorten and, eventually all telomere functions will be lost and the chromosomes will become unstable (7, 8). However, a compensatory mechanism is provided by telomerase, which adds telomeric repeats onto the 3’ DNA end of chromosomes (9–12).

Although the exact structure of the 3’-overhang varies between species, the presence of such overhangs is both conserved and believed to be essential for maintenance of chromosome end structure and function. In fact, a 3’-end of at least six nucleotides together with its associated telomere-binding factors is thought to participate directly in forming a specialized telomere structure (13, 14) (e.g. a “t-loop”). In human cells, chromosome ends with long G-rich 3’-overhangs that vary from 100 to 280 nucleotides have been detected (15, 16). In these cells, the rate of telomere shortening during cell doublings depends on the size of the overhang (17). In mammals, RNA priming events are thought to occur about every 100–200 bp during lagging-strand synthesis. This is roughly consistent with the rates of telomere shortening of 40–200 bp per cell division that has been observed in cultured human cells (11, 18). A possible explanation is that human cells lack the ability to position the final RNA primer at the very end of the chromosome. Several lines of evidence suggest that the normal DNA replication machinery generates the C-rich complement of the telomerase extended G-strand (4, 19) and that telomeric G- and C-strand syntheses are coordinately regulated (20, 21). Because telomerase requires a 3’-overhang, current models suggest that both chromosomal termini should contain G-rich 3’-tails. Evidence has been provided that Tetrhyymena thermophila rDNA molecules have overhangs on both telomeres (22). Consequently, the blunt termini that have been generated by leading-strand synthesis would need to be processed by a 5’ to 3’ exonuclease to erode the C-rich parental template (23, 24–27), although this remains controversial (15, 16, 28).

We have recently developed a novel protocol, which we call T-OLA,1 to examine the nature of the chromosomal DNA termini in cell cultures and tissues (29). With this method, we found quite unexpectedly that the 5’ C-rich end of the leading-strand template remains for a time unreplicated in proliferating cells, e.g. it persists as a 5’ C-rich tail during S phase. This result suggests that the replication fork may stall before reaching the very end of the chromosome. This in turn could lead to excessive shortening during replication of the lagging strand because the ability to position the RNA primer near the 3’-end of the lagging G-rich parental strand would be compromised. This scenario would account for both the long G-rich tails and the accelerated telomere shortening thought to accompany them. In addition, if the uncopied 5’ C-rich tail gets eroded by the process that normally exposes the G-rich tail on fully rep-

1 The abbreviations used are: T-OLA, telomeric oligonucleotide ligation assay; TRF, TTAGGG repeat-binding factor.
labeled leading-strand telomeres, then telomere shortening would also be further enhanced.

EXPERIMENTAL PROCEDURES

Cell Cultures and DNA Extraction—High molecular weight DNA (30) was obtained from human foreskin fibroblasts cultured in Eagle’s basal medium plus 10% fetal calf serum and from HeLa cells grown in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. HeLa cells were halted at the G/S boundary by a double treatment with 2 mM thymidine. Cells were harvested 0 and 4 h after the release. Resting fibroblasts were obtained either by serum starvation or by harvesting the cells that were maintained for at least 48 h in low serum medium (2%) after they reached confluence. Cell starvation was produced by seeding 5 x 10^6 cells in a 175-cm² flask with 30 ml of 10% fetal calf serum Eagle’s basal medium. Proliferating fibroblasts were harvested from 50% confluent flasks. Synchrony was monitored by flow cytometric measurements of DNA distribution. Typically, the proliferating HeLa and fibroblast cell cultures presented respectively 75% and 40% of cell population in S phase.

Telomeric Oligonucleotide Ligation Assay—T-OLA was performed as previously described with minor modifications (29). Briefly, oligonucleotides were phosphorylated by T4 polynucleotide kinase. A mixture of 30 μl containing 5 pmol of oligonucleotide and 50 pmol of [γ-32P]ATP (3000 Ci/mmol, 10 μCi/ml), 70 μM Tris, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol, and 20 units of T4 polynucleotide kinase was incubated for 40 min at 37 °C. 0.3 μl of 0.1 μl unlabeled ATP and a further 10 units of kinase were then added, and the reaction was continued for 15 min. Labeled oligonucleotides, [(CCCTAA)₅] and [(TTAGGG)₅], were sodium acetate/ethanol-precipitated and dissolved in an appropriate volume of water. Hybridization and ligation were conducted in a volume of 20 μl containing 5 μg of undenatured DNA, 0.5 pmol of oligonucleotide probe, 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin. All ingredients (except ligase) were placed into 0.5-ml PCR tubes and incubated at 33 °C for 12–14 h. Subsequently, 150 units of T4 DNA ligase was added, and the reaction was extended for a further 4 h. Reactions were ended by adding 30 μl of water and by phenol/chloroform extraction. Samples were precipitated with ethanol and dissolved in 6 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Electrophoresis Analysis of Reaction Products—Reaction products were analyzed on denaturing 6% acrylamide sequencing gels. In addition, reaction samples were resolved on non-denaturing 1% agarose gels to check for DNA quantitation and consistency between observed hybridization signal to high molecular weight DNA and T-OLA products. For analysis of ligated products under denaturing conditions, half of the volume was mixed with 4 μl of formamide stop solution. Samples were heated at 90 °C and immediately quenched on ice before loading 2 μl onto the gel. The remaining 3 μl were diluted in water and in a glycerol loading buffer to a final volume of 10 μl and run on a non-denaturing 1% agarose gel for 4 h at 90 V in Tris-acetate buffer. The gels were dried on nylon membrane (Stratagene) and exposed to autoradiography film. The images were acquired by 1D Image Analysis Software (Eastman Kodak Co.). Because the intensity of each band depends on both the frequency of the specific tail and on its length, the intensity of each band depends on both the frequency of the specific tail and on its length, the intensity of each band was divided by the number of concatenated oligonucleotide probes expected to be in the band so that the resulting relative intensities would be proportional to the relative tail frequency. This value was then normalized to the total intensity and plotted both as relative frequency and as length distribution of the tails.

Enzymatic DNA Modification—To remove the 3' single-stranded DNA, Escherichia coli Exonuclease I was used. DNAs were incubated in 10 mM Tris HCl, pH 8.0, 1 mM EDTA, 10 mM MgCl₂, 20 mM KCl, and 10 mM 2-mercaptoethanol and 1 unit/μl of enzyme Exonuclease I (U. S. Biochemical Corp.) for 24 h at 37 °C. For cleaving 3'-recessed but not 3'-protruding ends of DNA, Exonuclease III was used. DNA was incubated in 50 mM Tris HCl, pH 8.0, 6.6 mM MgCl₂, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 1 unit/μl Exonuclease III at 37 °C for 30 min. To remove the single-stranded regions, both in 5'→3' and 3'→5' directions, the DNA was incubated with 0.2 unit/μl Exonuclease VII (U. S. Biochemical Corp.) in 50 mM Tris HCl pH 7.9, 50 mM potassium phosphate buffer, pH 7.6, 9.3 mM EDTA, and 10 mM 2-mercaptoethanol at 37 °C for 1 h. To remove the 5' single-stranded DNA, the DNA was incubated with T7 (Gene6) Exonuclease (0.6 unit/μl) (U. S. Biochemical Corp.) in 50 mM Tris HCl pH 8.1, 5 mM MgCl₂, 20 mM KCl, and 5 mM 2-mercaptoethanol at 37 °C for 20 min. The reactions were stopped, the mixtures were extracted with phenol-chloroform, and the DNA was precipitated and dissolved in H₂O.

RESULTS

In the OLA, two oligonucleotides are designed to hybridize in exact juxtaposition to the target DNA sequences, permitting their covalent joining by a DNA ligase. Because human telomeres are composed of repetitions of a 6-nucleotide sequence, in frame hybridized complementary oligonucleotide ligation of a telomeric sequence is expected to produce a ladder of concatenated oligonucleotides whose lengths are comparable with the target sequences. If single-stranded telomeric regions are present in native DNA, they will be replenished by in-frame oligonucleotides that will be covalently joined by ligase. Our experimental approach for measuring the length of telomeric overhangs is based on the ligation of oligonucleotides to undenatured DNA using oligonucleotides designed to hybridize at high stringency, and in frame, with telomeric repeats (T-OLA). The sensitivity and specificity of this method have been extensively evaluated in a previous report (29) in which the 3'-overhang length distribution of various human cell cultures was reported. In the process of analyzing the length of the 3'-overhang of various mammalian cell lines and tissues, we unexpectedly detected in some cell cultures a ladder of products also when an oligonucleotide complementary to the C-rich strand was used. Although the length distribution of the single-stranded segments was very similar to that obtained probing...
for the G-rich strand, the total amount was variable but persistently lower, ranging from 1/3 to 1/10 (absent in some tissues). However, the total amount of C-rich single-stranded DNA appeared to correlate with the proliferative status of the tested cells, e.g., it was higher in fast proliferating cells. The results obtained with HeLa cells, foreskin human fibroblasts, and circulating leukocytes are shown in Fig. 1.

Because the G-rich telomeric strand is always synthesized as the leading strand, incomplete leading-strand replication should leave a 5′-rich overhang. The nature of the single-stranded DNA was investigated by treating the DNA with some specific exonucleases. Enzymatic treatment of DNA prior to T-OLA enables a series of predictions to be tested. A 3′ tail is expected to be specifically digested by *E. coli* Exonuclease I but will be insensitive to Exonuclease III. Conversely, a 5′-tail will be insensitive to Exonuclease I treatment but will be augmented by Exonuclease III. A 5′-exonuclease such as T7 (Gene 6) Exonuclease will increase the amount of 3′-overhang, lengthen the G-rich tail, and remove the 5′-overhang. Exonuclease VII, which is a strict single-strand directed enzyme with

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**Fig. 2. Effects of nuclease treatments on the T-OLA reaction.** A, predicted effects of enzymatic treatments on T-OLA. The enzymatic effects able to discriminate between 5′-tail (a) or 3′-tail (b), and internal gaps are indicated. B, observed effects of enzymatic treatments. Native, undigested DNA; ExoI, Exonuclease I; 5′Exo, T7 (Gene6) Exonuclease; Exo VII, Exonuclease VII; Exo III, Exonuclease III. The results obtained from enzymatic treatments of DNA extracted from proliferating cells (in this case HeLa cells) are shown. Compare quantity and quality of the ladder products with untreated DNA. The predicted and observed effects are consistent with 3′-G-rich and 5′-C-rich overhangs.

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**Fig. 3. G-rich 3′- and C-rich 5′-overhangs in resting and proliferating HeLa and fibroblasts.** A, T-OLA products. The G- and the C-strand ladders are obtained by probing HeLa cell DNA in G1/S and S phases, respectively with [(AATCCC)]2 and [(TTAGGG)]2 oligonucleotides. Resting (R) and proliferating fibroblast cells (P) were also studied. B, comparison of the quantity and quality of the tails. The histograms are deduced by quantitative analysis carried out with 1D Image analysis software (Kodak). The relative amounts and the length distributions of the G- and the C-tails in the two cell cycle phases are plotted as relative intensity. The lighter patterns indicate the percentage of tails shorter than 90 nucleotides, the dark part the percentage of tails longer than 90 nt.
at the G1/S boundary and at full S phase (75% of the cell in S phase). Samples from resting fibroblasts, obtained either by cell starvation or by maintaining confluent monolayers in low serum, were compared with samples derived from high proliferating fibroblasts (40% of cells in S phase). The results are shown in Fig. 3. Comparable amounts and lengths of 3’ G-rich tails are present in the two proliferative conditions both of HeLa and fibroblasts, albeit with somewhat higher amounts in S phase. In contrast, substantial amounts of the 5’ C-strand tail are present only in S phase. In addition, far more of the 5’ C-strand tails are skewed toward longer lengths than the G-strand. Fig. 3 also shows that very long G-rich and C-rich tails are present during the log phase of proliferating cells. In contrast, the amount and length of the G-rich tails are slightly reduced in resting cells, whereas the C-rich tails are virtually absent.

DISCUSSION
We show for the first time that in addition to long 3’-overhangs of the G-rich strand, long 5’-overhangs of the C-rich strand are transiently present at the telomere end of chromosomes of proliferating cells. We also show a direct correlation between 5’ C-rich tails and the proliferative rate of the cell cultures. To account for the above findings, we propose that the replication fork dissolves when the last possible RNA primer is assembled on the telomere (Fig. 4). Evidence suggests that a stretch of single-stranded DNA up to the unwinding point is present on the lagging arm, whereas the helicase occupies a part of the unwound parental leading strand (31). Consequently, the newly synthesized leading strand will not be extended up to the unwinding point; the DNA replication machinery will prime the lagging template strand close to the unwinding point and ahead of the 3’-end of the nascent leading strand. The coordinated synthesis of the leading and lagging strands will leave the parental C-strand uncopied until a new Okazaki fragment is initiated on the G-rich strand. Presumably, the DNA replication machinery will be able to complete leading-strand synthesis only if the parental G-rich strand is primed at the very chromosome end of a blunt-end telomere or at a region corresponding to the end of the C-rich strand of a telomere with a 3’-overhang. Priming occurring more centromeric will produce a C-rich 5’-tail of the leading strand telomere. The length of the single-stranded G-rich overhang might thus represent the distance from the last priming event during lagging-strand synthesis at the end of the chromosome.

It has been proposed that the natural chromosome ends are sequestered by the t-loop structure that is promoted by TRF1 and TRF2 and requires single-stranded extension of the TTAGGG sequence (14). Then, the leading strand telomeres have to transit from a 5’-overhang to a 3’-overhang. Recent experiments conducted using a dominant-negative mutant of TRF2 revealed that a high number of mitotic cells exhibited end-to-end chromosomal fusion between leading-strand telomeres (32). If the remodeling and capping process is impaired, a not-yet-eroded C-rich 5’-tail may find itself in the right polarity to pair to a newly generated 3’-overhang G-rich tail. In this case, the erroneous repair that leads to covalent end-joining between leading telomeres will be favored.

It has been argued that the nub of the chromosomal-end replication problem lies in the inability of DNA polymerase to complete synthesis of the leading strand (23). Conventional DNA polymerases cannot synthesize the extreme 5’-ends of a blunt end DNA molecule. Even if an RNA primer was paired with the extreme 3’-end of its DNA template, removal of this last RNA primer would give rise to a daughter molecule with a 5’-terminal gap. Lagging-strand synthesis would not necessarily be a problem as long as the RNA primer is positioned on the
3'-overhang. However, the leading strand will lose its 3'-overhang upon replication. Consequently, if a cell replicates without telomerase, it will divide into two daughter cells having lagging telomeres very similar to the parental chromosomes and with an overhang as long as the distance from the 3'-overhang to the last Okazaki fragment and a leading telomere shortened of the 3'-overhang. Human chromosomes have a single-stranded 3'-overhang of up to 300 nucleotides that roughly corresponds to the length of the Okazaki fragment of eukaryotic cells. Our data support a model in which telomere shortening is primarily caused by the inability to position the last RNA primer on the parental G-rich strand beyond the double-stranded region of the chromosome end. We propose that this is a consequence of the stall of the replication fork before reaching the very chromosome end. Thus, the C-rich parental strand that was already shorter with respect to the parental G-rich strand would require a further step to be replicated up to the end. A remodelling process occurring before this step would further increase telomere shortening (Pathway 2 of Fig. 4).

Acknowledgments—We thank A.V. Furano for helpful comments and suggestions and M. Foiani for helpful discussion on DNA replication.

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*J. Biol. Chem.* 2003, 278:2136-2140.
doi: 10.1074/jbc.M208939200 originally published online November 14, 2002

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