Normal human chromosomes have long G-rich telomeric overhangs at one end

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Telomeres protect the ends of linear chromosomes from degradation and abnormal recombination events, and in vertebrates may be important in cellular senescence and cancer. However, very little is known about the structure of human telomeres. In this report we purify telomeres and analyze their termini. We show that following replication the daughter telomeres have different terminal overhangs in normal diploid telomerase-negative human fibroblasts. Electron microscopy of those telomeres that have long overhangs yields 200 ± 75 nucleotides of single-stranded DNA. This overhang is four times greater than the amount of telomere shortening per division found in these cells. These results are consistent with models of telomere replication in which leading-strand synthesis generates a blunt end while lagging-strand synthesis produces a long G-rich 3′ overhang, and suggest that variations in lagging-strand synthesis may regulate the rate of telomere shortening in normal diploid human cells. Our results do not exclude the possibility that nuclease processing events following leading strand synthesis result in short overhangs on one end.

[*Key Words:* Telomeres; DNA replication; chromosome structure; cellular senescence; aging]

Received July 31, 1997; revised version accepted September 5, 1997.

Telomeres are the specialized ends of linear chromosomes that are involved in a variety of functions, including meiotic chromosome segregation, chromatin silencing, and protecting the ends of the chromosomes from degradation or end-to-end fusion (for review, see Blackburn 1994; Zakian 1995; Greider 1996). In most organisms, telomeres are composed of repetitive sequences in which the strand with its 3′ end at the terminus is G-rich and may extend beyond the DNA duplex to form a single-stranded G-rich overhang. In humans, telomeres contain up to several thousand repeats of the sequence TTAGGG (Moyzis et al. 1988; Cross et al. 1989). Because of the requirement for an RNA primer, DNA polymerases are unable to replicate the extreme 3′ end of a parental DNA strand (Watson 1972; Olovnikov 1973) and, in the absence of compensatory mechanisms, telomeres shorten with each cell division. The ribonucleoprotein telomerase provides such a compensatory mechanism. Telomerase contains reverse transcriptase motifs (Lingner et al. 1997), and using its RNA component as a template (Greider and Blackburn 1989), it can add repetitive sequences to the 3′ end of the chromosomes. Eliminating the RNA component of telomerase prevents this activity and results in telomere shortening in organisms ranging from yeast to humans (Singer and Gottschling 1994; Blasco et al. 1995; Feng et al. 1995). Telomerase activity can be detected in the vertebrate testis (Prowse and Greider 1995; Wright et al. 1996), and telomere length is maintained in the germ line (Cooke and Smith 1986; Hastie et al. 1990; de Lange et al. 1990). However, telomerase activity is repressed in most human tissues during development (Wright et al. 1996) and progressive telomere shortening is then observed (Hastie et al. 1990; Lindsey et al. 1991). This shortening has been proposed to serve as a mitotic clock that counts cell divisions and ultimately results in cellular senescence (de Lange et al. 1990; Greider 1990; Harley et al. 1990; Harley 1991; Wright and Shay 1995). The ability to maintain telomere length may be important in cancer formation, as approximately 85% of all human primary tumors express telomerase activity (for review, see Shay and Bachetti 1997).

The detailed structure of telomeric ends has been determined in hypotrichous ciliates such as *Oxytricha nova,* where a double-stranded region of 28 bp of TTTTGGGG repeats is followed by 14 nucleotides of a G-rich single-stranded overhang (Klobutcher et al. 1981). In *Saccharomyces cerevisiae,* although a longer single-stranded region can be transiently observed in late S phase, during most of the cell cycle any G-rich overhangs that are present are shorter than a 30-nucleotide detection limit (Wellinger et al. 1993). The loss of ~5 bp per division in yeast lacking telomerase RNA is consistent with a model in which both ends of the yeast telomere have an ~10-nucleotide G-rich overhang (Zakian 1995). Recent models for the action of telomerase have empha-

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sized the need for processing of the blunt end generated by leading strand synthesis so that it can be a substrate for telomerase, with subsequent processing events generating chromosomes with symmetrical telomeres containing short G-rich overhangs (Lingner et al. 1995; Lingner and Cech 1996; Wellinger et al. 1993, 1996). These models have a working assumption that there is a primase activity that can position an RNA primer at the extreme 3′ end of the chromosome. Such a primase activity has been found in *O. nova* (Zahler and Prescott 1988).

In contrast to yeast telomeres which lose only a few base pairs per division in the absence of telomerase, telomeres from normal diploid human cells have been found to shorten at rates varying between 40 and 200 bp per division (Harley et al. 1990; Counter et al. 1992; Shay et al. 1993; Vaziri et al. 1993). There are at least three hypotheses to explain the much greater losses in human cells. Exposure to oxygen levels >20% causes premature senescence in human fibroblasts, and it has been proposed that unrepaired oxidative damage causes the one-step loss of long stretches of telomeric repeats (von Zglinicki et al. 1995). This hypothesis predicts that the rate of loss of telomeric DNA under normoxic conditions would represent the average between slow rates of shortening on most chromosomes and rapid losses on some damaged chromosomes. A second hypothesis is that processing events involving the nucleolytic degradation of one or both strands would cause increased rates of shortening in human telomeres (Makarov et al. 1997). There is good evidence for a variety of processing mechanisms at telomeres. Different mutations in the yeast single-stranded telomeric binding protein cdc13p can cause the massive nucleolytic degradation of the C-rich strand (Garvik et al. 1995) or a failure of yeast telomerase to maintain telomere length (Nugent et al. 1996). The appearance of transient ⩾30-nucleotide overhangs on both ends of yeast chromosomes does not require yeast telomerase (Wellinger et al. 1996), and a nuclease able to digest G4 tetrastrand structures has been identified (Liu and Gilbert 1994). These observations suggest that specific nucleolytic processing of telomeres occurs in yeast. Nucleolytic processing is also seen in ciliates. The G-rich strand added by telomerase to the newly fragmented macronuclear DNA in hypotrichous ciliates is initially longer than in mature telomeres (Roth and Prescott 1985; Vermeesch and Price 1994), and the preferential pause site used by telomerase in vitro is not found at the end of ciliate telomeres synthesized in vivo (Klobutcher et al. 1981; Henderson et al. 1988; Shippen-Lentz and Blackburn 1989; Greider 1991). A third hypothesis is that human cells lack the ability to position the final RNA priming event at the very end of the chromosome. RNA priming events are thought to occur about every 100–600 bp during lagging strand synthesis in mammals (Anderson and DePamphilis 1979; DePamphilis 1993; Waga and Stillman 1994). 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. The length of the single-stranded G-rich overhang might thus represent the distance between the last priming event during lagging strand synthesis and the end of the chromosome.

As a first step in distinguishing between these models, we have developed techniques for purifying human telomeres and examining their structure. Our results demonstrate that the telomeres generated by leading versus lagging strand DNA synthesis are different and suggest that each chromosome has one telomere with a long G-rich overhang and one that is either blunt or has a short G-rich overhang. We provide the first direct electron microscopic measurement of the single-stranded region in telomeres from normal diploid human cells and find a 200 ± 75-nucleotide overhang. The rate of telomere shortening of 50 bp per division in these cells is consistent with models in which shortening results from overhangs produced by lagging strand synthesis. Our results do not support models of telomere shortening in which the primary mechanism is either oxidative damage or nucleolytic processing.

Results

Purification of human telomeres

Human telomeres were purified based on the ability of biotinylated oligonucleotides complementary to the G-rich telomeric repeat to anneal to the G-rich overhang in otherwise double-stranded DNA (Shay et al. 1994). Following annealing, DNA/oligonucleotide complexes were bound to streptavidin-coated magnetic beads and washed, and the telomeres were eluted and analyzed on agarose gels. Figure 1A demonstrates the sequence specificity of this purification. Although the telomeres in human placental DNA can be retrieved using biotinylated oligonucleotides containing four or six C-rich telomeric repeats (CTR_4 and CTR_6), neither six copies of the G-rich repeat (GTR_6) nor a non-telomeric oligonucleotide (ClaHin) were able to bind telomeres. The failure of the G-rich repeat to purify telomeres suggests that the binding of telomeres by the C-rich oligonucleotide is not due to strand invasion or gaps in the double-stranded DNA but, rather, is dependent on the presence of the G-rich 3′ overhang. Digestion of the DNA with exonuclease I resulted in a fourfold reduction in recovery and confirmed that most of this purification required a single-stranded overhang (Fig. 1B). We suspect that the presence of non-canonical structures such as G-quartets involving some of the overhangs may block exonuclease I activity, leaving some exonuclease-resistant single-stranded regions intact and thus available for hybridization to the biotinylated C-rich oligonucleotides. The minimal overhang that could be recovered with this technique was determined using an artificial telomere constructed by ligating a linearized 5-kbp plasmid to short double-stranded fragments containing variable numbers of TTAGGG repeats as single-stranded 3′ extensions. Overhangs containing as few as 12 nucleotides could be recovered (Fig. 1C).

The average efficiency of purification of telomeres [bound ÷ (bound + unbound)] using DNA from normal

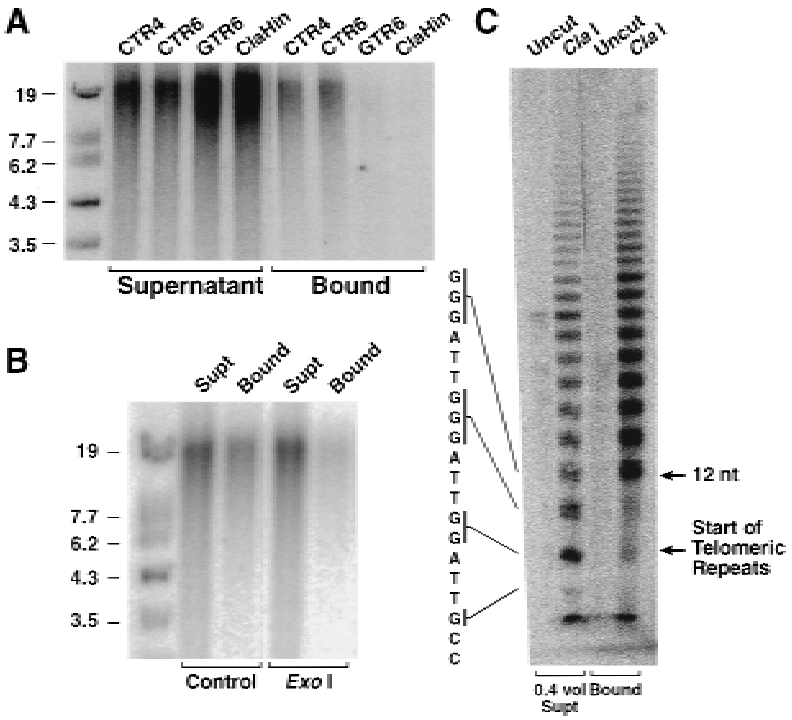

Figure 1. Purification of telomeres. (*A*) Sequence specificity of the purification of telomeres. *Hin*fI-digested human placental DNA was annealed to various biotinylated oligonucleotides, and telomere/oligonucleotide complexes were captured with streptavidin-coated magnetic beads. The DNA remaining in the supernatant vs. that bound to the beads was then analyzed on agarose gels and probed with a ^{32}P-labeled $(TTAGGG)_4$ oligonucleotide. CTR_4 and CTR_6 contain four and six copies of the C-rich terminal repeat (CCCTAA), GTR_6 contains six copies of the G-rich terminal repeat (TTAGGG), and ClaHin is a nontelomeric biotinylated oligonucleotide. Only the C-rich oligonucleotides complementary to the G-rich telomeric overhang were able to retrieve the double-stranded placental telomeres. (*B*) Purification requires single-stranded overhangs. Treatment of the DNA with the single-stranded exonuclease Exo 1 (1 U/μg) largely abolished the ability to retrieve telomeres. Noncanonical G structures (Henderson 1995; Kipling 1995) may make a small fraction of the overhangs resistant to complete digestion. (*C*) Purification requires ≥12 bases of overhang. A 5-kbp artificial telomere containing single-stranded G-rich overhangs of variable lengths was annealed to a biotinylated C-rich oligonucleotide and purified using streptavidin-coated magnetic beads. The material bound to the magnetic beads or remaining in the supernatant is large and does not enter a denaturing polyacrylamide gel (uncut lanes). The radioactive telomeric repeats were released by digestion with an enzyme that cuts the plasmid just before the start of the telomeric repeats (*Cla*I). Sequences containing as few as 12 nucleotides of G-rich overhangs can be recovered even if they are part of a 5-kbp-long artificial telomere.

diploid BJ human foreskin fibroblasts was 33% ± 15% (18 experiments). If fresh biotinylated C-rich oligonucleotide was annealed to the unbound fraction, an additional 10% ± 5% (8 experiments) of the original telomeres could be recovered. Only 2% ± 2% (7 experiments) of the original telomeres were bound following a third cycle of purification. The total recovery following three cycles of purification (33% + 10% + 2% = 45%) suggested that only half of the telomeres might have long G-rich overhangs.

Daughter telomeres do not have similar overhangs

The ability to purify telomeres containing overhangs allowed us to test the hypothesis that the G-rich overhang results from the gap between the last Okazaki priming event of lagging strand synthesis and the end of the chromosome. This model predicts that a blunt end is produced by leading strand synthesis, and thus the newly synthesized G-rich daughter strand would be present on a blunt-ended telomere and would not be purified by techniques that require overhangs (Fig. 2A). The daughter strands in normal diploid BJ human foreskin fibroblasts were labeled by growing cells for zero, one, or four divisions in the presence of 5-bromodeoxyuridine (BrdU). This generated unsubstituted (Thy:Thy), hemisubsituted (Thy:BrdU), and fully substituted (BrdU:BrdU) DNA. The telomeres with long overhangs were then purified, melted, and the BrdU-containing strands recovered using anti-BrdU antibodies. Figure 2B demonstrates that C-rich and G-rich daughter strands were not uniformly distributed among telomeres that have long overhangs. Unsubstituted telomeres (Thy:Thy, containing only thymidine in both strands after zero divisions in BrdU) were not retrieved by the anti-BrdU antibodies, whereas both C-rich and G-rich strands were recovered with equal efficiency from telomeres that had incorporated BrdU into both strands (BrdU:BrdU, after four divisions in BrdU). Mostly C-rich strands were bound by anti-BrdU antibodies in the hemisubstituted telomeres with long overhangs (Thy:BrdU, containing BrdU in only the daughter strand after one division in BrdU), indicating that the G-rich strand was the parental strand (and thus lacked BrdU after one round of DNA synthesis).

Table 1 presents the results from six experiments involving nine different samples of hemisubstituted DNA. On average, 5.4(±2.8) times as much C-rich as G-rich strands were bound by the anti-BrdU antibodies. This bias for BrdU incorporation into the C-strand in telomeres with long overhangs shows that these telomeres represent a population from one and not both ends of the chromosome.

Electron microscopic examination of telomeric overhangs

We measured the length of the telomeric overhang with electron microscopy by visualizing bacteriophage T4

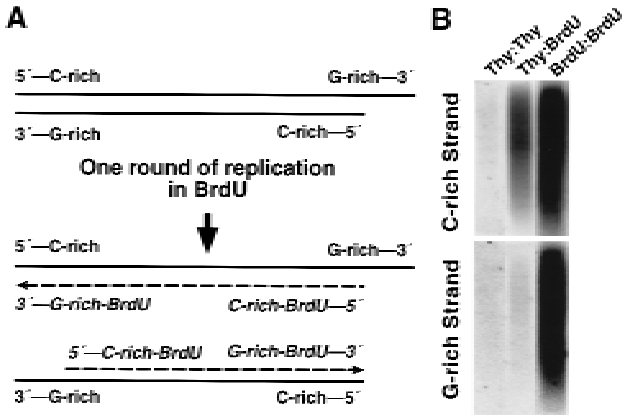

Figure 2. Telomeres with long overhangs contain newly synthesized C-rich daughter strands. (*A*) Schematic model for telomere replication. This model postulates that lagging-strand synthesis leaves a 3′ overhang of the parental G-rich strand. Following one round of replication in BrdU, labeled G-rich strands are present in blunt-ended telomeres, whereas labeled C-rich strands are present in telomeres that have overhangs. (*B*) Retrieval of BrdU-labeled daughter strands. Telomeres from BJ fibroblasts, in which both strands contained thymidine (Thy:Thy), only one strand contained BrdU after a single round of replication (Thy:BrdU), or both strands contained BrdU after four rounds of replication (BrdU:BrdU), were purified using biotinylated C-rich oligonucleotides, melted, and then precipitated with anti-BrdU antibodies. The antibody-bound DNA was then released by boiling in SDS, analyzed on agarose gels, and probed with oligonucleotides specific for each strand. The amount of purified telomeres used in each sample was not identical, as the efficiency of magnetic bead purification varied between experiments. The exposure of each lane has been adjusted to represent equivalent amounts of input telomeres (antibody bound + unbound for each strand). The newly synthesized (BrdU-containing) strand on those telomeres that contained long overhangs was the C-rich and not the G-rich strand.

gene 32 protein (gp 32) bound to single-stranded DNA in tungsten-shadowed preparations. Size standards for the quantitation of the length of single-stranded telomeric overhangs were prepared that contained cloned telomeric repeats as either terminal overhangs or internal single-stranded gaps. Figure 3A shows an example of the images obtained with an internal 450-nucleotide gap, and Figure 3B shows the linear relationship between the length of the gp32-coated region and the number of nucleotides for both internal and terminal single-stranded regions. Tracings of the protein-coated region yielded a value of 0.42 ± 0.02 nm/nucleotide, very close to previously published reports of 0.46 nm/nucleotide (Delius et al. 1972; Wu and Davidson 1975). Analysis of overhang-containing BJ fibroblast telomeres coated with T4 gp32 (Fig. 3C) yielded an average single-stranded overhang length of 200 ± 75 nucleotides (Fig. 3D). This is an order of magnitude greater than that found in organisms such as ciliates and yeast (Klobutcher et al. 1981; Wellinger et al. 1993) and consistent with recent estimates for human telomeres based on indirect biochemical techniques (Makarov et al. 1997; McEllingott and Wellinger 1997).

Telomere shortening in fibroblasts

Models of chromosome replication in the absence of telomerase in which only one telomere contains a long G-rich overhang predict that the size of the overhang should be four times the amount of shortening per doubling (Fig. 4, step 1). BJ fibroblast telomeres shorten by

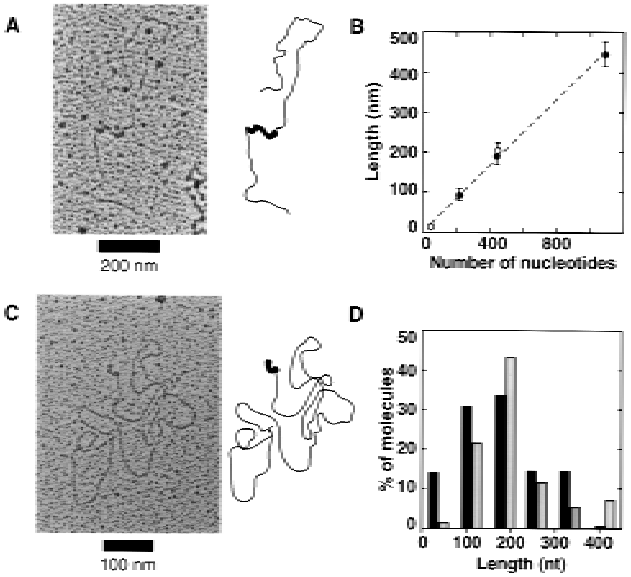

Figure 3. T4 gp32 decoration of single-stranded DNA and telomeric overhangs. (*A*) The single-stranded region of linearized plasmid DNA containing a 450-nucleotide gap of single-stranded telomeric repeats was decorated with the single-strand binding T4 gp32. Initial magnification, 100,000×. (*B*) Linear relationship between size standards and measured lengths. Different lengths of single-stranded gaps (●) or overhangs (○) coated with gp32 were examined. The 48- and 450-nucleotide single-stranded regions contained G-rich telomeric repeats; the 200- and 1000-nucleotide gaps contained plasmid sequences. Except for the 48-nucleotide overhang, 40–70 molecules of each type were examined. It was difficult to distinguish the very short decorated region from background for the 48-nucleotide overhang sample, and we consider 50 nucleotides of overhang to be the limit of detection for this technique. Error bars indicate 1 standard deviation (S.D.). (*C*) Purified BJ fibroblast telomere decorated with gp32. Initial magnification, 25,000×. (*D*) Histogram of the length of the gp32-decorated regions of purified telomeres from BJ fibroblast DNA. A total of 108 and 69 molecules from population doubling level (PDL) 20 (solid bar) and 87 (shaded bar) were examined. Between 70% and 80% of the molecules purified on the basis of having G-rich overhangs had one decorated end. The remaining undecorated molecules may represent fragments of broken telomeres. None of the telomeres was decorated at both ends. Average overhang lengths (±S.D.) were 157 ± 69 nucleotides for PDL20 and 226 ± 88 nucleotides for PDL87 fibroblasts. A higher background of free T4 gp32 in the PDL87 preparation may have compromised our ability to detect the shortest overhangs in that sample.

~50 bp per division in culture (Fig. 5), which is one-fourth of the 200-nucleotide overhang we observed. Models for telomere shortening in which nuclease processing generates symmetrical overhangs at both ends of the chromosome predict that the size of the overhang should be twice the rate of shortening per division (Fig. 4, step 2), which is not supported by our data.

Discussion

To our knowledge, the only rigorous evidence for symmetrical overhangs on both ends of a chromosome comes from studies of hypotrichous ciliates, in which the small and well-defined telomere length has permitted the direct sequencing of both telomeric strands of

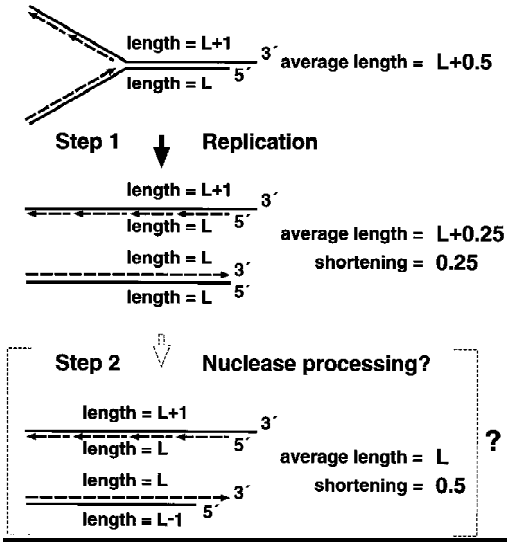

Figure 4. Telomere size changes after chromosome replication in the absence of telomerase. A replication fork is shown proceeding into a telomere of length L with a 3′ G-rich overhang 1 unit long. The size of the telomere before replication is the average of the two strands, which is [(L + 1) + L] ÷ 2 = L + 0.5 units. Lagging strand synthesis is illustrated as a series of discrete Okazaki fragments that would be joined together to form a continuous strand. Following replication, lagging strand synthesis would leave a long 3′ overhang; leading strand synthesis would generate a blunt end. After replication is complete (step 1) the average size of the four strands would be [(L + 1) + L + L + L] ÷ 4 = L + 0.25 units. The net shortening after replication would be (L + 0.5) − (L + 0.25) = 0.25, implying that the rate of telomere shortening should be one-quarter of the length of the G-rich overhang. A recent model (Makarov et al. 1997) has been postulated, in which extensive nuclease processing produces symmetrical long overhangs (step 2). If this were to occur, the average size of the four strands would be [(L + 1) + L + L + (L − 1)] ÷ 4 = L. The net shortening after replication and processing would thus be (L + 0.5) − L = 0.5, suggesting that the rate of telomere shortening in the absence of telomerase should be one-half the length of the G-rich overhang. Our data indicate that the rate of shortening (50 bp/division) is one quarter the length of the overhang (200 ± 75 nucleotides) in BJ fibroblasts.

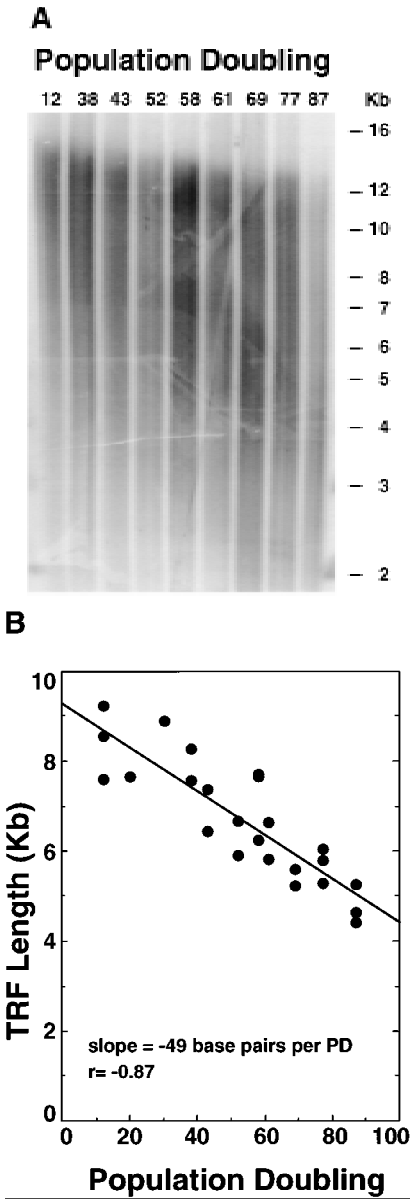

Figure 5. Telomere shortening in BJ fibroblasts. (*A*) Terminal restriction fragment (TRF) gel of DNA from BJ foreskin fibroblasts at different mean population doubling levels. (*B*) Rate of telomere shortening. Data from three different TRF gels using DNA prepared from two different lifespan studies are shown. The average rate of shortening was 49 bp/population doubling.

end-labeled total DNA (Klobutcher et al. 1981). The larger size and variable length of telomeres in other organisms has prevented a comparable analysis of their telomeres. The data in yeast showing the temporary circularization of linear plasmids owing to the presence of transient overhangs demonstrates that overhangs might be present on both ends of some of the chromosomes some of the time but does not directly address whether or not the overhangs are symmetrical or uniform. Our data show that the presence of long overhangs on only

one end of the chromosome in normal human fibroblasts differs from the symmetrical ends found in the hypotrichous ciliates.

Anti-BrdU antibodies recognized the C-rich but not the G-rich strand in telomeres purified based on their long overhangs from cells after a single cycle of DNA replication. This demonstrates that these telomeres contain a daughter strand synthesized from the G-rich telomeric template. Yeast origins of replication are internal to the telomere (Raghuraman et al. 1997). Assuming that the origins for telomere replication are also internal in vertebrates, our results show that long overhangs are present in telomeres that are produced by lagging-strand synthesis.

The 200 ± 75-nucleotide overhang we observed in BJ fibroblasts is sufficient to fully explain the rate of telomere shortening of 50 bp per division found in these cells using calculations based on models in which the telomere at one end of the chromosome is blunt and the one at the other end has a long G-rich overhang. These observations do not support the hypothesis that the rate of shortening represents the average of a few telomeres, which suffer oxidative damage and lose kilobases of telomeric sequences, and the majority of telomeres, which lose only very few nucleotides because of short unreplicated overhangs (von Zglinicki et al. 1995).

The biotinylated C-rich oliogonucleotides we have used are unable to bind single-stranded G-rich overhangs of less than ~12 nucleotides with sufficient stability to recover artificial telomeres 5 kbp long. We are thus unable to determine whether the telomeres resistant to purification are blunt ended or have very short overhangs. The presence of short overhangs would be consistent with the concept that proteins recognizing a single-stranded overhang might be required to cap the telomeres and prevent them from being degraded (Gottschling and Cech 1984; Gottschling and Zakian 1986). The BrdU labeling data does not exclude the possibility that leading and lagging strands might be processed differently, so that only one end is packaged into a G-quartet-like structure that is stable to the DNA isolation procedure and resistant to hybridization to the biotinylated C-rich oligonucleotides. Under this circumstance, one end might have a long but inaccessible G-rich overhang. However, the relationship between the measured length of the overhang and the observed rate of shortening (Figs. 3–5) argues against long overhangs on both daughter telomeres.

Makarov et al. (1997) recently reported that human telomeres contain long G tails at both ends of the chromosome. The strand-replacement technique (PENT) that these workers used to detect G-rich overhangs does not distinguish between long and short overhangs, and thus their conclusion that human cells contain symmetrical overhangs is not justified. In addition, their claim that 85% of human telomeres have G-rich overhangs may be in error. This assertion is entirely dependent on PhosphorImager scans of the relative intensities of three bands: full-length C-rich strands (C_o), newly synthesized replacement strands primed from the G-rich overhang (C_s), and the original C-strand being trimmed back as replacement synthesis progresses (C_t). Their quantitation showed that the intensity of C_o was ~15% of the sum of $C_s + C_t$, and they thus concluded that 85% of the telomeres had overhangs. However, their data (Fig. 5 in Makarov et al. 1997) indicate that they underestimated the amount of large DNA present. For example, even assuming that a 12.5-kb telomere digested with *Hin*fI contains 2.5 kb of subtelomeric DNA that lacks repeats (Levy et al. 1992), the number of telomeric repeats in a 12.5-kb C_o and a 10-kb C_t strand should be approximately three times the number in a 2.5-kb C_s strand. Rather than finding a threefold greater signal intensity in the large DNA probed with the G-rich telomeric repeat, their data show an approximately equal intensity of the small 2.5-kb C_s strand. Their quantitation of the fraction of telomeres that lacked available overhangs and were resistant to replacement synthesis (the fraction that was the C_o strand) could thus be off by a factor of three or more. We suspect that these results may be explained by an inefficient transfer of large DNA to the membrane in the vacuum blotting procedure they employed.

Much recent evidence implicates single-strand nucleases in telomere processing in telomerase-expressing model organisms (Wellinger et al. 1993, 1996; Garvik et al. 1995; Linger et al. 1995; Nugent et al. 1996), raising the possibility that human telomeres might be processed to have uniform G-rich overhangs. The data in the present report provide direct evidence that this is not the case in a telomerase-negative cell strain. Leading and lagging strand synthesis results in distinct telomeric structures in normal diploid human fibroblasts. Our results do not formally exclude the possibility that asymmetric overhangs could be generated by nuclease activity. We consider this unlikely, as an additional mechanism to restrict the digestion to the newly synthesized C-rich strand and not the parental C-rich strand would be required.

The presence of telomerase activity in most human tumors has generated much excitement concerning the potential efficacy of anti-telomerase therapies for the treatment of cancer. Understanding what regulates the rate of telomere shortening and how to manipulate it may provide the tools to increase the effectiveness of telomerase inhibitors in preventing the regrowth of tumor cells.

Materials and methods

Purification of telomeres

Double-stranded genomic DNA was digested overnight in 0.25 U/µg of *Hin*fI to free the telomeric repeats from most of the subtelomeric sequences. In a typical experiment, 30 µg of DNA in a final volume of 30 µl was then adjusted to 1× SSC/1% Triton X-100, mixed with 1 pmole of a biotinylated oligonucleotide, annealed for 15 min each at 65°C, 55°C, 45°C, 35°C, and room temperature, and combined with 3 µl of washed streptavidin-coated magnetic beads (10 mg/ml suspension, Dynal Inc.) that had been preincubated for at least 1 hr in 5× Denhardt's solution. The DNA–bead suspension was rotated end over end

at 2 rpm and 4°C overnight. The magnetic beads were drawn to the side of the tubes using a rare earth magnet (Edmund Scientific), and the supernatant removed and saved. The beads were resuspended and washed twice with 100 µl of 1× SSC/1% Triton X-100. The bound telomeres were eluted from the beads by melting the oligonucleotide/telomere interaction at 65°C for 10 min in 30 µl of 0.1× SSC/1% Triton X-100.

The fraction of telomeres recovered [bound ÷ (bound + unbound)] was quantitated from PhosphorImager scans of agarose gels probed with labeled $(CCCTAA)_4$ oligonucleotide. In some cases, the unbound fraction was subjected to additional cycles of purification. The percent of telomeres recovered during each cycle of binding was normalized to the original amount of input telomeres. For example, if 35% of the telomeres were recovered during cycle one, then only 65% of the telomeres would be left as the input to cycle two. A recovery of 20% of these telomeres would then represent 13% (0.2 × 0.65 = 0.13) of the original telomeres.

Technical points in this protocol include the following considerations. Forty micrograms of human DNA contains approximately 1 fmole of telomeres, so that 1 pmole oligonucleotide is in vast excess for even relatively long overhangs. One microliter of magnetic beads can bind at least 1 pmole of biotinylated oligonucleotide as measured by its ability to retrieve radioactive $(TTAGGG)_4$ oligonucleotide annealed to the biotinylated CCCTAA oligonucleotides. Although the rate of formation of magnetic bead/small oligonucleotide complexes is very rapid (1-µl beads can clear 1 pmole of oligonucleotide from 1 ml in ~15 min), the rate of binding to the large telomere/oligonucleotide hybrids is very slow. The amount of telomeres bound to 1 pmole of biotinylated oligonucleotide and 3 µl of beads after an overnight rotation in 30 µl was about twice as great as when 1 µl of beads was used, with a minimal increase observed using 10 µl of beads. Three microliters of magnetic beads was used, as larger amounts increased background binding slightly.

Artificial telomeres

Large DNA fragments with single-stranded G-rich overhangs were constructed by ligating variable numbers of telomeric sequences to a linearized plasmid. Single-stranded ladders of TTAGGG repeats were produced by the asymmetric PCR amplification of a cloned 450-bp telomeric insert using [α-^{32}P]dGTP and ddG. The ~100 bp of plasmid sequences between the forward primer and the start of the telomeric repeats was then made double-stranded, digested with *Sal*I, and ligated to a 5-kbp plasmid digested with *Xho*I. These enzymes have compatible ends. The resulting artificial telomeres were then gel purified and tested for their ability to bind biotinylated C-rich sequences.

Anti-BrdU precipitation of telomeres

Fibroblasts were grown in 30 µM BrdU for 20 hr (less than one doubling) to produce hemisubstituted DNA and for 1 week in 8 µM BrdU to produce DNA labeled in both strands. Deoxycytidine (200 µM) was included under both conditions to reduce toxic effects of BrdU. Purified telomeres from 10 µg of total genomic DNA were melted at 99°C for 3 min, quick chilled, and incubated with 5 ng of anti-BrdU antibody (Becton-Dickinson) for 60 min at room temperature in 25 µl of PBS containing 1% Triton X-100. Antibody–DNA complexes were then recovered following a 1-hr incubation with 15 µl of protein–G agarose beads (Boehringer Mannheim) and washed twice for 15 min each in PBS/1% Triton X-100. The DNA was then eluted from the beads at 99°C for 3 min in TE buffer (10 mM Tris, 1 mM EDTA at pH 8) containing 1% SDS. Input, bound, and unbound fractions were analyzed on duplicate agarose gels probed with either labeled C-rich or G-rich telomeric probes. Approximately 75% of the input telomeres were recovered (anti-BrdU bound + unbound) for each strand. The fraction of each strand bound by anti-BrdU antibodies was determined from PhosphorImager scans of each lane using the formula % Retrieved = {anti-BrdU bound ÷ (bound + unbound)} × 100.

Electron microscopy

Gapped linear DNA (100 ng) or telomeric DNA (140 ng) was incubated with 200 ng of T4 gene 32 protein (Delius et al. 1972; Wu and Davidson 1975) for 5 min at room temperature in 50 µl of 10 mM HEPES (pH 7.5), 100 mM NaCl, and 2.5 mM $MgCl_2$. Glutaraldehyde (Sigma) was added to a final concentration of 0.1% and incubated for 5 min at room temperature. The cross-linking reaction was quenched by the addition of an equal volume of 10 mM Tris-HCl and 1 mM EDTA. DNA was suspended in a buffer containing 2.5 mM spermidine and applied to glow-discharged, thin carbon films supported on copper grids (Griffith and Christiansen 1978), rinsed twice in double-distilled water, dehydrated in a graded series of ethanol solutions, briefly stained with 0.1 mM uranyl acetate in 90% ethanol, and air-dried. Samples were rotary shadowcast at an angle of 7° using evaporated tungsten wire in a vacuum evaporator cryopumped to $<10^{-6}$ Torr. Size standards were prepared by annealing single-stranded phagemid containing different numbers of telomeric repeats to the complementary strand of the plasmid vector backbone. This hybrid DNA was then restriction digested to produce linear DNA molecules containing known lengths of either internal or terminal regions of single-stranded telomeric repeats.

Terminal restriction fragment analysis

DNAs isolated from cells at different population doublings throughout their cultured lifespan were digested with a mixture of six restriction enzymes (*Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Msp*I, and *Rsa*I; Rogalla et al. 1994), analyzed on 0.5% agarose gels, and probed with a labeled $(CCCTAA)_4$ probe. Signal intensity is proportional to the number of telomeric repeats using this probe. Average telomere length was determined from PhosphorImager scans after normalizing for this effect using the formula Σ(Intensity) ÷ Σ(Intensity ÷ Length) (Harley et al. 1990).

Acknowledgments

This work was supported by grants from the National Institutes of Health (NIH) (to W.E.W. and S.D.L), the AlliedSignal corporation (W.E.W. and J.W.S.), and the Welch Foundation (W.E.W.). V.M.T. was funded in part by an NIH Oncology Training Grant. We thank Jennifer Cuthbert for valuable scientific discussions, and Ia Dac-Korytko and Martha Liao for technical support.

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Normal human chromosomes have long G-rich telomeric overhangs at one end

Woodring E. Wright, Valerie M. Tesmer, Kenneth E. Huffman, et al.

Genes Dev. 1997, **11:**
Access the most recent version at doi:10.1101/gad.11.21.2801