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The terminal DNA structure of mammalian chromosomes

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In virtually all eukaryotic organisms, telomeric DNA is composed of a variable number of short direct repeats. While the primary sequence of telomeric repeats has been determined for a great variety of species, the actual physical DNA structure at the ends of a bona fide metazoan chromosome with a centromere is unknown. It is shown here that an overhang of the strand forming the 3′ ends of the chromosomes, the G-rich strand, is found at mammalian chromosome ends. Moreover, on at least some telomeres, the overhangs are ≥ 45 bases long. Such surprisingly long overhangs were present on chromosomes derived from fully transformed tissue culture cells and normal G_0-arrested peripheral leukocytes. Thus, irrespective of whether the cells were actively dividing or arrested, a very similar terminal DNA arrangement was found. These data suggest that the ends of mammalian and possibly all vertebrate chromosomes consist of an overhang of the G-rich strand and that these overhangs may be considerably larger than previously anticipated.
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Introduction

The ends of eukaryotic chromosomes have special properties when compared with ends created by random breakage. Native ends, or telomeres, are stable structures not prone to degradation, recombination or fusions with other chromosome ends (Muller, 1938; McClintock, 1939). Furthermore, functional telomeres are not recognized by DNA damage recognition systems even though formally, a chromosomal end constitutes a double-stranded DNA break (Sandell and Zakian, 1993). These properties of eukaryotic telomeres are usually identified as the essential 'chromosome-capping' function of telomeres (for reviews, see Blackburn, 1994; Zakian, 1995; Greider, 1996). A second essential function of the telomeres stems from the fact that a DNA-end cannot be completely duplicated by the conventional DNA replication machinery (Olovnikov, 1971; Watson, 1972). Rather, telomeric sequences are replenished by telomerase, a ribonucleoprotein that uses a sequence within its associated RNA as template (Greider and Blackburn, 1985; Yu *et al.*, 1990; for reviews, see Blackburn and Greider, 1995; Greider, 1996).

Both of these essential functions of the telomeres are intimately related to the DNA sequences and structures at the chromosomal termini. Telomeric DNA of virtually all eukaryotic organisms consists of short, tandemly repeated sequences (Blackburn and Greider, 1995). The primary sequence and organization of these repeats from many species are clearly related as the repeats usually contain clusters of three or more G residues and the strand containing these clusters, hereafter called the G-rich strand, always forms the 3′-end of the chromosome (Blackburn and Greider, 1995). The number of these repeats at chromosomal ends varies greatly between different organisms and also between different telomeres of the same organism. For all vertebrates, including humans, the repeat is d(TTAGGG)$_n$ (Moyzis *et al.*, 1988; Meyne *et al.*, 1989) and individual telomeres may contain a few kilobases (kb), such as in some transformed human cell lines, or up to >100 kb, such as in some mouse cells, of the repeats in double-stranded form (de Lange *et al.*, 1990; Kipling and Cooke, 1990; Starling *et al.*, 1990). Most of this DNA is organized in nucleosomes and only the most distal portion could be in a special, non-nucleosomal chromatin conformation that may be analogous to the special structure found at yeast or *Tetrahymena* telomeres (Blackburn and Chiou, 1981; Wright *et al.*, 1992; Tommerup *et al.*, 1994; Lejnine *et al.*, 1995).

However, virtually nothing is known about the actual DNA configuration at the very ends of the chromosomes of any plant, fungal or metazoan species. The ends of the acentric DNA molecules contained in the macronucleus of protozoan ciliates such as *Oxytricha* and *Euplotes* are formed by a 12- to 14-base overhang of the G-rich strand over the C-rich strand (Klobutcher *et al.*, 1981). End-labeling studies of linear rDNA molecules isolated from *Tetrahymena* and *Didymium* also suggested a similar end structure with a 12- to 16-base overhang of the G-rich strand (Henderson and Blackburn, 1989). Transient and larger extensions of the G-rich strand have been demonstrated to occur during telomere replication in yeast (Wellinger *et al.*, 1993a,b) and during *de novo* telomere formation in *Euplotes* (Roth and Prescott, 1985; Vermeesch and Price, 1994). These long, single-stranded tails are the predicted products of telomerase-mediated telomere repeat addition, although other mechanisms also contribute to their formation (Dionne and Wellinger, 1996; Wellinger *et al.*, 1996). It is thought that the polymerase α/primase complex is filling in these transient single-stranded DNA extensions and this mechanism could yield a telomere structure with a short overhang of the G-rich strand (Zahler and Prescott, 1989; Greider, 1996).

Indirect evidence for the nature of the DNA structure required at the ends of the chromosomes is derived from the analysis of terminus-specific proteins. Telomere end-binding proteins have been isolated and characterized

from ciliate species such as *Oxytricha* and *Euplotes* (Gottschling and Zakian, 1986; Price and Cech, 1989; Price, 1990) and more recently, similar activities have been identified in *Tetrahymena*, *Xenopus* and yeast (Cardenas *et al.*, 1993; Sheng *et al.*, 1995; Lin and Zakian, 1996; Nugent *et al.*, 1996). These activities bind specifically to single-stranded DNA probes comprising the G-rich strand of the species-specific telomeric repeats and most require an unpaired, single-stranded 3′-end (Gottschling and Zakian, 1986; Cardenas *et al.*, 1993; Sheng *et al.*, 1995). Furthermore, the proteins from the hypotrichous ciliates can, when reconstituted with DNA molecules resembling the natural termini of these species, protect the DNA from chemical modification and nuclease digestion (Gottschling and Zakian, 1986; Price and Cech, 1989; Raghuraman *et al.*, 1989; Price, 1990; Gray *et al.*, 1991). Thus, it has been postulated that a telomeric DNA end-structure comprising a short overhang of the G-rich strand over the C-rich strand is conserved among the eukaryotes, and that this structure is required for the association of terminus-specific proteins forming the 'cap' (Henderson and Blackburn, 1989; Lingner *et al.*, 1995).

In order to gain a more detailed knowledge of the terminal DNA structure of mammalian chromosomes, we analyzed human and mouse telomeres using indirect labeling studies and a primer-extension protocol. The data demonstrate that, in all the cell types studied, overhangs of the G-rich strand over the C-rich strand can be detected. At least on some telomeres derived from non-dividing human peripheral leukocytes and on telomeres derived from HeLa cells, overhangs of $\geqslant 45$ bases were found. Thus, our results confirm the current model for the terminal DNA structure for mammals and furthermore demonstrate that the extensions of the G-rich strand are considerably longer than previously anticipated.

Results

Detection of single-stranded extensions on the G-rich strand of human chromosomes

In order to probe the terminal DNA structure of mammalian telomeres, a sensitive non-denaturing in-gel hybridization method was developed (Dionne and Wellinger, 1996). The advantages of this method are that terminal restriction fragments (TRFs) of virtually any size can be analyzed, relatively small probes can be used, background hybridization to double-stranded DNA is minimal, and the DNA can be denatured after the first hybridization to detect total, double-stranded DNA (Dionne and Wellinger, 1996, and see below). Thus, while 5 pg of single-stranded DNA containing vertebrate telomeric repeats are readily detected, even 20 ng of the same DNA in double-stranded form does not yield any signal, indicating a specificity of $>$1000-fold for single-stranded DNA over double-stranded DNA (Dionne and Wellinger, 1996, and data not shown).

Using this technique, we first analyzed the TRFs derived from HeLa cells, a fully transformed human cell line that contains telomerase activity (Morin, 1989). We reasoned that, irrespective of the constitutive structure of the DNA termini, if telomere replication proceeded via a transient appearance of single-stranded G-rich strands as was shown in yeast cells (Wellinger *et al.*, 1993a,b; Dionne and Wellinger, 1996), we could detect at least those extensions on the telomeres of actively dividing cells. When the DNA from HeLa cells was subjected to the non-denaturing in gel analysis, hybridization to an end-labeled $d(CCCTAA)_3$ probe revealed a smeary signal in the area corresponding to DNA molecules of 2–4 kb (Figure 1A, lane 4). No signal was detected when the DNA was pretreated with *Escherichia coli* exonuclease-I prior to TRF analysis (Figure 1A, lane 5). This enzyme is a single-strand-specific exonuclease that degrades DNA from the 3′-end with very little single-strand DNA endonuclease activity (Lehmann and Nussbaum, 1964). Thus, a mixed-in single-stranded circular M13 DNA was not susceptible to the enzyme treatment (Figure 1A; EtBr-stained gel). However, when the DNA in the gel was denatured and the gel reprobed with the same probe, the smear migrating at 2–4 kb remained detectable in both, exonuclease I-treated and non-treated DNA, indicating that the bulk of the DNA was not affected by the exonuclease digestion. In addition, DNA fragments of specific sizes were detected following DNA denaturation (Figure 1A, lanes 6 and 7). These fragments indicate internal blocks of double-stranded telomeric sequences present in the genome of the HeLa cells used here. When HeLa DNA was hybridized to an oligo derived from opposite strand, $d(TTAGGG)_3$, hybridization to genomic DNA was only detected once the DNA had been denatured (Figure 1B, lanes 2 and 5). Treating the DNA with BAL31, an exonuclease that degrades DNA from a double-stranded end inwards and that was used extensively to analyze the terminal location of telomeric sequences (Yao and Yao, 1981; Moyzis *et al.*, 1988), demonstrated that the smeary signal migrating between 2–4 kb corresponded to the TRFs in the HeLa cell line used here (Figure 1C, lanes 3–5). Note that after 5 min of BAL31 digestion, the TRFs were shortened by ~1 kb and single-stranded terminal extensions were no longer detected (Figure 1C, lane 3).

These results indicate that at least some telomeres of actively growing HeLa cells carry single-stranded extensions of the G-rich strand. Overhangs of $<$12 bases of either strand would not have been detected in our experiments (data not shown). Therefore, we cannot exclude the possibility that some telomeres may have very short overhangs ($<$12 bases) or are blunt-ended.

Strand-specific nicks have been reported to occur in the C-rich strand of the terminal telomeric repeats of *Tetrahymena* (Blackburn and Gall, 1978). If such nicks would also occur on mammalian telomeric repeat tracts, they would create stretches of unligated repeats of the C-rich strand that could dissociate from the G-rich strand during DNA isolation. This would lead to artifactually extended G-rich strands. In an attempt to test for this possibility, end-labeled oligonucleotide STRD2 was annealed to oligonucleotide STRD1 (see Materials and methods and Figure 5 for the structure of the oligonucleotides) and then mixed with HeLa cells at the time of cell disruption or incubated without cells. DNA isolation was carried out as before and the DNA obtained was analyzed by native polyacrylamide gel electrophoresis. The results indicated that: (i) about the same amount of input oligomer was recovered from the mock isolation as was with from the isolation with the HeLa cells; and (ii) the oligonucleotides recovered with the total HeLa genomic DNA were in the annealed input form (data not shown).

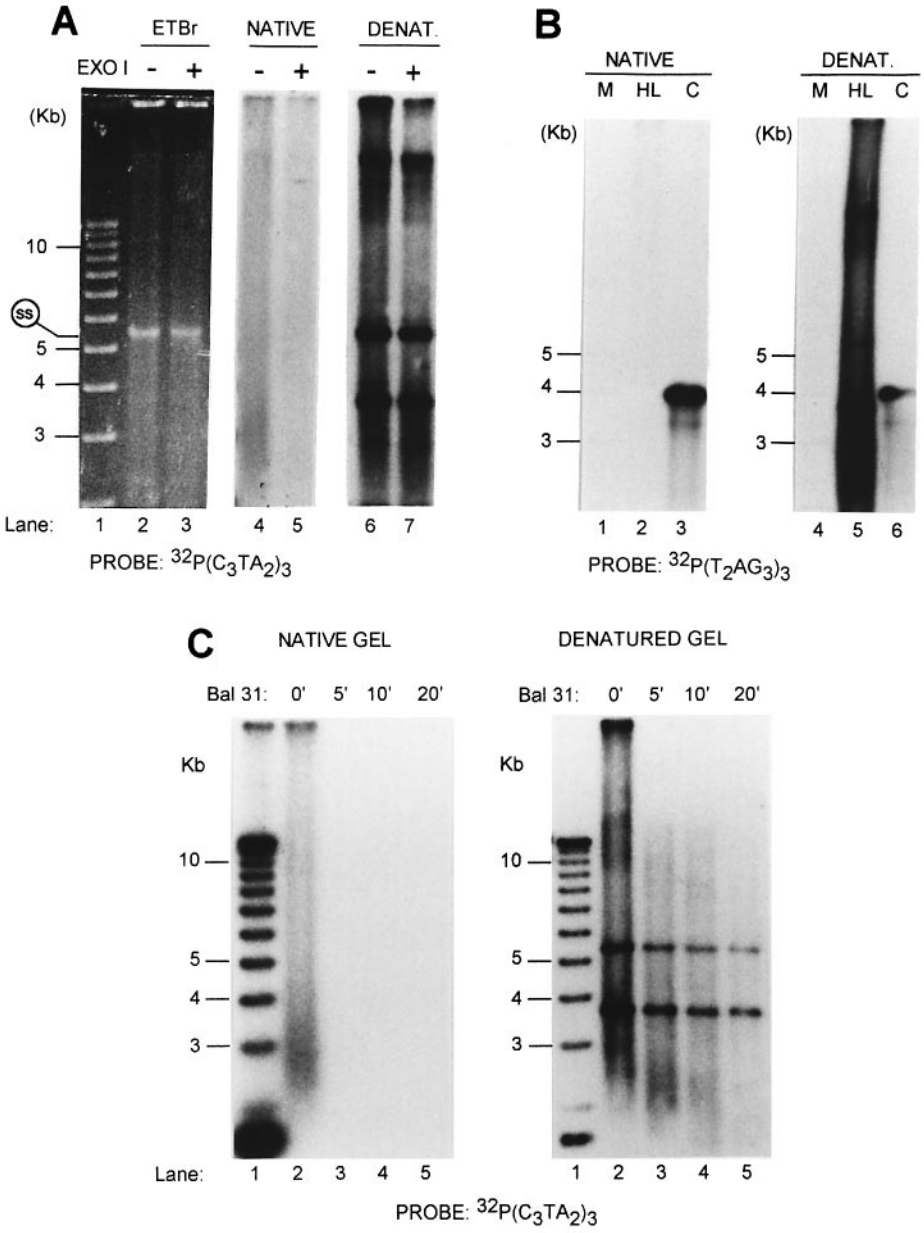

Fig. 1. Detection of single-stranded extensions on the G-rich strand of HeLa telomeres. (**A**) 5 µg of total genomic DNA was treated with *E.coli* exonuclease-I (lanes 3, 5 and 7; marked +) or mock-treated (lanes 2, 4 and 6; marked –), then digested with *Hin*fI and *Rsa*I and analyzed by agarose gel electrophoresis. The band visible in the ethidium bromide-stained gel and migrating at ~5.6 kb is mixed in single-stranded circular M13 DNA and served to show that the exonuclease had no single-strand endonuclease activity. The gel was first hybridized to an end-labeled $d(CCCTAA)_3$ probe without denaturing the DNA (Dionne and Wellinger, 1996, native gel). After the appropriate exposures had been obtained, the DNA was denatured in the gel and the gel rehybridized to the same probe (DENAT.). (**B**) 5 µg of genomic HeLa DNA was treated in the same way as in (A) except that an end-labeled probe of the opposite strand for the telomeric repeats, $d(TTAGGG)_3$, was used. Lane 1 (M): end-labeled DNA size marker; lane 2 (HL): HeLa DNA; lane 3 (C), 1 ng of linearized and denatured p16R DNA was loaded as positive control. (**C**) BAL31 treatment of the DNA. 10 µg of genomic HeLa DNA was treated with BAL31 for the times indicated, then digested with *Hin*fI and *Rsa*I and analyzed by non-denaturing in-gel hybridization to the telomeric $d(CCCTAA)_3$ probe (left panel, NATIVE GEL). The DNA was subsequently denatured in the gel and rehybridized to the same probe (right panel, DENATURED GEL). Lane 1: end-labeled DNA size marker; lane 2: mock treated DNA; lanes 3–5: DNA was digested with the BAL31 nuclease for 5, 10 and 20 min, respectively.

These data demonstrated that oligonucleotides of $\geqslant$17 bases stay associated in double-stranded form throughout the DNA isolation procedure, consistent with previous results obtained with *Tetrahymena* (Henderson and Blackburn, 1989) and yeast cells arrested in G_1 or in G_2/M phases of the cell cycle (Wellinger *et al.*, 1993b; Dionne and Wellinger, 1996). However, formally we cannot exclude that the strand-specific nicks occurred much more closely spaced than was previously shown for the rDNA of *Tetrahymena* (Blackburn and Gall, 1978), yielding much shorter (<15 bases) unligated oligonucleotides of the C-rich strand.

Detection of the extensions is not dependent on the presence of telomerase activity

There is mounting evidence that there is no or insufficient telomerase activity to maintain the lengths of the terminal repeat tracts in most somatic human tissue cells (Harley

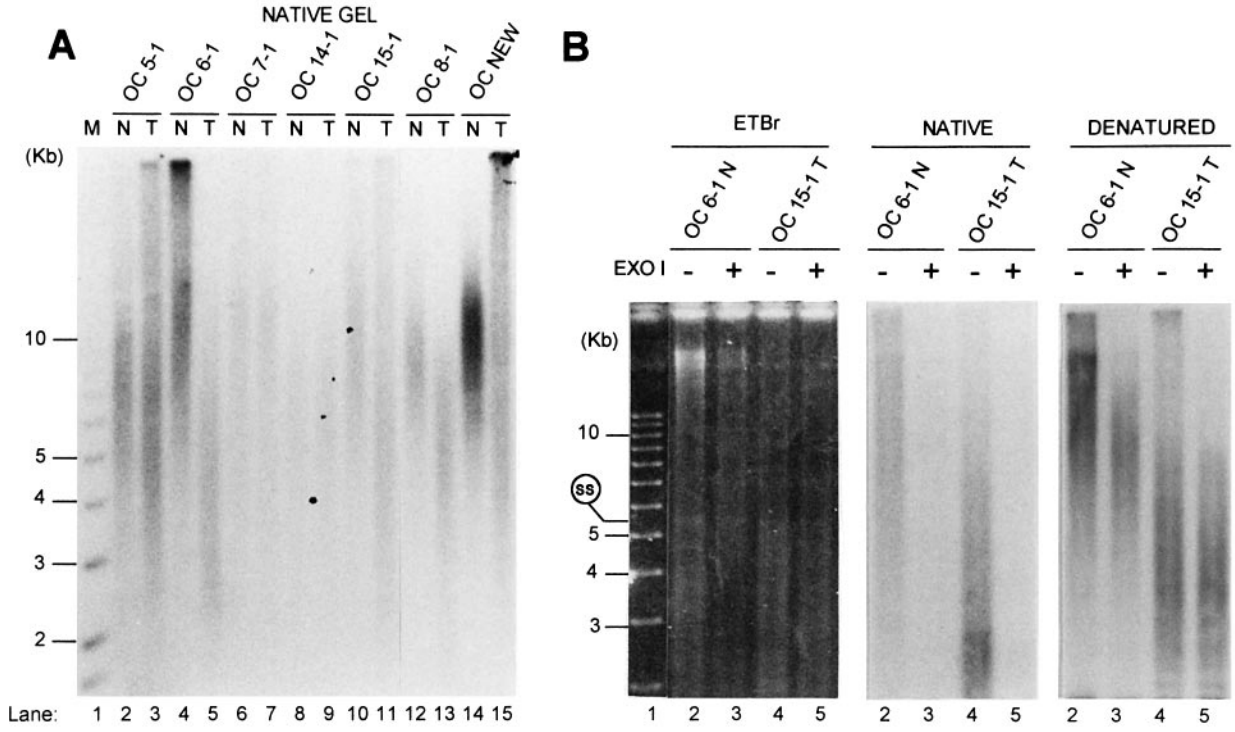

Fig. 2. Overhangs of the G-rich strand are present on telomeres derived from telomerase-positive and telomerase-negative cells. (**A**) DNAs derived from human ovarian carcinoma samples and adjacent normal cells were obtained from C.Counter and S.Bacchetti (see Counter *et al.*, 1994 for description and telomerase assays). The DNAs were digested and analyzed by native in-gel hybridization using the $d(CCCTAA)_3$ as probe as described in Figure 1A. Lane 1 (M): end-labeled DNA size marker. Lanes 2, 4, 6, 8, 10, 12 and 14 (labeled N at top): DNAs derived from normal, telomerase-negative cells. Lanes 3, 5, 7, 9, 11, 13 and 15 (labeled T at top): DNAs derived from tumor cells in which telomerase was active. Above each pair, the actual biopsy identification is indicated (Counter *et al.*, 1994). Upon rehybridization of the same gel after the DNA had been denatured, an indistinguishable pattern of hybridization signals was obtained. Probing the DNA with the $d(TTAGGG)_3$ oligo under native conditions did not yield any signal (data not shown). (**B**) A representative DNA sample derived from normal cells (OC 6-1 N, lanes 2 and 3) and a representative sample derived from a tumor (OC 15-1 T, lanes 4 and 5) were treated with *E.coli* exonuclease-I (lanes 3 and 5; marked +) or mock-treated (lanes 2 and 4; marked –) and analyzed as described in Figure 1A.

et al., 1990; Hastie *et al.*, 1990; Allsopp *et al.*, 1992; Counter *et al.*, 1992). However, cells derived from many tumor biopsies have readily detectable telomerase activity and in these cells, the terminal repeat tracts remain relatively stable (Counter *et al.*, 1992, 1994; Kim *et al.*, 1994). Thus, differences in DNA structure may exist between cells expressing active telomerase and cells lacking telomerase activity. When DNA isolated from ovarian carcinoma cells containing telomerase activity was analyzed by the native in-gel hybridization and compared with DNA derived from adjacent normal tissue lacking telomerase activity (Counter *et al.*, 1994), no significant difference was found (Figure 2A). The signals observed in the native gel generally correlated with the signal intensities in the denatured gel and minor differences may reflect DNA loading differences (data not shown). Furthermore, control experiments performed with a probe of the opposite strand did not detect any single-stranded repeats of the C-rich strand (data not shown). Because treatment of the DNA with *E.coli* exonuclease-I eliminated the signal (Figure 2B), we conclude that terminal extensions of the G-rich strand exist at the ends of chromosomes, irrespective of whether the cells contained telomerase or not.

Extensions are detected in non-replicating, normal human cells

Since in yeast, long extensions of the G-rich strands are only observed in S-phase (Wellinger *et al.*, 1993a,b; Dionne and Wellinger, 1996), it was possible that we were detecting only telomere replication intermediates with long extensions that were produced during S-phase. Human peripheral leukocyte populations are quiescent, generally containing <2% dividing cells (Buchkovich and Greider, 1996). Thus, in order to minimize the fraction of possible replication intermediates in our preparations, DNA was isolated directly from peripheral leukocyte cells, digested with restriction enzymes and analyzed as described above. Again, an exonuclease-I-sensitive smeary signal corresponding to 9–12 kb in size was observed (Figure 3). We conclude that in human cells, extensions of the G-rich strand occur in a cell cycle-independent manner and therefore are part of the constitutive DNA structure at chromosome ends. In addition, extensions of the G-rich strands were detected in DNA derived from a transformed mouse cell line (data not shown), suggesting that such extensions are a conserved feature of mammalian chromosomes.

The G-strand extensions are longer than 30 bases and some are at least 45 bases long

In order to obtain an estimate of the lengths of the observed overhangs, native in-gel hybridizations with increasing stringencies were performed. Four identical gels each containing 10 μg of restriction-digested HeLa DNA were hybridized to C-strand oligonucleotide probes of increasing lengths (Figure 4). After hybridization, the

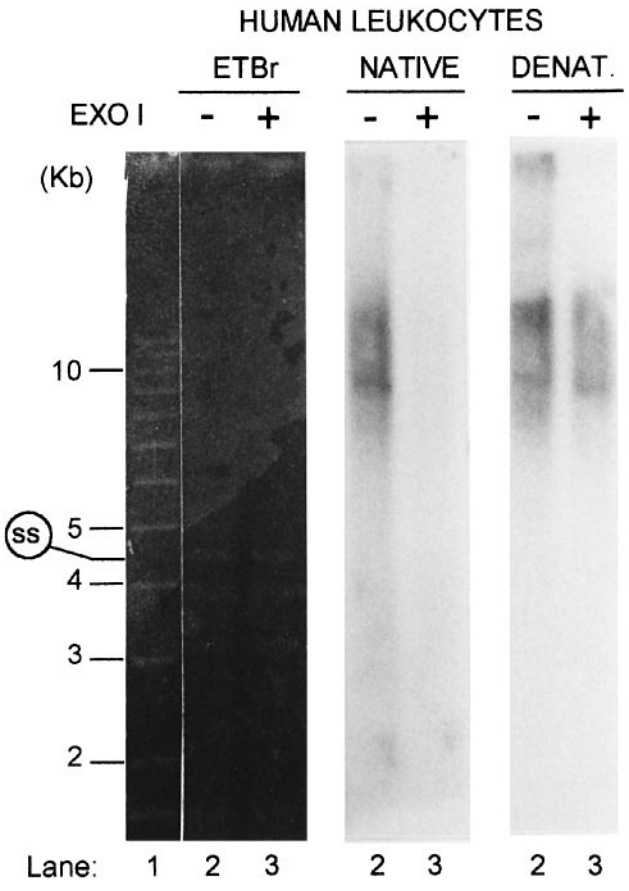

Fig. 3. G-strand extensions are detected on telomeres derived from non-dividing human leukocytes. DNA derived from total peripheral leukocytes was treated as described in Figure 1 for detection of single-stranded DNA comprising telomeric repeats in a native gel probed with end-labeled d(CCCTAA)$_3$. Lane 1: end-labeled DNA size marker. Exonuclease-I treatment (lane 3; marked +) and mock treatment (lane 2; marked –) were done in the same way as for HeLa DNA (Figure 1). Note that in this DNA the terminal restriction fragments are 8–12 kb in size.

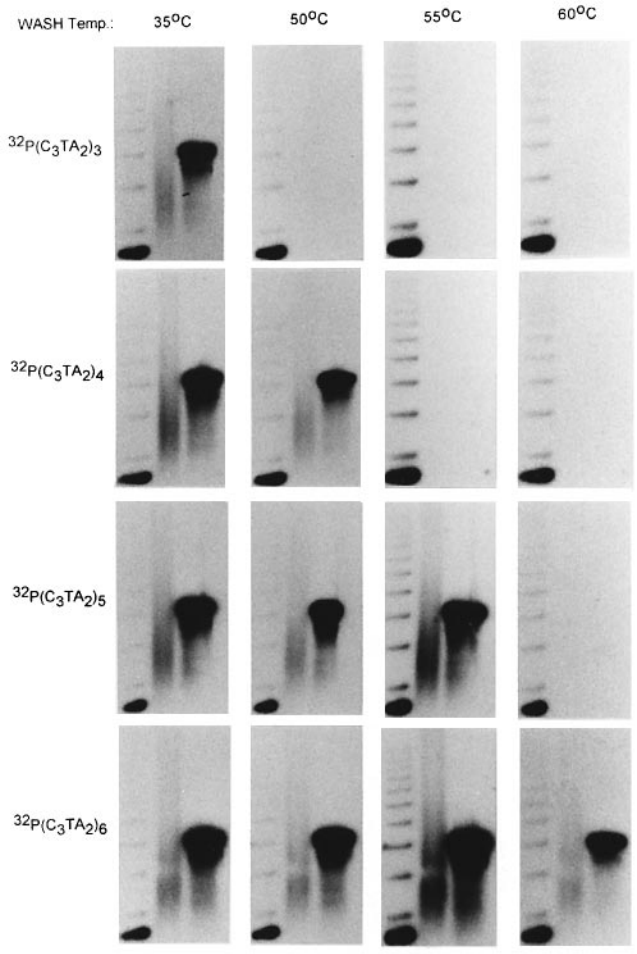

Fig. 4. Extensions on HeLa genomic DNA were at least 30 bases long. Four identical gels were run, each containing end-labeled size marker DNA in the first lane, 10 µg of HeLa genomic DNA digested with *Hin*fI and *Rsa*I in the second lane and 1 ng of linearized and denatured p16R DNA in the third lane. The gels containing undenatured genomic DNA were then hybridized individually to oligonucleotide probes consisting of three, four, five or six telomeric repeats of the C-rich strand (probes indicated at the left of the panels). All the gels were first washed in 0.25×SSC at 35°C (washing temperatures indicated at top of panels). After an appropriate exposure of the gels was obtained, the gels were rewashed at the next higher temperature and re-exposed. Note that exposure time for all four gels after each individual washing was equal.

gels were washed at increasing temperatures and an exposure obtained after each washing. While the probe consisting of three telomeric repeats remained hybridized after washes of up to 35°C, washes of 45°C and above completely dissociated the probe from the telomeric end-fragments and the positive control DNA (Figure 4). Probes consisting of four repeats remained hybridized up to 50°C, but were completely dissociated at 55°C. Furthermore, the probe consisting of five repeats was washed off at 60°C (Figure 4). However, a probe of six repeats, d(CCCTAA)$_6$, remained hybridized to the TRFs and the positive control even at 60°C (Figure 4). When the signals obtained for the probe containing five or six telomeric repeats were quantified using a PhosphorImager, the ratios of the signals obtained for the HeLa TRFs over the signal for the positive control DNA were virtually the same at 30°C and at 55°C (Table I). Thus, since no preferential loss of signal from the HeLa TRF DNAs as compared with the positive control DNA was observed (see also Figure 4), the vast majority of the extensions detected must be longer than 30 bases.

In order to obtain a minimal estimate for the longest extensions, a direct method based on primer-extension protocols was developed. An end-labeled oligo comprising three repeats of the C-rich strand was annealed to DNA with extensions in solution and the primer then extended with a polymerase that is unable to perform strand displace-

Table I. Quantification of the signal intensities for the terminal single-stranded extensions observed on HeLa DNA

Probe	TRF/control ratio at 30°C	TRF/control ratio at 55°C
d(CCCTAA)5	0.858	0.924
d(CCCTAA)6	1.264	1.043

Non-denaturing in gel-hybridization to *Hin*fI- and *Rsa*I-digested HeLa DNA was carried out as described in Figure 4 using the indicated probes. 1 ng of linearized and denatured p16R DNA was used as the control. The signal corresponding to the terminal extensions and the control DNA were quantified using a PhosphorImager.

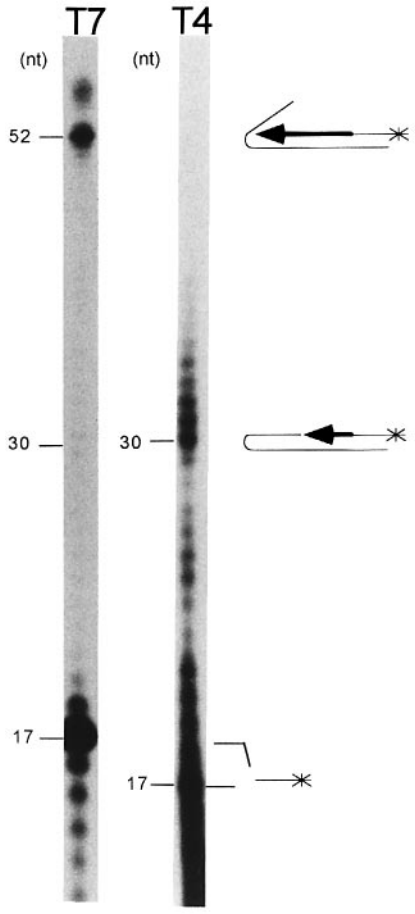

Fig. 5. No strand displacement occurs in the primer extension protocol using T4 DNA polymerase. End-labeled oligonucleotide STRD2 was annealed to STRD1 in solution and then extended either using a T7-derived DNA polymerase (Sequenase™) (lane marked T7) or T4 DNA polymerase (lane T4) using the primer extension protocol described in Materials and methods. Products were analyzed on 12% denaturing polyacrylamide gel. If there was no strand displacement, STRD2 should be extended to fill in the gap, yielding a product of 30 nucleotides (see lane T4). If the polymerase displaces the non-templating strand, synthesis will proceed through the double-stranded portion of the hairpin formed by STRD1, but stop at the first C-residue due to the absence of dGTP in the elongation reaction mixture. This will yield a product of 52 nucleotides (see lane T7). The signal above 52 nucleotides in lane T7 is derived from end-labeled STRD1 that was included in this experiment and is not observed if only STRD2 is labeled (see lane T4 and data not shown).

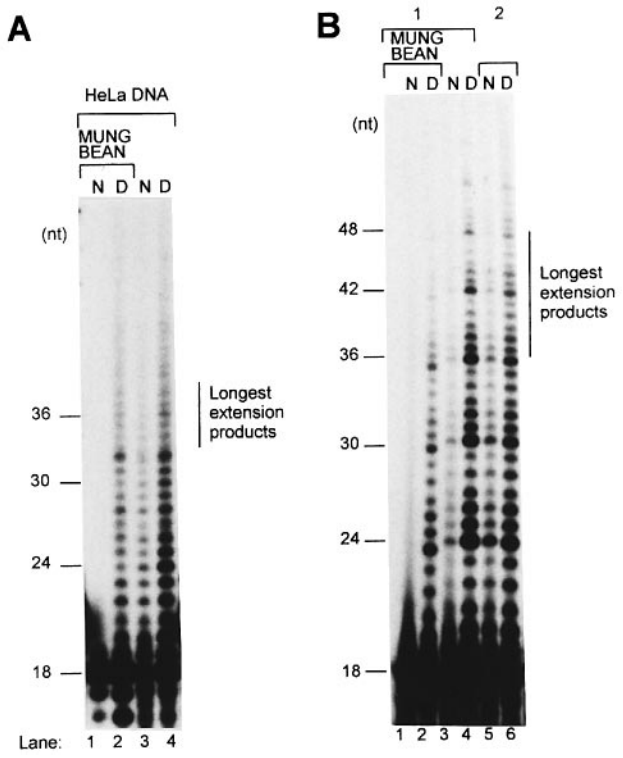

Fig. 6. The extensions of the G-rich strand can be at least 45 bases. (**A**) The primer extension protocol using end-labeled $d(CCCTAA)_3$ (see Materials and methods and Figure 5) was applied to native genomic DNA derived from HeLa cells. In lanes 1 and 2 (marked MUNG BEAN), total DNA was first treated with mung bean nuclease to remove all single-stranded DNA, including the extensions of the G-rich strand. Lanes 1 and 3 (labeled N at top): DNA was not heat-denatured prior to primer annealing. Lanes 2 and 4 (labeled D at top): DNA was heat denatured prior to primer annealing as positive control. On the native DNA (lane 3), extension products of up to 45 bases were observed (see also HeLa DNA on Figure 7). (**B**) The same experiment was conducted with DNA derived from non-dividing human leukocyte DNAs. The mung bean nuclease control (lanes 1 and 2) was as in (A). Numbers 1 and 2 at top of the gel denote two independent DNA preparations from two different individuals.

ment. In control experiments, in which a defined oligo with a 3′-overhang of the G-rich strand was present, T4 DNA polymerase did effectively fill in the gap between the annealed primer and the single-strand to double-stranded DNA boundary, consistent with reported characteristics of this enzyme (Kornberg and Baker, 1992; Figure 5, lane T4). Note that the oligonucleotides were modeled such that STRD1 has the same base composition as vertebrate telomeric DNA with a short double-stranded portion and a single-stranded extension of the G-rich strand. The oligonucleotide STRD2 will anneal to the 3′-most nucleotides of the single-stranded area of STRD1, yielding a double-stranded molecule with a 13-base gap. However, the use of a polymerase derived from the T7 phage (Sequenase™) resulted in a displacement of the non-templating strand (Figure 5, lane T7).

Next, we used this assay to measure the extensions present on telomeres derived from HeLa cells and non-replicating leukocyte cells. When the DNA was pretreated with mung bean nuclease to remove all single-stranded DNA from the telomeres, no extension products were detectable (Figure 6A, lane 1; Figure 6B, lane 1). As a control, the nuclease-treated DNA was denatured by heating prior to primer annealing and in this case, signals for extension products were detected (Figure 6A, lane 2; Figure 6B, lane 2). Virtually indistinguishable results were obtained when *E.coli* exonuclease-I was used instead of the mung bean nuclease (data not shown). For native DNA, extension products of up to 45 bases were detected for HeLa DNA, as well as for DNA derived from leukocytes (Figure 6A, lane 3; Figure 6B, lanes 3 and 5). Furthermore, comparable results were obtained with DNAs derived from telomerase-positive carcinoma cells and adjacent telomerase-negative cells (Figure 7). The signals observed had a periodicity of six bases, irrespective of whether denatured or native DNA was used in the assays (Figures 6 and 7). In these experiments, the input labeled oligonucleotide was in an ~1000-fold excess over the calculated number of telomeres. Therefore, on very long tracts of single-stranded telomeric repeats such as those

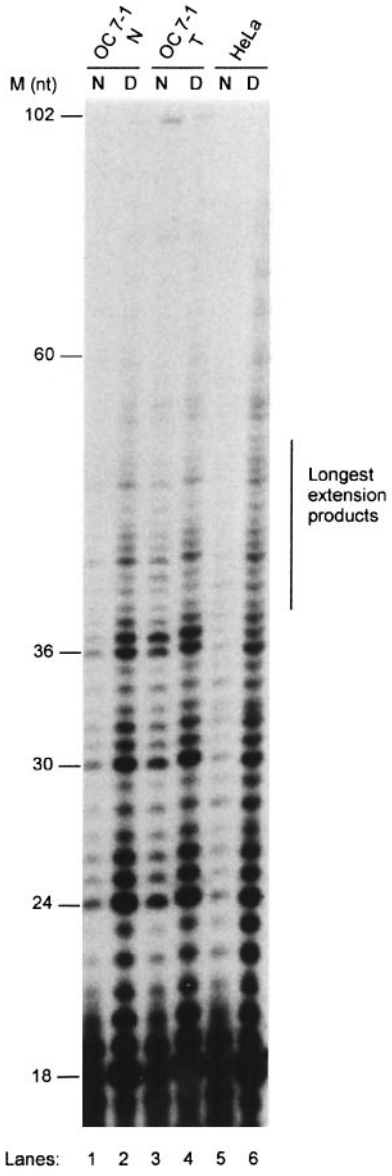

Fig. 7. Extensions of at least 45 bases can be detected on telomerase-positive and telomerase-negative cells. DNA derived from ovarian carcinoma (OC 7-1 T; lanes 3 and 4) and adjacent normal cells (OC 7-1 N; lanes 1 and 2) (see Counter *et al.*, 1994, for description of samples) were subjected to the primer extension protocol. As a control, DNA derived from HeLa cells was also included in this experiment (HeLa; lanes 5 and 6). Lanes 1, 3 and 5 (labeled N at top): native untreated DNA was used; lanes 2, 4 and 6 (labeled D at top): DNAs were denatured by heat prior to primer extension. The longest extension products observed for all native DNAs were ~45 nucleotides.

present in the denatured DNA samples, more than one molecule hybridized to a given single-stranded tract with a random spacing between the annealed oligos being a multiple of six bases. Thus, the longest extension products that were observed for the native DNA containing the extensions reflect a minimum length of the extensions. Since even when using a denatured DNA the signals observed were no longer than 60–70 bases (Figures 6 and 7), the extensions could in fact be much longer. However, removal of the single-stranded extensions by exonuclease-I did not result in a significant shift of the signal for the TRFs as measured by in-gel hybridization (see for example, Figure 1A), which may suggest an upper limit of a few hundred bases for the maximal lengths. We conclude that at least some of the telomeres derived from a variety of cells, including normal peripheral leukocytes, contained an overhang of the G-rich strand of at least 45 bases.

Discussion

Previous data derived from the analysis of acentric DNA from unicellular ciliates suggested a model for the DNA configuration of the ends of eukaryotic chromosomes with a short, 12- to 18-base overhang of the G-rich strand (Klobutcher *et al.*, 1981; Henderson and Blackburn, 1989). In the cases where it was studied in detail, these overhangs were necessary for the binding of terminus-specific proteins that are thought to form the chromosomal ‘cap’, a structure that is essential for chromosome stability (Gottschling and Zakian, 1986; Price and Cech, 1989; Raghuraman *et al.*, 1989; Price, 1990; Gray *et al.*, 1991; for reviews, see Blackburn and Greider, 1995; Lingner *et al.*, 1995). Recent evidence from yeast indicates that indeed, extensions of the G-rich strand can be created at the end of S-phase on all telomeres, irrespective of whether the telomeres were replicated by leading or lagging strand synthesis (Wellinger *et al.*, 1996). Furthermore, the extensions were also found to occur in cells that were lacking telomerase activity (Dionne and Wellinger, 1996). Thus, the recreation of overhangs of the G-rich strand at least at the ends replicated by leading synthesis cannot occur due to incomplete replication and is independent of the elongation activity of telomerase (Dionne and Wellinger, 1996; Wellinger *et al.*, 1996).

Here, we demonstrate that extensions of the G-rich strand do occur on telomeres of mammalian cells. The overhangs were found to be present in cells that contain telomerase and in cells lacking telomerase activity (Figures 2 and 7). Furthermore, the extensions were also detected in resting normal human leukocyte cells, suggesting that they are part of the constitutive DNA structure at human telomeres and not merely replication intermediates (Figures 3 and 6). Surprisingly, the data also show that virtually all extensions detected by our assays are longer than 30 bases, and at least some are longer than 45 bases (Figures 4, 6 and 7). Formally, we cannot exclude that a fraction of the telomeres in the various cells analyzed had extensions that were shorter than 12 bases, had no extensions at all or had short ($<$12-base) overhangs of the C-rich strand. Thus, our data are also consistent with the possibility that there are at least two populations of telomeres in a cell: those that have relatively long overhangs of $>$30 bases, and those that have very short overhangs of either strand or are blunt-ended.

At least for hypotrichous ciliates, chromosomal ‘capping’ is thought to occur via the same protein–DNA interactions on all telomeres (see for example, Klobutcher *et al.*, 1981; Gottschling and Cech, 1984; Gottschling and Zakian, 1986; Price, 1990). We therefore favor a model in which all telomeres in any particular species have a similar terminal DNA structure. For the mammalian cells analyzed here, this structure may be composed of a $>$30-base overhang of the G-rich strand. It is noteworthy that the length of the extensions could be very close to the

estimated amount of DNA lost from the termini of human chromosomes in every cell division (40–100 bp; Harley *et al.*, 1990; Allsopp *et al.*, 1992). It is thus tempting to speculate that telomerase-independent activities that reform the terminal DNA structure, i.e. exonucleases or helicases combined with single-stranded endonucleases, may be conserved elements of telomere maintenance (Lingner *et al.*, 1995; Wellinger *et al.*, 1996). Such telomere processing would thus explain the rates of sequence losses in telomerase-negative human cell lines. It is striking that telomerase-independent mechanisms to reform a terminal DNA structure with an overhang of the G-rich strand may be present in yeast and mammals—species that are very distant in evolutionary terms. This is consistent with the hypothesis that the re-formation of ends with an overhang of the G-rich strand is an essential process occurring in each cell cycle, since it will provide the proper substrate for the binding of proteins making up the 'cap'.

The overhangs detected here are significantly longer than the overhangs present on ciliate macronuclear DNA molecules. This suggests that, at least on those chromosomes with such long (≥ 45-base) G-strand overhangs, the terminal DNA–protein complex forming the 'cap' must be considerably different than the one described for the ciliates. Furthermore, this model predicts that telomere-specific single-strand DNA binding proteins analogous to the yeast *Cdc13* gene product could have important implications for the regulation of telomere maintenance and hence, chromosome stability (Garvik *et al.*, 1995; Lin and Zakian, 1996; Nugent *et al.*, 1996).

Materials and methods

Cells and DNA samples

HeLa cells and CB3 cells, a transformed mouse erythroleukemic cell line (Ben-David *et al.*, 1992), were grown as adherent cell cultures in DMEM supplemented with 10% fetal calf serum and in α-MEM supplemented with 5% fetal calf serum, respectively. At the time of harvest, HeLa cells were 60% in G_1, 15% in S and 25% in G_2/M as judged from FACS analysis (data not shown). Peripheral human leukocytes were isolated by density centrifugation in Ficoll and sodium diatrizoate using HISTOPAQUE®-1077 (Sigma) and established procedures to remove red blood cells (Boyum, 1968; Buchkovich and Greider, 1996). The isolation of the human ovarian carcinoma biopsies and the adjacent normal cells was described previously (Counter *et al.*, 1994).

Except for the carcinoma samples, high-molecular weight DNA was obtained from all cell sources by cell lysis in TNE (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA), adding SDS to 0.5–1% final concentration and incubating the lysate with 100 µg/ml proteinase K for 3 h at 37°C. The DNA was phenol/chloroform extracted and precipitated in ethanol. The resuspended pellets were treated with RNase and the DNA purified over a Sephadex G-50 column as described (Sambrook *et al.*, 1989). Leukocyte DNA samples were a gift of N.Chalhoub and J.-P.Thirion, and the human carcinoma DNAs were isolated as described (Counter *et al.*, 1994) and obtained from C.Counter and S.Bacchetti.

DNA analysis

Total genomic DNA was digested with *Hin*fI and *Rsa*I and loaded onto 0.5% agarose gels. Gels were subjected to electrophoresis and processed for native in-gel hybridizations as described (Dionne and Wellinger, 1996). Gels were washed at the indicated temperatures in 0.25×SSC and exposed to Kodak XAR5 X-ray films at –70°C. After appropriate exposures had been obtained, the DNA was denatured in the gels by soaking the gels in 150 mM NaCl/0.5 M NaOH for 30 min at 20°C. The gels were then soaked for 20 min at 20°C in 150 mM NaCl/0.5 M Tris–HCl, pH 8.0, for neutralization, and rehybridized using the same conditions as before. Note that denatured gels required significantly shorter exposure times than non-denatured gels to yield comparable signals. This is expected, however, since many more telomeric repeats are available for hybridization. For clarity and ease of comparison, exposures with comparable signals were chosen for the figures (Figures 1–3). In cases where the signals after the native in-gel hybridization was quantified, a Molecular Dynamics PhorphorImager™ SF with the MD ImageQuant software (version 3.3) was used (Johnston *et al.*, 1990).

For removal of the 3′ single-stranded DNA, *E.coli* exonuclease-I was used (Lehmann and Nussbaum, 1964; Wellinger *et al.*, 1993b). DNAs were incubated in appropriate buffer (Wellinger *et al.*, 1993b) and digested using 1 U/µl of enzyme for 24 h at 37°C. In all assays, single-stranded, circular M13 DNA was mixed in with the actual DNA prior to nuclease digestion to control for non-specific endonuclease activity. In control experiments, the exonuclease activity was readily detectable but no endonuclease activity was found (Dionne and Wellinger, 1996). For some experiments, mung bean nuclease (0.5 U/µl in 30 mM Na-acetate, 50 mM NaCl, 1 mM $ZnCl_2$ and 5% glycerol; 5 min at 37°C) was used. BAL31 treatment of the DNA was carried out as described previously (de Lange *et al.*, 1990; Wellinger *et al.*, 1992).

Oligonucleotides, control DNAs and primer-extensions

All oligonucleotides were obtained from a commercial source (Gibco-BRL) and were end-labeled using T4 polynucleotide kinase using standard techniques (Sambrook *et al.*, 1989). The control oligonucleotides used to show that there was no strand displacement were STRD1: 5′-CCATATCCTCACCCTATCCTTTGGATAGGGTGAGGATATGGTT-AGGGTTAGGGTGATGTTTAGGGTTAGGG-3′ and STRD2: 5′-CCC-TAACCCTAAACATC-3′. Note that on STRD1, nucleotides 1–19 form an inverted repeat with nucleotides 23–42 (underlined in the sequence) and the oligo can therefore form a hairpin structure such as outlined in Figure 5. The sequence of the protruding 3′-end is modeled such that it very closely resembles the expected sequence of a 3′-overhang on mammalian chromosomes. STRD2, which is complementary to the last 17 bases of STRD1, therefore is very similar to the sequence of the C-rich strand of telomeric DNA.

For the primer extension experiments, a modified method that was initially developed for oligonucleotide-mediated mutagenesis was used (Zoller and Smith, 1987; Sambrook *et al.*, 1989). End-labeled $d(CCCTAA)_3$ was mixed with 1–5 µg of total genomic DNA at ~1000:1 molar ratios of oligo:estimated number of telomeric DNA ends in PE1 buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol). For the control reactions, a 1:1 molar ratio of STRD2:STRD1 was mixed in PE1. Hybridization was carried out at 20°C for 12 h. The annealed primers were then extended in PE2 buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 7.5 mM dithiothreitol) in the presence of 0.5 mM dCTP, dATP and TTP with 3.5 U of T4 DNA-polymerase. The mixture was first incubated at 0°C for 5 min, then at 20°C for 5 min, and finally at 37°C for 60–90 min (Sambrook *et al.*, 1989). Reactions were stopped by a phenol/chloroform extraction; the DNAs precipitated and the products analyzed by denaturing polyacrylamide gel electrophoresis.

Plasmid p16R was obtained by digesting pSty11NotT1.6 (Zhong *et al.*, 1992; Hanish *et al.*, 1994, obtained from J.P.Hanish and T.de Lange) with *Bgl*II and *Bam*HI, which liberates an ~1.6 kb fragment containing two blocks of ~800 bp of vertebrate telomeric repeats. This fragment was then inserted into the *Bam*HI site of pVZ1 (Henikoff and Eghtedarzadeh, 1987). p16R was linearized using *Bam*HI and 1 ng was boiled in DNA loading buffer just prior to loading onto agarose gels as a positive control. In all gels, 1 kb ladder DNA (Gibco-BRL) was used as a DNA size standard.

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Note added

While this work was under review, another group reported results that fully agree and confirm our data presented here (Makarov *et al.*, 1997).

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