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Mammalian Telomeres End in a Large Duplex Loop

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Summary

Mammalian telomeres contain a duplex array of telomeric repeats bound to the telomeric repeat–binding factors TRF1 and TRF2. Inhibition of TRF2 results in immediate deprotection of chromosome ends, manifested by loss of the telomeric 3′ overhang, activation of p53, and end-to-end chromosome fusions. Electron microscopy reported here demonstrated that TRF2 can remodel linear telomeric DNA into large duplex loops (t loops) in vitro. Electron microscopy analysis of psoralen cross-linked telomeric DNA purified from human and mouse cells revealed abundant large t loops with a size distribution consistent with their telomeric origin. Binding of TRF1 and single strand binding protein suggested that t loops are formed by invasion of the 3′ telomeric overhang into the duplex telomeric repeat array. T loops may provide a general mechanism for the protection and replication of telomeres.

Introduction

DNA genomes tend to be circular. Bacterial genomes, plasmids, bacteriophages, and mitochondrial DNAs are usually circular. Even T phages, while linear, likely replicate through circular intermediates. Similarly, many eukaryotic DNA viruses, such as SV40, polyoma, and hepatitis B virus, have circular genomes, and like λ, Epstein–Barr virus is circular in its episomal state. In contrast, eukaryotic cellular chromosomes are by and large linear, a deviation from the norm that may have allowed the advent of meiosis (Naito et al., 1998).

A major drawback of linear chromosomes is associated with the presence of DNA ends in the eukaryotic nucleus. Telomeres are specialized terminal elements, composed of tandem repetitive sequences and specific proteins, that appear to obviate these problems. In many eukaryotes, telomeric repeat tracts are maintained by a telomere-specific reverse transcriptase, telomerase, that can counteract the loss of terminal sequences during DNA replication (Greider and Blackburn, 1985; reviewed in Nugent and Lundblad, 1998). However, other solutions to the end replication problem have been documented, generally involving some form of recombination of tandem terminal repeats (Morin and Cech, 1988; Lundblad and Szostak, 1989). The end replication problem of human telomeres has received particular attention for its possible significance to aging and cancer (de Lange et al., 1990; Harley et al., 1990; Hastie et al., 1990; Counter et al., 1994; reviewed in de Lange and DePinho, 1999). Maintenance of the telomeric TTAGGG repeats at human chromosome ends, either by telomerase (Bodnar et al., 1998) or an alternative mechanism (ALT; Bryan et al., 1997), is essential for long-term replicative survival of cells in vitro, and a telomerase-deficient mouse strain displays several phenotypes consistent with impaired tissue homeostasis (Lee et al., 1998; Rudolph et al., 1999).

Despite the importance of telomere maintenance, it has become increasingly clear that telomeres do not simply function as a buffer zone that prevents loss of essential sequences. A large body of evidence dating back to the work of Muller and McClintock (Muller, 1938; McClintock, 1941) is more consistent with the view that the telomeric complex allows cells to distinguish random DNA breaks and natural chromosome ends. Whereas broken chromosomes activate DNA damage checkpoints (Weinert and Hartwell, 1988; Sandell and Zakian, 1993) and are repaired, telomeres are not detected as DNA ends.

Based on work with unicellular organisms, the molecular mechanism of this capping function has been proposed to either depend on a specific DNA structure in the most terminal single-stranded portion of the telomere (e.g., G quartets; Williamson et al., 1989) or to require proteins bound to the 3′ telomere end (e.g., the *Oxytricha nova* telomeric protein; Gottschling and Zakian, 1986; Horvath et al., 1998). However, there is no evidence in support of G–G base-paired structures at telomere termini in vivo, and a terminus-specific protein has yet to be isolated from mammalian cells (reviewed in de Lange, 1996). Instead, duplex telomeric DNA–binding proteins have recently emerged as key players in the capping of mammalian chromosome ends.

The duplex array of TTAGGG repeats at mammalian telomeres is bound by two related proteins, the TTAGGG repeat–binding factors TRF1 and TRF2 (Chong et al., 1995; Bilaud et al., 1997; Broccoli et al., 1997). Inhibition of TRF2 results in immediate activation of the ATM/p53–dependent DNA damage checkpoint pathway, leading to cell cycle arrest and apoptosis (Karlseder et al., 1999). The exposure of chromosome ends to DNA damage checkpoints is not due to the loss of the duplex TTAGGG repeat array (van Steensel et al., 1998; Karlseder et al., 1999; J. Karlseder and T. d. L., unpublished data). Thus, the presence of telomeric DNA at chromosome ends per se is not sufficient for telomere function. Although the telomeric repeat array remains largely intact, the single-stranded protrusion of TTAGGG repeats found at all mammalian telomeres (Makarov et al., 1997) is lost upon inhibition of TRF2, suggesting that the G strand overhangs are important for telomeric protection

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(van Steensel et al., 1998). Furthermore, the unmasked telomeres eventually undergo covalent fusion, most likely reflecting the inappropriate exposure of unprotected telomere termini to ligases and other DNA repair activities (van Steensel et al., 1998; A. Smogorzewska and T. d. L., unpublished data). Based on these data, it was proposed that the TTAGGG repeats collaborate with TRF2 to sequester chromosome ends from the many cellular activities that threaten their integrity and safeguard against the inappropriate activation of DNA damage checkpoints by natural chromosome ends. However, the mechanism by which TRF2 acts to protect and mask chromosome ends is not known.

Here we present evidence that the sequestration of telomere ends by TRF2 may be achieved through an architectural change in the conformation of telomeric DNA. This structural solution to the chromosome end problem is deduced from the finding that the telomeric DNA can be isolated as large duplex loops, called t loops, in which the terminus is embedded within the double-stranded part of the telomeric tract. TRF2 can generate t loops in vitro, and its in vivo function is consistent with t loops as the main mechanism by which mammalian cells mask natural chromosome ends.

Results

TRF2 Generates T Loops on a Linear Telomeric DNA Model In Vitro

Our preliminary work with purified TRF2 and plasmid DNAs suggested that TRF2 might pair single-stranded and double-stranded DNA segments if both contained TTAGGG repeats (J. D. G. and T. d. L., unpublished data). To explore this further, a linear telomeric DNA model was constructed containing several kilobases of double-stranded tandem TTAGGG repeats grown from one end of a 3 kb segment of unique sequence DNA. The ends of the DNA were resected with a 5′ exonuclease to generate a 100–200 nt 3′ overhang of TTAGGG repeats, and the fraction of DNAs with an overhang and its length were monitored by addition of *E. coli* single strand binding (SSB) protein and electron microscopy (EM) examination (Experimental Procedures). Approximately 35%–40% of the molecules carried the expected 3′ overhang. The telomeric DNA model closely approximates the two known features of mammalian telomeric DNA, several kilobases of duplex TTAGGG repeats ending in a long 3′ tail of the G-rich strand.

The telomeric DNA model was incubated with baculovirus-derived purified human TRF2 and the complexes prepared for EM. As shown in Figure 1A, lariat- or lasso-like molecules, referred to here as t loops, were observed, and a large TRF2 protein complex was present at the loop–tail junction in all cases (the term "tail" will refer to the linear duplex DNA appendage attached to the circular portion of the molecule). Removal of the 3′ overhang from the model telomere with mung bean nuclease eliminated all SSB binding, as seen by EM, and in two experiments, addition of TRF2 resulted in 7% (n = 100) and 0% (n = 150) lasso-like molecules, revealing a requirement for the single-stranded TTAGGG repeats. The frequency of t loop formation on the model DNA by TRF2 was strikingly high (17% and 20% in two separate experiments, n = 100 for each) given that only 35%–40% of the model DNAs carried long 3′ TTAGGG repeat overhangs. Incubations of the DNA in the absence of protein resulted in 1%, 3%, and 5% lasso-like structures in three separate experiments, suggesting that t loops are a genuine product of the interaction of TRF2 with the telomeric substrate. In contrast to TRF2, addition of the related protein TRF1 resulted in only 6% t loops, a frequency of t loop formation that may not be significantly higher than what is observed in absence of protein. Furthermore, TRF1 was never observed at the tail–loop junction, indicating that the binding of TRF2 to t loops is a highly specific event.

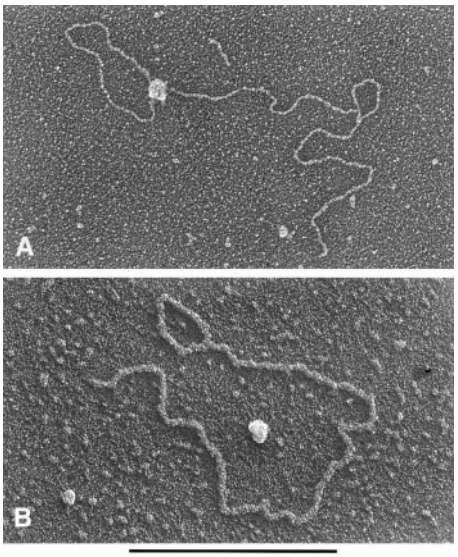

Figure 1. T Loops Generated In Vitro by Human TRF2

A telomeric DNA model containing 3 kb of unique sequence DNA followed by ~2 kb of repeating TTAGGG sequence with a 150–200 nt 3′ G strand overhang was incubated with human TRF2 protein. In (A), the sample was directly adsorbed to the carbon EM support followed by rotary shadowcasting with tungsten. In (B), following incubation with TRF2, the sample was exposed to psoralen and UV followed by deproteinization, surface spreading with cytochrome C, and rotary shadowcasting with platinum–paladium. Shown in reverse contrast. Bar is equivalent to 1 kb.

The requirement for a 3′ overhang in TRF2-mediated t loop formation suggested that this structure could depend on the invasion of the single-stranded TTAGGG repeats into the duplex part of the telomeric tract. In this case, psoralen cross-linking of the DNA strands is predicted to preserve the t loops after removal of protein. By constrast, if the t loops are held together by TRF2–DNA interactions only, the cross-linking of DNA strands is not predicted to preserve the t loops.

Accordingly, we next analyzed TRF2-induced t loops by psoralen cross-linking of the DNA (Hanson et al., 1976) followed by deproteinization and spreading of the DNA with cytochrome C ("Kleinschmidt" method) (Kleinschmidt and Zahn, 1959). Both approaches have been used extensively separately and in combination (Cech and Pardue, 1976; Hanson et al., 1976), and ways of employing them that avoid artifactual DNA associations are well understood. The psoralens HMT (4′-hydroxymethyl trioxalen) and AMT (4′-aminomethyl trioxalen)

intercalate into a duplex DNA and upon UV irradiation preferentially cross-link T residues of opposite strands. Thus, each TTAGGG repeat in the telomeric repeat tract contains potential cross-linking sites, and conditions were established that created a cross-link in HeLa DNA every few 100 bp (Experimental Procedures).

The Kleinschmidt EM technique is particularly suitable for the analysis of t loops, since the strong surface tension at the air–buffer interface forces DNA molecules (which have been thickened 5- to 10-fold and greatly stiffened) to spread out so that the strands seldom cross over themselves. Furthermore, DNA ends are held apart and DNAs known to be linear are seldom observed to form loops or lassos.

When the telomeric DNA model was cross-linked with AMT in the absence of protein, the frequency of lasso-like structures and circles varied from 0%–6% ($n = 100$) in four experiments, likely reflecting accidental juxtapositioning. However, when the telomere model DNA was treated with AMT after incubation with TRF2, the deproteinized products showed t loops at a high frequency (17%, 19%, and 25% in three experiments, $n = 100$ for each) (Figure 1B). As expected, very few t loops (4% and 0%, respectively, $n = 50$ in two experiments) were observed in parallel incubations with two control proteins, TRF1 and tankyrase (Bianchi et al., 1997; Smith et al., 1998). These results argue that TRF2 can promote a looped structure in telomeric DNA that involves the invasion of the 3′ telomeric overhang into the duplex DNA. Further detailed analysis of the generation of in vitro t loops by TRF2 will be described elsewhere (R. M. S., T. d. L., and J. D. G., in preparation).

The formation of t loops by TRF2 in vitro suggested a mechanism by which TRF2 and telomeric repeats might function together to sequester chromosome ends. To address this possibility, the presence of t loops at chromosome ends in vivo was studied.

Isolation of Telomeric DNA for Analysis of In Vivo Telomere Structure

To enrich telomeric DNA for EM visualization of in vivo telomeric structures, we employed a unique feature of telomeric TTAGGG repeat tracts—their lack of recognition sites for restriction endonucleases. As described previously (de Lange et al., 1990), human telomeric DNA can be separated from bulk genomic DNA when the DNA is cleaved with fine-cutting enzymes and subsequently subjected to size fractionation. To determine the feasibility of this approach for EM analysis, we first employed a HeLa subclone (HeLa1.2.11) (van Steensel et al., 1998) that has telomeres in the 25 kb range. Protein-free total genomic DNA was digested with HinfI and RsaI and fractionated on a large Biogel A5m gel-filtration column (Experimental Procedures; see Figure 2A for profile of HeLa S3 DNA). Hybridization of a telomere-specific TTAGGG repeat probe to fractions across the elution profile showed that nearly all of the telomeric DNA was present in the excluded fractions, while the bulk of the genomic DNA (ranging from 100 to 600 bp) was included and eluted after the telomeric DNA (Figures 2B and 2C). Psoralen cross-linking did not affect the isolation or fractionation of the telomeric fragments from the bulk genomic DNA.

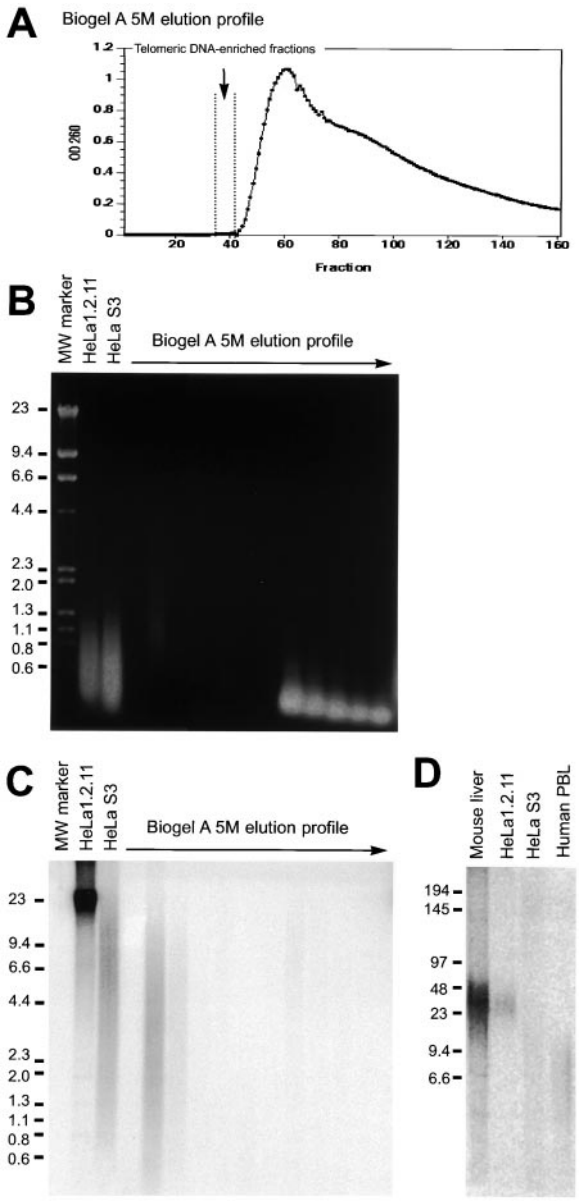

Figure 2. Enrichment for Telomeric DNA by Gel Exclusion Size Fractionation

(A) Elution profile of HeLa DNA cleaved with HinfI and RsaI and size fractionated over Biogel A5m (Experimental Procedures).

(B) Ethidium bromide–stained agarose gel of HinfI/RsaI–digested genomic DNA from HeLa1.2.11 and HeLa S3 cells run next to fractions (pools of ten fractions) from a Biogel A5m elution of the HinfI/RsaI–cleaved HeLa S3 DNA shown in (A).

(C) Autoradiograph representing identification of telomeric TTAGGG repeat–containing fragments on a Southern blot of the gel shown in (B). The probe is an 800 bp TTAGGG repeat fragment labeled with Klenow fragment and [α-^{32}P]dCTP using a C strand–specific primer.

(D) Autoradiograph of a CHEF gel of HinfI/RsaI–cleaved DNA from the indicated sources, blotted and hybridized to identify telomeric restriction fragments as in (C). All MWs are shown in kb.

EM examination of the enriched telomeric DNA revealed the presence of DNA fragments ranging in size from 5 to 30 kb. The small amount of this DNA was

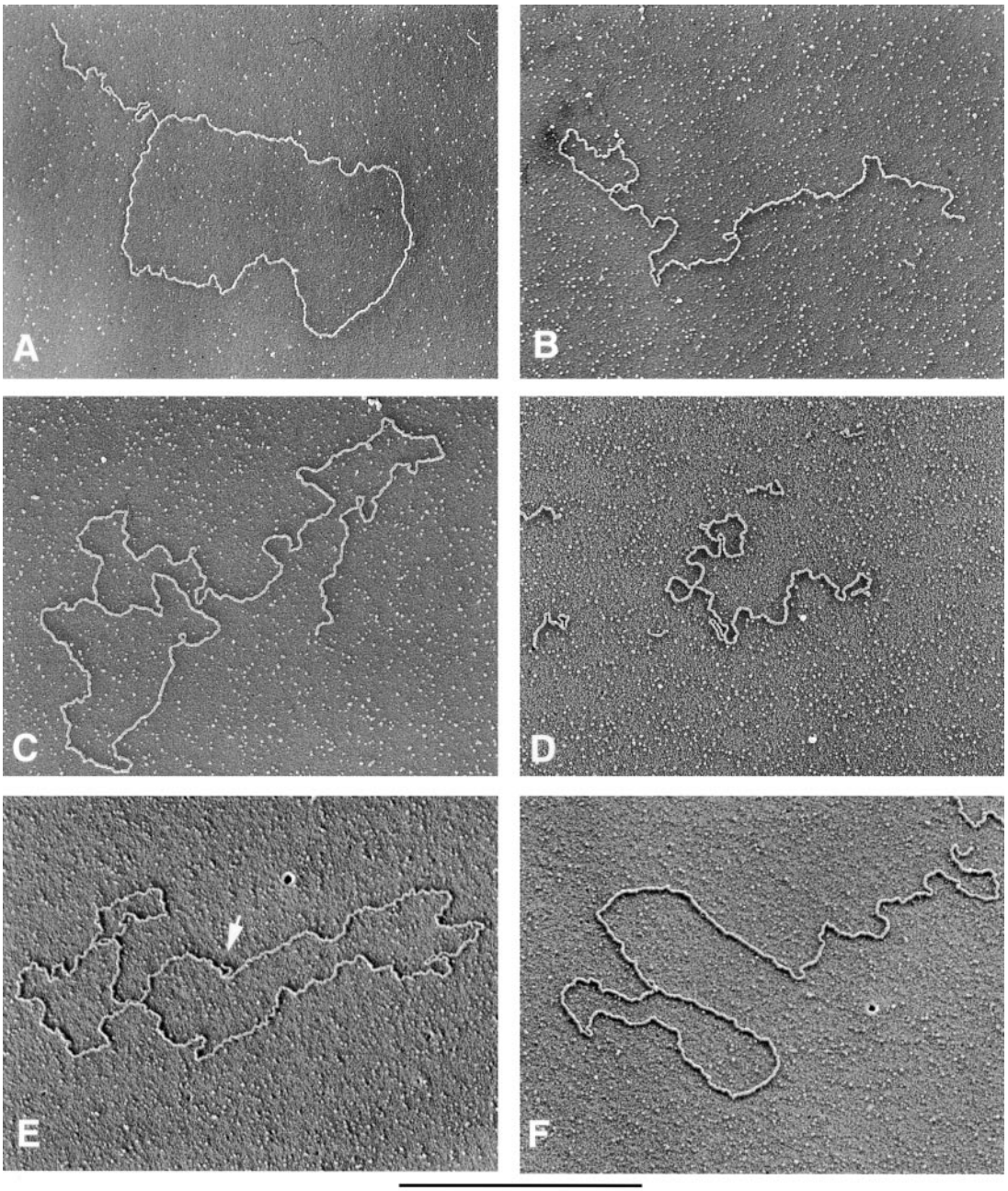

Figure 3. Visualization of Mammalian T Loops

Telomeric DNA from HeLa cell clone 1.2.11 (A–C), human PBLs (D), and mouse liver (E and F) was isolated by size fractionation following psoralen/UV treatment of nuclei, deproteinization, and restriction cleavage. Arrow in (E) points to the short (300 bp) tail. The DNA was spread on an air–buffer interface with cytochrome C protein followed by rotary shadowcasting with platinum–paladium. Shown in reverse contrast. Bar is equivalent to 5.0 kb.

consistent with the presence of the expected amount of high-molecular-weight telomeric DNA (a few micrograms of telomeric restriction fragments should be present in the 3–5 mg genomic DNA fractionated on the column). As described below, these excluded fractions were found by TRF1 staining to consist of 85%–90% telomeric DNA, indicating that the telomeric DNA was enriched about 1000-fold by this procedure. Examination of the included fractions by EM revealed the expected short linear DNAs also observed by agarose gel electrophoresis (Figure 2B).

This study employs four different sources of mammalian DNA chosen for their different telomere lengths (Figure 2D and below). A recently subcloned HeLa cell line (HeLa1.2.11) has long telomeres, whereas much shorter telomeres are present in the standard HeLa cell line S3. In addition, we used DNA derived from mouse liver and normal human peripheral blood leukocytes (PBLs), rep-

Table 1. T Loop Dimensions Correlate with Telomere Lengths

Cell Type	Telomeric Restriction Fragments Range (Mean) in kb	T Loop Dimensions (kb) Loop	Tail	Loop + Tail	
HeLa clone 1.2.11	15–40 (23)	13.8 ± 6.2	8.5 ± 6.6	22.4 ± 6.9	n = 58
HeLa clone S3	3.5–20 (10)	5.8 ± 3.5	5.2 ± 3.0	10.1 ± 2.8	n = 37
Mouse liver	10–50 (25)	18.2 ± 6.2	0.92 ± 1.7	19.2 ± 5.9	n = 38
Human PBL	3.5–15 (7)	2.9 ± 1.9	5.4 ± 3.0	8.3 ± 3.0	n = 27

resenting untransformed mammalian cells with long telomeres and short telomeres, respectively.

Frequent T Loops in Telomere-Enriched DNA

The telomere-enriched fractions from psoralen cross-linked HeLa nuclei revealed a striking abundance of t loops (Figures 3A–3C). In some cases the tail was not visible, resulting in a circle. The fraction of molecules arranged into t loops was scored in 15 different experiments (using the two different HeLa lines) and found to range from 15%–40%. In this study, "percentage of t loops" will be taken to mean the fraction of molecules ≥5 kb arranged into lassos or circles. In a titration experiment, the frequency of t loops was reduced severalfold as the concentration of AMT was decreased 5- and 10-fold. Less than 1 molecule in 1000 was arranged as a double lasso (both ends folded back to generate a molecule consisting of two circles joined by a linear segment), indicating that the t loops are not the result of DNA ends being sticky and fortuitously associating with an internal site during the purification or EM steps.

Although t loops were more abundant in psoralen cross-linked preparations, they could also be observed without this treatment. EM analysis of the excluded fractions of non-cross-linked HeLa preparations showed that 95%–98% of the DNA was linear with no noteworthy structures at either end. However, 2%–5% of the DNA molecules carried t loops (results from seven experiments, each involving scoring >100 molecules). While this is a relatively small number, when HeLa or mouse genomic DNA was randomly sheared into 5–20 kb lengths and then prepared for EM as above, less than 0.25% of the molecules (n = 1000) were found arranged into lasso structures. Thus, telomeric DNA is highly enriched (10- to 20-fold) for t loops even without cross-linking.

T Loops in Primary Human and Mouse Cells

To determine whether t loops occur in primary cells, mouse liver and human blood leukocytes were examined as above. Nuclei were prepared from fresh mouse livers, psoralen cross-linked, and the DNA processed to enrich for telomeric restriction fragments. Examination of telomere-enriched fractions revealed frequent large t loops (Figures 3E and 3F) similar to those from HeLa cells, although the tails tended to be shorter in the mouse liver DNA than in preparations from human cells. T loops were also present in telomere-enriched psoralen cross-linked DNA from human PBLs, isolated from blood freshly drawn from a fairly normal donor (J. D. G.) (Figure 3D). The abundance of t loops in the mouse preparations ranged from 15% to 20% of the DNA ≥5 kb (n = 300) in three experiments and was ~15% (n = 300) in the human PBL DNA. As described below, however, the dimensions of these t loops were severalfold smaller than those isolated from mouse liver.

T Loop Sizes Correlate with Telomere Lengths

If the t loops represent telomeres, the size of these molecules might be expected to correlate with telomere length. To explore this possibility, we measured the dimensions of t loops in the four cell types with distinctly different telomere lengths, shown in Figure 2D. Although the exact lengths of human telomeric repeat tracts cannot be determined accurately by genomic blotting (reviewed in de Lange, 1995), HeLa1.2.11 telomeres appear to range from 15–40 kb (mean 23 kb), whereas HeLa S3 and normal human PBL telomeres are shorter, displaying a mean telomeric restriction fragment length of 10 and 7 kb, respectively (Table 1). *Mus musculus* telomeric restriction fragments have a much wider size distribution, ranging from 5–60 kb (Kipling and Cooke, 1990; Zijlmans et al., 1997), with the bulk migrating at 25 kb in agarose (CHEF) gels (Figure 2D; Table 1).

Psoralen cross-linked, telomere-enriched DNAs from the four cell types were examined by EM, and sequentially encountered t loops were photographed and their lengths determined. The data in Figures 4A–4C and Table 1 present values obtained from 58 t loops derived from the HeLa subclone with long telomeres (HeLa1.2.11). The contour length of the circular segment of the t loops in these cells varied from 3 to 25 kb with approximately two-thirds of the values lying between 12 and 18 kb (mean of 13.8 ± 6). The t loop tails varied from 0 to 12 kb (mean 8.5 ± 6 kb), and the length of the entire t loop (summation of tails and circles) yielded a value of 22.4 ± 7 kb.

Similar measurements were carried out for the HeLa S3 cell t loops as well as for those from mouse liver and human PBLs. Histograms representing the sizes of the circular t loop segments (Figures 4D–4F) reveal an obvious difference that correlates with telomere lengths in these cells. The mouse liver t loops have the largest circles, the HeLa S3 cells have substantially smaller circular segments, and the t loops from human PBLs have smaller circles still. The mean lengths of the circular segment plus the tail (entire t loops) also correlated with telomere lengths, with the mouse liver t loops greater than 20 kb, HeLa S3 t loops around 10 kb, and the PBL t loops measuring around 8 kb (Table 1).

The good agreement between the length of the t loops and the size of telomeres argued that t loops represent telomeric DNA, as contrasted to some other DNA that

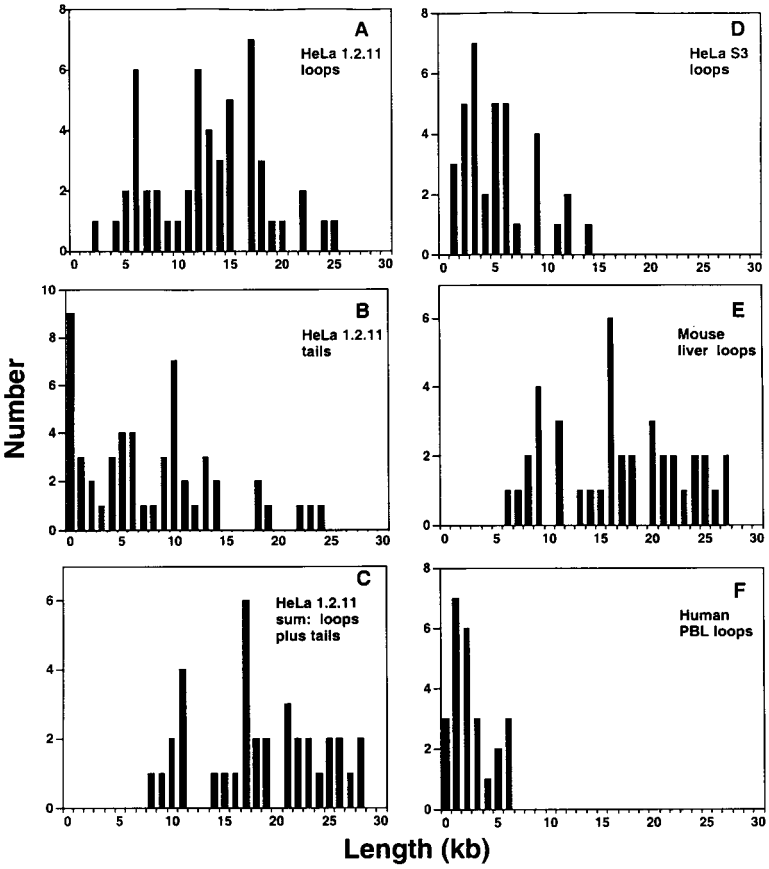

Figure 4. T Loop Dimensions

Micrographs such as those in Figure 3 were taken sequentially as t loop molecules were encountered at the EM in preparations from from HeLa1.2.11 (A–C), HeLa S3 (D), mouse liver (E), and human PBL (F) sources. Histograms present loop sizes (A, D–F), tail length (B), and the sum of the loop plus the tail (C). O class refers to molecules measuring between 0 and 1 kb.

survived the cleavage and size fractionation steps. The large size of the t loops from the HeLa1.2.11 cells and mouse liver sources makes it unlikely that they arose from the end of the telomeric DNA looping back after deproteinization. If that had occurred, the loop size would follow the Shore and Baldwin distribution (Shore et al., 1981) for DNA circularization, a distribution that peaks between 200 and 500 bp, not 14,000 to 18,000 bp as observed here. Thus, the t loops were most likely formed in situ by the action of cellular proteins.

T Loops Reflect Cross-Linking of Telomeres In Situ

The higher abundance of t loops in DNA from cells that have been psoralen treated and their reduced preservation at lowered psoralen concentrations suggested that the t loops represent a noncovalent arrangement in vivo that can become resolved during standard isolation of genomic DNA. To further exclude the possibility that the cross-linking somehow induced t loops following deproteinization, we performed a mixing experiment using HeLa cells with different telomere lengths. First, deproteinized non-cross-linked telomeric restriction fragments were isolated from the HeLa subclone with long telomeres (HeLa1.2.11). This "unlooped" DNA (<4% t loops) was then mixed with nuclei from HeLa S3 cells, which carry shorter telomeres (see above), allowing us to distinguish between t loops from the two sources of DNA. This mixture was then psoralen cross-linked, enriched for telomeric sequences as before, and molecules examined by EM to determine the relative frequency of small and large t loops.

In the resulting mixture, t loops of the size class consistent with HeLa S3 cells were about 10-fold more abundant (5.1%, n = 330) than t loops of the size of HeLa1.2.11 cells. The mean circle contour of the smaller circles in this mixing experiment was 6.3 ± 4 kb. This was not statistically different (by student t test analysis) from the mean for the S3 HeLa cells (5.8 ± 3.5) but was statistically different from 13.8 ± 6.2, the mean value of the HeLa1.2.11 cells. The lowered frequency of t loops in the mixture was consistent with the dilution of the HeLa S3 DNA by the additional HeLa1.2.11 DNA. These observations show that while cross-linking of DNA in chromatin in HeLa S3 yielded t loops, as expected, few if any t loops were induced in the naked HeLa1.2.11 DNA present in the same experiment. An additional test demonstrated that in the mixing experiment above, t loops do not get degraded when incubated with HeLa nuclei (data not shown). Taken together, these experiments argue that the telomeric DNA must be complexed by protein into chromatin in situ for the psoralen/UV treatment to yield t loops.

T Loops Contain Duplex Telomeric DNA

To assess whether the DNA sequence in the t loops is indeed an array of duplex TTAGGG repeats, we employed the unique sequence specificity of the double-stranded telomeric repeat binding–factor TRF1. TRF1

contains a Myb-related C-terminal domain and binds to telomeric DNA as a dimer (Chong et al., 1995; Bianchi et al., 1997; van Steensel and de Lange, 1997). The specificity of TRF1 for double-stranded TTAGGG repeat arrays is well documented, and closely related sequences (such as TTTAGGG or TTAGGC repeats) are generally a very poor binding substrate, as is single-stranded telomeric DNA (Zhong et al., 1992; Hanish et al., 1994; Chong et al., 1995).

Previous EM analysis of TRF1 DNA complexes revealed that short arrays of telomeric DNA (e.g., 6 or 12 repeats) recruit a single ball of protein, representing a TRF1 tetramer (Griffith et al., 1998). When TRF1 is allowed to bind to long telomeric tracts at saturating protein concentrations, the protein coats the telomeric DNA along its length, forming a 10 nm thick smooth array of bound proteins. Even at high TRF1 to DNA mass ratios, no TRF1 binding was observed by EM on nontelomeric sequences, making EM visualization of TRF1 binding a well-defined direct assay for the presence of telomeric DNA.

To apply this test, t loop–containing fractions from HeLa cells were incubated with baculovirus-derived purified human TRF1, cross-linked with glutaraldehyde, and visualized by EM (Experimental Procedures). At saturating TRF1 concentrations, TRF1-coated telomeric DNA filaments were visible and readily distinguishable from protein-free DNA on the same grids (Figure 5A). The appearance of naked and TRF1-bound DNAs side by side argued that the TRF1-coated species contained substantial regions of TTAGGG repeats.

At low protein concentrations, scattered TRF1 complexes were observed on some molecules (Figure 5B), whereas higher TRF1 concentrations yielded a large number of thick filaments. Some of the thick filaments were spread well enough to reveal a t loop arrangement (Figures 5C and 5D), and in the examples shown, the circular segment of the t loops is thick and TRF1 coated, while the tail is thin, protein free–appearing DNA. Presumably, in these molecules the telomeric DNA had looped back to anneal close to the junction of the telomeric and subtelomeric sequences, resulting in a tail devoid of telomeric repeats and hence not bound by TRF1. In the majority of TRF1-coated molecules, the thick filament was twisted about itself, making it difficult to trace a clear contour even though the presence of a circular form was highly suggestive. Addition of saturating amounts of TRF2 to such large molecules led to severe collapse and multimolecule tangles. The specific binding of TRF1 to t loops was further substantiated by an immunodepletion experiment in which the presence of t loops was reduced $\sim$7-fold upon incubation of the DNA–TRF1 complexes with an anti-TRF1 antibody (Experimental Procedures).

Although their twisted appearance made scoring the fraction of TRF1-bound t loops difficult, it was simple to score the fraction of thick protein-containing DNA as contrasted with thin, uncoated DNA. In HeLa DNA highly enriched for t loops, 80% of the large molecules judged to be $\geq$5 kb were bound by TRF1 (n = 100). When an aliquot of the same fraction was spread for EM in the absence of TRF1, 35% of the DNA $\geq$5 kb was in t loops. In an identical but independent experiment, the fraction most enriched for telomeric DNA scored as 85%–90% telomeric DNA based on TRF1 binding (n = 100), and 40% of this fraction was comprised of t loops. Thus, in the latter experiment, a minimum of 63% (and most likely all) of the t loops contained telomeric DNA.

SSB Reveals a Displacement Loop at the Loop–Tail Junction

The t loops can be explained by a model in which the single-stranded TTAGGG repeat overhang of the telomere folds back and undergoes limited strand invasion to form a displacement loop with the duplex part of the telomeric repeat tract. The resulting displacement loop at the junction is expected to contain up to 300 nt of single-stranded TTAGGG repeats. To explore the presence of single-stranded DNA in the t loop junctions, we employed the *E. coli* SSB. HeLa t loops were incubated with SSB, and the complexes were prepared for EM by directly adsorbing them onto thin carbon foils and shadowcasting with tungsten. Inspection of the samples showed that 35% of the t loops (n = 30) had one or several SSB protein complexes at the loop–tail junction (Figures 5E and 5F). Since each SSB complex (tetramers and octamers) would be expected to associate with 75–150 nt (Chrysogelos and Griffith, 1982), this suggests that SSB-bearing sites contain a single-stranded segment of possibly 75 to 200 nucleotides. It is possible that the junctions that did not stain with SSB contained some single-stranded character but not enough to bind a tetramer or octamer of SSB.

Discussion

Based on chromosome analysis in X-ray irradiated flies, Muller proposed the term telomeres and surmised that they are required to "seal" chromosome ends (Muller, 1938; see Gall, 1995 for discussion). The data presented here reveal an unexpected structure present in vivo at mammalian telomeres, called the t loop, that is proposed to represent the "sealed" chromosome ends that Muller referred to. The t loop is a large duplex loop-back structure most likely formed through the invasion of the single-stranded telomeric 3′ overhang into the duplex telomeric repeat array. In vitro, t loops can be formed by TRF2, a telomeric protein known to be required for the protection of mammalian chromosome ends. We propose that t loops represent the basic mechanism by which the telomeric nucleoprotein complex sequesters the natural ends of chromosomes from DNA damage checkpoints, DNA repair enzymes, and telomerase.

The Structure of T Loops

A proposal for the in vivo configuration of telomeric DNA at mammalian chromosome ends based on the data reported here is presented in Figure 6A. Inspection of the in situ configuration of human and mouse telomeric DNA indicates the presence of large loops formed by folding back the end of the telomere. The circular segment of the loops is composed of duplex telomeric DNA, as identified by binding of TRF1. The 3′ overhang of single-stranded TTAGGG repeats appears to be inserted into the duplex telomeric tract, resulting in displacement of the TTAGGG repeat strand at the loop–tail junction. Based on binding with SSB, the displacement loop of

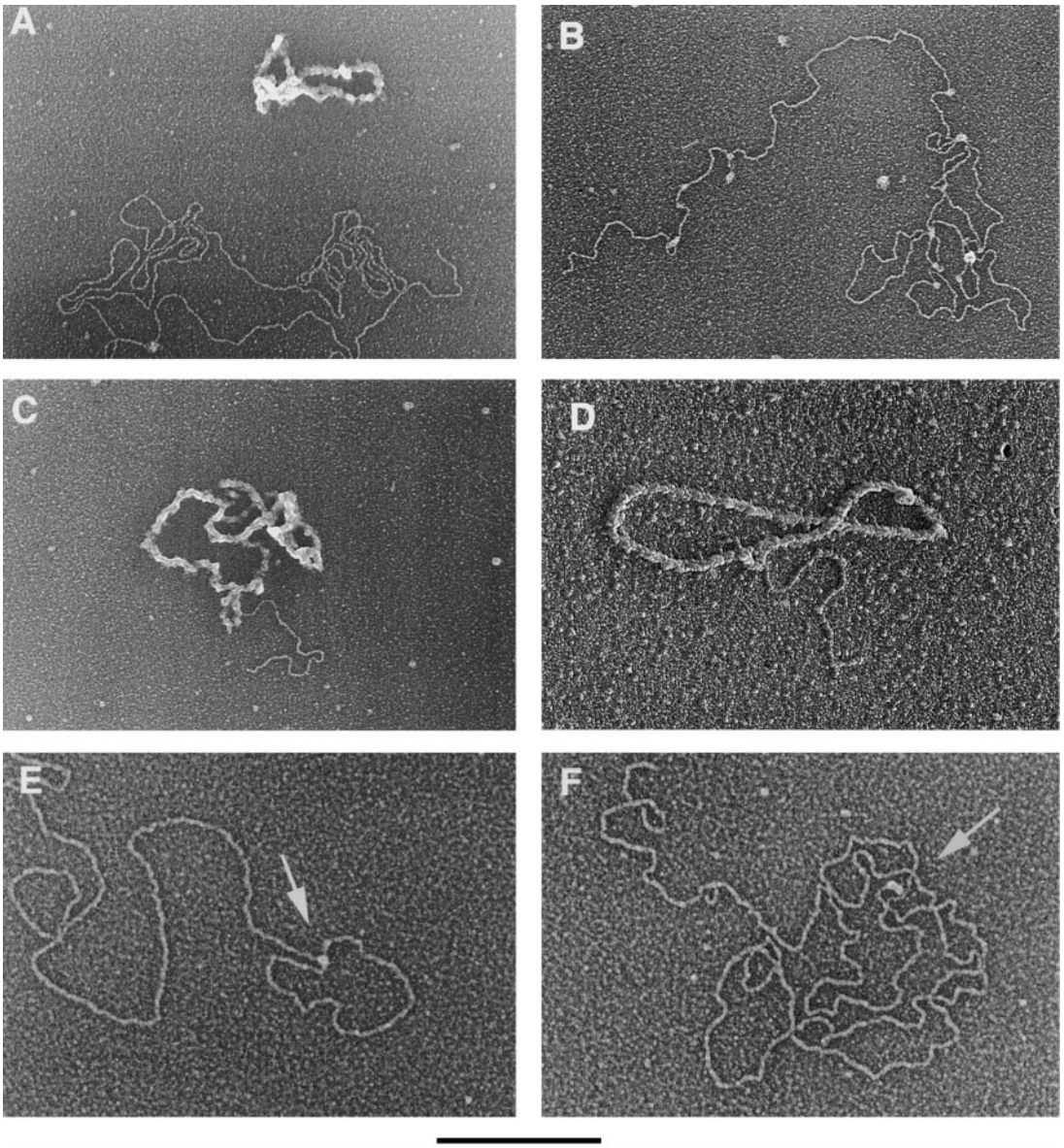

Figure 5. Staining T Loops with TRF1 and SSB Protein

A telomere-enriched fraction of DNA from a size fractionation of psoralen- and UV-treated HeLa1.2.11 cell DNA that contained ~40% t loops was incubated with human TRF1 protein at high (A, C, and D) or low (B) concentration. The same DNA was independently incubated with *E. coli* SSB protein (arrows point to SSB particles) (E and F). Samples were prepared for EM by fixation followed by either direct adsorption to thin carbon foils and tungsten shadowcasting (A–C, E, and F) or surface spreading with cytochrome C (D) and shadowcasting with platinum–paladium. Shown in reverse contrast. Bar is equal to 1 kb.

TTAGGG repeats is deduced to be in the order of a few hundred nucleotides in many of the t loops. Although the exact site of the 3′ end invasion point was not established, in most molecules the loop is very large (many kilobases) and in some cases clearly encompasses the whole telomere. There is a close correlation between the length of the telomeric repeat array and the size of the t loops. The question of whether t loops always reach back to the "base" of the telomere (the junction between telomeric and subtelomeric DNA) in primary cells and how their lengths vary with age needs to be addressed further.

Formation of T Loops

In vitro data indicates that TRF2 promotes t loop formation. A model telomeric substrate composed of a large segment of duplex TTAGGG repeats terminating in a 100–200 nt 3′ overhang of the same sequence was used here to study TRF2–telomere complexes. The telomeric DNA model was converted into a large loop upon incubation with purified TRF2, and all of the resulting t loops carried TRF2 protein at the loop–tail junction. A challenge will be to determine what type of biochemical activity allows TRF2 to promote in vitro telomeric loop formation. While TRF2 is known to bind to duplex telo-

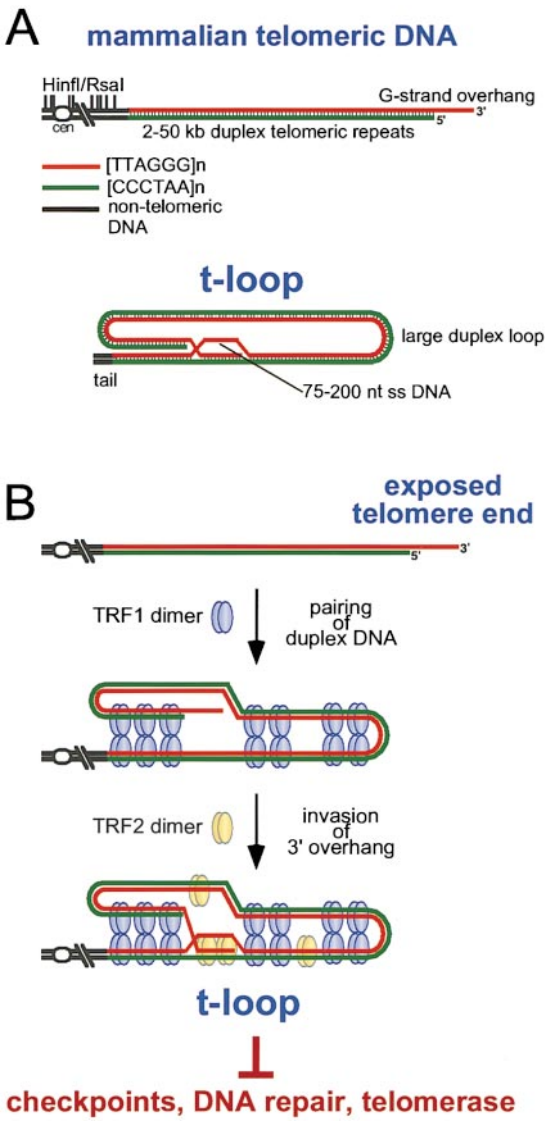

Figure 6. Proposed Structure, Formation, and Function of T Loops

(A) The DNA structure at the ends of mammalian chromosomes and a description of the proposed configuration of t loops.

(B) Speculative scheme depicting a possible mode of t loop formation based on the in vitro biochemical activities of TRF1 and TRF2. T loops are proposed to mask telomere termini from cellular activities that can act on DNA ends. See text for discussion.

meric tracts (Broccoli et al., 1997; Bianchi, 1999), a DNA end is not required for this interaction, and single-stranded TTAGGG repeat arrays (representative of the 3′ telomeric overhang) have not been found to form a complex with TRF2 under incubation conditions similar to that used here (Broccoli et al., 1997; Bianchi, 1999; A. B. and T. d. L., unpublished data; R. M. S. and J. D. G., unpublished data). One possibility is that TRF2 binding causes partial unwinding of the duplex telomeric repeat array, thus allowing strand invasion by the 3′ terminus. However, the finding of TRF2 bound at the tail–loop junction suggests that its binding might stabilize the displacement loop, and this activity could also contribute to the induction and maintenance of t loops in vitro and in vivo.

These studies illuminate the possibility that TRF2 protects human telomeres through the catalysis of an architectural change involving the invasion of the 3′ telomere terminus into the duplex part of the telomere (Figure 6B). In this regard, it is of interest that TRF1 also has the ability to engineer the conformation of telomeric DNA. TRF1 was inferred to induce a shallow bend in duplex TTAGGG repeats (Bianchi et al., 1997), but perhaps more pertinent to the current results is the ability of TRF1 to pair telomeric tracts in vitro (Griffith et al., 1998). Since telomeres are not paired or clustered in human somatic cells (Luderus et al., 1996), intratelomeric synapsing of TTAGGG repeat arrays was suggested to induce a coiled structure at telomeres (Griffith et al., 1998) (illustrated in Figure 6B). The presence of t loops at telomeres in vivo now suggests a role for the TRF1-induced intratelomeric pairing. A synaptic complex of parallel or antiparallel paired TTAGGG repeat arrays could facilitate the strand invasion of the 3′ overhang and stabilize t loops. A scenario for a possible synergistic role of TRF1 and TRF2 in t loop formation is presented in Figure 6B. According to this model, both TRF1 and TRF2 stimulate t looping at telomeres, with TRF1 coiling the duplex telomeric tract on itself, allowing TRF2 to promote the invasion of the 3′ end. A variety of other cellular proteins might be expected to promote t loop formation (e.g., helicases) or stabilize/regulate their persistence (e.g., single-stranded binding proteins).

T Loop Model for Telomere Function

A major function of telomeres is to sequester chromosome ends from the DNA damage response pathway and to prevent inappropriate DNA repair at these sites (for instance, ligation). Recent progress reveals a key role for TRF2 in this capping function (van Steensel et al., 1998; Karlseder et al., 1999). Inhibition of TRF2 results in activation of a double strand break checkpoint that includes signaling through ATM and p53 and induces apoptosis in some cells. In addition, loss of TRF2 renders chromosome ends sensitive to a nonhomologous end-joining pathway resulting in dicentric chromosomes and anaphase bridges. Interestingly, one of the earliest events in TRF2-deficient cells is the disappearance of the G strand overhang from telomere termini, suggesting that TRF2 is required for the maintenance of these telomeric tails. These results also raise the possibility that the G strand overhang is critical to the protective activity of telomeres. The presence of t loops at mammalian telomeres now provides an explanation for these diverse findings.

We propose a t loop–based model for the sequestration of natural chromosome ends (Figure 6B). According to this proposal, the telomere terminus is normally not exposed to the cell but rather embedded within the double-stranded part of the telomere through formation of the t loop. Cellular activities that might act on the ends of linear DNAs are proposed to be incapable of acting on telomeric ends that are sequestered in t loops. Opened t loops, perhaps resulting from inhibition of TRF2 or through the loss of the G tail, would be expected to induce the activation of DNA checkpoints, as observed in cells expressing a dominant-negative allele of

TRF2 (Karlseder et al., 1999). T loops may also fail to form in cells with very short telomeres, and this deficiency may contribute to genome instability as well as the cellular phenotypes (senescence and crisis) associated with telomere shortening (reviewed in de Lange, 1995). This model explains how cells can distinguish randomly broken DNA from natural chromosome ends, since random breaks are unlikely to form t loops efficiently.

One aspect to be explored further is the similarity of the t loop junction to a DNA replication intermediate, a feature that might be crucial to the mechanism by which checkpoints ignore this structure. Specifically, the invasion of the 3′ telomere terminus in the duplex telomeric tracts could create a configuration resembling a (stalled) replication fork. In this structure, the 3′ telomere terminus would be topologically equivalent to the end of leading strand synthesis. Additional invasion of the 5′ end of the telomere terminus (the end of the C-rich strand) would create a structure analogous to the end of lagging strand synthesis. Whether DNA replication factors (e.g., PCNA, DNA polymerases, RPA) are a component of the telomeric complex and their possible role at telomeres in mammals warrants further consideration.

Although mammalian systems may have limited predictive value for telomere biology in other organisms, the possibility that t loops are a more general mode by which chromosome ends are sequestered is not excluded. T loop formation critically depends on the only common essential feature of telomeres, the presence of tandem repeat arrays. In this regard, structures resembling t loops were observed more than 20 years ago (Goldbach et al., 1979) at the ends of *Tetrahymena* mitochondrial DNA, later shown to be composed of tandem repeats (Morin and Cech, 1988). Thus, it is a possibility that t loops are not confined to the ends of eukaryotic chromosomes. An interesting exception is found in the macronuclei of hypotrichous ciliates, where telomeres are probably too short to loop back (<50 bp; Klobutcher et al., 1981) and are tightly covered by a protein that buries their 3′ termini (Horvath et al., 1998). Since homologs of this telomere protein are not found in other eukaryotes, this radically different mode of telomeric protection may be related to the remarkable abundance of telomeres (10 million per nucleus) in these organisms (reviewed in Price, 1999).

The Role of T Loops in Telomere Maintenance

The embedded telomere terminus as present in t loops is not expected to be a good substrate for telomerase. As such, t loop formation may provide a mechanism for the regulation of telomere maintenance by telomerase. In this regard, TRF1 is known to be a negative regulator of telomere maintenance in human cells (van Steensel and de Lange, 1997), and TRF2 was recently found to have a similar activity (A. Smogorzewska, A. B., B. van Steensel, and T. d. L., in preparation). These findings could be explained if t loops limit the access of telomerase to the 3′ end of the telomere (Figure 6B). If t loops prevent telomerase from elongating the telomere, their transient resolution and unfolding could be essential for telomerase-mediated telomere maintenance. Interestingly, the binding of TRF1 to telomeric DNA can be inhibited by tankyrase, a telomeric poly(ADP)ribose polymerase (Smith et al., 1998), providing a possible regulatory pathway for t loop formation or maintenance. In addition, t loops could be opened by the replication forks, allowing transient access of telomerase to the telomere end. It will be of interest to determine the cell cycle regulation of t loops and the timing of telomere elongation by telomerase.

Other mechanisms for the maintenance of telomeric DNA, not involving telomerase or other reverse transcriptases, have been proposed to explain observations on telomerase-negative eukaryotic cells. One feature of t loops that is particularly interesting in this regard is the invasion of a 3′ end into duplex DNA. Work in T4 phage (Luder and Mosig, 1982; Kreuzer and Morrical, 1994) has shown how strand invasion of exactly the form illustrated here for telomeric DNA (Figure 6A) can prime new DNA synthesis. Such an event, followed by appropriate cleavages and gap filling, could give rise to a large increase in telomere size. The t loop junction also resembles half of a Holliday junction, and branch migration in the centromeric direction followed by degradation of the single strand DNA segments generated could give rise to substantial losses of telomeric sequences.

Conclusion

The t loop model for telomere function (Figure 6B) proposes an architectural solution to many of the problems posed by chromosome ends. Further experimental testing of this proposal should shed light on the role of t loops in protection and synthesis of telomeres in mammals and other eukaryotes.

Experimental Procedures

Preparation of Nuclei, Psoralen Photocross-Linking, and DNA Purification

Mouse liver (10 g) was homogenized in 100 ml homogenization buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 0.1 mM EGTA, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2% NP-40 [Sigma Inc.], and 5% sucrose) using a Waring Blendor for 2 min at full speed. The homogenate was filtered through fine mesh cloth, and the nuclei were collected at 5000 g for 10 min, washed several times, and resuspended in 3 ml of cross-linking buffer (15 mM Tris [pH 7.4], 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.1 mM EGTA, and 0.25 M sucrose). HeLa cell nuclei were isolated by suspending 3×10^8 cells in 20 ml of homogenization buffer for 10 min on ice followed by centrifugation for 15 min at 1300 g, washed in homogenization buffer, and suspended in 3 ml of cross-linking buffer. Human PBL nuclei were isolated from buffy coat cells by douncing on ice in 9 ml of homogenization buffer, collected at 1300 g for 15 min, and then washed and resuspended as above.

To 3 ml of nuclei in cross-linking buffer, AMT (or HMT as specified) (Sigma Inc.; 10 mg/ml stock dissolved in DMSO) was added to a concentration of 250 μg/ml. The mixture was spread on a 100 mm plastic petri dish on ice and stirred for 30 min while exposed to a 365 nm UV light bulb at a distance of 2 cm. Nuclear suspensions were treated with proteinase K in the presence of SDS, and DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The deproteinized sample was suspended in 9 ml of 10 mM Tris (pH 7.5), 1 mM EDTA (TE) and cleaved with RsaI (1250 units) and HinFI (1250 units) in a buffer of 10 mM Tris, 10 mM $MgCl_2$, 30 mM NaCl, 1 mM DTT, and 100 μg/ml of bovine serum albumin for 12 hr at 37°C. During the final hour, RNase (Pharmacia) was added to 20 μg/ml. The sample was then extracted one time with phenol:chloroform:isoamyl alcohol, precipitated with ethanol, and suspended in 3 ml of TE. The sample was applied to a 2.5 × 100 cm Biogel A5M

column (Biorad) and eluted at a flow rate of 0.2 ml/min. Fractions of 0.6 ml were taken and the OD_{260} of each determined. Genomic blotting for telomeric DNA was done as described (Broccoli et al., 1996; Luderus et al., 1996).

To verify that the psoralen/UV treatment cross-linked the DNA in situ, aliquots of HeLa1.2.11 or S3 DNA from the Biogel A5m size fractionations were heated to 95°C, quick-cooled, and electrophoresed on agarose gels. A single interstrand cross-link in a DNA fragment allows the two strands to rapidly reanneal after heating. With no psoralen/UV treatment, DNA from all Biogel A5m fractions was reduced to fast-migrating single-stranded DNA following heating. When the nuclei were treated with 250 μg/ml AMT, the DNA appeared heat resistant until the average size was ~100 bp, at which point it was rendered single stranded (data not shown). With 50 and 25 μg/ml AMT, this transition occurred at 100–200 bp and ~300 bp, respectively.

Generation of a Telomeric DNA Model and the Formation of Model DNA–Protein Complexes

A model telomere DNA consisting of 1–2 kb of repeating TTAGGG sequences grown from one end of a 3 kb unique sequence DNA was generated (R. M. S., J. Merker, S. Michalowski, and J. D. G., in preparation). The 5′ ends of the DNA were resected 100–200 nt by incubation with T7 gene 6 exonuclease (USB) for 8 min at 16°C. When the model DNA was incubated with SSB protein (purified by the method of Chase et al. [1980]), 40% showed a short SSB tract equivalent to a 100 to 200 nt overhang; the remaining 60% did not stain with SSB, suggesting that they had overhangs less than 50–75 nt.

DNA–protein complexes were assembled in 20 mM HEPES (pH 7.5), 100 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA. Telomeric DNA (2 μg/ml) was incubated with TRF2 (4 μg/ml) (Bianchi, 1999), TRF1 (4 μg/ml) (Bianchi et al., 1997), or tankyrase (8 μg/ml) (Smith et al., 1998). Each of the proteins contained a His tag and was purified by Ni^{+} chromatography of baculovirus-produced protein. All incubations were for 30 min on ice.

Immunodepletion of T Loops

A DNA fraction from HeLa cells with 35% t loops was incubated with TRF1 and a TRF1-specific, affinity-purified polyclonal antiserum (#371, van Steensel and de Lange, 1997). This resulted in the TRF1–DNA filaments being sequestered into large aggregates, presumably through antibody cross-linking. Incubation with the antibody alone did not generate such aggregates. There remained some protein-free DNA on the grids, which contained only 5% t loops as contrasted to the 35% prior to addition of TRF1 and antisera.

Electron Microscopy

TRF1–DNA complexes were formed by incubation of t loop–containing fractions with 1 to 10 μg/ml of human TRF1 in a buffer containing 20 mM HEPES (pH 7.5), 75 mM KCl, 0.1 mM EDTA for 20 min at 21°C, followed by the addition of glutaraldehyde to 0.5% for 10 min. T loop DNA was stained with SSB protein by incubating the DNA with SSB at a concentration of 1 μg/ml for 20 min on ice followed by addition of glutaraldehyde to 0.6% for an additional 10 min.

The droplet variation of the Kleinschmidt method (Kleinschmidt and Zahn, 1959) was used for surface-spreading DNA. A 50 μl aliquot of DNA (or DNA bound by TRF1) in TE was mixed with ammonium acetate (pH 7.9) to a final concentration of 0.25 M. Cytochrome C (Sigma Inc.) was added to 4 μg/ml and the drop placed on Parafilm for 90 s. A parlodion-covered EM grid was touched to the drop and then dehydrated through two washes of 80% ethanol followed by air drying and rotary shadowcast with platinum–paladium (80:20). To directly visualize DNA with bound proteins, the samples were prepared as described (Griffith and Christiansen, 1978).

Samples were examined in a Philips CM12 instrument. Lengths were measured by projecting images onto a Summagraphics digitizing tablet coupled to a Macintosh computer programmed with software developed by J. D. G. Images for publication were scanned from film using a Nikon LS4500 film scanner and the contrast adjusted using Abobe Photoshop.

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