t-loops at trypanosome telomeres

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Mammalian telomeres form large duplex loops (t-loops) that may sequester chromosome ends by invasion of the 3' TTAGGG overhang into the duplex TTAGGG repeat array. Here we document t-loops in Trypanosoma brucei, a kinetoplastid protozoan with abundant telomeres due to the presence of many minichromosomes. These telomeres contained 10–20 kb duplex TTAGGG repeats and a 3' TTAGGG overhang. Electron microscopy of psoralen/UV cross-linked DNA revealed t-loops in enriched telomeric restriction fragments and at the ends of isolated minichromosomes. In mammals, t-loops are large (up to 25 kb), often comprising most of the telomere. Despite similar telomere lengths, trypanosome t-loops were much smaller (~1 kb), indicating that t-loop sizes are regulated. Coating of non-cross-linked minichromosomes with Escherichia coli single-strand binding protein (SSB) is observed t-loops in enriched telomeric restriction fragments and at the ends of isolated minichromosomes. In mammalian t-loops and several cross-linked minichromosomes had t-loops at both ends. These results support t-loops and their prerequisite 3' tails can be formed on the products of both leading and lagging strand synthesis. We conclude that t-loops are a conserved feature of eukaryotic telomeres.

Keywords: telomere/t-loop/trypanosome

Introduction

The advent of linear chromosomes in eukaryotes was accompanied by the acquisition of specialized terminal structures that preserve chromosome ends. Most eukaryotic telomeres feature a tandem array of short repeats and a 3' overhang (Wellinger and Sen, 1997). These telomeres are maintained by telomerase, which adds telomeric repeats to the 3' end of the chromosome (Greider and Blackburn, 1985; Nugent and Lundblad, 1998). Telomerase-mediated telomere elongation is the predominant mechanism by which eukaryotes compensate for the failure of lagging strand synthesis to complete the replication of terminal sequences.

Telomeres protect chromosome ends against degradation and end-to-end fusion, and they prevent inappropriate activation of checkpoint pathways that respond to chromosome breaks (Muller, 1938; McClintock, 1941; Sandell and Zakian, 1993; van Steensel et al., 1998; Karlseder et al., 1999). This capping function is mediated by telomere-associated proteins. The stability of Saccharomyces cerevisiae telomeres depends on Cdc13p, a single-stranded telomeric DNA binding protein that protects telomeres from degradation and prevents activation of the RAD9 checkpoint pathway (Garvik et al., 1995; Lin and Zakian, 1996; Nugent et al., 1996). Similarly, hypotrichous ciliates have short (~20 nucleotides (nt)) telomeric overhangs are bound by a single-strand binding protein, but the in vivo function of this complex has not been established (Gottschling and Cech, 1984; Gottschling and Zakian, 1986; Price, 1999). Mammalian telomeres are protected by TRF2, which binds along the duplex array of telomeric TTAGGG repeats (Bilaud et al., 1997; Broccoli et al., 1997; Smogorzewska et al., 2000). Interference with TRF2 function results in immediate deprotection of telomeres, as evidenced by loss of the telomeric 3' overhang, formation of end-to-end chromosome fusions, activation of an ATM/p53-dependent DNA damage checkpoint, cell cycle arrest and apoptosis (van Steensel et al., 1998; Karlseder et al., 1999).

Electron microscopy (EM) of mammalian telomeric DNA has revealed large duplex loops (t-loops) at chromosome ends in vivo (Griffith et al., 1999). t-loops were stabilized by cross-linking the DNA in chromatin with the psoralen 4’aminomethyltrioxalen (AMT) and UV light. Telomeric DNA was enriched by gel-filtration chromatography after digestion of the bulk chromosomal DNA with frequently cutting restriction enzymes that do not cleave TTAGGG repeats. t-loops are composed of duplex TTAGGG repeats and vary with the length of the telomeric repeat array from 1 to 25 kb. Single-stranded DNA binding protein (SSB) from Escherichia coli bound to the tail-loop junction, indicating that there is a segment of single-stranded DNA at this site, most likely formed by strand displacement upon invasion of the 3' overhang into the duplex telomeric repeat tract. t-loops were observed frequently in a variety of mammalian DNA sources, including HeLa cells, mouse liver, HT1080 cells and primary peripheral blood leukocytes. A recent report showed loops at the ends of amplified micronuclear chromosomes in Oxytricha fallax (Murti and Prescott, 1999). These loops were not found in DNA from macronuclei, in which telomeres are extremely short and have a protein complex bound to the 3' overhang.

Although the mechanism by which t-loops are formed in vivo has not been established, both TRF2 and the related telomeric protein TRF1 have biochemical features suggestive of a role in loop formation and/or maintenance. TRF1 can loop and pair telomeric DNA, and TRF2
promotes the formation of t-loops from a model telomere substrate (Bianchi et al., 1997, 1999; Griffith et al., 1998, 1999). In addition, proteins involved in DNA recombination and repair have been proposed to contribute to t-loop dynamics. An example is the Rad50–Mre11–Nbs1 recombinational repair complex that binds to TRF2 and interacts with mammalian telomeres (de Lange and Petrini, 2000; Zhu et al., 2000).

T-loops could provide an architectural solution to the problems posed by telomeres by hiding the telomere terminus in the duplex part of the telomere, preventing activation of DNA damage checkpoints and protecting chromosome ends from inappropriate DNA repair (Griffith et al., 1999). In addition, insertion of the 3' terminus would presumably block telomerase from adding repeats to the chromosome end. Therefore, the rate at which t-loops are refolded after DNA replication might contribute to the regulation of telomere length.

To determine whether t-loops are conserved, we employed the advantages of trypanosome genomes. The Trypanosomatidae are protozoan parasites, of which Trypanosoma brucei is best known for the phenomenon of antigenic variation, allowing extracellular survival in the bloodstream of its mammalian host. Each bloodstream form (BF) cell expresses 10 million copies of a single species of variant surface glycoprotein (VSG) (Cross, 1975). The expressed VSG gene (VSG) is invariably positioned at a telomere (de Lange and Borst, 1982), at the end of a polycistronic transcription unit called an expression site (ES). Only one ES is transcriptionally active at any time, and antigenic variation is achieved either by gene conversion of the transcribed VSG by one of the several hundred VSGs dispersed around the genome, or by coordinated activation/inactivation of different telomeric ESs (Cross, 1996; Rudenko et al., 1998). ESs are not transcribed in the non-infectious insect stage (procyclic form, PF) of the life cycle.

Trypanosome telomeres contain long TTAGGG repeat arrays, which grow by 9–12 bp per cell division (Bernards et al., 1983; Blackburn and Challoner, 1984; van der Ploeg et al., 1984), presumably through the action of telomerase (Cano et al., 1999). Transcriptionally active telomeres grow slightly faster and are more susceptible to truncations than inactive ones (Pays et al., 1983; Horn and Cross, 1997), suggesting complex mechanisms of telomere maintenance and protection. Position effects within the ES (Rudenko et al., 1995; Horn and Cross, 1997), chromatin remodeling and developmentally regulated repression effects close to telomeres suggest a role for telomeres in regulating antigenic variation (Navarro et al., 1999).

Trypanosoma brucei contains 11 large chromosome pairs (Melville et al., 1998, 2000), ranging from 1 to >5 Mbp, which carry the essential genes, and ~100 minichromosomes, of 25–150 kb, which are predominantly composed of tandem 177 bp repeats (Weiden et al., 1991). Minichromosomes have canonical telomeres at both ends and some telomeres carry silent VSG genes that can contribute to the expressed VSG repertoire through transposition to ESs in the large chromosomes (van der Ploeg et al., 1984; Weiden et al., 1991). Minichromo-somes represent an abundant source of telomeres, which were used in this study to address the evolutionary conservation of t-loops.

Results

Trypanosoma brucei telomeres carry ~10–20 kb of TTAGGG repeats and have a 3' overhang

It was previously shown that T. brucei telomeres are composed of TTAGGG repeats and that most TTAGGG repeats in the trypanosome genome occupy terminal sites, based on their sensitivity to exonuclease treatment of intact genomic DNA (Blackburn and Challoner, 1984; van der Ploeg et al., 1984). In order to determine the median length of the TTAGGG repeat arrays in the T. brucei line used in this study, we applied a technique previously used to measure the length of human telomeres (Saltman et al., 1993). The rate at which the exonuclease Bal31 removes TTAGGG repeat hybridization signal is compared with the rate at which the enzyme shortens terminal DNA fragments. In T. brucei, this approach is facilitated by the availability of probes for subtelomeric VSG genes and the detailed knowledge of the restriction maps of these loci, allowing precise measurements of Bal31 digestion rates on well-defined terminal restriction fragments.

Digestion of DNA from BF and PF T. brucei with frequently cutting enzymes yielded telomeric fragments in the 10–20 kb range (Figure 1A), suggesting that the telomeres contain long arrays of TTAGGG repeats. To measure the length of the telomeric repeat array directly, intact PF DNA was treated with Bal31 exonuclease for increasing times, digested with HinI–AluI–RsaI and hybridized to a TTAGGG repeat probe (Figure 1B). Quantification of the TTAGGG repeat signal at each time point indicated that the exonuclease removed ~2.6% of the TTAGGG repeat signal per minute. The rate at which Bal31 shortened the telomeric fragment that carries VSG 221 was determined in parallel (Figure 1B; see Figure 1C for restriction map). The same shortening rate was found for the telomeric restriction fragment carrying VSG 121 (Figure 1B; see Figure 1C for restriction map). As expected, Bal31 did not affect chromosome-internal restriction fragments, such as those carrying non-telomeric copies of VSG 121 (Figure 1B). Comparison of the two rates (Figure 1D) showed that Bal31 removed ~10% of the TTAGGG repeat signal in the time needed to shorten the telomere by 1.5 kb, implying that the average length of the TTAGGG repeat array was ~15 kb. This value for the length of the TTAGGG repeats array was in agreement with the median length for the telomeric fragments observed in DNA digested with HinI and RsaI (Figure 1A), which are expected to remove most of the subtelomeric sequences from the terminal fragments.

We next determined whether T. brucei chromosome ends carry an overhang of the TTAGGG repeat strand. Mammalian chromosomes have up to 200 nt of single-stranded TTAGGG repeats at their 3' termini (Makarov et al., 1997; McElligott and Wellingler, 1997; Wright et al., 1997; van Steensel et al., 1998; Huffman et al., 2000) and this overhang is presumed to be important for the formation of t-loops. The presence of single-stranded TTAGGG repeats can be assessed by annealing labeled
C-strand-specific oligonucleotides to genomic DNA. Using this approach, we found that native DNA from *T. brucei* contained single-stranded TTAGGG repeats (Figure 1E). The signal was not detected in DNA digested with *E. coli* exonuclease I, which is specific for 3' single-stranded tails, or in a control hybridization with an oligonucleotide representing the G-rich telomeric strand, consistent with the signal being derived from 3' single-stranded TTAGGG tails. Furthermore, the signals were present on large fragments (>10 kb) in DNA that was digested with *AluI*±*HindIII*±*RsaI*, as would be expected if the G-tails are present at the ends of the trypanosome telomeres. The presence of single-stranded telomeric tails was corroborated by EM analysis of minichromosomes coated with *E. coli* SSB (see below). These data indicate that *T. brucei* telomeres resemble human telomeres in both the length of the TTAGGG repeat array and the presence of a 3'[TTAGGG]<n overhang.

### t-loops in *T. brucei* telomeric DNA from procyclic and bloodstream forms

The telomeric repeat arrays of *T. brucei* are sufficiently long to allow their isolation by differential size fractionation after digestion of genomic DNA with frequently cutting restriction endonucleases (see Figure 1A). We previously employed this approach to isolate telomeric DNA from the human genome, for cloning and for EM visualization (de Lange et al., 1990; Griffith et al., 1999). Furthermore, the TTAGGG sequence of trypanosome telomeres, like their human counterparts, lends itself to cross-linking with psoralen (AMT) and UV light, which cross-links T residues on opposite strands at AT steps, potentially stabilizing t-loops during their isolation. Accordingly, PF or BF *T. brucei* were permeabilized with digitonin and treated with AMT and UV light. Following deproteinization, the DNA was cleaved with *AluI*–*HindIII*–*RsaI* and size fractionated on a Bio-Gel A-15m...
Fractions containing large DNA fragments were then prepared for EM by spreading on a denatured film of cytochrome c protein, followed by rotary shadowcasting. EM examination revealed the presence of long DNA molecules is the early eluting (high molecular weight) fractions of the Bio-Gel column, and many of these molecules contained loops at one end. In five experiments, the fraction of long linear DNAs (>10 kb) containing a loop at one end varied from 8% to as much as 25% (n >100 for each experiment), which is a frequency of t-loops very similar to that observed in mammalian telomeric restriction fragments. The structure of trypanosome t-loops appeared to be similar to that of t-loops from mammalian cells, containing a single terminal loop of variable size and a variably sized unforked tail. Examples of trypanosome t-loops are shown in Figure 2, where the loops vary in size from 6.3 (A) to 0.63 kb (F). No difference was observed in t-loop frequency or structure in DNA from BF and PF trypanosomes.

**Trypanosome t-loops are small**

Measurement of loop contour lengths of t-loops in enriched telomeric restriction fragments showed a wide variety of sizes ranging from as small as 0.3 kb to as large as 8 kb. However, >65% of the loops were quite small (<1.5 kb) and the median length of the 48 t-loops from telomeric restriction fragments was ~1.1 kb. Similarly, t-loops at the ends of isolated minichromosomes (see below) showed a range in loop sizes from as small as 570 bp to as large as 8.4 kb, with a median value of 1.0 kb for 21 loops analyzed. The combined data on the size range of the t-loops in both types of DNA preparations are given in Figure 3. Overall, the median size of the loops was 1.1 kb, and 42 out of 69 t-loops analyzed were very small, ranging between 0.5 and 1.0 kb. Furthermore, a number of trypanosome t-loops measured <500 bp. Large t-loops (>3 kb) were rare in both the enriched telomeric restriction fragments and in isolated minichromosomes.
The level of resolution of the surface spreading method employed here is such that circles of <150±200 bp would frequently appear as balls rather than small loops or donuts. Examination of the minichromosomes by directly adsorbing the samples to carbon supports and rotary shadow-casting in the absence of denatured protein allowed a higher resolution inspection of the DNA ends. However, even using this technique, no examples of circles smaller than those detected by surface spreading were observed (data not shown). Nonetheless, it remains possible that some very small loops were present and not scored in these experiments.

t-loops and single-stranded tails at both ends of T. brucei minichromosomes

We next asked whether t-loops can occur at both ends of a chromosome. Trypanosome minichromosomes are sufficiently small to allow their visualization as intact molecules, allowing inspection of both ends of each chromosome (Weiden et al., 1991). To isolate minichromosomes for this purpose, permeabilized trypanosomes were treated with psoralen and UV, gently lysed, deproteinized, and sedimented through a 5–20% sucrose gradient. Gradient fractions containing minichromosomes were identified by gel electrophoresis, under conditions that separate minichromosomes from larger chromosomes, followed by detection of telomeric DNA with a TTAGGG repeat probe. Using this approach, fractions were identified that were highly enriched for minichromosomal DNA (Figure 4A and B). These fractions appeared to lack DNA derived from the larger chromosomes because they did not contain detectable amounts of an abundant 50 bp repeat element that is present upstream of ESs on the larger chromosomes (Melville et al., 1998) (compare fractions to total DNA in Figure 4C).

The enriched minichromosomal fractions were analyzed by EM and found to contain linear DNA molecules ranging from 20 to 50 kb. This size range is about half that expected from measurements by EM and gel electrophoresis (Figure 4), suggesting that some of the minichromosomal DNAs were broken during isolation. However, EM analysis showed that 14 out of 144 molecules contained a small t-loop at one end. In a second experiment, 23 out of 115 large molecules showed a loop at one end. Thus, overall, ~15% of the ends had a t-loop. In these experiments we found four minichromosomal DNAs that carried t-loops at both ends (Figure 5) and a fifth double-looped minichromosome was found in a third experiment. If the t-loop frequencies in our preparations were primarily determined by the extent to which t-loops were lost during DNA isolation due to incomplete cross-linking or breakage, we would expect that the frequency of double-looped molecules would be ~2.25% (15% of 15%), predicting approximately six double-looped molecules in the 259 DNAs that were examined. This number is in reasonable agreement with the four double-looped molecules that were observed, suggesting that t-loops often occur at both ends of minichromosomes.

A 3′ overhang of single-stranded telomeric repeats is likely to be a prerequisite for t-loop formation. However, based on the mechanism of DNA replication, 3′ overhangs are not expected to occur at chromosome ends formed by leading strand DNA synthesis and there are conflicting reports on whether both ends of human chromosomes have a single-stranded tail (Makarov et al., 1997; Wright et al., 1997).

To address this issue we used E. coli SSB to query the status of the DNA at the ends of trypanosome minichromosomes (Figure 6). Trypanosoma brucei minichromosomes were prepared by lysis of PF cells and sucrose gradient sedimentation in the presence of sarcosyl without AMT and UV treatment. Aliquots were then chromatographed over Bio-Gel A-15m to remove the detergent and the minichromosomes incubated with E. coli
SSB protein to bind any single-stranded DNA. Single tetramers or octamers of SSB bound along the length or at the ends of otherwise duplex DNA can be distinguished by EM, and represent the presence of ~75 (single tetramer) or 150 nt (octamer) of single-stranded DNA (Chrysogelos and Griffith, 1982). The minimum length of a single-stranded DNA overhang that will allow binding of an SSB tetramer has not been established. Thus, overhangs less than ~75 nt may be missed using this approach. Following preparation of the complexes for EM, examination of 138 minichromosomes judged to be >50 kb revealed that 70% showed no SSB on either end, 23% showed SSB binding at one end and 7% had SSB at both ends (Figure 6).

To evaluate the length of the overhang, the number of SSB tetramers bound at an end was counted. For the minichromosomes with SSB bound at just one end or at both ends, 71 and 70%, respectively, of the ends showed from one to three tetramers bound, suggestive of overhangs in the range of 75–225 nt. The remaining 30% of the ends showed longer SSB-bound tracts ranging up to ~500 nt. Thus, consistent with the annealing data in Figure 1, trypanosome chromosome ends contain substantial regions of single-stranded DNA and these overhangs can occur at both ends of the same chromosome.

Discussion

This report documents the presence of t-loops at chromosome ends in trypanosomes. Although they have the same sequence and overall length, trypanosome telomeres had loops that were significantly smaller than those of mammalian telomeres, indicating that t-loop sizes are determined by a specific mechanism. A significant fraction of isolated intact trypanosome minichromosomes had two t-loops and carried single-stranded overhangs at both ends, showing that telomeres generated by both leading and lagging strand synthesis can be remodeled into t-loops. Generation of the 3’ overhang for t-loop formation at the end duplicated by leading strand synthesis must involve post-replicative modification since the replication product is predicted to be blunt. Our findings, together with the demonstration of t-loops in mammals and ciliates, indicate that they are a conserved feature of eukaryotic telomeres and their presence at both ends of a chromosome is consistent with a requirement for t-loops in the function of all telomeres.

Trypanosome t-loops are relatively small

The trypanosome telomeres analyzed in this study are composed of 10–20 kb of TTAGGG repeats. Human telomeres have a very similar structure, containing a duplex TTAGGG repeat array in the 5–20 kb range. Despite these similarities, the size distribution of the t-loops observed in these two species was significantly different. Trypanosome telomeres often had small t-loops (median size 1.1 kb), whereas human telomeres very rarely showed t-loops in that size range. For instance, HeLa cells with telomeres in the 20 kb range had t-loops with a median size of 14 kb and <2% of the t-loops were <1 kb. The smallest human t-loops were observed in cells with telomeres composed of ~5 kb TTAGGG repeats, but these t-loops were still significantly larger (median 3 kb) than those of trypanosomes. Similarly, the loops observed at the ends of micronuclear chromosomes of O.fallax were much larger [5–10 kb loops (Murti and Prescott, 1999)] than trypanosome t-loops and more comparable to those of mammalian cells. Although it is not clear what determines the size of the t-loops, the data suggest that there is an active process involved in establishing or maintaining t-loops of a specific size.

Conservation of t-loops

This study focused on telomeres in trypanosomes because of their experimental advantages and because they represent a very ancient lineage. Trypanosomes probably branched off >500 million years ago, long before the origin of their metazoan hosts (Stevens and Gibson, 1999). The molecular biology of these highly diverged protozoa is quite distinct from the perceived...
norm as represented by yeast, plants and mammals. For instance, trypanosomes have a specialized organelle for glycolysis, their mitochondria contain an unusual network of small circular DNAs, mitochondrial RNAs are edited, and nuclear mRNAs are formed by trans-splicing. Within this context, the conservation of t-loops in trypanosomes stands out as highly significant and predicts that t-loops play an essential role at telomeres in many eukaryotes.

The previous demonstration of looped structures at the ends of *O.fallax* micronuclear chromosomes (Murti and Prescott, 1999) is in agreement with the proposal that t-loops are highly conserved. Interestingly, this organism also provides an example of functional telomeres that lack t-loops. The macronuclear DNA of *Oxytricha* and other hypotrichous ciliates is formed by extensive fragmentation and processing of the micronuclear genome, resulting in amplified small DNA fragments each carrying one gene. These gene-sized molecules are all endowed with short telomeres that contain <50 bp of duplex telomeric DNA and a short single-stranded overhang (Price, 1999). Given their extreme short size, it was anticipated that these telomeres would lack t-loops, (Griffith *et al.*, 1999) a prediction consistent with the EM analysis. Instead, the ends of the macronuclear DNAs may be protected by the tenaciously bound protein complex (Gottschling and Zakian, 1986; Horvath *et al.*, 1998; Price, 1999).
Collectively, the presence of t-loops in organisms as diverged as mammals, ciliates and Kinetoplastidae indicates that this aspect of telomere structure is highly conserved.

**t-loops at yeast telomeres?**

The finding of t-loops in diverged eukaryotes has raised the question of whether they occur in budding yeast, where telomeres have been characterized extensively. A t-loop-like structure was proposed by Li and Lustig (1996) as an intermediate in the rapid deletions that can occur when yeast telomeres are excessively long. Telomere folding (without strand invasion) was also proposed by McEachern and Blackburn (1995) to explain the mechanism of telomere length regulation in *Kluyveromyces lactis*, and Zakian and Ptashne and their colleagues proposed a fold-back structure for telomeres in *S. cerevisiae* based on studies of transcriptional regulation of subtelomeric genes (de Bruin et al., 2000, 2001). Similarly, based on the ability to cross-link the telomeric DNA binding protein Rap1p to subtelomeric Y′ elements, Grunstein, (1997) proposed that yeast telomeres form a higher order structure in which the telomere is folded back along the subtelomeric DNA. Technical limitations of the current t-loop assays have hindered direct examination of yeast telomere structure.

A significant difference between yeast telomeres and those of trypanosomes and mammals is that yeast telomeres appear to lack long single-stranded protrusions (except for a short window late in S phase (Welling et al., 1993)). Such telomere tails are presumed to be required for the strand invasion that creates the t-loop, although different scenarios can be envisioned. Furthermore, t-loop formation in mammals has been proposed to depend on the telomeric protein TRF2, and a recent comparison of the mammalian and yeast telomeric complexes has suggested that budding yeast has lost the genes encoding both TRFs (Li et al., 2000). Interestingly, the major yeast telomeric DNA binding protein Rap1p has the ability to promote the pairing of single-stranded telomeric DNA with duplex repeat tracts in vitro (Gilson et al., 1994), an activity that could be indicative of a role in higher order remodeling of telomeric DNA.

So far, the protein components of trypanosome telomeres have not been identified. Specifically, it will be of interest to determine whether trypanosomes have TRF and Rap1p orthologs. Given the ease of gene targeting in trypanosomes and the abundance of their telomeres, trypanosomes could become a fruitful system for telomere biology once telomeric proteins are in hand.

**t-loops and telomeric tails at both ends of each chromosome**

Several trypanosome minichromosomes showed t-loops at both ends. The frequency of double-looped molecules was high enough to suggest that t-loops are formed at DNA ends created by both lagging and leading strand synthesis. The two modes of DNA synthesis are predicted to generate different ends. Lagging strand synthesis generates a 3′ overhang with a length that depends primarily on the site where primase synthesized the last RNA primer; removal of the RNA primer could contribute an additional 8–12 nt to the protrusion. By contrast, leading strand synthesis should result in a blunt end and formation of a 3′ overhang was therefore suggested to require a nuclease. This dilemma was previously recognized in the context of tests for the presence of 3′ overhangs at both ends of each chromosome and there are conflicting reports on the terminal structure of human chromosome ends (Makarov et al., 1997; Wright et al., 1997). Our data are compatible with the view that all chromosome ends carry a 3′ overhang, as an overhang is likely to be required for the maintenance of t-loops. Indeed, EM analysis of minichromosomes with bound SSB showed frequent occurrence of single-stranded DNA (presumably the G-strand overhang) at both chromosome ends. There are several mechanisms by which the end created by leading strand synthesis could acquire a 3′ overhang. Telomerase could synthesize the overhangs, they could be generated by an (unknown) 5′→3′ exonuclease, or the newly generated blunt end could invade the duplex part of the telomere and the 3′ end could then be extended by the replication machinery. Regardless of the mechanism of their formation, the presence of overhangs and t-loops at both ends of trypanosome chromosomes further corroborates the idea that t-loops are required for the protection of all chromosome ends.

**Materials and methods**

**Trypanosomes**

Molteno Institute Trypanozoon antigenic type 1.2 (MITat 1.2), clone 221a, derived from strain 427 was used. Mice were infected by intraperitoneal injection and observed until the parasitemia reached approximately 108 parasites/ml. A total of 108 parasites were purified through DEAE-cellulose as described (Cross, 1975), centrifuged gently and suspended in ice-cold trypanosome dilution buffer, TDB (5 mM KCl, 80 mM NaCl, 1 mM MgSO4·7H2O, 20 mM Na2HPO4·2H2O, 20 mM glucose). Procyclic forms of the same strain were cultured at 27°C in SDM-79 supplemented with fetal bovine serum, to a concentration of 107 parasites/ml. A total of 109 parasites were collected, washed and resuspended in ice-cold phosphate-buffered saline (PBS) pH 7.3 (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH2PO4·7H2O, 1.4 mM KH2PO4).

**Genomic blotting**

To isolate genomic DNA, ~5×108 parasites were resuspended in TNE (10 mM Tris pH 7.4, 10 mM EDTA, 100 mM NaCl) and lysed in TNES (10 mM Tris pH 7.4, 100 mM NaCl, 10 mM EDTA, 1% SDS) in the presence of 100 µg/ml proteinase K. After overnight incubation with proteinase K at 37°C, and phenol/chloroform extractions, DNA was precipitated with isopropanol and resuspended in TE (10 mM Tris pH 7.5/1 mM EDTA). RNase A treatment, phenol/chloroform extractions and isopropanol precipitation followed. Bst31 digestions were performed as described elsewhere (de Lange and Borst, 1982). At each time point, the reaction was stopped by increasing the temperature to 65°C for 10 min and DNA was purified using Sepharose Microspin columns (Amersham). For telomere blots, DNA was digested overnight with the restriction enzymes Aldh, HinF1, RsaI, MboI or XmaI, and size fractionated as described (de Lange and Borst, 1982). Telomeric restriction fragments were detected using a probe containing TTAGGG repeats and labeled as previously described (de Lange, 1992). The sequence of the 50 bp repeat was obtained from a plasmid provided by P.Borst (pBL-50), and a probe consisting of one repeat (GTGTAATTCCCGTAC-TAAAGATTTACATCAGGGGTGTCGATGACTGT) was synthesized and end labeled. Probes for VSGs 221 and 121 were labeled by standard methods. Signals were quantified using ImageQuant software and PhosphorImager data.

**Permeabilization, cross-linking and telomeric DNA preparation for EM**

BF or PF trypanosomes (5×108) washed in TDB or PBS, respectively, were resuspended in resuspension buffer (15 mM Tris–HCl pH 7.4,
15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.25 mM sucrose) and incubated on ice in the presence of 40 μM digitonin (Sigma) for 5 min. Trypanosomes were collected by spinning at 3000 r.p.m. for 30 s in a microcentrifuge, and treated for cross-linking and DNA extraction following scaled-down adaptations of previously published protocols. Specifically, 50 μl of a solution of AMT (Sigma; 5 μg/ml in H2O) were added to 1 ml of permeabilized cells. Trypanosomes were stirred slowly and exposed to UV light (350 nm) for 30 min. An equal volume of TNE-15 was added and supplemented with 200 μg of proteinase K. The samples were incubated at 55°C for 2 h. After two extractions with phenol/chloroform and precipitation of the DNA with isopropanol, the DNA was resuspended in TNE and treated with RNase A (20 μg/ml) for 1 h at 37°C. DNA was extracted twice with phenol/chloroform, collected by precipitation with isopropanol, dissolved and digested with RsaI–HindIII–AluI. The digest was phenol extracted, ethanol precipitated and resuspended in TE.

**Sucrose gradients and pulsed-field rotating gel electrophoresis of minichromosomal DNA**

The procedure was adapted from the protocol of Weiden et al. (1991). Briefly, digitonin-permeabilized trypanosomes cross-linked with psoralen/UV as above were resuspended in 100 μl of TNE and immediately lysed by adding 1 ml of lysis solution [200 mM EDTA, 1% sodium lauryl sarcosinate (SLS), 0.5 mg/ml proteinase K] and incubating for 2–3 h at room temperature. The lysate was loaded onto a 35 ml linear 5–20% sucrose gradient in 25 mM Tris pH 7.5, 1% SLS. Gradients were centrifuged at 25°C for 16 h at 10 000 r.p.m. in an SW28 ultracentrifuge rotor. Fractions of 2 ml were collected from the bottom of the gradient. Aliquots of 30 μl from each fraction were mixed with 30 μl of 1.6% low melting point agarose at 65°C and loaded onto a 0.8% agarose gel. Electrophoresis was carried out at an angle of 120°. 1–2 s linear ramp, and a constant voltage of 180 V for 15 h at 13°C in a rotating agarose gel electrophoresis (RAGE) apparatus (Stratagene).

**Gel chromatography of T. brucei telomeric restriction fragments**

Following restriction of total cross-linked T. brucei DNA, the DNA was precipitated with ethanol and resuspended in 10 mM Tris pH 7.5, 1 mM EDTA at a concentration of ~200 μg/ml and the sample incubated on ice for 10 min. Glutaraldehyde was then added to 0.6% for 5 min at room temperature. The lysate was loaded onto a 35 ml linear 5–20% sucrose–sarcosyl gradient in 25 mM Tris pH 7.5, 1% SLS. Gradients were centrifuged at 25°C for 16 h at 10 000 r.p.m. in an SW28 ultracentrifuge rotor. Fractions of 2 ml were collected from the bottom of the gradient. Aliquots of 30 μl from each fraction were mixed with 30 μl of 1.6% low melting point agarose at 65°C and loaded onto a 0.8% agarose gel. Electrophoresis was carried out at an angle of 120°. 1–2 s linear ramp, and a constant voltage of 180 V for 15 h at 13°C in a rotating agarose gel electrophoresis (RAGE) apparatus (Stratagene).

**Staining of minichromosomes with SSB**

Aliquots of T. brucei minichromosomes in sucrose–sarcosyl were chromatographed over 2 ml columns of Bio-Gel A-5m equilibrated in Bio-Gel A-5m in the same buffer. The chromatography was controlled with a Pharmacia Gradifrac apparatus. The DNA profile was determined by absorbance readings at 260 nm and fractions containing the telomeric restriction fragments were prepared for EM.

**EM methods**

To examine cross-linked T. brucei minichromosomes separated by sucrose sedimentation, the pooled DNA fractions were chromatographed through 2 ml columns of Bio-Gel A-5m (Bio-Rad Inc.) equilibrated in TE to remove the sucrose and detergent. Following cross-linking and processing, DNA was precipitated with ethanol and dissolved in TE. Cross-linked DNA samples in TE were prepared for EM by spreading on a denatured film of cytochrome c using the droplet variation of the method of Kleinschmidt as described (Griffith et al., 1999). The grids were air dried and rotary shadowcast with platinum/paladium (201), and examined in a Philips CM12 instrument at 40 kV. DNA lengths were measured by projecting images on EM sheet film onto a Summagrams digitizing tablet attached to a Macintosh computer. The images were stored with software developed in the Griffith laboratory. Images for publication were scanned using a Nikon LS4500 film scanner, and the contrast adjusted and images arranged into figures using Adobe Photoshop software.

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