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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) often revealed 3′ overhangs at both telomeres and several cross-linked minichromosomes had t-loops at both ends. These results suggest that 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 that 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 *VSG*s 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 *Bal*31 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 *Bal*31 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 *Bal*31 exonuclease for increasing times, digested with *Hin*fI–*Alu*I–*Rsa*I 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 *Bal*31 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, *Bal*31 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 *Bal*31 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 *Hin*fI and *Rsa*I (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 Wellinger, 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

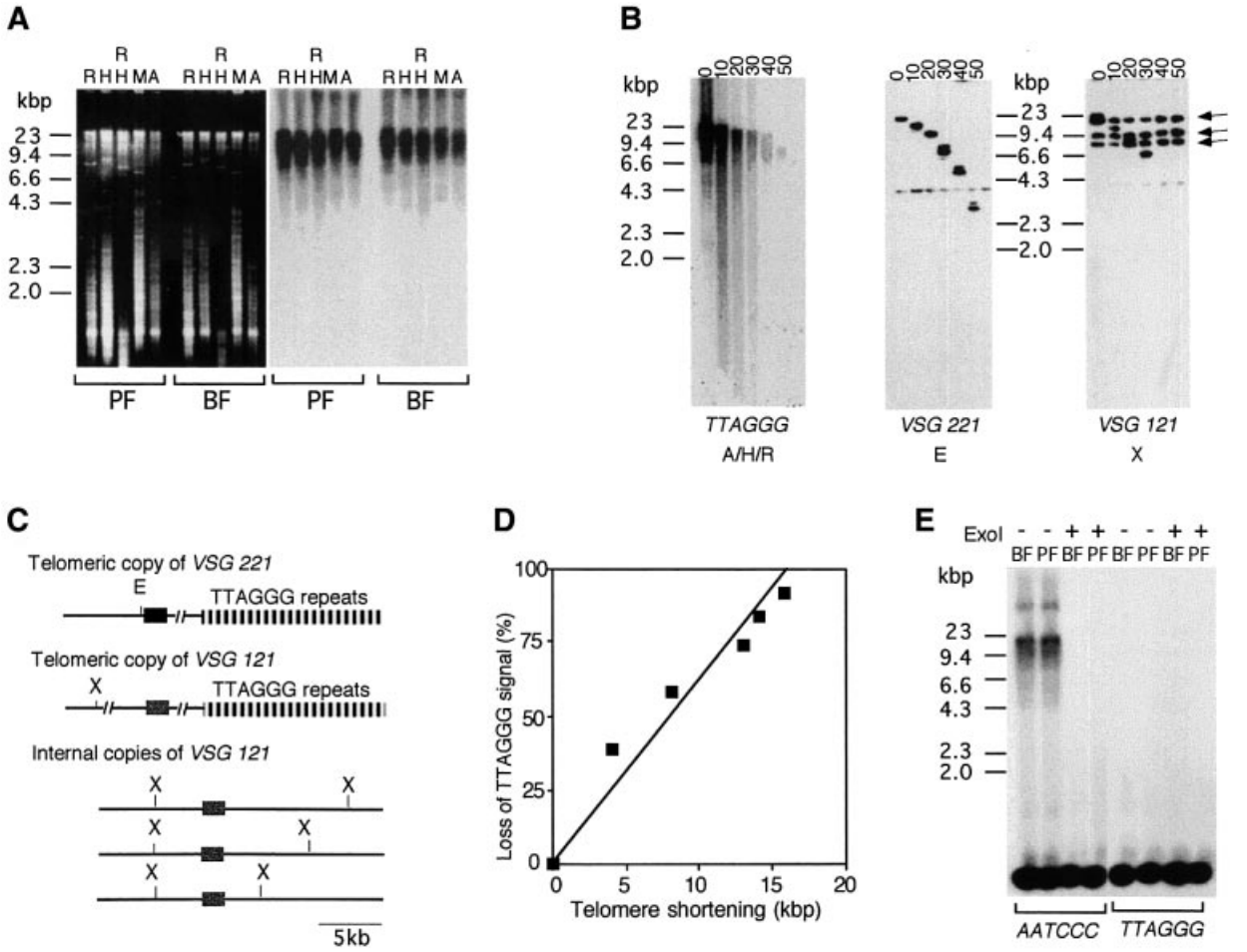

Fig. 1. Analysis of *T.brucei* telomeric DNA. (**A**) Southern blotting analysis of telomeric restriction fragments in DNA from PF and BF trypanosomes. The DNAs were digested with *Rsa*I (R), *Hin*fI (H), *Rsa*I–*Hin*fI (RH), *Mbo*I (M) or *Alu*I (A). The left panel shows the ethidium bromide; the right panel shows a Southern blot probed with a $(TTAGGG)_{27}$ probe. (**B**) *Bal*31 digestion of trypanosome telomeric DNA. Intact genomic DNA from BF trypanosomes was treated with Bal31 for the indicated times (in minutes) and digested with *Alu*I–*Hin*fI–*Rsa*I (left panel), *Eco*RI (middle) or *Xmn*I (right panel) and probed as indicated below the panels. The arrows indicate three non-telomeric *VSG 121* fragments. (**C**) Restriction maps of the telomeric *VSG 221* and *121* loci and the chromosome internal *121* genes. E, *Eco*RI; X, *Xmn*I. (**D**) Graph of the rate at which *Bal*31 removed the TTAGGG repeat signal in (B) plotted against the rate at which the exonuclease shortened the *VSG 221* telomeric *Eco*RI fragment in (B). (**E**) Overhang assay. DNA from BF and PF was digested with *Alu*I–*Hin*fI–*Rsa*I after treatment with (+) or without (–) *E.coli* exonuclease I. DNA was incubated with radiolabeled single-stranded $[TTAGGG]_4$ or $[AATCCC]_4$ probes as indicated and separated by agarose gel electrophoresis (van Steensel *et al*., 1998). The gel was dried and exposed on a PhosphorImager (Molecular Dynamics). The signal at the front represents free probe.

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 *Alu*I–*Hin*fI–*Rsa*I, 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 *Alu*I–*Hin*fI–*Rsa*I and size fractionated on a Bio-Gel A-15m

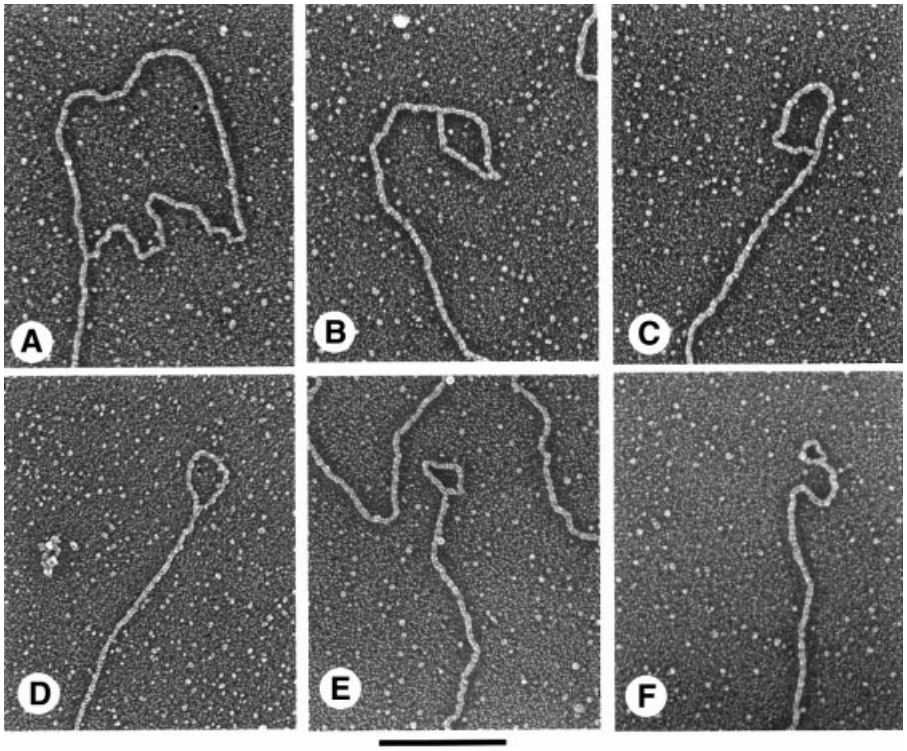

Fig. 2. Visualization of t-loops from *T.brucei* DNA photo-cross-linked with AMT and UV light. Trypanosomes were permeabilized with digitonin and treated with AMT and UV light, followed by endonuclease cleavage of purified DNA and isolation of the telomeric restriction fragments by gel filtration. DNA fragments were prepared for EM by spreading on a denatured film of cytochrome *c* protein and rotary shadowcasting with platinum:paladium. Shown in reverse contrast. *t*-loops shown in (**A**–**F**) measured 6.3, 1.75, 1.5, 1.2, 0.99 and 0.63 kb, respectively. Bar is equivalent to 1 kb.

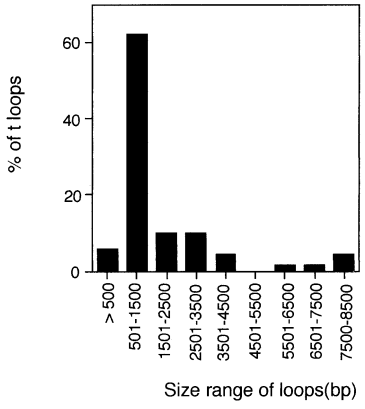

Fig. 3. Size distribution of trypanosome t-loops. Bar graph depicting the size distribution of *T.brucei* t-loops. The data were obtained from measurements of 48 t-loops in enriched telomeric restriction fragments (examples shown in Figure 2) and from measurements of 21 t-loops at the ends of minichromosomes (examples shown in Figure 5).

matrix. 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% ($n \geqslant 100$ 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.

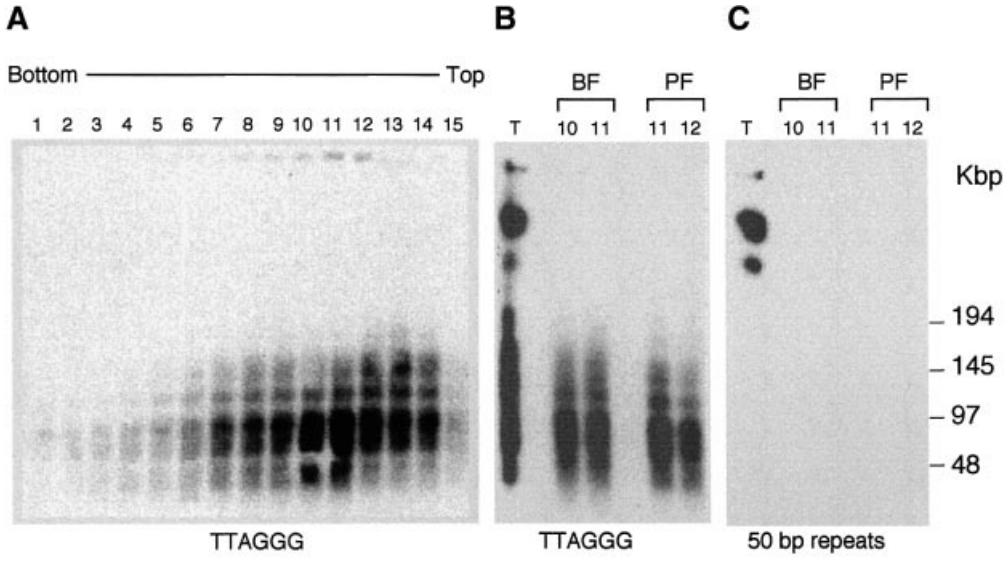

Fig. 4. Isolation and analysis of minichromosomes. (**A**) Southern blot of fractions from sedimentation of BF trypanosome chromosomal DNA through a linear 5–20% sucrose gradient. Fractions were analyzed by rotating agarose gel electrophoresis (RAGE) and probed with $[TTAGGG]_{27}$. Two fractions from BF chromosomes (10 and 11) and two fractions from a parallel sedimentation of PF chromosomes (11 and 12) are compared alongside input PF DNA prior to sedimentation (T). Blots were probed with TTAGGG repeats (**B**) or a 50 bp repeat probe (**C**).

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 shadowcasting 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*

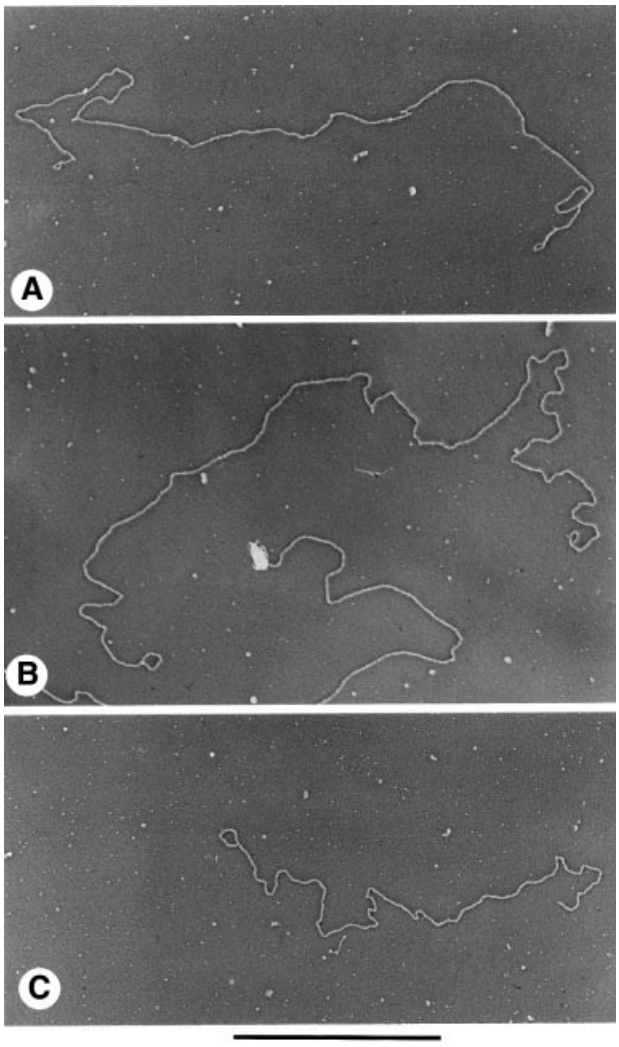

Fig. 5. t-loops in *T.brucei* minichromosomes. Minichromosomes enriched by sucrose gradient sedimentation were prepared for EM as described in Materials and methods. The minichromosome in (**A**) measures 28.7 kb, and the loops at the left and right ends measure 650 and 710 bp, respectively. The minichromosome in (**B**) is 30.5 kb, with loops of 1310 and 790 bp at the left and right ends, respectively. The molecule in (**C**) is 21.1 kb (most likely a broken minichromosome) and the loop at the left end is 1470 bp. Shown in reverse contrast. Bar is equivalent to 5 kb.

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 $\geqslant$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 $\leqslant$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

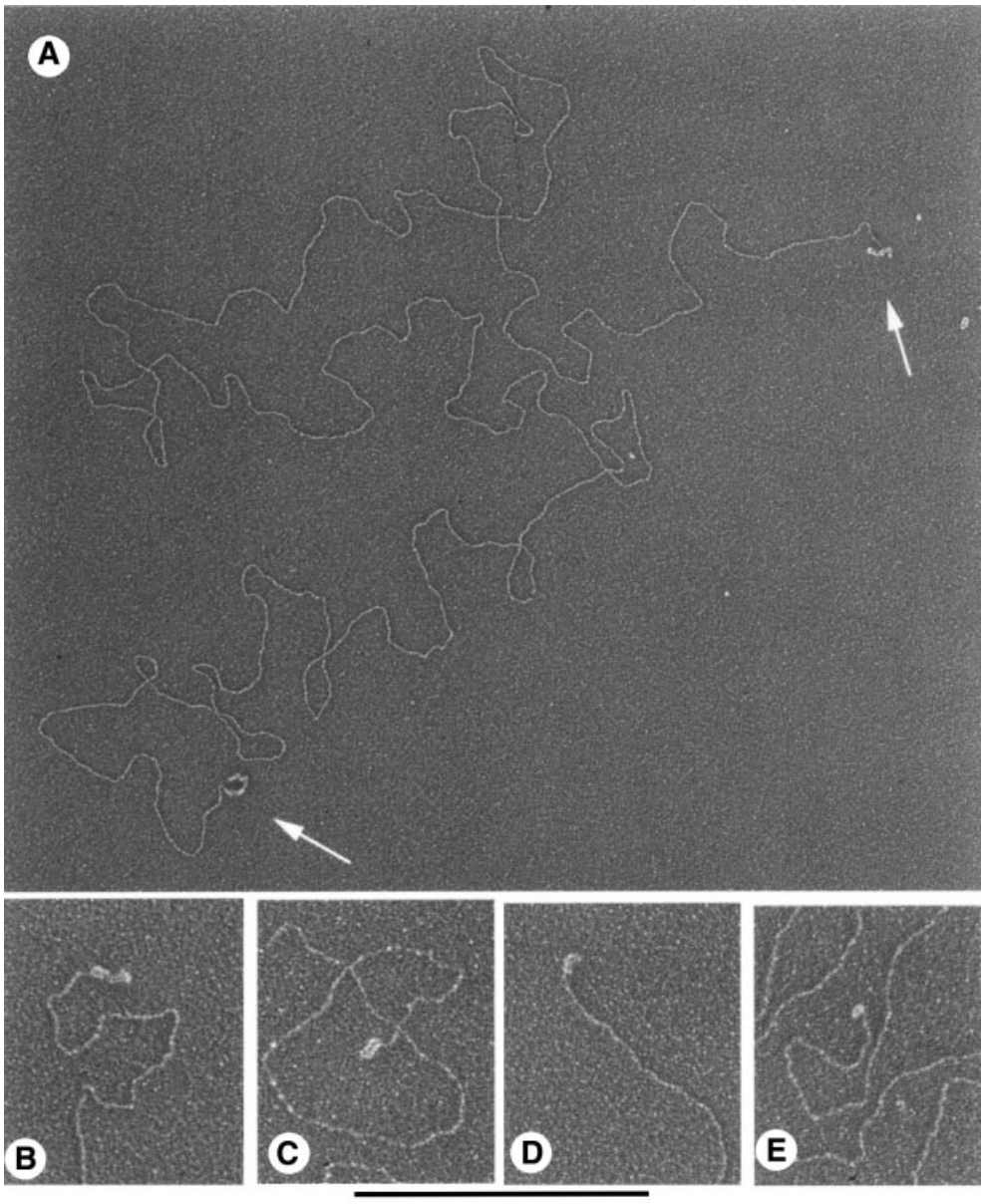

Fig. 6. *Trypanosoma brucei* minichromosomes with single-strand overhangs stained with SSB. Non-cross-linked minichromosomes enriched by sucrose gradient sedimentation were incubated with *E.coli* SSB and prepared for EM as described in Materials and methods, including adsorption to thin carbon foils, dehydration and rotary shadowcasting with tungsten. (**A**) A 31 kb minichromosome with single-stranded overhangs at both ends. The overhangs on this molecule are longer than most and were selected for greater visibility at low magnification. (**B–E**) Individual minichromosome ends with SSB bound. The size of the particle in (E) corresponds to a single SSB tetramer. Shown in reverse contrast. Bar equals 0.5 μm (A) and 0.27 μm (B–E).

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 (Wellinger *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 ~5 × 10^8 trypanosomes/ml. A total of 10^9 trypanosomes 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 $MgSO_4 \cdot 7H_2O$, 20 mM Na_2HPO_4, 2 mM $NaH_2PO_4 \cdot 2H_2O$, 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 10^7 trypanosomes/ml. A total of 10^9 trypanosomes 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 $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4).

Genomic blotting

To isolate genomic DNA, ~5 × 10^8 trypanosomes 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. *Bal*31 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 Sephacryl MicroSpin columns (Amersham). For telomere blots, DNA was digested overnight with the restriction enzymes *Alu*I, *Hin*fI, *Rsa*I, *Mbo*I or *Xmn*I, and size fractionated and blotted 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 (GTGTACTTGCCTGTAC-TAAAAGTATTCTTACAGGGGTTGCAGTATACTGT) 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 × 10^8) 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 H_2O) 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 TNES 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 *Rsa*I–*Hin*fI–*Alu*I. 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 100 mM EDTA, 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 at an angle of 120°, 1–12 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 applied to a 20 ml column of Bio-Gel A-5m equilibrated in the same buffer. The chromatography was controlled with a Pharmacia Gradifract apparatus. The DNA profile was determined by absorbance readings at 260 nm and fractions containing the telomeric restriction fragments were prepared for EM.

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 20 mM HEPES pH 7.5/1 mM EDTA. *Escherichia coli* SSB was added to 1 µg/ml and the sample incubated on ice for 10 min. Glutaraldehyde was then added to 0.6% for 5 min at room temperature and the sample chromatographed over a second Bio-Gel column to remove the free protein and fixative. Minichromosomes in the excluded fractions were prepared for EM by direct adsorption onto glow-charged carbon films in the presence of spermidine, washed, air dried and rotary shadowcast with tungsten (Griffith and Christiansen, 1978).

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 (20:1), and examined in a Philips CM12 instrument at 40 kV. DNA lengths were measured by projecting images on EM sheet film onto a Summagraphics digitizing tablet attached to a Macintosh computer programmed 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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