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Identification of the telomere in *Trypanosoma cruzi* reveals highly heterogeneous telomere lengths in different parasite strains

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ABSTRACT

Here we describe the cloning and characterisation of the *Trypanosoma cruzi* telomere. In the Y strain, it is formed by typical GGGTTA repeats with a mean size of ~500 bp. Adjacent to the telomere repeats we found a DNA sequence with significant homology to the *T.cruzi* 85 kDa surface antigen (gp85). Examination of the telomere in nine *T.cruzi* strains reveals differences in the organisation of chromosome ends. In one group of strains the size of the telomere repeat is relatively homogeneous and short (0.5–1.5 kb) as in the Y strain, while in the other, the length of the repeat is very heterogeneous and significantly longer, ranging in size from 1 to >10 kb. These different strains can be grouped similarly to previously existing classifications based on isoenzyme loci, rRNA genes, mini-exon gene sequences, randomly amplified polymorphic DNA and rRNA promoter sequences, suggesting that differential control of telomere length and organisation appeared as an early event in *T.cruzi* evolution. Two-dimensional pulsed field gel electrophoresis analysis shows that some chromosomes carry telomeres which are significantly larger than the mean telomere length. Importantly, the *T.cruzi* telomeres are organised in nucleosomal and non-nucleosomal chromatin.

INTRODUCTION

Telomeres are composed of G-rich DNA sequences ending with a 3' single-stranded overhang. The telomeric DNA is associated with a specific set of proteins and these complexes are important for the maintenance of chromosome integrity, avoiding degradation and preventing unwanted fusion events. Telomeres are also involved in nuclear architecture, control of gene expression, tumour formation and senescence (1). The DNA sequence of telomeres was first identified in 1978 in the ciliated protozoan *Tetrahymena thermophila* and consists of the hexamer repeat sequence TTGGGG. Similar G-rich repeat sequences have since been found at chromosome ends of a wide phylogenetic range of eukaryotic organisms (reviewed in 2).

The cell replication machinery is incapable of replicating the complete sequence of the genome and a few bases would therefore be lost from the end of the chromosomes with each cell division (3,4). In most cases, this loss is compensated for by the activity of a specialised reverse transcriptase, named telomerase, which is formed by protein and RNA molecules (5). This enzyme adds *de novo* telomere repeats to the chromosome ends using its RNA component as a template. There are other mechanisms involved in the maintenance of chromosome integrity in eukaryotic cells, although it appears that telomerase is the most commonly employed. For example, *Drosophila* uses a reverse transcriptase and a retrotransposon element to cap the chromosome ends (6). Homologous recombination can also maintain chromosome integrity in some immortalised human cells which do not express telomerase (7).

Among the protozoan flagellates, the Trypanosomatide family includes genera that are important human and animal pathogens. Understanding the mechanisms that control growth could lead to important targets to combat the respective diseases. Although some progress has been made in the study of telomeres and the subtelomeric regions in parasites such as *Trypanosoma brucei* (8) and *Leishmania* species (9), the physical ends of chromosomes have not been studied in great detail in *Trypanosoma cruzi*, the etiological agent of Chagas' disease. About 18 million people are infected with *T.cruzi* in Latin America (10) and some of these individuals will develop chronic cardiomyopathy and gastrointestinal complications, such as megacolon (11). *Trypanosoma cruzi* has a complex life cycle involving both insect and vertebrate hosts (12). During its life cycle, *T.cruzi* alternates between highly proliferative forms, which require telomere maintenance of its linear chromosomes to remain viable, and non-proliferative but infective stages.

To initiate studies on the role of telomere and chromosomal stability in *T.cruzi*, we have cloned and characterised the

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T.cruzi telomere. Unexpectedly, we found that the strains studied can be divided into two distinct groups based on telomere length, demonstrating differential telomere length regulation in *T.cruzi*.

MATERIALS AND METHODS

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this study appear in the GenBank nucleotide sequence database with the accession number AF117957.

Parasite culture and DNA preparation

Trypanosoma cruzi strains and clones used in this paper are: clones Dm28c (13), CL Brener (14) and Teh CL 2 (15). Strains Y (16), Tulahuen (17), CL (18), F (19), G (20), Mont 17 (21) and Berenice (22). Epimastigote forms were grown at 28°C in liver infusion medium (20,23) containing 10% fetal bovine serum. Genomic DNA was extracted from log-phase cultures of 1×10^8 epimastigote forms as previously described (24).

Cloning of the *T.cruzi* telomeric sequence

Total DNA (1 µg) was digested with the restriction endonuclease *Alu*I (Boehringer) and size fractionated on a 1% TAE agarose gel. The region of 0.6–2 kb that hybridised with *T.brucei* telomeric sequence, provided by S. Beverley, Washington University, was cut out of the gel and purified using the QIAquick gel extraction kit (Qiagen). The 3' overhanging ends of the telomere were filled in using T4 DNA polymerase (Boehringer) and the fragments were cloned into the pUC18/*Sma*I dephosphorylated vector (Pharmacia). The ligation products were transformed in One Shot super competent cells (Invitrogen) and the colonies were screened using the *T.brucei* telomeric probe. Positive clones were selected and sequenced using the AmpliCycle sequencing kit (Perkin-Elmer).

Southern blot analysis and hybridisation conditions

Genomic DNA from epimastigote stage parasites was digested with restriction enzymes and separated on a 1% agarose gel by electrophoresis. The gel was stained with ethidium bromide, photographed, exposed to UV light (260 nm) for 5 min and blotted under alkaline conditions to a nylon filter (Hybond N^+, Amersham). For Dot blot analysis we used 1 and 0.1 µg of genomic DNA (25). DNA probes were labelled with [α-^{32}P]dATP using the Megaprime DNA labelling system (Amersham). Hybridisation was performed in 7% SDS, 0.5 M $NaPO_4$ pH 7.2, 1% BSA at 65°C. Blots were washed twice at high stringency in 0.1× SSC, 0.1% SDS at 65°C. The *T.cruzi* α/β-tubulin probe was prepared from the *Bam*HI fragment of the pDC1 plasmid, provided by Y. Yara Traub-Czeko, Instituto Oswaldo Cruz, RJ, Brazil (26). The poly(A)-binding protein (PABP) gene probe was used as described (14).

Two dimensional pulsed field gel electrophoresis (2D-PFGE)

Agarose blocks of epimastigote stage *T.cruzi* parasites were prepared and PFGE was performed using the CHEF apparatus (Pharmacia) at 18°C in 0.5× TBE. Chromosomes were fractionated on 1.2% agarose (Chromosome grade, Bio-Rad). The electrophoresis conditions were performed as described (27). 2D-PFGE was performed as described previously (28). Chromosomes were separated in the first dimension by PFGE as above, and the lane containing the chromosomes was excised and digested with *Hpa*II, an isoschizomer of *Msp*I. The DNA was then fractionated by electrophoresis on 1.2% agarose gels at 100 V for 1 h in 0.5× TAE.

Preparation of nuclei and micrococcal nuclease digestion

Nuclei from 1×10^9 Y and CL Brener strain epimastigote forms were resuspended in 9 ml of 10 mM potassium glutamate, 0.25 M sucrose, 1 mM $CaCl_2$. The parasites were subjected to N_2 cavitation at 500 psi for 3×30 min (Parr Instruments). The nuclei were collected by centrifugation at 12 500 *g* and resuspended in 1 ml of the same buffer. The 250 µl aliquots of nuclei from 2.5×10^8 parasites were subjected to digestion with 0, 1.5, 15 and 150 U of micrococcal nuclease (Pharmacia) for 3 min. The reaction was terminated by the addition of 10 mM Tris (pH 7.0), 5 mM EDTA, 0.1% SDS. DNA was purified by Proteinase K treatment (Boehringer) at a final concentration of 20 µg/ml for 30 min at 37°C and phenol–chloroform extraction and precipitation in ethanol. The DNA was digested with 100 µg/ml RNase A (Boehringer) for 20 min at 37°C. The digested DNA was run on a 1.5% agarose gel and transferred to nylon membrane and hybridised as previously described.

RESULTS

Isolation and characterisation of the *T.cruzi* telomere

Initial characterisation of the telomere was performed by measuring telomere length by Southern blot analysis of the terminal restriction fragment (TRF) (29). Genomic *T.cruzi* DNA was digested with various frequently cutting restriction enzymes followed by hybridisation with a probe corresponding to *T.brucei* telomeric sequence. This technique relies on the fact that telomere repeats lack recognition sites for most restriction enzymes. Thus digestion with four-base cutting restriction enzymes liberates the terminal telomere repeat array. As shown in Figure 1A, the telomere sequence hybridises with broad 'fuzzy' bands of DNA digested with *Alu*I, *Msp*I, *Rsa*I or *Sau*3AI, respectively. *Msp*I digestion produced the smallest range of fragments, also obtained when all four enzymes were used together. This result suggests that the mean telomere length of *T.cruzi* Y strain is ~500 bp and that the *Msp*I site is the closest to the telomere repeat arrays. We decided to use *Alu*I digested DNA, which gave rise to a larger TRF, in order to clone part of the subtelomeric region or telomere associated sequences (TAS). We screened a genomic library of *Alu*I digested Y strain genomic DNA with the *T.brucei* telomeric repeat that cross hybridises with all *T.cruzi* chromosomes (27).

We isolated a positive clone, named Tc-Y-Tel1, of 1049 bp, which contained a stretch of identical hexamer repeats (GGGTTA) the same as the telomere repeats of *T.brucei*. The telomere repeat motif of the clone was present in 120 copies (Fig. 1C). Bal31 sensitivity provides evidence that the sequences recognised by the probe are located at the chromosome ends of the *T.cruzi* Y strain (data not shown). In the subtelomeric region we found a short area of 57 bp showing 82% identity to the gene encoding the 85 kDa surface glycoprotein gp85 (30). Some members of this polymorphic gene family have been previously shown to be located in the subtelomeric regions of *T.cruzi* (31). A DNA probe of sequences

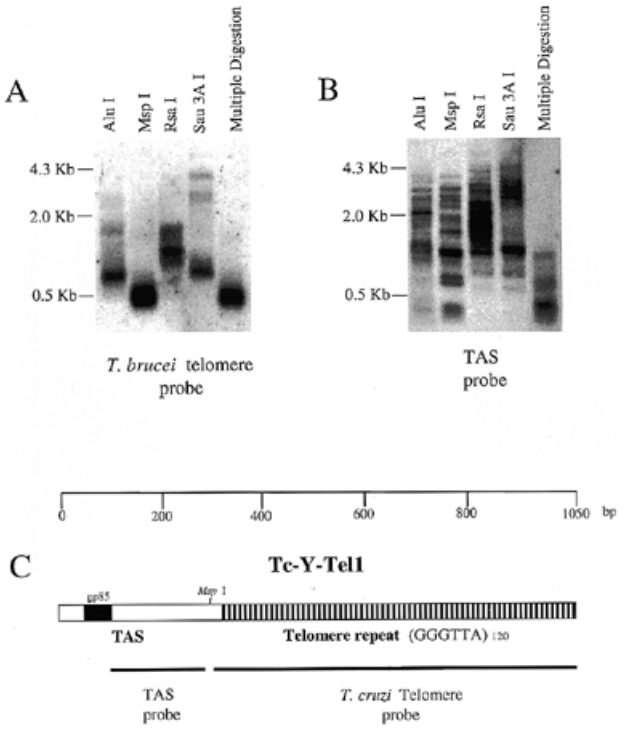

Figure 1. Characterisation of the *T.cruzi* telomere. (**A**) TRF analysis of genomic DNA of the Y strain telomere using various frequently cutting enzymes. The multiple digestion was performed using restriction enzymes *Alu*I, *Msp*I, *Rsa*I and *Sau*3AI. The blot was hybridised with the *T.brucei* telomeric probe. (**B**) The same Southern blot was stripped and rehybridised with the TAS from Tc-Y-Tel1. (**C**) Schematic representation of the cloned telomeric region for *T.cruzi*: clone Tc-Y-Tel1 (accession no. AF117957). The sequence was composed of TAS and telomere repeats. The telomere consists of the unique repeat GGGTTA whereas the subtelomeric segment is non-repetitive and contains a region homologous to the multigene family gp85 of *T.cruzi* (30).

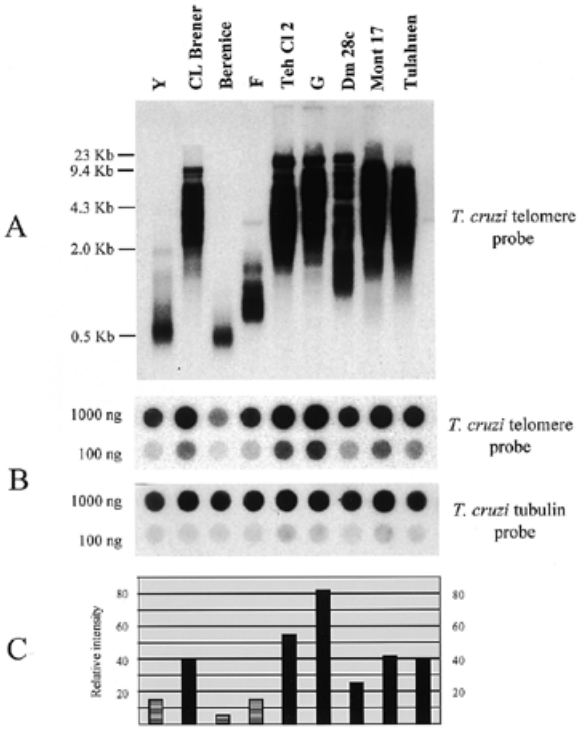

Figure 2. TRF analysis of the nine *T.cruzi* strains. (**A**) 500 ng of genomic DNA from each strain was digested with *Msp*I and run on a 0.8% TAE agarose gel. Southern blot analysis was performed using the *T.cruzi* telomeric probe. (**B**) Dot blot quantification of telomere signal using 1000 and 100 ng of genomic DNA from each strain hybridised with *T.cruzi* telomeric and α/β-tubulin probes. (**C**) Quantification of the telomere hybridisation signal normalised with tubulin using the PhosphorImager ImageQuant Molecular Dynamics program. Hatched bars represent the strains with short telomeres and solid bars represent the strains with long telomeres.

adjacent to the telomere repeats of Tc-Y-Tel1 hybridises to multiple bands of variable size in the Y strain (Fig. 1B), indicating that this sequence exists in many copies in the genome of the Y strain.

Telomere length is variable in different *T.cruzi* strains

As part of the characterisation of the *T.cruzi* telomeres, we used the Y strain telomere repeat probe to investigate the organisation of telomeres in different *T.cruzi* strains. The DNA was isolated from logarithmically growing epimastigote forms of several *T.cruzi* strains and digested with *Msp*I to liberate the TRF. Figure 2A shows that the size of the telomere differs greatly between strains. In the strains Y, Berenice and F, *Msp*I digestion yielded short and relatively homogeneous 'fuzzy' bands ranging in size from 450 to 1500 bp (short telomere group). In the second group of strains, the telomere probe hybridised to a large number of heterogeneous bands ranging from 1 to >10 kb (long telomere group). The same size pattern was found when the DNA was digested with four restriction enzymes together, as described in Figure 1. The hybridisation signal obtained with the telomeric probe under high stringency was analysed by dot blot. As shown in Figure 2B, there is a difference in hybridisation intensity with the telomeric repeat probe. To quantify these differences more accurately, the hybridisation signal was normalised with that obtained by hybridisation with the α/β-tubulin probe. The relative

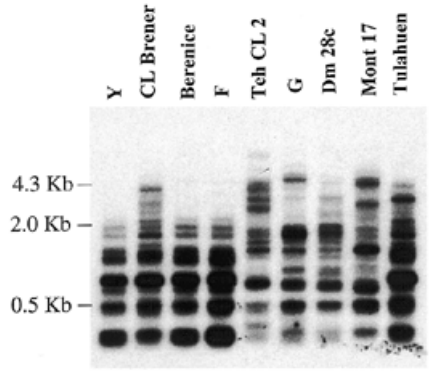

Figure 3. Southern blot fingerprint analysis from the nine *T.cruzi* strains. *Hpa*II digested genomic DNA was separated on a 1% agarose gel and hybridised using the repetitive subtelomeric probe TAS from Tc-Y-Tel1 (see Fig. 1C).

intensity measured by phosphorimager analysis is graphically represented in Figure 2C showing that parasite strains with longer telomeres also present a stronger hybridisation signal. However, the strength of the signal does not correlate strictly to values estimated by the mean telomere length, as is illustrated in the case of clone Dm28c. The telomere repeat hybridisation signal of Dm28c is roughly double that of the Y strain, although the apparent telomere length has been calculated to differ by a factor of 5–10 times between the two strains. A possible explanation

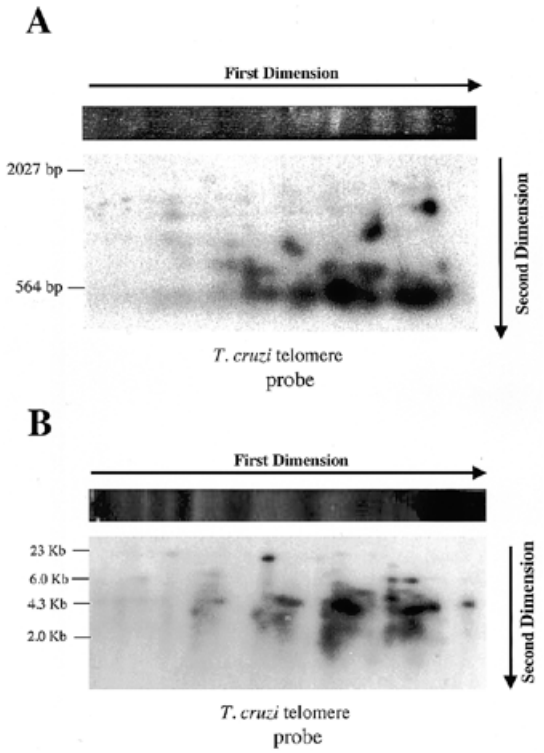

Figure 4. Telomere length of individual chromosomes. Chromosomes from Y strain (**A**) and CL Brener clone (**B**) parasites were separated by PFGE, a lane of ethidium bromide stained chromosomes was cut from the gel (first dimension) and digested with *Hpa*II an isoschizomer of *Msp*I which digests efficiently agarose embedded DNA. The digested chromosomes were migrated in the second dimension by electrophoresis on a 1.2% agarose gel, transferred to a nylon membrane and hybridised with the *T.cruzi* telomere probe.

could be that strain-specific, telomere-adjacent sequences without the restriction enzyme sites used in this study do exist in the strains CL Brener, Teh CL2, G, Dm28c, Mont 17 and Tulahuen, adding to the length and heterogeneity of the telomere restriction fragments. To confirm that the hybridisation signal corresponds to the telomeric sequence PFGE was run with the same nine *T.cruzi* strains used in Figure 2. The telomere repeat probe hybridised to all the chromosomes of the different strains and the intensity of the signal in each strain correlates to the apparent length of the telomere (data not shown).

The subtelomeric probe from Tc-Y-Tel1 was used to investigate the hybridisation profile in all nine *T.cruzi* strains used in this study. Figure 3 shows DNA fingerprint analysis of *Hpa*II digested genomic DNA probed with the TAS region (for details see Fig. 1C). Interestingly, the strains with short telomeres show identical band sizes with the TAS probe, whereas the strains with large telomeres show a unique and more complex fingerprint pattern. These data suggest that the subtelomeric organisation of the Y, Berenice and F strains are closely related to each other, whereas the remaining strains are distinguished by a unique polymorphic pattern.

Several chromosomes carry telomeres significantly larger than the mean telomere length

To further the study of *T.cruzi* telomere structure, we measured the telomere length of the individual chromosomes of the Y and CL Brener strains, representing the long and short telomere groups. 2D-PFGE was performed using 5×10^6 parasites. The chromosomes were separated in the first dimension by PFGE and the whole lane was cut out of the gel. The intact chromosomes were digested in the agarose with the restriction endonuclease *Hpa*II, which is an isoschizomer of *Msp*I. Digested chromosomes were separated in the second dimension and hybridised with the *T.cruzi* telomeric probe. In the Y strain, most of the telomeres of the different chromosomes are in the range of 500 bp. However, some chromosomes have significantly larger telomeres with sizes up to 1.6 kb (Fig. 4A). For the CL Brener clone, most of the individual telomeres are in the size range of 4 kb, as shown in Figure 4B. Some longer telomeres were observed, ranging up to 10 kb and also some shorter telomeres of ~1 kb. These data illustrate a high degree of heterogeneity in the telomere length of *T.cruzi*.

Chromatin organisation of telomeres in *T.cruzi*

We then studied the chromatin organisation of the *T.cruzi* chromosomes since unusual, non-nucleosomal packaging of the telomeric sequences in *Tetrahymena*, yeast and human chromosomes has been reported (32–34). Micrococcal nuclease digestion, which cleaves the DNA between each nucleosome, was used to digest the DNA from isolated nuclei obtained from 1×10^9 Y and CL Brener parasites. The usual nucleosomal ladder was obtained after gel electrophoresis of both parasite strains as shown in Figure 5A. The multimeric series of DNA bands was transferred to a nylon membrane and hybridised with a gene probe coding for PABP which is present in two copies in the parasite genome and with the *T.cruzi* telomeric probe (Fig. 5B and C). The ladder obtained with the PABP gene probe was as expected for normal nucleosomal organisation in both the Y strain and CL Brener, showing a spacing of ~190 bp (35). However, when the blot was hybridised with the telomeric probe, a smear and some bands resembling the nucleosomal banding pattern were observed with the Y strain. Similar data were observed with CL Brener, however, the nucleosomal-like banding pattern was more continual than in the Y strain. To confirm that these bands are due to nucleosomal organisation within the telomere, MNase digested DNA of CL Brener was digested with *Alu*I, *Rsa*I, *Sau*3A and *Msp*I in order to separate the telomere from the subtelomeric region. The DNA was migrated once again by gel electrophoresis and hybridised with the telomeric probe (Fig. 6). The nucleosomal pattern was found to remain unchanged confirming that the bands observed are due to the presence of several nucleosomes in the telomere of the CL Brener clone. Hybridisation of the same blot with the PABP probe demonstrated that the chromosome internal DNA had been digested leading to many small irregular bands.

DISCUSSION

In this paper, we have studied the telomere of *T.cruzi* and found that it is organised in multiple repeats of the sequence GGGTTA, as in other kinetoplastids and some higher eukaryotes such as humans and mice (2). Analysis of TAS revealed the presence of a sequence homologous to a member of the 85 kDa gene family immediately adjacent to the telomere repeats. The presence of this sequence in such close proximity to the telomere confirms previously published results showing subtelomeric location of these genes (30,31) and suggests that members of this gene family may be commonly found in the TASs. This idea is supported by a recent analyis of a subtelomeric region

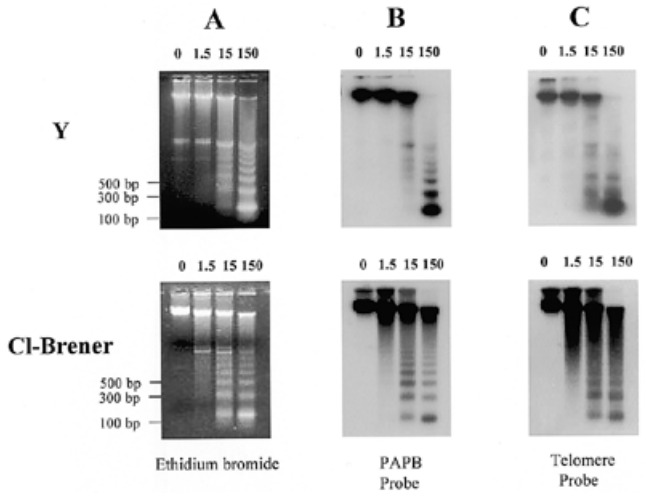

Figure 5. Microccocal nuclease analysis of chromatin organisation. Nuclei from 10^9 Y strain and CL Brener clone parasites were isolated and digested with microccocal nuclease at the concentrations (units/reaction tube) indicated, for 3 min at 30°C. (**A**) Ethidium bromide staining of the digested DNA reveals a ladder of multimeric nucleosomes. The digested DNA was transferred to a nylon membrane and subsequently hybridised with the gene probe PABP (**B**) and with the *T.cruzi* telomere probe (**C**).

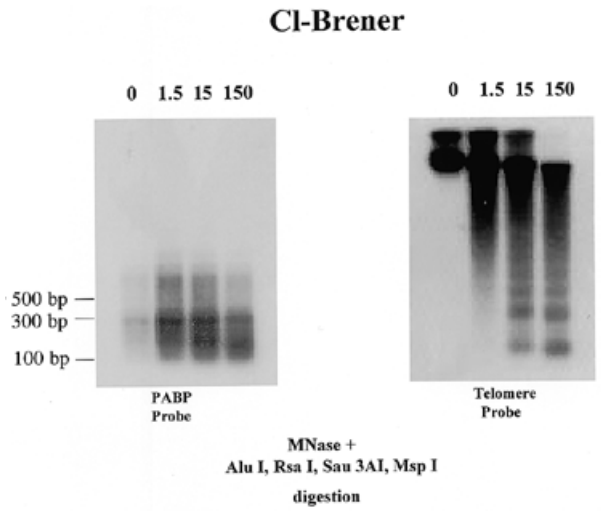

Figure 6. The *T.cruzi* telomere is organised in nucleosomal and non-nucleosomal chromatin. TRF analysis of genomic DNA prepared from nuclei of the CL Brener strain treated with MNase. Purified DNA was digested with the restriction enzymes *Alu*I, *Rsa*I, *Sau*3A and *Msp*I and hybridised with a PABP and telomere probe. The telomere probe gives a superposition of two distinct signals, a smear along the lane and nucleosomal pattern.

of the CL Brener strain, in which the authors show the presence of an entire gp85 gene adjacent to the telomere repeats (36). Gene sequences are not normally found in such close proximity to the telomere. However, in *Giardia lamblia* rDNA sequences are found directly associated with the telomeres (37,38). Furthermore, genes coding for variant antigens and virulence factors involved in the evasion of the host immune system during infection and pathogenesis, are often found in subtelomeric regions of the genome. Important examples are the genes involved in antigenic variation in *T.brucei* (8) and *P.falciparum* (39). There is much evidence showing that subtelomeric sequences are highly recombinogenic and involved in genome plasticity in different organisms (40). A possible explanation is that gp85 sequences in *T.cruzi* have become associated with the telomeres by recombination events such as chromosome breakage and repair by telomerase in a similar mechanism to that found in *P.falciparum* (41).

By studying Micrococcal nuclease digestion of isolated *T.cruzi* nuclei we found a typical nucleosomal ladder as previously described (42). Multimeric nucleosome bands hybridise with a PABP gene probe; a distinct pattern was, however, observed for the telomere. The telomeric probe revealed a smear superposed to nucleosomal-like bands in the Y and CL Brener strains, demonstrating unusual chromatin organisation of the telomeric DNA. The number of bands observed in CL Brener is significantly higher compared to the Y strain and suggest that the number of telomeric nucleosomes depends on the length of the number of telomere repeats. Similar results have been described in the telomeres of *Tetrahymena* and human cells (34,43).

Our findings suggest the existence of two groups of strains based on the length of the telomeres. One group contains short telomeres which are relatively homogeneous in size while the other has longer telomeres more heterogeneous in length. Chiurillo *et al.* have demonstrated that the 'long' telomeres of CL Brener are composed of the same repeat sequence and that the subtelomeric region does not contain degenerate telomeric repeats (36). This observation is unexpected since this type of size difference has only been observed in telomeres of distinct species. In the *Plasmodium* species, for example, the mean telomere length can vary from ~1 to 6 kb, whereas within the same malaria species only minor differences have been observed (Figueiredo *et al.*, manuscript in preparation). These data point to the prevalence of two genetically different *T.cruzi* groups. Our observation is consistent with several other results showing that *T.cruzi* strains are genetically polymorphic (44) and that the species can be divided into two major phylogenetic lineages (45). This grouping is based on rRNA sequence analysis (45,46), spliced leader RNA and rRNA promoter studies (47,48), RAPD and MLEE analysis (49,50) and polymorphic microsatellite repeats (51). Although there is a general consensus that these two distinct groups are separated by a large evolutionary distance (50), there is some controversy over the precise interpretation of the results for some strains; for example, CL Brener and Tulahuen (50,52).

Trypanosoma cruzi strains that fall into identical groups based on their telomere size generally belong to the same group based on the other criteria as described above. However, we found that CL Brener and F do not follow this principle (Table 1). One possible explanation is that there was genetic convergence for one or several of the markers analysed that appeared later in evolution. It has been suggested that there is an association of lineage 1 strains with domestic reservoirs and lineage 2 strains with animal reservoirs (53). Nevertheless, a larger number of strains has to be studied in order to verify whether there is some association of different strains and/or genotypes with virulence, since the clinical manifestations of Chagas' disease vary from almost completely asymptomatic to fatal.

In conclusion, we describe the molecular structure of the chromosome ends of *T.cruzi*. We also show that the telomere repeats are packed in a chromatin structure clearly distinct

Table 1. *Trypanosoma cruzi* lineages defined by telomere organisation and rRNA sequences

Trypanosoma cruzi isolates	Telomere length[a]	Phylogenetic lineage[b]
Y	S	1
Berenice	S	1
F	S	2
CL Brener	L	1
Teh CL 2	L	nd
G	L	2
DM 28c	L	2
Mont 17	L	nd
Tulauhen	L	2

[a]Telomere length classification: S, small telomeres; L, long telomeres.
[b]The phylogenetic lineages defined by rRNA sequence (45,54). nd, not determined.

from the nucleosomal organisation of genes found in coding regions of the genome. This finding points to the possible existence of epigenetic phenomena at chromosome ends of *T.cruzi*. Given the striking differences found in the telomere organisation between different strains, it is possible that the two different groups, defined by long and short TRFs, may be associated with correspondingly different biological properties.

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