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Chromosomal size conservation through the cell cycle supports karyotype stability in *Trypanosoma cruzi*

Mario Galindo[a,*], Valeria Sabaj[a], Ingrid Espinoza[a], Gabriela Cecilia Toro[a], Jaqueline Búa[b], Jose Grenet[c], Remigio López-Solís[a], Andres Mariano Ruiz[b], Norbel Galanti[a,*]

[a] *Program of Cellular and Molecular Biology, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, University of Chile, Santiago, Chile*
[b] *National Institute of Parasitology Dr. Mario Fatala Chabén, Buenos Aires, Argentina*
[c] *Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, USA*

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Abstract The *Trypanosoma cruzi* karyotype shows an extensive chromosomal size polymorphism. Absence of condensed mitotic chromosomes and chromatin fragility are characteristic features of *T. cruzi* which would allow DNA breaks and chromosomal rearrangements during cell proliferation. We have investigated by pulsed field gel electrophoresis (PFGE) eventual changes in chromosomal size during exponential and stationary phases of *T. cruzi* epimastigotes in culture, in G0 trypomastigotes and throughout the cell cycle in synchronized epimastigotes. *T. cruzi* molecular karyotype was stable throughout the cell cycle and during differentiation. Thus, the chromosomal size polymorphism previously reported in *T. cruzi* contrasts with the stability of the molecular karyotype observed here and suggests that chromosomal rearrangements leading to changes in chromosomal size are scarce events during the clonal propagation of this parasite.
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1. Introduction

The protozoon *Trypanosoma cruzi* has a complex multiclonal structure [1]. An outstanding feature of this parasite is that its chromatin does not condense into mitotic chromosomes during cell division [2]. Thus, the *T. cruzi* karyotype can be analyzed by pulsed field gel electrophoresis (PFGE) but not by classical cytogenetic techniques. Analysis of the molecular karyotype in different strains and clones of *T. cruzi* using that technique shows differences of up to 50% in the size of genetically equivalent chromosomes [3], a fact suggesting the occurrence of major chromosomal rearrangements during the evolution of this parasite. It has also been proposed that *T. cruzi* undergoes clonal-type propagation by cell proliferation with rare events of genetic recombination [4].

Some structural features of *T. cruzi* chromatin could provide the foundation for chromosomal rearrangements. Thus, *Trypanosoma* chromatin shows a greater sensitivity to micrococcal nuclease and a lower compactness, when compared to mammalian chromatin [5]. Besides, proliferative epimastigote forms exhibit limited chromatin compaction with respect to the non-proliferative epimastigotes and trypomastigotes (Go cells) [6,7]. Finally, *Trypanosoma* chromosomes are subjected both to dynamic spatial reorganization during DNA replication [8] and to traction forces by a mitotic spindle during chromosomal segregation [9]. Together, these findings suggest that DNA breaks and chromosomal rearrangements could occur during proliferative expansion in *T. cruzi*, particularly during DNA replication and mitosis.

On the other hand, a source of non-biological but rather methodological artifacts could explain chromosomal variability as studied by PFGE. Classical cytogenetic techniques require metaphase arrested cells; in contrast, samples for PFGE molecular karyotyping are usually obtained from asynchronic cultures, which include cells in different phases of the cell cycle. Particularly, in PFGE separations the partially replicated chromosomes in S-phase are expected to migrate to different positions as compared to non-replicating and to fully replicated chromosomes, what could represent artefactual variants.

We analyzed the molecular karyotype and the migration of specific chromosomes by PFGE in epimastigotes at the exponential and stationary phases of growth, in synchronized parasites progressing through S and G2/M and in differentiated G0 trypomastigotes. Our results show significant chromosomal size conservation in *T. cruzi* thus supporting karyotype stability during cell proliferation and differentiation of this parasite.

2. Materials and methods

2.1. Cell culture

T. cruzi epimastigotes, strain Tulahuen, were grown as previously described [10]. Growth rate was assessed by cell counting. Trypomastigotes, the non-proliferative forms of *T. cruzi*, were obtained from clone DM 28C epimastigotes by in vitro transformation, as described by Contreras et al. [11] and purified following De Sousa [12].

2.2. Cell synchronization

Epimastigotes were synchronized at the G1/S boundary as described [10] and harvested at selected times after DNA synthesis stimulation.

2.3. Cytofluorometric analysis

Distribution of cells at specific cell cycle stages was evaluated by cytofluorometric analysis as described [13]. Cytofluorometric measurements of DNA were performed using a Zeiss microdensitometer.

*Corresponding authors. Fax: +56 2 7373158.
E-mail addresses: mgalindo@med.uchile.cl (M. Galindo), ngalanti@med.uchile.cl (N. Galanti).

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2.4. DNA synthesis analysis

DNA synthesis was estimated by measuring [^{3}H]-thymidine incorporation into DNA following Rojas and Galanti [14].

2.5. Chromosome preparation and pulsed field gel electrophoresis

T. cruzi chromosomes were prepared as previously described [15]. Chromosomes were separated by PFGE in 0.5× TBE buffer at 14 °C and 180 V using a CHEF DRIID apparatus (Bio-Rad) and a two step PFGE running condition consisting in a 70 s pulse time for 18 h followed by a 150 s pulse time for 24 h [16]. *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were used as size markers. Gels were stained with 0.1 μg/ml ethidium bromide in 0.5× TBE, washed in distilled water and photographed under UV light.

2.6. Southern Blot analysis

Chromosomes were transferred from gel to nylon membranes (Hybond-N$^+$, Amersham), using standard procedures [17]. Tc13 antigen [18] and H3 histone [19] gene probes were labelled with [α-^{32}P]dCTP (Amersham) using a random priming labelling kit (Rediprime, Amersham). Blots were hybridized under previously described conditions [16]. After hybridization, membranes were washed at high stringency with 0.1× SSC (150 mM NaCl, 15 mM sodium citrate) plus 0.1% SDS at 65 °C. Hybridized membranes were exposed to BioMax MR film (Kodak).

3. Results

3.1. Chromosomal size stability during cell proliferation and differentiation

Fig. 1 shows that epimastigotes grew from day 1 to day 6 (exponential phase) and reached the stationary phase at day 7 (Fig. 1A). The low level of [^{3}H]-thymidine incorporation into DNA at day 12, as compared to day 5 (Fig. 1B), is in agreement with no further increase in cell number between days 7 and 14 (stationary phase) (Fig. 1A). Coincidentally, the percentage of cells in S phase drops from 45% at day 5 to 30% at day 12, as estimated by DNA/cell content (Fig. 1C).

When molecular karyotypes of exponential growing and stationary phase epimastigotes were analyzed by PFGE and ethidium bromide staining, no changes in chromosomal size were observed (Fig. 2A, lanes 1–14). The migration of individual chromosomes was tested by Southern Blot analysis using probes that hybridize to chromosomes of 2000–2200 kbp (Tc13 antigen) and 1150 kpb (H3 histone) genes (Fig. 2B and C). No additional hybridization signals were observed in these specific chromosomes during the exponential and

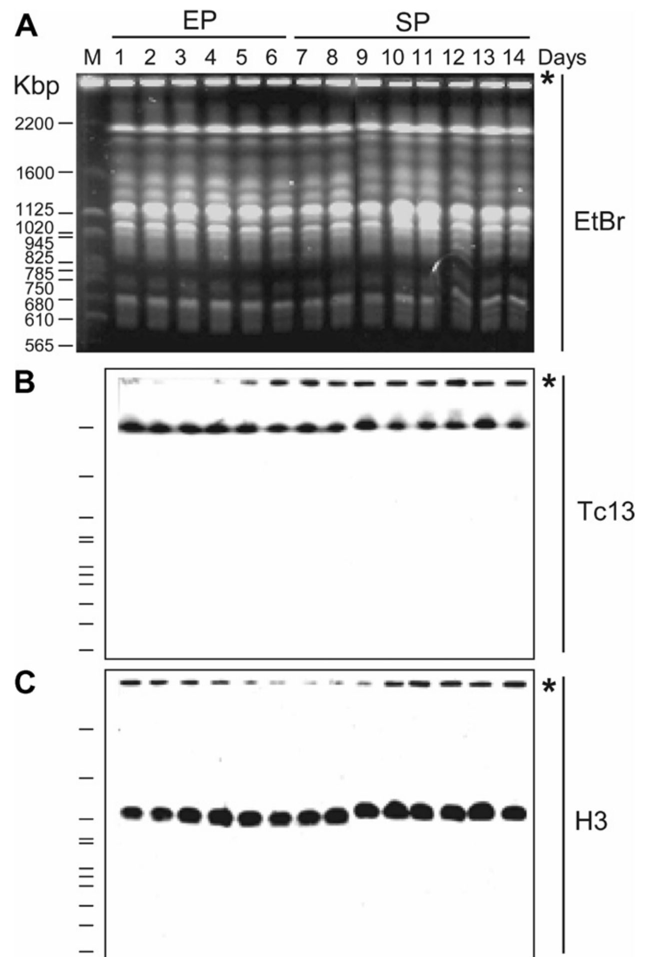

Fig. 2. Molecular karyotype of *T. cruzi* epimastigotes during cell growth in culture. Cells were harvested at different days of growth and chromosomes were separated by PFGE. Days of culture are indicated at the top of the gel. Exponential phase (EP); stationary phase (SP). (A) Ethidium bromide staining. M: size marker chromosomes (*Saccharomyces cerevisiae*). (B and C) Chromosomes from the gel shown in panel A were transferred to a nylon membrane and hybridized with Tc13 antigen (panel B) or H3 histone (panel C) probes, respectively. Hybridization signals at the top of gel in panels B and C (*) correspond to material trapped in the agarose plugs. Lines at the left margin of panels B and C indicate size markers, as shown correspondingly in panel A.

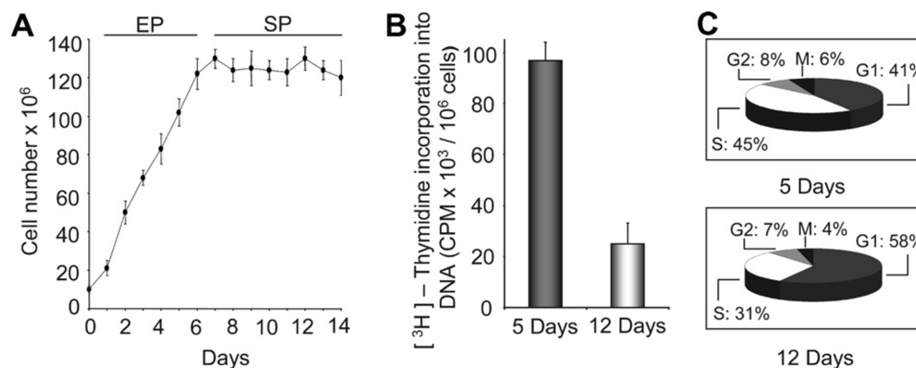

Fig. 1. Proliferative status of *T. cruzi* epimastigotes. (A) Growth curve of epimastigotes. Exponential (EP) and stationary (SP) phases are indicated. (B) [^{3}H]-thymidine incorporation into epimastigotes DNA. (C) Fraction of cells in G1, S, G2 and M phases of the cell cycle at days 5 and 12 of culture expressed as a percentage of the total number of cells.

stationary phases, thus indicating that changes in chromosomal size do not occur. These results point to a significant karyotipe stability throughout different phases of epimastigote growth in culture.

Karyotype stability was also studied during *T. cruzi* differentiation from epimastigote to trypomastigote, a non-proliferative form of the parasite. Proliferative activity of these cells was confirmed by [^{3}H]-thymidine incorporation into DNA (Fig. 3A). Again, chromosome sizes in exponentially growing epimastigotes were found to be similar to that of G0 trypomastigotes (Fig. 3B).

3.2. Chromosome size stability during DNA replication

Epimastigotes were synchronized at the G1/S transition point by using HU. G1-arrest was reflected by the very low level of [^{3}H]-thymidine incorporation observed after 24 h incubation in HU (Fig. 4). Following HU washout and stimulation with fresh medium containing 20% FBS, cells progressed into S phase within 4 h (Fig. 4). Then, [^{3}H]-thymidine incorporation decreased by 8 and 12 h, indicating the end of the S phase and the beginning of the G2/M phase.

PFGE molecular karyotype of synchronized epimastigotes was found to be stable through the S phase (Fig. 5A, lanes 4–6) and in G2/M (Fig. 5A, lane 7). However, an increase in fluorescence intensity of the chromosomal bands was observed at 12 h after HU washout and serum stimulation (end of the S

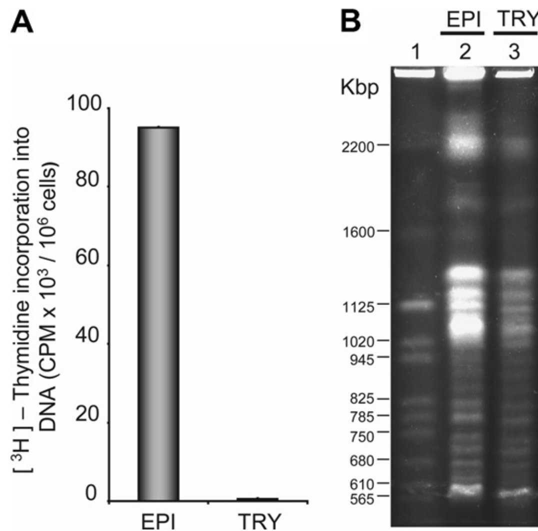

Fig. 3. Molecular karyotype of *T. cruzi* epimastigotes and trypomastigotes. (A) Incorporation of [^{3}H]-thymidine into DNA of both epimastigotes (day 5 of culture) and trypomastigotes. (B) Chromosomes from *Saccharomyces cerevisiae* (lane 1), *T. cruzi* epimastigotes clone DM 28c at day 5 of culture (lane 2) and trypomastigotes (lane 3) were separated by PFGE and stained with ethidium bromide.

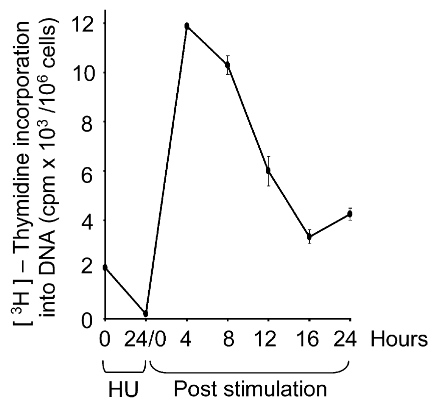

Fig. 4. DNA synthesis in synchronized *T. cruzi* epimastigotes. Epimastigotes were incubated with 20 mM HU for 24 h and subsequently stimulated to progress in the cell cycle by means of HU washout and addition of fresh medium plus 20% FBS. Before (0 h) and after (24 h/0) the HU treatment as well as at the indicated times after serum stimulation (4, 8, 12 and 16 h) cells were incubated with [^{3}H]-thymidine for 1 h. Radioactivity incorporated into DNA was measured as described in Section 2.

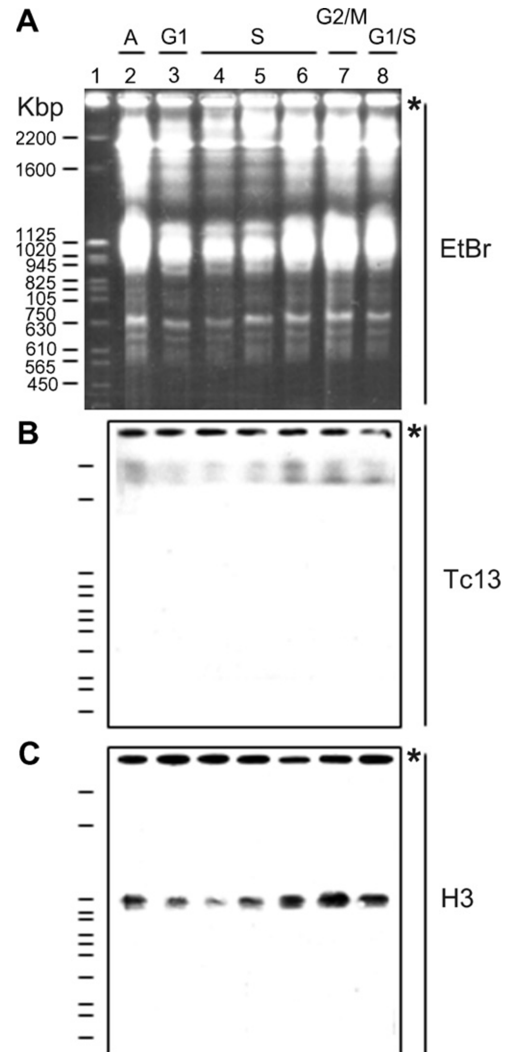

Fig. 5. Molecular karyotype of *T. cruzi* epimastigotes throughout the cell cycle. (A) Chromosomes from asynchronic epimastigotes at day 5 of culture (lane 2), as well as chromosomes from both cells arrested in G1 phase (24 h in HU) (lane 3), cells in S phase (4, 8 and 12 h after serum stimulation) (lanes 4–6) and cells in G2/M phase (16 h after serum stimulation) (lane 7) were separated by PFGE and stained with ethidium bromide. Chromosome size markers (*Saccharomyces cerevisiae*) (lane 1). (B and C) Chromosomes from the gel shown in panel A were transferred to a nylon membrane and hybridized with Tc13 antigen (panel B) or H3 histone (panel C) probes, respectively. Hybridization signals at the top of gels in panels B and C (*) correspond to material trapped in the agarose plugs. Lines at the left margin of panels B and C indicate size markers, as shown correspondingly in panel A.

phase) (Fig. 5A, lane 6), probably as a consequence of the completion of DNA replication. The increase in fluorescence reaches a maximum at 16 h after HU washout (Fig. 5A, lane 7; G2/M). To determine chromosomal size changes eventually undetected by ethidium bromide staining, we analyzed two individual chromosomes through the cell cycle by southern blot analysis, probing for Tc13 and H3 histone genes (Fig. 5B and C). Consistent with the above mentioned results, chromosome size was found to be stable when analyzed by PFGE in synchronized cells throughout the cell cycle. Again, increases of the hybridization signals were observed in parallel to the chromosomal duplication in S phase and the subsequent progression into G2/M. The fact that the chromosomal pattern is constant during the S and G2/M phases suggests that *T. cruzi* chromosomes are highly stable during the cell cycle.

4. Discussion

In this work, we show that *T. cruzi* karyotype is stable at the level of individual chromosomal size during the exponential and stationary phases of growth, in the cell cycle and during differentiation from epimastigotes to trypomastigotes. These observations are consistent with previous reports showing that the molecular karyotype is constant after successive and prolonged passages of 'vitro' cultures [20].

Absence of condensed chromosomes during mitosis and presence of a physically and enzymatically fragile chromatin are characteristics of *T. cruzi*. Its chromatin presents histone proteins highly divergent when compared to those of higher eukaryotes [21–24]. This fact would suggest a weaker nucleosomal interaction with DNA, what could explain chromatin fragility in this parasite and the breaking of chromosomes when tensed during karyokinesis [2]. Indeed, in *Parascaris* and *Ascaris* development, a loss of heterochromatin was associated with chromosomal fragmentation during mitosis in somatic cells [25]. However, our results indicate that the molecular karyotype of *T. cruzi* does not present changes in the chromosomal size during mitosis, as evaluated by PFGE. This result suggests the occurrence of an efficient process of chromosome segregation without DNA fragmentation. Consequently, in spite that *T. cruzi* displays a fragile chromatin structured by divergent histones, this is stable to the traction forces occurring during mitosis.

Molecular karyotype is highly polymorphic among different strains and clones of *T. cruzi*, what may result from both genetic recombination by nuclear hybridization, chromosomal rearrangement and other sources of molecular karyotype variability, such as expansion/contraction of tandem repeats having taken place during the evolution of this parasite [3,26–28]. However, published data are congruent with a predominant long-term clonal evolution in *T. cruzi* with only occasional genetic recombination by nuclear hybridization [27,29,30].

Regarding the occurrence of chromosomal rearrangements, it has been reported that two *T. cruzi* DTUs (Discrete Typing Units) (lineages IId and IIe) correspond to hybrid lineages stabilized by subsequent clonal propagation [29,30], whereas the major lineages (*T. cruzi* I and *T. cruzi* II) are estimated to have diverged around 10 million years ago [29]. Analysis of chromosome size polymorphism between these two major lineages strongly supports the occurrence of only an ancient chromosome breakage or a chromosome fusion event in *T. cruzi* [26,27]. Coincidentally, minor chromosomal rearrangements, such as inversion or transposition, have been described in *T. cruzi* [20].

The analysis of tandem repeat units in *T. cruzi* chromosomes (telomeric sequences, satellites and genes) show the presence of different numbers of those repeats among different strains and stocks of *T. cruzi*. Thereby, gradual expansion/contraction of the tandem repeat sequences has also been proposed as a theoretical explanation of chromosomal size changes [20,26,27,31]. Thus, the minor chromosomal rearrangements and the low frequency of these changes are consistent with the clonal evolution model previously proposed for *T. cruzi*.

Consequently, the paradox between chromosome size stability and chromosome size polymorphism can be conciliated by a predominant long-term clonal evolution in *T. cruzi* with only occasional evolutionary events of chromosomal rearrangements and expansion/contraction.

Finally, a space-functional organization of the nucleus with respect to DNA replication [8] and a position effect respect to transcriptional activity [32] have been described in *T. cruzi* and *T. brucei*. Therefore, genetic recombination by nuclear hybridization, chromosomal rearrangements and expansion/contraction of tandem repeats would be limited by the conservation of a space-functional nuclear organization in *T. cruzi*.

On the other hand, because chromosomes are usually obtained from asynchronous cultures, an unexplored possibility in *T. cruzi* is that the polymorphism observed in the molecular karyotype could be explained by the occurrence of chromosomal size variants corresponding to incompletely replicated chromosomes during the S phase. However, we did not observe any difference in the size of chromosomes between cultures obtained from exponential and stationary phases or between epimastigotes (proliferative form) and trypomastigotes (non-proliferative form). Moreover, using synchronous cultures we did not observe any additional chromosomal band during the S phase, which could correspond to individual chromosomes in a progressive stage of replication. Additionally, in these synchronized cultures, the comparison of the molecular karyotype of cells in G1 phase and that of cells at the end of the S phase shows that chromosome size does not change after DNA synthesis. Only variations in the intensity of the bands and hybridization signals were observed which are consistent with a reliable duplication of the chromosomes occurring during S phase.

Our results show that the *T. cruzi* molecular karyotype is highly stable throughout the cell cycle, supporting the karyotypic stability of different lineages and stocks of this parasite in a clonal evolution model.

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