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Differences in the Nuclear Chromatin Among Various Stages of the Life Cycle of *Trypanosoma cruzi*

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Abstract *Trypanosoma cruzi* is the etiological agent of Chagas. Although the nuclear chromatin of this parasite is organized in the form of nucleosome filaments, its chromatin is physically and enzymatically fragile, and no condensation into chromosomes occurs during mitosis. All previous investigations have been carried out with epimastigote form in its proliferate stage. It is not known whether these differences in chromatin structure are also found in the non-proliferate stationary epimastigote forms and in tissue derived trypomastigotes. Our results confirm that chromatin of logarithmic epimastigotes presents limited compaction when increasing salt concentrations from 1 to 100 mM NaCl, and no 30-nm fibers were formed. Contrary to these results, non-proliferative forms of the parasites showed a pattern of compactation similar to that observed in rat liver chromatin, where solenoids of 30-nm fibers are formed at 100-mM NaCl. In accordance with these results, digestion of the nuclear chromatin with DNase I revealed that the chromatin of logarithmic phase epimastigotes was more accesible to the enzyme. We conclude from these results that structural differences in the chromatin exist not only between *T. cruzi* and higher eukaryotes but also among various forms of the parasite. The functional significance of these differences are currently under investigation. J. Cell. Biochem. 84: 832–839, 2002.

Key words: chromatin; histones; nucleosome; *Trypanosoma cruzi*

The hemofalgellate *Trypanosoma cruzi* is the etiological agent of Chagas disease in humans and affects more than 20 million people in endemic areas of Central and South America [Moncayo, 1993]. This intracellular parasite changes morphologically and biochemically through its complex life cycle as an adaptation to different environmental changes. During its life cycle, the parasite has four major developmental stages: epimastigotes, metacyclic trypomastigotes, blood stream trypomastigotes, and amastigotes. In the vertebrate host, *T. cruzi* multiplies as intracellular amastigotes that then transform into blood stream trypomastigotes forms. When they are released, the invasive bloodstream trypomastigotes infect host cells, and the parasite continues its life cycle of transformation and multiplication. When the hematophagous reduvid insects feed on blood infected with circulating trypomastigotes, these, in turn, differentiate into epimastigotes that multiply in the midgut of the insect. Subsequently, in the hindgut, epimastigotes differentiate into metacyclic trypomastigotes, which can infect mammalian host cells when the excrements of the infected bugs come in contact with the skin of humans [De Souza, 1984]. The complex changes observed during the life cycle of *T. cruzi* could be the results of adaptative modifications in gene expression

Abbreviations used: BAC, benzyldiemethylalkylammonium chloride; EDTA, ethylen diamine-tetraacetic acid; LIT, liver infusion tryptose; MEM, minimun essential media; PBS, phosphate buffered saline; PMSF, phenyl-methyl-sulphonyl-fluoride; TLCK, tosyl-lysine chloromethyl ketone.

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[Gonzãlez-Pino et al., 1999], which might be accompanied by modifications of chromatin structure [Reverol et al., 1997].

In eukaryotes, the basic repeating structural unit of chromatin, the nucleosome, consists of a DNA strand 146–200 base pairs (bp) long wrapped around a group of small basic histone proteins. Two pairs of each histone, H2A, H2B, H3, and H4, form the octamer and H1 is located outside the nucleosomal core, protecting 20 bp of DNA. The latter is essential for the folding of chromatin into a 30 nm fiber [Garcúa-Ramúrez et al., 1992; van Holde and Zlatanova, 1996; Bradboury, 1998; Zlatanova et al., 1998; Ornberg and Lorch, 1999].

Histones are highly conserved throughout evolution. However, several variations in the structure of core and H1 histones, between higher and lower eukaryote, have been reported [Hecker and Gander, 1985]. The chromatin of *T. cruzi* and other lower eukaryotes is also organized in nucleosomes of about 200 bp. However, the nuclear organization of *T. cruzi* as well as that of other trypanosomatids differs from that of higher eukaryotes in the persistence of a nuclear membrane and the lack of chromatin condensation during mitosis [Hecker et al., 1994]. The condensation of the nucleosome filaments that occurs in higher eukaryotes at NaCl concentrations of 10–100 mM with the formation of compact solenoids (30-nm fibers) is not observed with the chromatin of trypanosomatids [Hecker and Gander, 1985]. Differences in the primary structure of *T. cruzi* histone proteins have been related to the unusual behavior of chromatin condensation observed in these parasites [Hecker et al., 1994].

All previous investigations on *T. cruzi* chromatin have been performed with the noninfective proliferative epimastigote form of the parasite grown in vitro in an axenic media similar in composition to the vectors midgut where these forms naturally propagate. However, at present it is unknown whether the differences found between *T. cruzi* epimastigote chromatin and that of higher eukaryotes is a characteristic of all the stages of the life cycle of the parasite.

With this purpose, we studied the pattern of chromatin condensation not only in the proliferative epimastigote form but also in the stationary phase epimastigote form as well as the tissue derived infective trypomastigotes.

MATERIALS AND METHODS

Growth of *Trypanosoma cruzi*

The proliferative forms of *T. cruzi*, originally isolated from a Venezuelan patient (EP), were grown at 27°C in LIT (liver infusion tryptose: 0.15 M NaCl; 5.36 mM KCl; 0.005 M Na_2HPO_4; 0.02 M glucose; 0.03 mM hemin; 1.5% Bactotryptose; 0.5% yeast extract; 0.5% liver broth; 10% heat inactivated fetal calf serum). The log-phase epimastigotes forms were collected at day 6 of culture. The stationary-phase forms were collected at day 12 of culture.

The trypomastigote forms of the parasite were obtained from infected VERO cells at 37°C in MEM media (minimum essential media, Sigma) according to Henríquez et al. [1981].

Purification of Nuclei

Nuclei from fresh *T. cruzi* were prepared in the cold and in presence of protease inhibitors (*Proteases Inhibitors*: 0.5 mM TLCK; 2 mM PMSF; 1 mM N-ethilmalemide; 0,5 TPCK) according to Astolfi Fihlo et al. [1980] with slight modifications. Parasites (1×10^9) were collected by low-speed centrifugation (500*g*, 10 min) and washed once with PBS 1× (PBS 1×: buffer phosphate, 0.2% KCl; 0.2% KH_2PO_4; 8% NaCl; 0.93% Na_2HPO_4 pH 7.0) and once in 0.5 ml of buffer A (*Buffer A*: 20 mM Tris-HCl pH 8.0; 1 mM $MgCl_2$; 250 mM sucrose; 0.01% spermidine; 0.005% spermine). Washed cells were resuspended in 2 ml of buffer B (*Buffer B*: 20 mM Tris-HCl pH 8.0; 1 mM $MgCl_2$; 5 mM KCl; 3 mM $CaCl_2$; 1.0% NP-40; 0.01% spermidine; 0.005% spermine; 15 mM-mercaptoethanol) and subsequentely lysed with 50 strokes with a teflon Dounce homogenizer. The lysate was centrifuged for 5 min at 500*g*. The supernatant was centrifuged for 10 min at 2,000*g* and the resulting pellet was resuspended in 1 ml of buffer B without NP-40 and diluted with 3 ml of buffer C (*Buffer C*: 20 mM Tris-HCl pH 8.0; 1 mM $MgCl_2$; 5 mM KCl; 0.2 mM $CaCl_2$; 2.0 M sucrose; 0.1% spermidine; 0.005% spermine; 15 mM-mercaptoethanol).

The aliquot was layered over 1 ml cushion of buffer C in a Beckman SW 50.1 rotor and centrifuged at 38,000 rpm for 60 min at 4°C.

Rat liver nuclei were obtained according Burgoyne et al. [1970]. Isolation and washing were carried out in Buffer A (60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 15 mM 2-mercaptoethanol, 15 mM tris-chloride,

pH 7.4) with additions as indicated. Liver from an adult rat was rapidly homogenized in at least 7 ml of buffer A-0.34 mM sucrose, 2 mM EDTA, 0.5 mM EGTA/gram of liver, and the homogenate was layered on a 0.33 volumes of buffer A-1.37 M sucrose, 1 mM EDTA, 0.25 mM EGTA, and centrifuged for 15 min at 16,000*g*. The nuclear pellet was dispersed in 7 volumes of the same buffer and centrifuged for 4 min at 75,000*g*; after one wash in buffer A-0.34 M sucrose by centrifuging for 15 min at 16.000*g*.

Nuclei were resuspended in suspension buffer (*Suspension buffer*: 60 mM KCl; 15 mM spermine; 0.5 mM spermidine; 15 mM Tris-HCl pH 7.4; 1.9 M sucrose; 0.1 mM Na_2EDTA) to a concentration of 50 A_{260} per milliliter for *T. cruzi* and to a concentration of 30 A_{260} per milliliter for rat liver according to Hecker and Gander [1985].

Preparation of Soluble Chromatin

Nuclei from rat liver and parasites were digested with 0.06 U per 80A_{260} per milliliter of DNase I at 37°C for 1 min. The digestion was stopped by adding 20 l of 0.1 M Na_2EDTA per milliliter and the nuclear suspension chilled on ice. The nuclei were centrifugated at 4,000*g* for 5 min. The nuclear pellet was resuspended in lysis buffer (1 mM Triethanolamine-HCl pH 7.4; 0.2 mM Na_2EDTA) and kept on ice for 30 min with repeated aspiration through a narrow Pasteur pipette. The insoluble material was removed by centrifugation at 4,000*g* for 5 min. The supernatant containing large fragments of soluble chromatin was used for analysis by electron microscopy [Thoma et al., 1979; Hecker and Gander, 1985].

Transmission Electron Microscopy (TEM)

Thin nuclei section. The Trypanosome and rat liver nuclei were prepared for thin-sectioning and electron microscopy according to Hecker and Gander [1985] with slight differences. In brief, an enriched fraction of nuclei was fixed in glutaraldehyde 2.5% in PBS 1 × at 4°C overnight. The fixed pellet was minced and fixed in osmium tetroxide 1%, 246 mosm/L in PBS 1 ×, 1 h at 4°C. After carefully washing the sample, it was dehydrated progressively with ethanol 50, 70, 80, 90, and 100% for 5 min at 4°C. Nuclei were washed first in propylene oxide:ethanol 1:1 followed by propylene oxide 100% for 15 min at 4°C each. The sample were embedded in Polibet 812 resin and polymerized at 60°C for 48 h. The sections were obtained in a Porte-Blum equipped with a diamond knife. The sections of 60 nm were stained with uranyl acetate and lead citrate [Reynolds, 1963].

Soluble chromatin. The fractions with large fragments of chromatin were divided into five aliquots and dialyzed against 5 mM TEA-Cl pH 7.4 and 0.2 mM Na_2EDTA containing 0 and 100 mM NaCl, respectively for 4 h at 4°C [Thoma et al., 1979]. Glutaraldehyde was then added to the dialysis buffer to a concentration of 0.2% for parasite chromatin or 0.1% for rat liver chromatin. The fixation was performed at 4°C for 6 h. The fixed chromatin was diluted in the corresponding fixation buffer to an A_{260} of 0.02–0.06 and BAC was added from a stock solution in water (0.2%) to a concentration of 0.2×10^{-4}% ($\approx 7 \times 10^{-4}$ mM) for 30 min. Five microliters of fixed chromatin was spread over a carbon-coated grid of 200 nm covered with collodion and carbon film. The adsorption was allowed for 5 min at room temperature. The grids were washed with bidistilled water and dried at room temperature. The grids were rotary-shadowed in a JEOL JEE-4C, using carbon-platinum evaporated at an angle of 7° at 10^{-5} torr. The grids were examined in a Philips CM-10 transmission electron microscope at a 16,000–20,000 ×.

DNase I Digestion and Agarose Gel Electrophoresis

Purified nuclei in suspension buffer were digested with 0.06 U of DNase I per 80 A_{260} per milliliter at 37°C for 1, 5, and 20 min. The digestion was stopped with 0.2 mM Na_2EDTA pH 7.4, and the solution was chilled on ice. The nuclei were resuspended in lysis buffer to an A_{260} of 80 per milliliter, and the lysis was performed on ice with repeated aspiration through a narrow Pasteur pipette for 30 min. The suspension was then digested with proteinase K 100 g/ml at 50°C overnight. The DNA from digested chromatin was purified by phenol-chloroform extraction. The fragments of DNA obtained were separated on a 1.5% agarose gel [Schlimme et al., 1993].

RESULTS

Nuclei Fine Structure

Nuclei were isolated from *T. cruzi* or rat cells and then prepared for electron microscopy as described in Materials and Methods. Electron microscopy thin section of the different

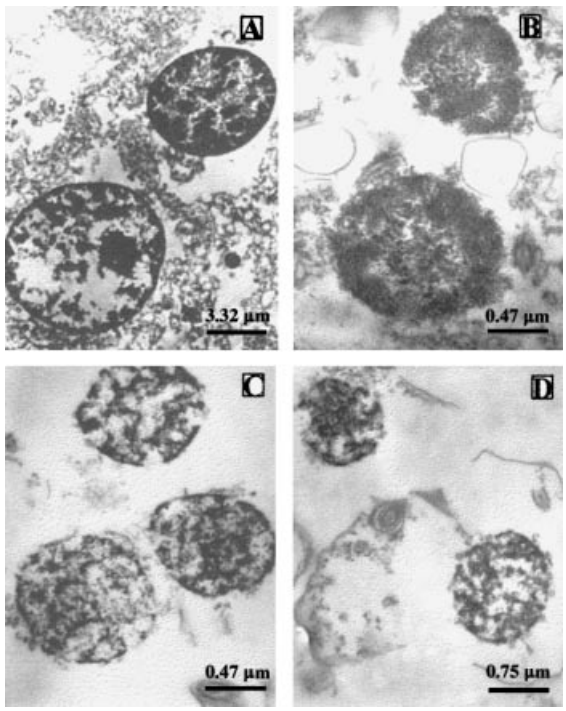

Fig. 1. Thin section of nuclei of rat liver and *Trypanosoma cruzi*. **A**: The chromatin from rat liver consists of electron dense fiber of 30 nm. **B**: The fibers of nuclear chromatin of epimastigote log-phase of *T. cruzi* is finer as compared to the rat. **C**: Stationary-phase and (**D**) cell culture derived trypomastigote form shown similar but not identical compacted regions of chromatin as compared as compared to the rat.

preparations are shown in Figure 1. Heterochromatin of rat liver nuclei consisted of electron dense fibers organized in loosely as well as densely-packed arrangements (Fig. 1A). Thin sections prepared from logarithmic epimastigotes nuclei showed chromatin fibers that were finer than rat heterochromatin, observing a looser network of electron dense material (Fig. 1B). Stationary phase epimastigotes (Fig. 1C) and trypomastigote forms (Fig. 1D) showed similar but not identical compacted regions of chromatin when compared to rat nuclei.

Compactation Pattern of Soluble Chromatin

Soluble chromatin was prepared from rat liver and *T. cruzi* epimastigotes by digestion of isolated nuclei with DNase I as described in Materials and Methods. Large chromatin fragments were centrifuged through a sucrose gradient containing 10 mM NaCl, dialyzed against concentrations of 0 or 100 mM NaCl, fixed and prepared for TEM. As can be observed in Figure 2B, at high salt concentrations (100 mM NaCl), rat liver chromatin condensed to higher order structures, forming dense tangles as previously described by Thoma et al. [1979]. Under identical experimental conditions, chromatin from logarithmic epimastigotes barely condensed and only loose tangles could be observed (Fig. 2C). In contrast, chromatin condensation into compact fibers was observed in stationary phase epimastigotes (Fig. 2D) and cell culture derived trypomastigotes (Fig. 2E). These results are comparable to that found in rat liver chromatin (Fig. 2B).

Nucleosomal Pattern After DNase I Digestion

T. cruzi and rat nuclei were prepared as described in Materials and Methods. The purified nuclei were digested with DNase I and the DNA was then prepared by phenol-chloroform extractions. The fragments were separated on agarose gels and are shown in Figure 3. The pattern of DNase I digestion of rat liver and of *T. cruzi* chromatin was similar yielding DNA fragments that were characteristic of the nucleosomal organization of chromatin. However, the degree of digestion with time varied between *T. cruzi* and rat liver and also between the different developmental forms of the parasite. Very little, if any, digestion of rat liver chromatin was observed at 1 min (Fig. 3, lane 1) and by 5 min, some digestion could be observed (lane 2). In logarithmic epimastigotes, there was almost complete digestion to mononucleosomes and dinucleosomes when chromatin was incubated with DNase I for 1 and 5 min (Fig. 3, lanes 4 and 5) meanwhile, the chromatin of stationary phase epimastigotes and cell culture derived trypomastigotes was digested to a lesser extent yielding ladders of DNA fragments ranging from oligonucleosomes (Fig. 3, lanes 7) to mononucleosomes (Fig. 3, lanes 8, 10, and 11). After 20 min of digestion, nucleosome ladders were still observed in rat liver and stationary epimastigote forms (lane 3), whereas chromatin of logarithmic epimastigote forms and trypomastigote forms were completly digested to mononucleosomes at this time of digestion (lanes 6 and 12).

DISCUSSION

There are several differences in the nuclei organization and chromatin structure of trypanosomatids compared to higher eukaryotes. Specifically, in trypanosomatids, chromosomes can not be observed during mitosis [Vickerman

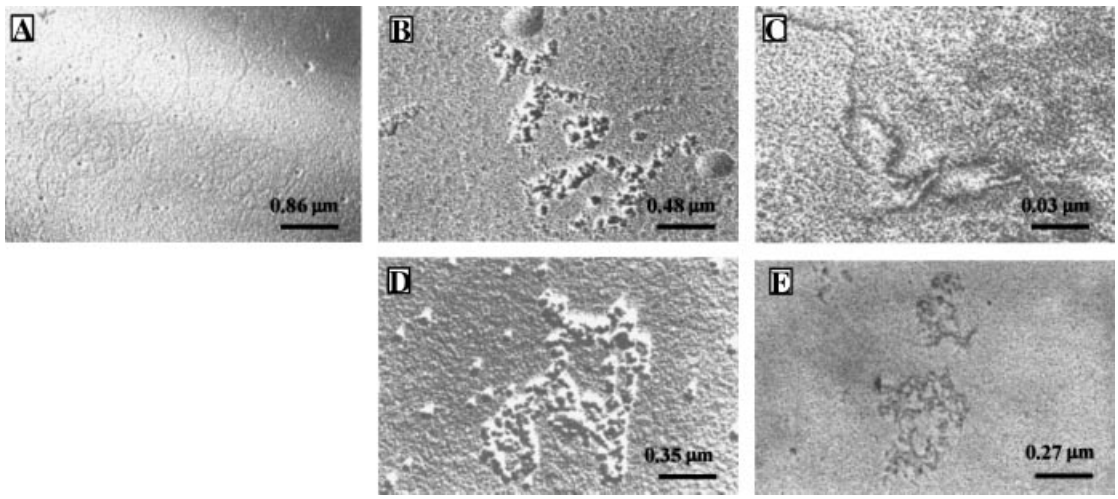

Fig. 2. Structure and condensation pattern of soluble chromatin of rat liver and *T. cruzi*. Aliquots of soluble chromatin from rat liver (**A**,**B**) and *T. cruzi* logarithmic forms (**C**) stationary forms (**D**) and cell culture derived trypomastigote forms (**E**), were dialyzed against: (A) 0 mM NaCl, (B–E) 100 mM NaCl. Then fixed in glutaraldehyde at 4°C for 6 h and prepared for transmission electron microscopy (see Materials and Methods).

and Preston, 1970] and electron microscopy of nuclei thin sections show the presence of loosely packed heterochromatin within the nucleus [Hecker and Gander, 1985]. Despite these important differences, the general organization of chromatin in trypanosomatids is similar although not identical to that observed in higher eukaryotic cells.

Astolfi Fihlo et al. [1980] showed that nucleosomes were the basic structural units of *T. cruzi* chromatin. The sedimentation of mononucleosomes in a sucrose gradient is similar to that observed for higher eukaryotes and the values reported for the repeat length of the DNA varies between 185 and 212 bp. The data obtained from *T. brucei* [Hecker and Gander, 1985], *Critidia*

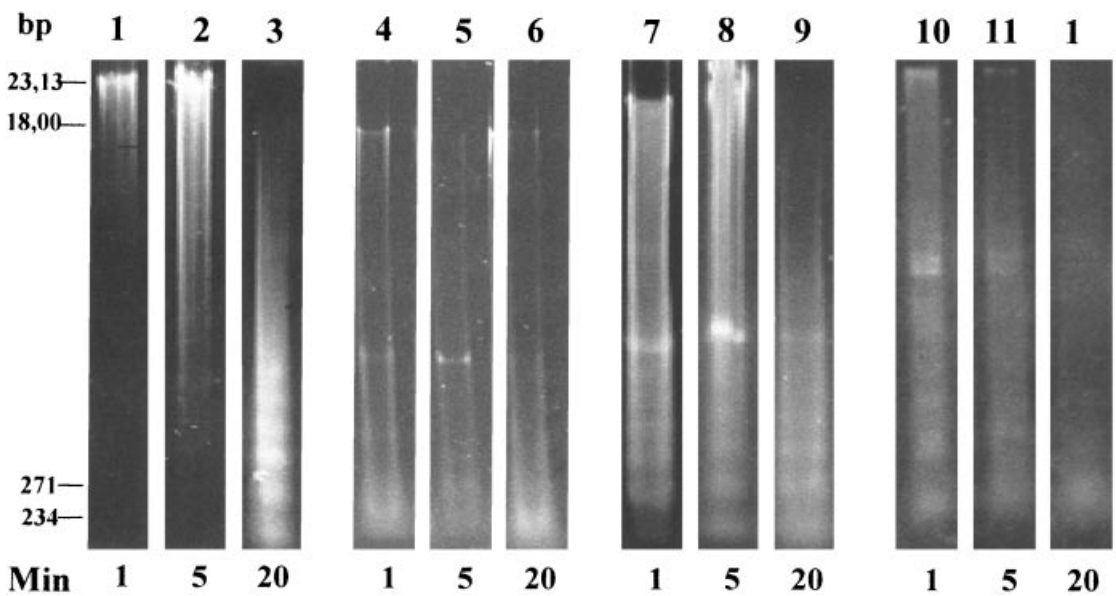

Fig. 3. DNA digestion pattern of chromatin of rat liver and *T. cruzi*. DNA purified from nuclear chromatin was digested with 0.06 U of DNase 1 for different length of time and separated by 1.5% agarose gel electrophoresis. Rat liver 9 (1–3). Epimastigotes of *T. cruzi* logaritmic form (4–6). Epimastigotes of *T. cruzi* stationary form (7–9). Cell culture derived trypomastigotes of *T. cruzi* (10–12).

fasciculate [Duschak and Cazzulo, 1990], and more recently for *T. congolense* [Hecker et al., 1995] confirms these results for other trypanosomatids. However, in contrast to the chromatin of higher eucaryotes that condenses to 30-nm fibers at high salt concentrations [Thoma et al., 1979], no condensation of soluble chromatin is observed in *T. cruzi* and other trypanosomatids in response to increasing salt concentrations [Hecker and Gander, 1985]. These observations are in agreement with results on the digestion of chromatin of parasite with nucleases were DNA fragments form ladders in agarose gels similar to those of rat liver. Parasites' chromatin is digested more rapidly indicating that it is more accesible to the enzyme.

These results suggest a different compactation pattern of trypanosome chromatin that can be explained in part by differences in the primary structure of the histones, the presence of histone variants and/or posttraductional modifications of H1 and/or core histones.

Histone proteins of *T.cruzi* have been extensively characterized by Toro and co-workers [Toro and Galanti, 1988, 1990; Toro et al., 1992, 1993] and Reverol et al. [1997]. These authors characterized *T.cruzi* histones by their banding pattern in different gel systems, their solubility properties, and amino acid composition. Their results confirm the presence of a full set of histone proteins in *T.cruzi* chromatin. *T. cruzi* histones can, however, be differentiated from higher eukaryotes histones on the basis of their primary sequence as well as their behavior upon separation in different gel systems. Specifically, they differ in the strong hydrophilic character and the low molecular weight of a H1-like protein [Toro and Galanti, 1988; Toro et al., 1992; Aslund et al., 1994], the divergent amino acid sequence in the amino terminal of H4 [Toro et al., 1992], and finally, the presence of a shorter amino terminal domain in *T.cruzi* histone H2B, where three out of the four possible acetylation sites present in histone H2B of higher eukaryotes are missing [Toro et al., 1993].

The unusual characteristics in the primary structure of *T. cruzi* histone may contribute to the observed abnormal behavior of chromatin condensation and contribute to changes in histone–histone as well as histone–DNA interactions [reviewed by Bender et al., 1992]. In this respect, *T. cruzi* histone H1 interacts weakly with DNA as compared to higher eukaryotes and is in line with results obtained with *T. brucei* [Burri et al., 1993]. A concentration of 380-mM NaCl completely removes histone H1 of *T. cruzi* and *T. brucei* whereas the dissociation of rat H1 starts at concentrations above 400 mM NaCl [Thoma et al., 1979].

As stated above, histone H1 of *T.cruzi* is more hydrophilic compared to higher eukaryotes. The protein runs far ahead of the core histones in Triton acid-urea gels [Toro and Galanti, 1988] which is probably due to its high lysine content. This, with the fact that histone H1 of *T. cruzi* has only the size of a core histone and that it lacks the globular domain and N terminal domain [Aslund et al., 1994], may contribute to explain its dissociation behavior.

However, despite the different characteristics of parasite H1, heterologous reconstitution of H1 depleted nucleosome filament has shown that histone H1 of rat liver can not induce a 30-nm solenoid structure on a *T. cruzi* nucleosome filament, but if histone H1 of *T, cruzi* is added to nucleosome filaments of rats, a condensation to a 30-nm fiber-like structure occurs [Schlimme et al., 1995]. This result indicates that not only the strong differences in the amino acids composition and sequence of histone H1 are responsible for the weak condensation of the chromatin of *T. cruzi* but also that properties of the core histones might be important. This will be in line with other authors who postulate a strong contribution of post-translational modifications of histone tails, such as acetylation phosphorylation, and ubiquitination in the modulation of chromatin structure and function [Li et al., 1993; Cheung et al., 2000]. In *T. cruzi*, Reverol et al. [1997] have demonstrated the presence of an unusually high concentration of ubiquitinated histone-like proteins.

All previous investigations have been carried out with proliferative epimastigote forms propagated in axenic media. However, it is currently unknown whether the differences found are characteristic of all the stages of the life cycle of *T. cruzi*. In the present work, we took advantage of the possibility of the in vitro growth of the different developmental stages of the parasite namely trypomastigotes and logarithmic and stationary epimastigotes. The growth curve of epimatigotes was used based on the well-established fact that the in vitro transformation of non-infective epimastigotes into infective metacyclic trypomastigotes appears to be triggered by stress resulting from the

nutritional exhaustion of the culture media at the stationary phase of the parasites growth curve [Contreras et al., 1985]. Therefore, the growth cycle of *T. cruzi* epimastigotes can be used as an in vitro model to study the molecular changes that take place in the parasite on nutritional depletion to further decipher the events that lead the parasite to differentiate into infective forms in vivo.

Our results show that when chromatin is prepared under identical conditions, differences in the level of condensation were found in the different stages of the in vitro life cycle of *T.cruzi*. As reported previously by Hecker and Gander [1985], the condensation of soluble chromatin into compact 30-nm solenoid was never observed in the proliferative epimastigotes at a salt concentration of 100 mM. However, condensation of chromatin was more pronounced in stationary epimastigotes and trypomastigotes, although never reaching the levels observed in rat chromatin. These results are in agreement with the results obtained when chromatin of the different forms was digested with DNase I for different lengths of time and with the results obtained with electron microscopy of thin sections of nuclei.

The differences observed in the compactation pattern of chromatin among different forms of *T.cruzi* are in line with previous work by Schlimme et al. [1993] in *Trypanosoma brucei*. These authors report differences in the level of condensation between procyclic culture forms and blood forms, observing a higher level of condensation in the latter forms. Differences in H1 as well as core histones with respect to amount, number of bands and banding pattern were reported by these authors between both parasite forms.

It has been suggested that differences in chromatin structure and condensation patterns might be associated with differences in gene expression [Edmonson and Roth, 1996; Cheung et al., 2000]. Specifically, differences in the DNase I digestion of chromatin have been attributed to the transcriptional activation of specfic genes. Tikoo et al. [1997] have studied DNase I digestion of rat liver nuclei to determine that chromatin that is preferentially digested was enriched with trancriptionally active genes. Our results are important because the differences observed in chromatin behavior during the growth curve of *T. cruzi* epimastigotes as well as in trypomastigotes might be associated with differences in transcription detected in these developmental stages of the parasite [Gonzãlez-Pino et al., 1999]. We are currently using specifc genes as probes together with DNase I digestion of chromatin *T.cruzi* in order to further investigate this matter.

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