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Unambiguous Identification of Histone H_1 in *Trypanosoma cruzi*

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Abstract The existence of histone H_1 has been questioned in Trypanosomatids. We report here the presence of a histone H_1 in the chromatin of *Trypanosoma cruzi*. This protein was purified by narrow-bore reversed phase HPLC and its amino acid composition analyzed and compared with histones H_1 from other species. Furthermore, the purified chromosomal protein was digested with proteases and the amino acid sequences of the resulting peptides were analyzed by the automated Edman degradation. The sequences obtained were found to present a high degree of homology when compared to the carboxy terminal domain of other known histones H_1.

Key words: histone H_1, chromatin, carboxy terminal domain, Trypanosomatids, narrow-bore

Histone H_1 plays a key role in chromatin structure, both at the level of the nucleosome particle itself and in the formation of the higher-order structure of the chromatin fibre [McClearly and Fasman, 1980; Thoma et al., 1979]. Historically, histone H_1 along with nucleosomal core histones have been regarded as part of a general mechanism that ensured a strong and stable repression of gene expression [Weintraub, 1984, 1985]. In addition histone H_1 might also be involved in controlling the transcriptional activity of individual genes [Zlatanova, 1990; Laybourn and Kadonaga, 1992].

Although the precise role of this very lysine rich histone H_1 remains unknown, it has been assigned many functions, even that of a recombinase [Kawasaki et al., 1989]. On the other hand, different regulatory properties of the chromatin would depend on the presence of the histone H_1 [Du Clos et al., 1991; Kas et al., 1989].

Histone H_1 is the less conserved among the histones; it presents multiple variants and subtypes [Lennox and Cohen, 1984] whose functions are not yet known. Moreover, this histone, found in most eukaryotic cells, is largely replaced in mature erythrocytes of birds, reptiles, amphibians, and fish by another class of lysine rich histone: H_5 [Smith et al., 1984]. A histone H_1° has also been described in mammals which presents intermediate characteristics between H_5 and H_1 [Cary et al., 1981].

On the other hand, the presence of histone H_1 has been questioned in yeast [Certa et al., 1984] and in Trypanosomatids since a protein with its characteristic electrophoretic mobility was not found [Rubio et al., 1980; Hecker and Gander, 1985; Hecker et al., 1989; Bender et al., 1992].

The presence of a histone H_1 in *Trypanosoma cruzi* seems of interest considering that *T. cruzi* chromatin does not condense into chromosomes during cell division [Solari, 1980]. However, compact heterochromatin is present during interphase in this parasite.

In previous studies, a histone with high electrophoretic mobility in acid-urea [Astolfi Filho et al., 1980; Rubio et al., 1980; Toro and Galanti, 1990] and in Triton-acid-urea gels [Toro and Galanti, 1988, 1990] was found in *T. cruzi* chromatin. This protein was soluble in 0.75 M perchloric acid and in 5% trichloroacetic acid, it was extracted from chromatin between 0.35 and 0.5 M NaCl, and presented cross-immunoreactivity with an antiserum raised in rabbits against sea urchin sperm histone H_1. It also presented the metachromasie characteristic of histone H_1 and could be purified to a high degree using histone

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H_1 preparative methods [Toro and Galanti, 1988, 1990].

All this data led us to postulate the presence of a histone H_1 with unusual characteristics in *T. cruzi,* similar to the histone H_1 from *Oxytricha* sp. which presents a high electrophoretic mobility in acid-urea gels [Caplan, 1975]. Interestingly, Tetrahymena shows a histone H_1 which also exhibits an unusual behavior in different electrophoretic systems [Gorovsky et al., 1974].

In this paper we confirm the presence of a chromosomal protein from *T. cruzi* presenting an elution behaviour similar to that of histone H_1 from calf thymus, when purified by narrow bore reversed phase HPLC. The amino acid composition of this protein showed great similarity when compared with histones H_1 from different sources. Peptides obtained with proteases from this protein presented an amino acid sequence with a high degree of homology when compared to the carboxy terminal sequence of histone H_1 from sea urchin gonads. It also presented the conserved motifs proper of most histones H_1 sequenced to date [Vanfleteren et al., 1988; Maeder and Bohm, 1991].

Our results show that a histone H_1 is present in *T. cruzi* chromatin. This protein shows most of the features previously described for this chromosomal protein in other species, except an unusual mobility in acid-urea and in SDS gels.

METHODS

Cell Culture

T. cruzi epimastigotes strain Tulahuén were grown in Diamond medium [Diamond, 1968] supplemented with 2.5% fetal calf serum at 28°C. Cells were routinely harvested by centrifugation at day 7 of culture, washed three times in phosphate-buffered saline (PBS), and used immediately.

Extraction of Histones and Purification of Histone H_1

Chromatin was obtained from calf thymus or from *T. cruzi* following the procedure of Stein et al. [1975], with some modifications [Toro and Galanti, 1990]. For the extraction of histones from chromatin, the procedure of Panyim and Chalkey [1969] was applied. In some experiments, a histone was obtained from *T. cruzi* chromatin by the procedure of Sanders [1977], which was designed for the purification of histones H_1 from different sources. The same procedure was applied to obtain histone H_1 from calf thymus.

Electrophoretic Procedures

Total *T. cruzi* histones (70 μg) or histones purified by HPLC, were loaded onto gels containing 15% acrylamide, 0.38% Triton DF-16, 0.9 N acetic acid, and 6M urea (TAU) as indicated by Alfageme et al. [1974].

Alternatively, total calf thymus histones (45 μg) and calf thymus histone H_1 (10 μg) were analyzed on a 15% acrylamide gel as described by Laemmli [1970], with the modifications of Thomas and Kornberg [1975].

HPLC Isolation of Calf Thymus and *T. cruzi* Histones

Histone separation was also performed by narrow-bore reversed phase high performance liquid chromatography (HPLC) on a 2.1 × 100 mm, 3 μm support (μ RPC C2/C18 SC2.1/10 from Pharmacia-LKB Biotechnology, Uppsala, Sweden). The freeze-dried *T. cruzi* total histones were dissolved in 0.15 M NaCl, 0.13% trifluoroacetic (TFA), and samples of 10 to 25 μg were injected to the column. The histones were chromatographed within 55 min at a flow rate of 100 μl/min and at room temperature. A multistep acetonitrile gradient was applied (solvent A: 0.13% TFA in water; solvent B: 0.13% TFA in acetonitrile). The concentration of solvent B was increased in the following order: 0 to 0 (during 5 min), from 0 to 40% (during 5 min), from 40 to 50% (during 40 min), and from 50 to 70% (during 5 min). The eluate was monitored at 215 nm. The fractions eluted were analyzed by TAU-PAGE.

Protease Digestions

The fraction eluted from HPLC, corresponding to the protein with high electrophoretic mobility in a TAU gel (band f, Fig. 2, insert) was incubated either with trypsin, V8 protease, or Lys C. For the tryptic digestion, histone f from *T. cruzi* (50 μg), was dissolved in 300 μl of 1.5 M NaCl and 20 mM Tris-Cl, pH 8.0; TPCK-treated trypsin (Worthington) was then added to obtain an enzyme/histone ratio of 1:100 (w/w). This solution was incubated at room temperature for 90 min. as indicated by Barbero et al. [1980]. For V8 protease digestion, 50 μg of histone f from *T. cruzi* was dissolved in 0.1 M ammonium bicarbonate and incubated at 37°C overnight at

an enzyme-substrate ratio of 1:60 (w/w) [Vanfleteren et al., 1988]. For the digestion with Lys C, the dry sample (20 μg) was dissolved in 50 μl of 8 M urea and sonicated. Then 50 μl of 0.1 M Tris-Cl, pH 9.0, were added at an enzyme-substrate ratio of 1:100 (w/w). The digestion was carried out at 37°C overnight.

After digestion, each reaction mixture was loaded onto the HPLC column where the enzymatic cleavage fragments were chromatographed within 80 min at a flow rate of 100 μl/min and at room temperature, with a linear acetonitrile gradient. The eluate was monitored at 215 nm.

Amino Acid Analysis

Samples were hydrolyzed for 24 h at 110°C in vacuum-sealed tubes, in 5.7 M HCl containing phenol (1 mg ml^{-1}). The hydrolyzates were analyzed, following the instructions of the manufacturer.

Amino Acid Sequencing

Selected peptides obtained by digestion with proteases and separated by HPLC were sequenced by automated Edman degradation in an Applied Biosystem Protein Sequencer, model 470A or 477A, both equipped with on-line PTH analyzer, model 120A (Applied Biosystems, Foster City, CA). The instruments were operated according to the manufacturer's instructions.

Sequence Comparisons

Each partial amino acid sequence obtained was screened against the Protein Identification Resource (PIR) of the National Biomedical Research Foundation with the FASTP program [Lipman and Pearson, 1985]. The final alignment presented was composed by hand.

RESULTS

It has been shown that histones H_1 and H_5 from goose and chicken are not retained and elute first in respect to the core histones when they are separated by reversed-phase HPLC [Helliger et al., 1988]. Using the same experimental conditions described for goose and chicken histones H_1, a C2/C18 column was calibrated with total calf thymus histones and with histone H_1 from calf thymus that was purified by the method of Sanders [1977].

As expected (Fig. 1) purified histone H_1 was excluded from this support (peak I, continuous line). When a total calf thymus histone preparation was passed through the column, the excluded fraction (peak I, dotted line) was lyophilized and analyzed by SDS gel electrophoresis. Figure 1 (insert) shows that histone H_1 from calf thymus is highly purified in this peak (lane I), clearly separated from other histones (peak II, III, and IV). Conclusively histone H_1 from calf thymus can be purified by reversed-phase HPLC as is histones H_1 from chicken and goose erythrocytes.

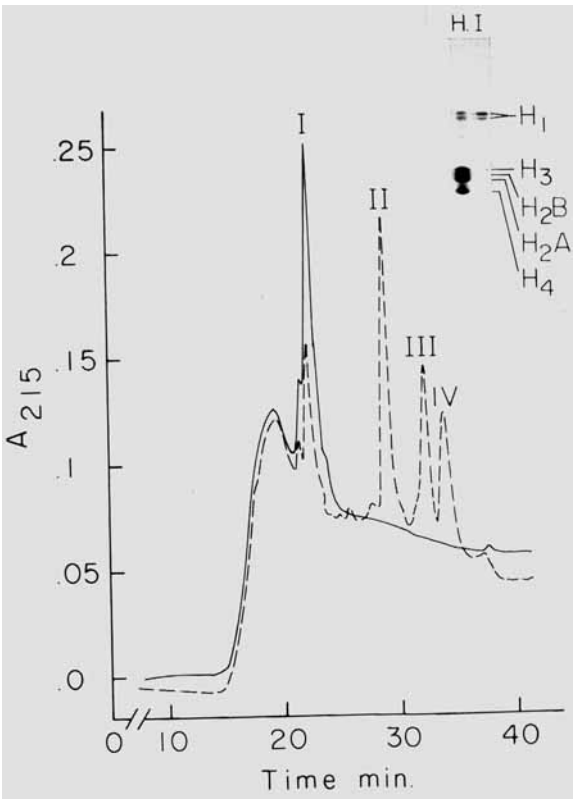

Fig. 1. Separation of histones from calf thymus by reversed-phase HPLC. A C2/18 column from Pharmacia was used. The flow rate was 100 μl/min using a multistep acetonitrile gradient as described in Methods. -----, total histones; ____, histone H_1 obtained by the Sanders technique. **Insert:** SDS-PAGE of calf thymus histones. H: Total histones from calf thymus. I: histone eluted in peak I.

Figure 2 presents the elution profiles of total histones from *T. cruzi* as fractionated using the column previously calibrated with total and H_1 calf thymus histones. The protein excluded from the column (peak I, dotted line) in the same zone as calf thymus histone H_1, presents a high electrophoretic mobility in Triton-acid-urea PAGE (Fig. 2, insert, lane I). We have previously characterized this protein (band f) as a histone H_1 considering its solubility in acids and in salt solutions, as well as its immunoreactivity with

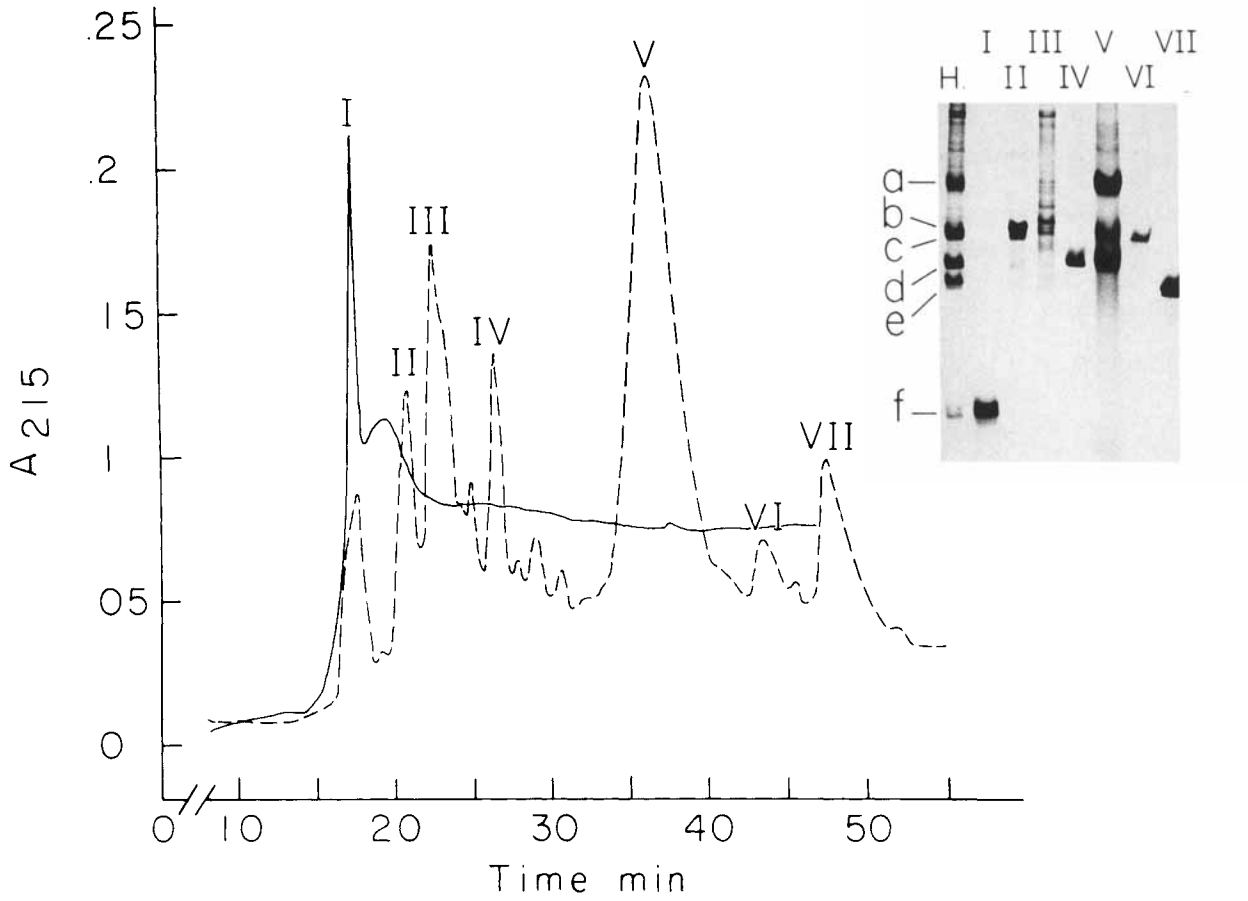

Fig. 2. Separation of histones from *T. cruzi* by reversed-phase HPLC. -----, total histones; ___, histone H_1 obtained by the Sanders technique. **Insert:** Triton DF-16-urea-acid PAGE. H: Total histones from *T. cruzi*. I–VII: Fractions from the HPLC column.

a heterologous antiserum raised in rabbits against sea urchin histone H_1 [Toro and Galanti, 1988, 1990]. Moreover, by purification of a histone H_1 from the *T. cruzi* chromatin using the Sanders procedure a protein is obtained which comigrates with band f [Toro and Galanti, 1988]. When this protein is passed through the C2/C18 column, it is also excluded appearing in the same position as peak I (Fig. 2, continuous line). Consequently, band f behaves as a histone H_1 when isolated by reversed-phased HPLC. By SDS-PAGE (Fig. 3, lane 4) this histone presents an electrophoretic migration similar to core histones from *T. cruzi* (Fig. 3, lane 3) or calf thymus (Fig. 3, lane 1), different from the electrophoretic behavior of calf thymus histone H_1 (Fig. 3, lane 2).

The protein eluted in peak I (Fig. 2, band f) was analyzed for its amino acid composition. Results are shown in Table I. Most aspects of the amino acid composition of band f corresponds to those described for histones H_1 from different species. It is highly enriched in lysine and presents the expected proportions of alanine and proline. The basic/acid and the lysine/arginine ratios are similar to calf thymus histone H_1, and in the range seen for other known histones H_1. The percentage of hydrophobic amino acids is coincident with calf thymus histone H_1 [Panyim et al., 1971] and different from Tetrahymena histone H_1 [Gorovsky et al., 1974], which has been described as a protein devoid of a globular domain [Wu et al., 1986].

The histone from *T. cruzi* showing physicochemical characteristics, immunological properties, and amino acid composition of a histone H_1 (band f, insert, Fig. 2) was transferred from the gel to a PVDF membrane for sequencing of its amino acids by the automated Edman degradation procedure. The results showed that the

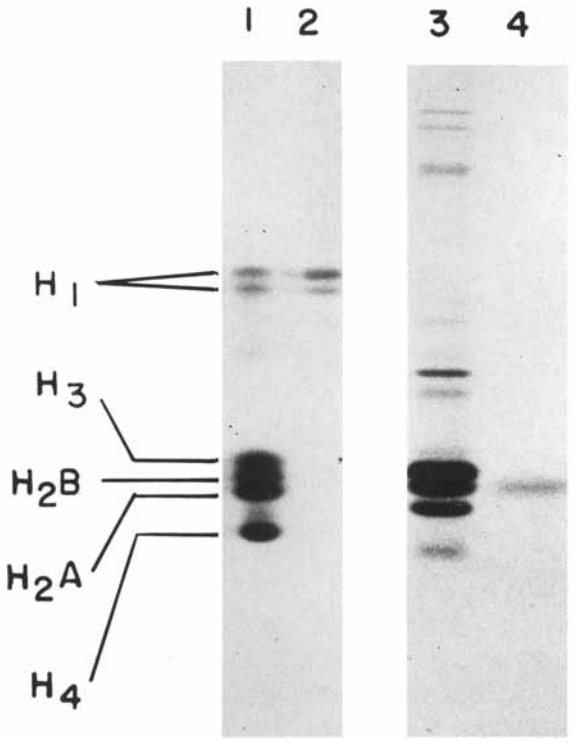

Fig. 3. SDS-PAGE. **Lane 1:** Total calf thymus histones. **Lane 2:** Calf thymus histone H_1. **Lane 3:** Total histones from *T. cruzi*. **Lane 4:** *T. cruzi* histone showing elution characteristics proper of H_1.

amino terminal end of this protein is blocked, a situation which is common to all histones H_1 known [Vanfleteren et al., 1988].

To sequence this protein, 100 μg of histone f was prepared from *T. cruzi* chromatin using the procedure of Sanders [1977]. This protein was further purified by reversed-phase HPLC as indicated in Figure 2 and digested alternatively with proteases Lys C, V8, or trypsin. The fragments obtained after each digestion were isolated by reversed-phase HPLC and 8 selected peptides were analyzed by the automated Edman degradation. Results are shown in Table II. The sequences of these 8 peptides correspond to motifs which are present in the carboxy terminal domain of all histones H_1 described so far. However, each motif is present in this domain in a different number of repeats, depending upon the species since they present cluster organization [Vanfleteren et al., 1988; Maeder and Bohm, 1991]. We do not know at present the number of each of the 8 repeats found in histone f in its carboxy terminal domain.

The amino acid sequences of each peptide were screened against the Protein Identification

TABLE I. Comparative Amino Acid Composition of *T. cruzi* Histone H_1

Amino acid	*Tetra-hymena*[a]	*Oxy-tricha*[b]	*T. cruzi*	Calf thymus[c]
Lysine	29.9	31.6	32.35	27.3
Histidine	2.3	0.5	1.3	0.0
Arginine	2.4	3.4	2.0	2.1
Aspartic acid	9.9	1.9	3.71	2.6
Threonine	8.6	4.1	1.79	6.0
Serine	7.5	6.9	5.9	6.9
Glutamic acid	7.3	1.2	2.6	4.7
Proline	6.1	5.1	10.97	8.4
Glycine	3.5	2.8	4.3	6.7
Alanine	14.0	29.2	26.5	23.4
Cysteine	0.0	0.0	ND	0.0
Valine	3.9	8.1	4.77	6.0
Methionine	Trace	0.0	ND	Trace
Isoleucine	2.3	4.3	0.8	0.8
Leucine	1.7	0.3	1.54	3.9
Tyrosine	0.5	0.2	0.7	0.6
Phenylalanine	0.4	0.2	0.5	0.6
Basic/acid	2.0	11.5	5.6	4.0
% Hydrophobic aa	22.8	42.3	34.8	35.3
Lysine/arginine	12.5	9.3	16.2	13.0

[a]From Gorovsky et al. [1974].
[b]From Caplan [1975].
[c]From Panyim et al. [1971].

TABLE II. Common Motifs of Histones H_1 Found in Histone f From *T. cruzi* Chromatin*

Enzyme used and fragment number	Motif
V8 I	AAKKAPKKAVKKAPKKK
V8 II	EAKKXAPKKAP
Lys C I	RAAPK
Lys C II	KPAVK
Lys C III	KKPAAAK
Trypsin I	KAAPK
Trypsin II	KAPKKAP
Trypsin III	KPAAK

*Sequences of selected peptides eluted from HPLC, corresponding to the digestion with proteases of the protein with high electrophoretic mobility in a TAU gel.

Resource (PIR) of the National Biomedical Research Foundation with the FASTP program [Lipman and Pearson, 1985]. The highest scores were obtained with histones H_1 from different sources. An alignment of these sequences with the carboxy terminal domain of histone H_1 from the gonads of the sea urchin *Parechinus* sp. (HSUR1P) is displayed in Figure 4. This alignment is essentially valid for the carboxy terminal domain of all histones H_1. Considering the

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          130
HSUR1P      KAKKAKAAAAKKARKAKAAAKRKAALAKKKAAAAKRKAAAKAKKAKKPKK
                 .::.       ::::.:       ::.::::: :.:::
T. cruzi         RAAPK      KAAPK        KKPAAAK KPAAK

HSUR1P      KAAKKAKKPAKKSPKKAKKPAKKSPKKKKAKRSPKKAKKAAGKRKPAAKK
            ::.:::  .::: :::: : : :       :  ::::        ::::.:
T. cruzi    KAPKKAP AAKKAPKKAVKKAPKKK  EAKKXAPKKAP       KPAVK

HSUR1P      ARRSPRKAGKRRSPKKARK
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Fig. 4. Alignment of the amino acid sequences of 8 peptides derived from *T. cruzi* histone H_1 (*T. cruzi*) with the carboxy terminal domain of histone H_1 from the gonads of the sea urchin *Parechinus* sp. (HSUR1P).

high degree of homology, the amino acid sequences obtained from histone f of *T. cruzi* correspond to the carboxy terminal domain of a histone H_1.

DISCUSSION

T. cruzi is an interesting model for the study of chromatin organization. It is supposed to be one of the oldest eukaryotes [McLaughlin and Dayhoff, 1973]. At the ultrastructural level the interphase nucleus is similar to those of higher eukaryotes. However, its chromatin does not condense into chromosomes during cell division [Solari, 1980]. Nucleosomal organization in this protozoan is fragile. To obtain the classical pattern of 200 bp DNA fragments observed in nucleosomes from higher eukaryotes, a low concentration of nucleases and a short period of incubation must be applied [Rubio et al., 1980]. On the other hand, the counterparts of H_2A and H_2B histones are extracted from *T. cruzi* chromatin at a lower salt solution concentration than the one needed for the extraction of the same histones from calf thymus [Toro, 1991]. Finally, histone H_4 of *T. cruzi* was found to present 50% substitutions in the amino terminal domain, being one of the least conservative histones H_4 known [Toro et al., 1992].

Taken together, these features of Trypanosoma chromatin may be interpreted as an intermediate model of the genetic organization which was tested during the evolution of eukaryotes and successfully maintained to date. This consideration, together with the fact that *T. cruzi* is a parasite which causes Chagas disease, has focused our interest on the systematic study of the organization and function of its chromatin.

Histone H1 was not found in Trypanosomes for many years [Rubio et al., 1980; Hecker and Gander, 1985; Hecker et al., 1989; Bender et al., 1992]. However, this chromosomal protein was looked for mainly by gel electrophoresis. It is necessary to consider that histones H1 are the most variable in amino acid composition and sequence and are very sensitive to intrinsic proteases. These facts and the extremely charged nature of this molecule, seem to conspire against a fixed mobility in SDS or in acid-urea gels for histones H_1 from different species. In any case, the high mobility of *T. cruzi* histone H_1 in acid-urea gels may have caused other authors to lose this protein, which may have run out of the gels. Moreover, in SDS gels it comigrates with the core histones (Fig. 3), making its identification a different task.

It is tempting to suggest that the high mobility of *T. cruzi* histone H_1 in acid-urea gels [Toro and Galanti, 1990] is due to a high number of positively charged groups. Indeed, the percentage of lysine residues in *T. cruzi* histone H_1 (Table I) is significantly higher than that observed in histone H_1 from calf thymus [Panyim et al., 1971] (see Table I). Moreover, *T. cruzi* histone H_1 presents a modest amount of histidine which is not present in calf thymus histone H_1, and the basic/acid ratio is higher in *T. cruzi* histone H_1 than in calf thymus.

The mobility of *T. cruzi* histone H_1 in SDS-PAGE at the level of calf thymus and of *T. cruzi* core histones should be taken cautiously. In the first place, the molecular weight estimation of histones in SDS gels is affected by other physicochemical characteristics of these molecules [Hamana and Iwai, 1974]. However, it is difficult to understand that the great differences we have observed in the mobilities of histones H_1 of calf thymus and *T. cruzi* may be solely explained on the bases of other characteristics different from their molecular weight. Consequently, the result shown in Figure 3 may indicate that histone H_1 of *T. cruzi* presents a lower molecular weight than histone H_1 from calf thymus. Indeed, Rizzo [1985] found that in Peridinium a doublet of histones supposed to be H_1 presented a higher migration than calf thymus histone H_1 in SDS gels, suggesting a lower molecular weight. A histone H_1 with a low molecular weight (119 residues) was also found in *Platynereis dumerilii* [Kmiecik et al., 1985]. Another interesting feature of *T. cruzi* histone H_1 is its percentage of alanine (26.5) very similar to calf thymus (23.4) and *Oxytricha* (29.2) and almost twice the percentage of this amino acid found in Tetrahymena (14% for the WH-6 strain, Table I, and 12.7% for the GL strain [Gorovsky et al., 1974]. Similarly, the total percentage of hydrophobic amino acids is also higher in *T. cruzi,* calf thymus, and *Oxytricha* sp. than in Tetrahymena (Table I). This is an intriguing observation considering that a globular region was not found in Tetrahymena [Wu et al., 1986].

Sequence analysis of histone H_1 from *T. cruzi* transferred to PVDF membranes showed that the amino terminal end of this protein was blocked. This result was not surprising considering that histones H_1 from different sources present modified N-terminal residues, N-acetylserine being the most common first amino acid [Vanfleteren et al., 1988].

When fragments derived from this protein by proteolysis were sequenced, a high degree of homology was found with the carboxy terminal domain of histones H_1 from different origins, showing an unambiguous identity with this histone. Several motifs greatly enriched in lysine, alanine, and proline were found. This result is in accordance with data showing that, depending upon the source of the histone H_1, the carboxy terminal domain comprises approximately 100 residues which are dominated by lysine (40 mol%), alanine (30 mol%), and proline (12 mol%) [Vanfleteren et al., 1988; Maeder and Bohm, 1991].

Taking into account our previous and present evidence, we conclude that a histone H_1 is present in *T. cruzi* chromatin showing the following characteristics: a) it is extracted from chromatin in 0.75 M PCA, 5% TCA, or 0.5 M NaCl [Toro and Galanti, 1988]; b) it presents cross-immunoreactivity with an antiserum against histone H_1 and possesses metachromasie proper of histones H_1 [Toro and Galanti, 1988]; c) its amino acid composition is similar to most histones H_1; d) its amino terminal residue is blocked; e) its carboxy terminal domain presents the motifs of amino acids expected for histones H_1; f) its migration in SDS gel suggests a molecular weight in the range of the core histones from calf thymus; g) its migration in acid-urea gels is far ahead from the core histones [Toro and Galanti, 1990]; and h) it is composed of three or four variants [Toro and Galanti, 1990; Toro, 1991].

The participation of histone H_1 in the mechanisms leading to chromatin condensation [Thoma et al., 1979] and in the down-regulation of transcription by RNA polymerase II [Laybourn and Kadonaga, 1991, 1992] in higher eukaryotes has been established.

Moreover, it has been found that axonemal microtubules of sea urchin sperm flagella are stabilized by histone H_1 [Multigner et al., 1992]. This observation is of importance considering that the participation of histones in the organization of nucleosomes has been related to the appearance of mitosis and intracellular cytoskeleton-based motility during evolution [Multigner et al., 1992]. Taking into account these facts and proposals, it has been suggested that microtubules and DNA probably use histone H_1 in their structural and functional regulation [Multigner et al., 1992].

The presence of histone H_1 in *T. cruzi,* which does not condense its chromatin into chromosomes during cell division, suggests that the role of this protein in the compactation mechanism of chromatin may have been overestimated and should be reviewed. The peculiar characteristics of *T. cruzi* chromatin may be related with other features as divergent core histones [Toro et al., 1992] and a singular histone H_1.

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