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Molecular characterization and transcription of the histone H2B gene from the protozoan parasite *Trypanosoma cruzi*

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Summary

The structure, genomic organization and transcrlptlon of the gene encoding hlstone H2B In the protozoan parasite Trypansosoma cruzl have been studied. This gene consists of a 746~nucleotide unit, tandemly repeated at least 18 times in each of two clusters. DNA probes corresponding to hlstones H2B and H3 hybridized to different chromosomes revealing that the genes coding for these two hlstones are not physl-cally linked in the genome of r. cruzl. The primary transcription product of the H2B gene Is processed by trans-splicing and polyadenylatlon. Inhibltlon of DNA synthesis with aphidicolin resulted in the reduc-tlon of histone H2B mRNA to undetectable levels in about two hours, suggesting that Its abundance is regulated throughout the cell cycle as it occurs in other eukaryotes. in addition, a concomitant inhibition of translation by cyclohexfmide reverted this effect indicating that de novo protein synthesis is required for **RNA** instability. **Hlstone mRNA abundance was dependent on the life-cycle stage of r. cruzl: abun-dant in amastigotes and epimastlgotes, the dividing forms in the host cell and the Insect vector, respec-tively, while undetected in trypomastigotes, the para-site's nondlvidlng life stage.**

Introduction

Trypanosoma cruzi is a haemoflagellated protozoan belonging to the order Kinetoplastidae, and is the causa-tive agent of Chagas' disease, which afflicts millions of people in the American continent (World Health Organiza-tion 1989). Its life cycle Includes several developmental stages (De Souza, 1984). Amastigotes and epimastigotes are replicative forms that are found in the cytoplasm of mammalian cells and in the midgut of invertebrate hosts, respectively. Trypomastigotes are nondividing forms responsible for the infectivity 01 the parasite towards a wide range of mammalian cells. Like all members of the family Trypanosomatidae, 7. cruzi exhibits a number of molecular and biochemical properties not, or rarely, found in other eukaryotes. Polycistronic transcription (Gonzalez ef *al.,* 1985; Muhich el al., 1988) trans-splicing (Murphy ef al., 1986) or mitochondrial RNA editing (Simpson el al., 1987) are phenomena that, together with organelles such as glycosomes and a unique kinetoplast-containing mitochondrion, reflect the ancient evolutionary divergence of these organisms.

Histones are basic proteins that are essential com-ponents of the structural framework that packages DNA in the nucleus of eukaryotic cells (Lewin, 1980). Four types of histones, H2A, H2B, H3 and H4, form an octamer core around which DNA is coiled to establish the funda-mental unit of chromatin, the nucleosome (Simpson, 1978). A fifth histone protein, Hl, binds to the linker region between nucleosomes. Histones can undergo post-translational, covalent modifications (Ajiro et al., 1985; Busch and Goldknopf, 1981; De Lange et al., 1973; Doenecke and Gallwitz, 1982; Levy-Wilsson, 1983). The resulting heterogeneity of histones is considered to be important for differences in chromatin structure which in turn may influence gene expression. In trypanosomatids, mitosis is characterized by the lack of condensed chromo-somes (Solari et a/., 1985). Few studies have been carried out on the chromatin structure and composition in these organisms, most of them in 7. cruzi (Solari et al., 1985; Rubio et a/., 1980). The nucleosomes have been described: they consist of a DNA repeating unit of about 200 bp and a set of core histones, which resemble those found in higher eukatyotes. Four proteins seem to be represented in the nucleosomal core histones of 7. cruzi (Astolfi el al., 1980; Toro and Galanti. 1990), *Trypano-soma brucei* (Bender, 1992), *Trypanosoma lewisi* (Elpidina et al., 1979) and *Crithidia fasciculata* (Duschak and Cazzulo, 1990). Their overall amino acid composition is similar to that of the histones from lower eukaryotes. The postulated absence of histone Hl in Trypanosomati-dae (Rubio et a/., 1980) is in contradiction to the recent

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description of proteins that are considered to be a subspecies of histone H1 in T. cruzi (Toro and Galanti, 1990) and in C. *fasciculata* (Duschak and Cazzulo, 1990).

The structures of genes and mRNAs encoding histones have been analysed in many organisms (Maxson *et* al., 1983). Histone genes are often clustered in genomic DNA but do not exhibit any single conserved topology. Their transcripts are not polyadenylated in higher eukaryotes but contain a conserved stem-loop structure at their 3' end (Wells and Kedes, 1986). On the other hand, mRNAs for 'basal' histones (a less abundant class of histones in eukaryotes) (Mannironi *et al.*, 1989) and for the histones from lower eukaryotes (fungi, ciliates and yeast) are polyadenylated and do not contain stem-loop structures (Osley, 1991). Typically, histone protein synthesis and mRNA abundance are coupled to the cell cycle: histone biosynthesis is restricted to the S phase, occurring simultaneously with DNA synthesis and chromosome replication (Marashi et *al.*, 1982). In general, three pathways regulate the production of histone mRNA in the cell cycle of higher eukaryotes (Baumbach et a/., 1987; Harris et *al.,* 1991; Heintz *et al.*. 1983; Sittman et *a/.,* 1983). The first one regulates transcription at the Gl-S phase boundary: as cells enter S phase, nascent histone mRNA synthesis increases three- to fivefold. The two remaining pathways act post-transcriptionally. One acts in the cytoplasm to control the half-life of histone mRNAs: in the absence of DNA synthesis, histone mRNAs are rapidly degraded. The second pathway acts in the nucleus to process histone pre-mRNAs (Stauber and Schumperli, 1988). In higher eukaryotes, the stem-loop at the 3'-untranslated end is required for both processing and degradation of histone RNAs (Bimstiel *et a/.,* 1985; Pandey and Marzluff, 1987). The minor polyadenylated 'basal' histone mRNAs are not regulated during the cell cycle (Wu and Bonner, 1981) and are not sensitive to the inhibition of DNA synthesis (Sittman et *a/.,* 1983). In contrast, polyadenylated histone mRNAs in yeast cells are cell-cycle regulated and destabilized in the absence of DNA synthesis (Lycan et *al.,* 1987; Xu et *a/.,* 1990).

To the best of our knowledge, three histone genes have been characterized to date from kinetoplastid protozoa: the genes coding for histone H2B from *Leishmania enriettii* (Genske et *a/.,* 1991) and for histone H2A from *Leishmania donovani infantum* (Soto *et al.,* 1992) and *T. cruzi* (Puefta *et* al., 1994). Their transcription products are polyadenylatec!. However, it is reported (Genske et *a/.,* 1991) that inhibition of DNA synthesis in *L. enriettii* does not decrease the relative level of histone H2B mRNA, suggesting that its abundance is not coupled to the *Leishmania* cell cycle.

We report here the cloning, molecular characterization and genomic organization of the gene coding for histone H2B in *T. cruzi* and show that its transcription product is polyadenylated. Inhibition of DNA synthesis results in decreasing histone H2B mRNA abundance to a very low level, suggesting that histone gene expression is coupled to the cell cycle in Trypanosoma. In accordance with this, while the H2B mRNA is readily detected in the life forms able to divide (epimastigotes in the insect vector and amastigotes in the mammal host), it can be barely detected in the non-dividing trypomastigotes.

Results

Cloning of a T. cruzi *histone H2B cDNA fragment*

Taking advantage of the fact that all mRNAs from *T.* cruzi start with the same 39 nucleotides (the spliced leader, added by *trans*-splicing to the primary transcription product of any gene) (De Lange et *al.,* 1984), we cloned the 5' ends of transcripts of interest by synthesizing cDNA using reverse transcriptase and primers specific for particular sequences, and then amplifying the cDNA by the polymerase chain reaction (PCR), using the same primer and a second one corresponding to the spliced leader sequence. Owing to the low-stringency conditions in the priming of the reverse transcriptase reaction and because in many instances we utilized degenerate first primers, it was not unusual to end up with more than one amplified product (as seen by agarose gel electrophoresis). By characterizing these bands we have obtained useful information on the 5' ends of numerous *T. cruzi* transcripts (J. A. Garcia-Salcedo et *a/.,* in preparation). In an experiment using the oligonucleotide described in the *Experimental procedures,* a 200 bp band was amplified, isolated, cloned in an *Eco*RV-linearized pBSKS- (plasmid p41 Ec) and sequenced. The amino-acid-derived sequence from this cDNA, starting at the first ATG downstream of the spliced leader, showed strong homology to H2B histones in protein databases. As shown below, this cDNA fragment does correspond to *T. cruzi* histone H2B (nucleotides 78-258 plus 30 nucleotides of the spliced leader).

Genomic organization of the T. cruzi *histone H2B gene*

The genomic organization of *T. cruzi* histone H2B gene was studied by Southern blotting and chromosomal blotting of genomic DNA using the cDNA probe. Figure 1A shows the bands obtained in a blot of DNA digested with a number of restriction enzymes. This pattern concurs with that of a tandemly repeated gene unit of about 750 bp. Almost all copies of the gene contain one site for *Xba*I and SacI, most have two PstI sites and there is an NcoI site that is polymorphic among the repeating copies and/or alleles. Finally, the enzymes *Kpn*I and *Hin*dIII do

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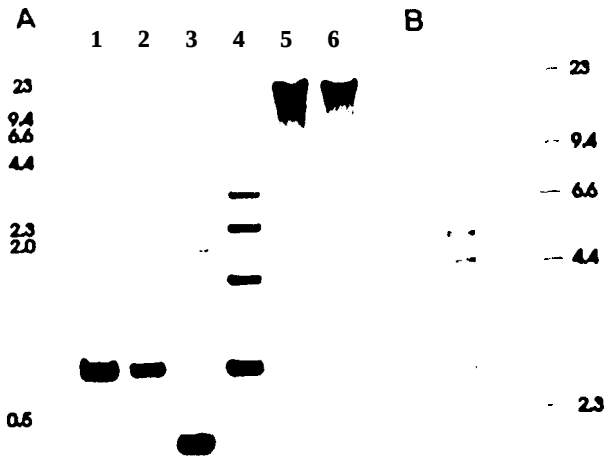

Fig. 1. Southern blot analysis of the histone H2B gene organization. Total DNA trom *T. cruzi* epimastigotes (1 μg) was digested with a number of restriction enzymes, electrophoresed. blotted and hybridized to digoxigenin-labelled histone H2B probe (insert from plasmid p41 Ec without spliced leader sequence).
A. Lanes l-6 correspond to digestions with *Xba*I, *Sac*I, *Pst*I, *Nco*I, *Kpn*I and *Hin*dIII, respectively.
B. Partial digestions with *Xba*I. Molecular size markers (shown at the sides in kb) are *Hin*dIII digests of lambda DNA.

not cut inside the cluster. To confirm this explanation and to estimate the number of repeating units in the histone H2B cluster, the experiment shown in Fig. 1 B was carried out. Partial digestions with *Xba*I produce bands differing in size by one copy. Since 17 bands can be unambiguously determined from the picture, 18 is probably the minimum number of repeats in the cluster.

T. cruzi chromosomes were separated by pulsed-field gradient electrophoresis, blotted and hybridized to the H2B probe (Fig. 2, lane 1) and to a probe corresponding to maize histone H3 (lane 2). While the H3 probe hybridizes to a chromosome of about 1.7 Mb, the H2B probe complements sequences in two very large chromosomes of different size. Although the most straightforward inference is that there are two unrelated genomic loci each containing a similar number (as judged by the intensity of the bands) of tandemly repeated copies of the histone H2B gene, it should be borne in mind that T. cruzi is a diploid organism that often exhibits size heterogeneity between allelic chromosomes (Gibson, 1986; Henriksson et *al.*, 1990). On the other hand, the histone H3 locus is not linked to the H2B loci or to the H2A locus that occurs in a 1 Mb chromosome (Puerta et *al.*, 1994) revealing that the genes coding for different histones are not grouped in the genome of *T. cruzi.*

Nucleotide sequence and transcription of the H2B gene

The H2B cDNA probe was used to screen a genomic library of T. *cruzi* DNA in lambda EMBL3 phage (González *et al.*, 1990). A positive clone was isolated that contained several copies of the H2B gene plus *14* kb of 5' upstream sequence (data not shown). Copy units were isolated by electrophoresis after *Xba*I digestion of phage DNA, purified, subcloned and sequenced. Also, by sequencing upstream of the first copy of the cluster in the genomic clone, we could determine the commencement of the repeat. Through direct comparison to the cDNA sequence, it was established that *trans*-splicing takes place at position 77 of the reported sequence, behind an acceptor AG; a pyrimidine track, postulated to determine the splice site (Layden and Eisen, 1988), appears at position 30 to 53. In the 5'-untranslated end, the sequence AACCA is repeated three times. Downstream, the first ATG (position 120) is followed by an open reading frame encompassing 112 codons (termination codon TAG at position 456) presenting the usual GC bias of *T. cruzi.* The amino-acidderived sequence shows the characteristics of eukaryotic histone H2B (see the next section).

Epimastigote RNA was fractionated on an oligo d(T)-cellulose column, subjected to Northern blotting and hybridized to the cDNA probe. A 670-nucleotide band appears only in the lane loaded with the polyadenylated fraction (Fig. 3A); thus, the H2B mRNA is polyadenylated, as are histone mRNAs of *Leishmania* (Genske, 1991; Soto *et al.*, 1991), *T. cruzi* H3 mRNA (our unpublished observation) and H2A mRNA (Puerta et al., 1994). Cytoplasmic RNA from different *T. cruzi* life stages was size-separated on formaldehyde-agarose gels, blotted and hybridized to the cDNA probe (Fig. 3B). A band of the expected size appeared in the lanes loaded with RNA from epimastigotes and amastigotes (i.e. the forms that are able to multiply), but not with trypomastigote

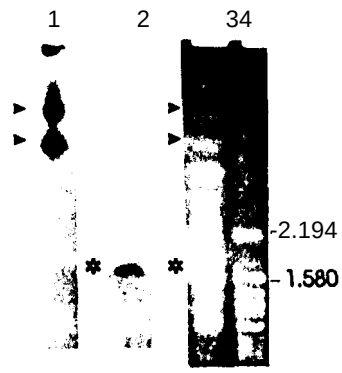

Fig. 2. Pulsed-field gel electrophoresis of *T. cruzi* chromosomes. After blotting, the filter was hybridized to the histone H2B probe (arrowheads, lane 1) or to a maize histone H3 probe (asterisk, lane 2). Hybridizing chromosomes are marked in an ethidium-bromide-stained lane (lane 3). Also stained, lane 4 displays the chromosomes of S. *cerevisiae*, strain S13, used as markers (the sizes of the two largest chromosomes are shown in kb). For me maize H3 probe the hybridization temperature was reduced to 40 C.

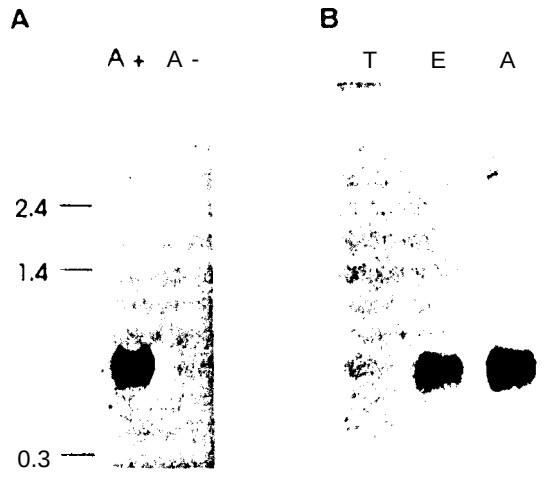

Fig. 3. Northern blot of *T. cruzi* cytoplasmic RNA.
A. Probing of poly-A⁺ (2 μg) and poly-A (10 μg) with an H2B probe.
B. Probing of poly-A⁺ RNA (2 μg) from trypomastigotes (T), epimastigotes (E) and amastigotes (A).

RNA, indicating that histone H2B RNA accumulates only in the life forms that are able to divide.

S1nuclease protection experiments (data not shown) were carried out to map the polyadenylation site of the H2B mRNA. The site could accurately be assigned to position 665 or 666 of the sequence; this was confirmed by isolating a full-size clone from a cDNA library, constructed using amastigote mRNA (Gonzdlez *et al.*, 1990), and sequencing through the poly-A tail. The H2B mRNA consists of the spliced leader, 42 nucleotides of 5′-untranslated region, 339 nucleotides of coding sequence, 206 or 207 nucleotides of 3′-untranslated end and a poly-A tail of some 50 residues. Comparison of the cDNA and the genomic sequences results in the following discrepancies: G for C (position 118) in good agreement with the polymorphic nature of the *NcoI* site (Fig. 1A), A for G (position 126) changes threonine to alanine in the amino-acid-denved sequence, G for T (position 243) changes alanine to serine. It is likely that the cDNA corresponds to a gene copy different from the genomic one sequenced.

T. cruzi *histone H2B*

The H2B gene codes for a 112-amino-acid protein (Fig. *4)* with a deduced molecular weight of 12 000 and the usual features of histones H2B: very basic (isolectric point 11.9) and slightly more lysine rich (11.6%) than arginine rich (9%). A putative nuclear localization signal (Moreland et *al.*, 1987) located between amino acids 15 and 23 is placed just before the transition from hydrophilic to hydrophobic in the hydropathic plot of the protein. The alignment of the *T. cruzi* H2B sequence to other histones H2B from a number of species reveals a 67% identity with L. *enriettii*, a *47%* identity with Saccharomyces cerevisiae, a 46% identity with *Caenorhabditis elegans.* a *46%* identity with sea urchin and a 45% identity with chicken H2B. As previously observed, the amino terminus is the least conserved part among H2B molecules.

Effect of DNA and protein synthesis inhibitors on H2B mRNA abundance

Except for the basal histones, the steady-state level of histone mRNAs in higher and lower eukaryotes is regulated by the cell cycle: histone mRNA concentrations rise by an order of magnitude as the cell enters the S phase, the result being an increase of both histone gene transcription

```
T.c:                                                    MATPKSSSANRKKGGKKSHRKPKRTWNVYINRSLKSINNH
L.e:                                                     MASQRLAPRKSSAAH.....S....VS....A..S.
T.t:                                                APKKAPA.AAEKKVKKAPTTE..NKK.RSE.FAI..FKV..QVHPD
S.c:                                             SSAAEKKPASKAPAEKK.AAKKTSTSVD...RSKVR.E.YSS..YKV..QTHPD
T.a:    PBKKPAAENKVEKAAEKTPAGKKPKAEKRLPAGKTASKEAGGEGKT.GRKKGSKAK.GVE.YKI..FKV..QVHPD
G.g:                                           PEPAKSAPAPKKGSK.AVTKTQK..D..RKKSR.ESYSI.VYKV..QVHPD
C.e:                                             APPKPSAKG.KKAKKTVTKP.D...RKKAR.ESYS...Y.V..QVHPD
D.M:                                             PPKTSGKAAKKAGKAQK.IT.TD..KK..R.ESYAI..YKV..QVHPD
S.p:                                           APTAQVAKKGSKKAVKGTKTAX....RN..R.ESYGI..YKV..QVHPD

MSMSGRTMKIVNSFVNDLFERIACEAATVVRVNKKRTLGARELQTAVRLVLPADLAKHAMAEGTKAVSHASS
.............Y...VM....M...SI..AH..L......V.........PE.............NSCR
VGI.KKA.N.M...I..S.....L.SSKL..F..R...SS..V....K.L..GE..R..IS......TKF..STN
TGI.QKS.S.L.....I.....T..SKLAAY...S.IS...I.....I..GE.....VS...R..TKY..STQA
IGI.SKA.S.M...I..I..KL.G.S.KLA.Y...P.ITS..I..S.....GE.....VS......TKFT.S
TGI.SKA.G.M.....I.....G..SRLAHY..RS.ITS..I.....L..GE.....VS......TKYT.SK
TGV.SKA.S.M.....V.....A..SRLAHY..RS.ISS..I.....I..GE.....VS......TKYT.SK
TGI.SKA.S.M.....I.....A..SRLAHY..RS.ITS..I.....L..GE.....VS......TKYT.SK
TGI.SPA.V.M.....I.....G..SRLAQY...S.ISS..I.....I..GE.....VS.....TKYTTSK
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Fig. 4. Amino acid sequence of histone H2B of *T. cruzi* (T.c.) and homology lo histones H2B from *L. enrietti* (L.e.); *Tetrahymena thermophila* (T.t.); *S. cerevisiae* (S.c.); *Triticum aesnvum* (T.a.); *Gallus gallus* (G.g.); C. *elegans* (C.e.); *Drosophila melanogaster* (D.m.) and *Strongylocentrotus purpuratus* (S.p.). Dots indicate identical amino acids. The EMBL database accession number for the *T. cruzi* histone H2B gene is X60962.

Table 1. Time course 01 effect of aphidicolin on H2B mRNA levels, DNA synthesis and protein synthesis.

Time	Percentage H2B mRNA	Percentage [^{3}H]-thymidine incorporation	[^{35}S]-methionine (c.p.m.)
0	100	100	40 684.4
15 min	73 5	—	—
30 min	65.6	43.2	—
45 min	53.1	—	—
60 min	—	34.0	—
75 min	90	15.1	—
2h	13.0	10.0	65 563.4
4h	13.5	—	—
5h		0.37	—
6h	13 3		
8 h	130	—	—
10 h	—	9.6	—
12h	26.0	—	—
14h	32.0	—	57 475.6
15h	—	7.8	—
18 h	47 0	—	—
20h	53.0	4.7	—
24h	169.0	—	—
26h	186.0	9.4	—
28h	205 7	—	—

DNA synthesis is expressed as per cent incorporation of [^{3}H]-thymidine.
Protein synthesis is expressed as total [^{35}S]-methionine incorporation into TCA-precipitable material.

and histone mRNA stability (Osley, 1991). Only in one case. the trypanosomatid *L. enriettii*, has it been reported that the RNA abundance for a non-vanant histone is not cell-cycle regulated (Genske et al., 1991). We have extended this study to *T. cruzi* HB2 mRNA by, in the absence of a protocol for synchronizing cells. using aphidicolin, an inhibitor of DNA polymerase. Incubation of *T. cruzi* epimastigotes in the presence of 5 μg ml^{-1} aphidicolin results in a 90% reduction in DNA synthesis by 2 h as measured by [^{3}H]-thymidine incorporation into acid-precipitable material, a level of inhibition maintained up to 26 h later (Table 1). Aliquots of the cell culture were obtained at different times and the relative amount of H2B RNA was measured by Northern blotting of total cytoplasmic RNA and hybridization to a H2B probe. As a control, the blots were also hybridized to a probe corresponding to 1f8, a tandemly repeated gene coding for a putative calcium-binding protein (Gonzalez *et* al., 1985). As shown in Fig. 5A, the H2B mRNA abundance declines steadily for the first 75 min and remains at basal level for about 4 h but then, surprisingly, increases continuously with a doubling time of about 3 h for at least 28 h (Fig. 5B, Table 1). The 1f8 band remained essentially unmodified throughout the time course of the experiment. These results suggest that the H2B mRNA level is

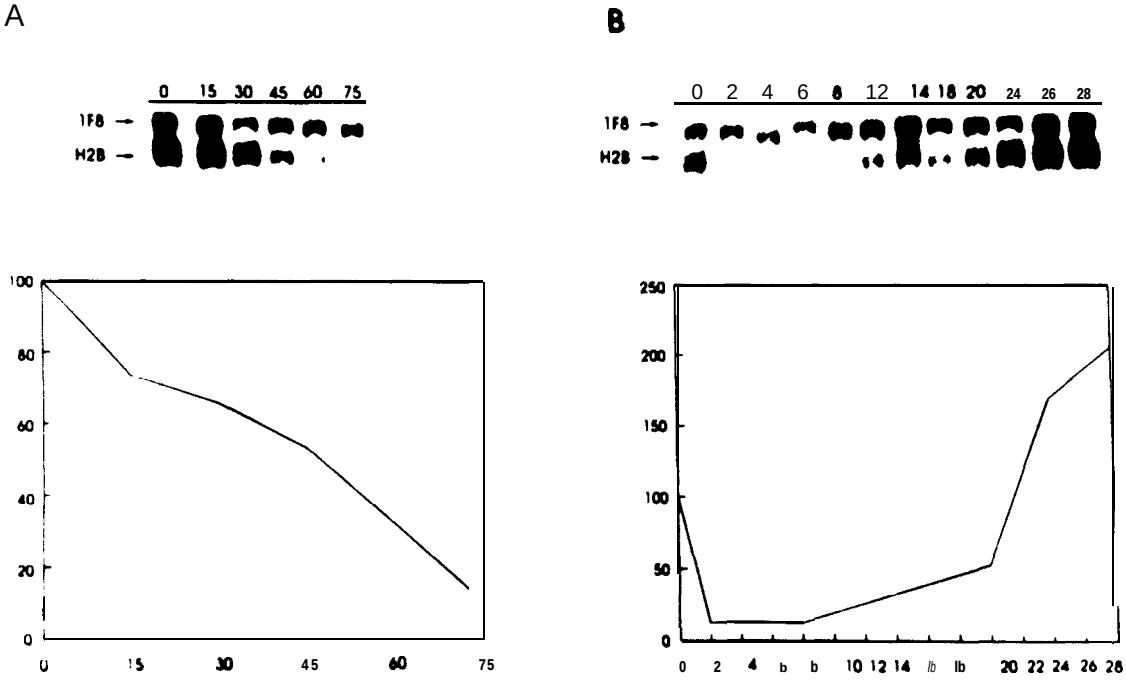


Fig. 5. Effect of aphidicolin on the relative abundance of H2B mRNA.
A A Northern blot of 15 μg total cytoplasmic RNA from epimastigotes cultured in medium supplemented with 5 μg ml^{-1} aphidrcolin. Culture aliquots were taken at 0. 15. 30. 45. 60 and 75 mln of addition of aphidicolin and hybndized with an H2B probe and a 1f8 probe as control, both of which were radioactively labelled. Below is a graphical representation of the relative abundance of transcripts; the ordinate axis is the percentage optical density of H2B mRNA with respect to the 1f8 control (t = 0 is 100%) at me indicated intervals.
B. The same expenment overa28 h time-course. Culture aliquots were taken at 0, 2. 4. 6. 8. 12. 14. 18, 20, 24, 26 and 28 h (note the recovery of mRNA levels in spite of the ongoing inhibition of DNA synthesis).

controlled by the cell cycle but in a way that is somehow different from that of other eukaryotic histone mRNAs (see the *Discussion*) as the regulation of histone H2B mRNA abundance seemed to be uncoupled from DNA replication, which was suggested by the fact that mRNA levels kept Increasing even though DNA synthesis was almost completely blocked (Fig. 5 and Table 1). It has been reported that the decline in H2B mRNA after the addition of aphidicolin is dependent on *de novo* protein synthesis (Stahl and Gallwitz, 1977). We find that cycloheximide, an inhibitor of protein synthesis by eukaryotic organisms, impedes the decline in histone H2B mRNA levels (Fig. 6). However, the observed accumulation of H2B mRNA at later times cannot be attributed to the absence of protein synthesis because [^{35}S]-methionine incorporation 14 h after the beginning of the expenment is 100% that of the control (Table 1). Furthermore, the inhibition of protein synthesis alone is enough to cause a marked increase in the relative levels of H2B mRNA when compared to β-tubulin mRNA (Fig. 6, lane B_{45}). This 'super-induction' of H2B mRNA is completely independent of the extent of DNA replication, as the addition of aphidicolin does not reduce the abundance of the H2B messenger (Fig. 6, lane B_{165}) when compared to the control. Table 2 shows the quantification of the experiment depicted in Fig. 6.

Table 2. Effect of aphidicolin and cycloheximide on protein synthesis, DNA synthesis and H2B mRNA abundance.

Treatment	Protein synthesis (%)	DNA synthesis (%)	H2B mRNA (%)
Control	100	100	100
Cycloheximide	24.6	51.0	200
Aphidicolin	07.7	12.6	13.0
Cycloheximide + aphidicolin	27.9	15.6	212.6

DNA synthesis is expressed as per cent of control cultures. H2B mRNA abundance is shown relative to β-tubulin abundance. Aliquots of cultures were taken at the times indicated by the subscripts in Fig. 6 (minutes) and treated as described in the *Experimental procedures.*

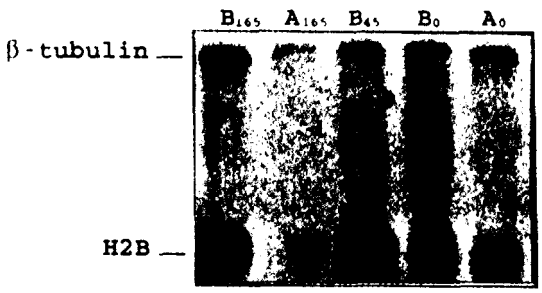
Discussion

Despite an early report to the contrary (Beck and Walker, 1964), the presence of histones associated with nuclear DNA has been firmly established in kinetoplastid protozoa such as C. *oncopelti* (Leaver and Ramponi. 1971) and *T.* cruzi (Astolfi *et* al., 1980). Physicochemical characterization of histone-like proteins suggested that trypanosomatid organisms contain a set of histone proteins similar to those found in other eukaryotes. The genes coding for histone H2B of *L. enriettii* (Genske et a/., 1991) and H2A of *L. donovani infantum* (Soto et a/., 1992) and T. cruzi (Puerta et *al.,* 1994) have been cloned and characterized.

We have extended the study of histone genes in Trypanosomatidae by cloning the H2B gene from *T. cruzi.* This gene codes for a 112-amino-acid protein whose homology to H2B histones decreases from *Leishmania* to vertebrates in agreement with the evolutionary divergence obtained using other methods (Michels and Opperdoes, 1991; Sogin et *al.,* 1986). In contrast to the *Leishmania* protein, it is as lysine rich as the H2B histones from other eukaryotes and contains a well-conserved nuclear localization signal (Moreland *et al.,* 1987) indicating that these signals are conserved in phylogenetically distant organisms.

The maximum parsimonious tree obtained (Fig. 7) revealed an extremely divergent nature for the H2B sequence from *T. cruzi.* Bootstrap confidence values were obtained not only in the comparison with other Protozoa (*T. thermophila*) but also with members of the same family (*L. enrietti*). An extreme divergence has also been observed for the histone H4 sequence from *T.* cruzi (Toro et *al.,* 1992). Both observations contrast with the well-known conservatism of histones throughout evolution and agree with the hypothesis of a clonal origin for these protozoa (Tibayrenc et al., 1986).

As much as 51% of *T. cruzi* DNA consists of infrequently repeated sequences (Lanar et *al.,* 1981); this fraction is believed to be made up of tandemly repeated genes. The H2B gene is repeated at least 18 times in a cluster that appears on two very large chromosomes of different mobility. As to whether these chromosomes form an allelic pair of different size or are different chromosomes will be determined once additional genetic markers become available. Allelic chromosomes of different size have been shown to exist in the genome of T. *cruzi* (Gibson and Miles, 1986; Henriksson *et al.,* 1990) but, if this is the case for the H2B-hybridizing chromosomes, their



Flg. 6. Effect of cycloheximide on the abundance of histone H2B mRNA.
A. A culture treated with 5 μg ml $^{-1}$ aphidicolin at t = 45 min.
B. A culture treated with 20 μg ml $^{-1}$ cycloheximide at t = 0, and 5 μg ml $^{-1}$ aphidicolin 45 min later. Cytoplasmic RNA extractions were performed at times indicated (in minutes) by the subscnpts. Each lane represents 15 μg cytoplasmic RNA. As a control, the membrane was hybridized to a probe specific for β-tubulin.

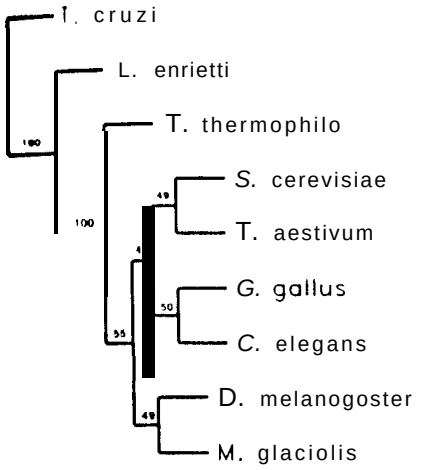


Fig. 7. **Unrooted phylogenetic** tree based on sequences of the **H2B histone, obtained** by maximum **parsimony**. Numbers **at** the nodes of **branches** represent bootstrap confidence values **obtained** from 100 **replications**.

difference in size would be surprisingly large. On the other **hand,** both an H2A probe and a maize H3 probe hybridize **to** a different *T.* cruzi chromosome, indicating that no linkage Is found among different **histone** genes in *T. cruzi*, as occurs in other eukaryotes.

Tandemly repeated genes are transcribed **polycistronically** in trypanosomatids (Gonzalez *et al.*, 1985; **Muhich** and Boothroyd, 1988; Murphy et a/., 1986). **Pre-mRNAs** are processed by ***trans*-splicing** and polyadenylation to produce translatable **RNAs** and intergenic sequences that are degraded. It is not surprising, then, that the **H2B mRNA** is polyadenylated, as polyadenylation is probably necessary for the processing of **pre-mRNA** transcribed from tandemly repeated genes. We have been able to determine the beginning of the **H2B** repetitive cluster and have found that ***trans*-splicing** of the **H2B pre-mRNA** takes place at the first AG in the repeated sequence. The significance of this, if any, is unknown at the moment, because, to the best of our knowledge, the beginning of any other cluster, which could be used for comparative purposes, is still to be characterized.

Inhibition of *T. cruzi* **DNA** synthesis by aphidicolin results in the reduction of **H2B mRNA** abundance to a basal level, suggesting that **histone** gene expression is coupled to the cell cycle, as is the case in yeast and higher eukaryotes. Accordingly, no **H2B mRNA** can be detected in the **trypo**mastigote stage of the parasite, which is unable to multiply. However, a progressive recovery in **H2B mRNA** abundance can be observed with time that cannot be imputed to the concomitant decrease in protein synthesis needed for the **mRNA** level decrease, nor to a reinitiation of DNA synthesis. Genske et ***al.* (1991),** after studying the **histone H2B** expression in L. ***enriettii*,** concluded that it is unrelated to the cell cycle because the addition of aphidicolin did not result in a reduction in **H2B mRNA** abundance. Although it cannot be ruled out that **leishmanial H2B** expressron Is regulated differently from its trypanosomal counterpart, our results might explain the apparent disagreement. The first time point reported in that study is taken 8 h after the addition of aphidicolin. By comparison to the curve in Fig. 5B, and bearing in mind the somehow faster metabolism and cell cycle of *Leishmania, 8* h may be sufficient for recovery of **H2B mRNA** levels.

The regulation of **mRNA** abundance involves transcriptional as well as post-transcnptional mechanisms, the latter acting both at the level of processing and at the level of messenger stability. **Peltz** and Ross (1987) have shown that the presence of free **histones** accelerates the degradation of their **mRNAs** in a cell-free system. They have argued that the accumulation of free **histones** in a cell in which DNA synthesis has been inhibited would be (at least partially) responsible for the concomitant decrease in their **respective mRNAs.** This would take place by a hypothetical interaction between the **histone** and its messenger which would render the **mRNA** more susceptible to nuclease attack. Interestingly, the 3' end of yeast **H2B mRNA** has been reported to confer cell-cycle regulation of its abundance by specific destabilization of reporter gene constructs (Xu *et al.*, 1990).

Formally, the hypothesis that transcriptional control of **H2B** expression is at work cannot be ruled out. Nevertheless, we feel inclined to speculate that **H2B mRNA** levels are regulated post-transcriptionally, in an **autogenous** mechanism in which **histone H2B** usage is a key regulatory element. The reasons for this belief are: first, if inhibition of DNA replication is taken as the sole stimulus necessary to stop transcription of the **H2B** genes, it is hard to explain why it is later reinitiated, whilst DNA replication (the original down-regulatory stimulus) remains blocked; second, a replication-coupled transcriptional control of **H2B mRNA** levels would not explain the superinduction of **H2B mRNA** observed when *de novo* protein synthesis is inhibited, as DNA replication inhibition cannot overcome the up-regulatory effect of protein synthesis inhibition (Fig. 6 and Table 2). The hypothesis that **histone H2B** is at least one of the regulatory agents of **H2B mRNA** abundance is suggested by the work of **Peltz** and Ross **(1987),** and also by the fact that this would explain the drop and subsequent increase in **H2B** levels — a decrease after **histone** requirements are diminished by DNA synthesis inhibitlon and a recovery after depletion of the cytoplasmic pool of the protein, a depletion which also must occur (and more rapidly) when protein synthesis is blocked with ongoing DNA replication, thus explaining the rapid 'superinduction' observed.

Both **S1** nuclease protection studies (data not shown) and computer predictions have revealed a very complex

secondary structure for the 3' end of the H2B mRNA, which could provide a framework for interactions of the type proposed. The relevance of this finding is currently under study in our laboratory.

Experimental procedures

Growth of parasites and isolation of nucleic acids

The Y strain of *T. cruzi* (Silva and Nussensweig, 1953) was used throughout this work. Epimastigotes were grown in liver infusion tryptose (LIT) medium supplemented with 10% heat-inactivated fetal calf serum at 26 C with gentle shaking (Castellani et al., 1967). Trypomastigotes were collected from the supernatant of infected monolayers of LLC-MK_2 epithelial cells (Andrews and Colli, 1982). Extracellular amastigotes were obtained by incubating recently released trypomastigotes in LIT medium at 37 C under 5% CO_2 for 24 h (Andrews *et al.*, 1987).

Cells were lysed with 1% NP40 in the presence of 10 mM vanadyl complexes (Sambrook *et al.*, 1989). After centrifugation, DNA was purified from the nuclear pellet using the SDS-Protelnase K method (Gross-Bellard *et al.*, 1973); cytoplasmic RNA was obtained from the supematant as reported (Gonzalez *et al.*, 1990). The polyadenylated fraction of the RNA was obtained by chromatography on an oligo(dT)-cellulose column (Aviv and Leder, 1972).

Southern, Northern and chromosomal blot analysis

T. cruzi DNA was digested with restriction enzymes and electrophoresed in agarose gels using TAE buffer containing ethidium bromide, depurinated in 0.25 M HCl for 15min and transferred to nylon membranes in 0.5 M NaOH (Sambrook *et al., 1989).* For molecular karyotype analysis (Schwartz and Cantor, 1984), approximately 8 x 10' epimastigotes per plug of low-melting-point agarose were treated with SDS and Proteinase K (Van der Ploeg et *al.*, 1984). Pulsed-field gel electrophoresis was performed in an LKB 2015 Pulsphor System apparatus using 1% agarose gels and TBE buffer maintained at 12°C. The running conditions were 350, 500, 750 and 1000 second pulse times for 24 h each at 84 V. Chromosomes were visualized by incubation with ethidium bromide, depurinated and transferred to nylon as above. RNA was denatured and size-separated by electrophoresis in 1% agarose gels containing formaldehyde in MOPS buffer, pH 7.0. Transfer to nylon membranes was carried out in 50 mM NaOH by capillarity.

Membranes were neutralized by washing with 2 x SSC and then incubated at 42°C for at least 3 h in prehybrfdization solution consisting of 50% formamide, 5 x SSC, 50mM sodium phosphate, pH 6.8, 5 x Denhardt's (50 x Denhardt's is 1% each of Ficoll 400, polyvinylpyrrolidone and bovine serum albumin) and 0.2% SDS. Probes were labelled using [α-^{32}P]-dCTP by the random primer method (Feinberg and Volgelstein, 1983). To the prehybridization solution was added denatured probe (2 x 10^6 c.p.m. ml-' of hybridization solution) and salmon sperm DNA (0.2 mg ml-' final concentration). Hybridization was carried out overnight at 42 C. After a final wash at 55 C in 0.1 × SSC and 0.1% SDS, the filters were exposed to XAR5 films (Kodak). Densitometric analyses were performed by scanning the autoradiograms with a BioRad 620 densitometer and the peak areas were automatically integrated using the BioRad 1-D Analyst software. When Indicated, the probe was labelled with digoxigenin-dUTP (Boehringer Mannheim) and hybridizations were carried out, as suggested by the manufacturer, at 68 C.

Library screening, subcloning and sequence analysis

Standard procedures (Sambrook *et al.. 1989)* were used to screen with radioactive probes and to isolate positive clones from an amastigote cDNA library constructed in lambda gtl 1, and a *T. cruzi* genomic library in lambda EMBL3 (González et al., 1990). Restriction or PCR-amplified fragments were subcloned into Bluescript (Stratagene) or Geminis (Promega) plasmids, used to transform *Escherichia coli* XL-1 Blue. For all purposes, plasmids were purified by the alkaline-SDS miniprep method (Birnboim and Doly, 1979) scaled up for 15 ml culture volume. Sequencing was performed on both strands by Sanger's dideoxy protocol (Sanger et al., 1977) using Klenow or phage T7 DNA polymerases. Sequence analysis was carried out using the University of Wisconsin GCG software package (Devereux et al., 1984).

Primer extension and PCR amplification

cDNA was synthesized from epimastigote poly-A' RNA by AMV reverse transcriptase using as primer the oligonucleotide 5'-GGNAYNCKNACYTCNCC-3'. The RNA template was hydrolysed with NaOH and the cDNA passed through a Sephadex G-50 spun minicolumn. PCR amplification was carried out using the same primer and a second one, 5'-CGCTATTATTGATACAGTTTCTG-3', in a thermocycler set at 1.5 min x 94 C, 1.5 min x 48 C and 3 min x 72°C for 30 cycles. Reaction products were separated by electrophoresis in agarose gels and the DNA from gel slices containing visible bands were isolated using GeneClean (Bio 101).

Measurement *of* the rate of DNA and protein *synthesis*

One-hundred microlitres of culture samples of 35 x 10^6 epimastlgotes per ml in DMEM plus 10% heat-inactivated fetal calf serum were pulsed for 30 min with 30 μCi ml^{-1} of [^{3}H]-thymidine or 10 μCi ml^{-1} [^{35}S]-methionine. The pulse was terminated by the addition of 1 ml of cold 10% trichloroacetic acid (TCA). The precipitate was filtered through glass microfibre filters and washed twice with 5 ml cold 5% TCA. The filters were dried at 60' C for 30 min and bound radioactivity was counted in 4 ml of scintillation liquid.

Phylogenetic tree construction

The H2B sequence from *T. cruzi* was compared to eight homologous sequences retrieved from the Swiss-Prot protein sequence database. The following taxa and accession numbers were used: *L. enrietti* accession number P27893; *Tetrahymena thermophila* accession number P08993; yeast

(*S. cerevisae*) accession number P02293; wheat (*Triticum aestivum*) accession number P27807; spiny starfish (*Marthasterias glacialis*) accession number P02285; nematode (*C. elegans*) accesston number P04255; fruit fly (*Drosophi/a melanogaster*) accession number P02283; and chicken (*Gallus gallus*) accession number P02279. Sequences were aligned using the overall multiple alignment algonthm (Higgins and Sharp, 1989); statistical confidence in the topologies was assessed using the bootstrap algonthm with 100 replications. To this end, the aligned sequences were subjected to analysis by several programs from the PHYLIP (Version 3.5c, March 1993) phylogeny inference package (Felsenstein, 1988). First, we used SEQBOOT to produce 100 bootstrapped data sets. Then, we ran PAOTPARS to obtain the maximum parsimony trees for each one of these data sets. Lastly, the program CONSENSE allowed us to obtain the majority rule consensus tree with bootstrap confidence values.

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