# Organization, transcription and regulation of the *Leishmania infantum* histone H3 genes

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The genomic organization and transcription of the genes encoding the histone H3 of the protozoan parasite *Leishmania infantum* have been studied. It was found that there are multiple copies of the histone H3 genes distributed in chromosomal bands XIX and XIV. The nucleotide sequence of two of the *L. infantum* H3 genes, each one located in a different chromosome, is reported. Although the nucleotide sequence of the coding region of both genes is identical, the sequence of the 3′ untranslated region is highly divergent. It was found also that there exist two different size classes of histone H3 transcripts, each one derived from a different gene, and that they are polyadenylated. The steady-state level of the transcripts dramatically decreases when the parasites enter the stationary phase of growth, suggesting a mode of regulation which is linked to the proliferation status of the cell. Unlike the replication-dependent histones, the *L. infantum* H3 mRNA levels do not decrease after treatment with DNA synthesis inhibitors. A comparative analysis of the sensitivity of the histone mRNA levels to DNA inhibition in the parasites *L. infantum* and *Trypanosoma cruzi* revealed the existence of different control mechanisms in histone expression in these two phylogenetically related protozoan parasites.

## INTRODUCTION

The protozoan parasites of the genus *Leishmania*, transmitted to vertebrate hosts by sand fly vectors, are responsible for a spectrum of severe diseases known as leishmaniasis. These parasites, as well as other related kinetoplastid protozoa, are considered to be among the most primitive eukaryotes [1]. Peculiar features of gene organization and expression, such as the presence of reiterated genes organized in tandem arrays expressed by polycistronic transcription [2–5], RNA processing via *trans*-splicing [6], and the transcriptional editing of mitochondrial mRNAs [7], probably reflect the ancient evolutionary divergence of these organisms.

Another specific feature of trypanosomatids is the lack of condensed chromosomes at any phase of their life cycle, in spite of their DNA being associated with all the classes of histones and being packed into nucleosomes. It has been suggested that the lack of condensed chromosomes may result from DNA–histone weak interactions [8,9]. This suggestion has been reinforced by the characterization of the *Leishmania* genes coding for histones H2B [10], H2A [11], H1 [12] and H3 [13], and the *Trypanosoma* genes coding for histones H1 [14], H3 [15], H2B [16] and H2A [17], which shows that important differences exist in the amino acid sequence of the trypanosomatid histones when compared with the consensus sequence of the histones of higher eukaryotes [18]. These differences are likely to be responsible for the differences in the biochemical properties of the parasite histones relative to those of higher eukaryotes [19,20]. In fact, as a reflection of their function, the histone regions which are involved in protein–DNA interactions for nucleosome formation are more conserved than the regulatory regions exposed outside of the nucleosomal particle.

Although the data on the genomic organization and expression of trypanosomatid histone-coding genes are still scanty, it has been shown that the histone transcripts are polyadenylated like those of the constitutively expressed histone variants of higher eukaryotes, but unlike the cell-cycle regulated histones of these organisms [21]. Stem–loop structures, similar to those implicated in the processing of the 3′-untranslated regions (UTRs) of the cell-cycle-inducible histone mRNAs of higher eukaryotes, have also been found in the 3′-UTRs of several trypanosomatid histone mRNAs [10,14–17,22]. However, regulation of the expression of the histone genes in trypanosomatids, and its relation to DNA replication, is not well understood [23]. In fact, while the expression of the H2B gene from *Leishmania enriettii* does not seem to be coupled to DNA replication [10], a certain relation appears to exist between the expression levels of the *Trypanosoma cruzi* H2B histone transcripts and DNA synthesis [16].

In previous reports, we described the isolation of a cDNA coding for the *Leishmania infantum* histone H3 by immnoscreening an expression library with a visceral leishmaniasis serum [13], and found that this cDNA hybridized with two different chromosomal bands of the parasite [24]. In the present study we show that the *L. infantum* H3 genes present in these two chromosomal bands are actively transcribed as two different size classes of polyadenylated transcripts. In addition, the effect of the inhibition of DNA synthesis on the steady-state levels of the *L. infantum* H2A and H3 transcripts has been analysed. For comparative purposes, the abundance of these histone transcripts upon inhibition of DNA synthesis was studied in a related trypanosome, *T. cruzi*.

## MATERIALS AND METHODS

### Parasites

Promastigotes of *L. infantum* (WHO code MHOM/FR/78 LEM75) were grown at 26 °C in RPMI 1640 medium (Gibco, Paisley, U.K.) supplemented with 10% (v/v) heat-inactivated

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The nucleotide sequence data reported in this paper appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X77591 and X95484.

Abbreviations used: PFGE, pulsed-field gel electrophoresis; UTR, untranslated region.

fetal calf serum (Flow Laboratories, Irvine, U.K.). Experimental cultures were initiated at $1 \times 10^6$ promastigotes $\cdot$ ml$^{-1}$ and subsequently harvested for study at different points during their transition from the logarithmic ($5 \times 10^6$–$9 \times 10^6$ promastigotes $\cdot$ ml$^{-1}$; days 2–3) to the stationary ($4 \times 10^7$–$6 \times 10^7$ promastigotes $\cdot$ ml$^{-1}$; days 6–7) phase of growth. Epimastigotes of *T. cruzi* (G strain) were cultured in liver infusion tryptose medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 26 °C.

### Library screening, subcloning and sequence analysis

An *L. infantum* cDNA expression library was made in λgt11, as previously reported [11]. LiB6 cDNA was isolated as described previously [13] and subcloned into the *Eco*RI site of the pUC18 plasmid. Probe UTR-I corresponds to the 64 nt DNA fragment obtained after *Apa*LI–*Eco*RI double digestion of the LiB6 cDNA. Also, an *L. infantum* genomic DNA library constructed in EMBL-3 [25] was screened with $^{32}$P-labelled nick-translated LiB6 cDNA by *in situ* plaque hybridization [26]. A hybridizing recombinant phage, called LiB6g-5, was chosen for detailed analysis with a variety of restriction enzymes. The 0.69 kb *Sma*I–*Sma*I fragment of LiB6g-5 recombinant phage was subcloned in the pUC18 cloning vector to obtain the clone pLiB6D. Probe UTR-II corresponds to the 450 nt DNA fragment obtained after *Pvu*II–*Sma*I double digestion of clone pLiB6D. Both strands of clone pLiB6D were sequenced by the dideoxy chain-termination method [27] using a Sequenase Kit (United States Biochemical Corporation). Analysis of the DNA and amino acid sequences was performed using University of Wisconsin Genetics Computer Group programs [28] and by accessing the GenBank and EMBL databases of protein and DNA sequences.

### Southern, Northern and chromosomal blot analysis

*L. infantum* DNA and RNA were isolated as previously described [29,30]. Promastigote total DNA was digested with a variety of restriction enzymes, subjected to electrophoresis in 0.8%-agarose gels and transferred to nylon membranes (Hybond-N, Amersham) by standard procedures [26]. Preparations of *L. infantum* genomic DNA for pulsed-field gel electrophoresis (PFGE) have been described previously [24]. PFGE samples were separated using contour-clamped homogeneous electric field electrophoresis (LKB, Pharmacia) at 15 °C with a 65–90 s ramping pulse at 170 V. Total RNA was separated according to size on 1%-agarose/formaldehyde gels [31] and electro-transferred to nylon membranes using an LKB system (Pharmacia). Hybridizations, either for DNA or RNA analysis, were performed in 50% (v/v) formamide, $6 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 0.1% (v/v) SDS and 0.25 mg $\cdot$ ml$^{-1}$ herring sperm DNA at 42 °C overnight. Final post-hybridization washes were performed in $0.1 \times$ SSC/0.2% (w/v) SDS at 50 °C for 1 h. For re-use, blots were treated with 0.1% (w/v) SDS for 30 min at 95 °C to remove the previously hybridized probe. Removal of the probe was verified by autoradiography.

### Drug treatments and [*methyl*-$^3$H]thymidine incorporation into DNA

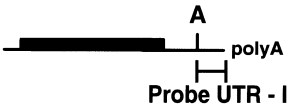
Inhibition of DNA synthesis by hydroxyurea was estimated by measuring [*methyl*-$^3$H]thymidine incorporation into DNA in hydroxyurea-treated cultures relative to untreated cultures. For this purpose, parasites in logarithmic phase of growth ($5 \times 10^6$ parasites $\cdot$ ml$^{-1}$) were incubated in the presence of 10 μCi $\cdot$ ml$^{-1}$ [*methyl*-$^3$H]thymidine (Amersham, 2.0 Ci mmol$^{-1}$) with (treated culture) and without 5 mM hydroxyurea (untreated culture). Aliquots of 100 μl from each one of the cultures (treated and untreated) were taken after 0, 2, 4, 6 and 8 h of incubation at 26 °C. Thymidine incorporation into DNA was determined using the MultiScreen assay system (Millipore) following the manufacturer's instructions. To study the effect of hydroxyurea on histone RNA levels, 50 ml of logarithmic-phase cultures ($5 \times 10^6$ cells $\cdot$ ml$^{-1}$) of either *L. infantum* promastigotes or *T. cruzi* epimastigotes were incubated in the presence of 5 mM hydroxyurea. After the addition of hydroxyurea, 10 ml aliquots were removed at 0, 4, 6 and 8 h from cultures for RNA extraction [30]. For RNA synthesis inhibition, 10 μg $\cdot$ ml$^{-1}$ actinomycin D (Sigma Corporation) was added to logarithmic *Leishmania* promastigote cultures. Aliquots (10 ml) were removed at 0, 1, 2, 4 and 6 h, and processed for RNA preparation [30].

## RESULTS

### Genomic organization of *Leishmania* histone H3 coding genes

As we previously reported [13], a cDNA clone coding for the *L. infantum* histone H3, named LiB6, was isolated after immuno-

**A**




**B**

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      gtttcactaccgccatccaacccccctgccactcccacccccaccgcaccacc  52

  1 M   S   R   T   K   E   T   A   R   A   K   R   T   I
    ATG TCC CGC ACC AAG GAG ACC GCC CGC GCG AAG CGC ACC ATC   94

 15 T   S   K   K   S   K   K   A   P   S   G   A   S   G
    ACG TCG AAG AAG AGC AAG AAG GCG CCG AGC GGG GCG TCC GGC 136

 29 V   K   R   S   H   R   R   W   R   P   G   T   C   A
    GTG AAG AGG TCG CAT CGC CGC TGG CGC CCG GGC ACC TGC GCG 178

 43 I   R   E   I   R   K   F   Q   K   S   T   S   L   L
    ATC CGC GAG ATC CGC AAG TTC CAG AAG AGT ACG AGC CTG CTG 220

 57 I   Q   C   A   P   F   Q   R   L   V   R   G   V   E
    ATC CAG TGC GCG CCG TTC CAG CGC CTG GTG CGA GGT GTC GAG 262

 71 R   Q   K   E   G   L   R   F   Q   S   S   A   I   M
    CGG CAG AAG GAG GGC CTG CGC TTC CAG AGC AGC GCT ATC ATG 304

 85 A   L   Q   E   A   T   E   A   Y   I   V   S   L   M
    GCG CTG CAG GAG GCG ACG GAG GCG TAC ATT GTG TCG CTG ATG 346

 99 A   D   T   N   L   A   C   I   H   A   K   R   V   T
    GCG GAC ACG AAC CTC GCC TGC ATC CAC GCG AAG CGC GTG ACG 388

113 I   Q   P   K   D   I   Q   L   A   L   R   L   R   G
    ATC CAG CCG AAG GAC ATC CAG CTG GCG CTG CGC CTG CGC GGT 430

127 E   R   H   *
    GAG CGC CAC TAG ggcgggcccgctctcccccccctcatagataccatgtt  480

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                                  ApaLI =======>   <=======
    gccgagagagtgagagggtcattgaaccctctagagcccgccaaaaaaaaaaa  586
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**Figure 1 Nucleotide sequence of the *Leishmania* cDNA clone LiB6**

(**A**) Schematic map of the cDNA clone LiB6 also showing the location of probe UTR-I. The coding region is marked by a solid box. (**B**) Nucleotide sequence and deduced amino acid sequence of LiB6 clone. The TAG stop codon is marked by an asterisk. Numbers to the right and left of the sequence indicate the nucleotide and amino acid positions respectively. The presence of inverted repeats with potential to form stem–loop structures is indicated by double lines under the sequence. The position of the *Apa*LI restriction site is underlined.

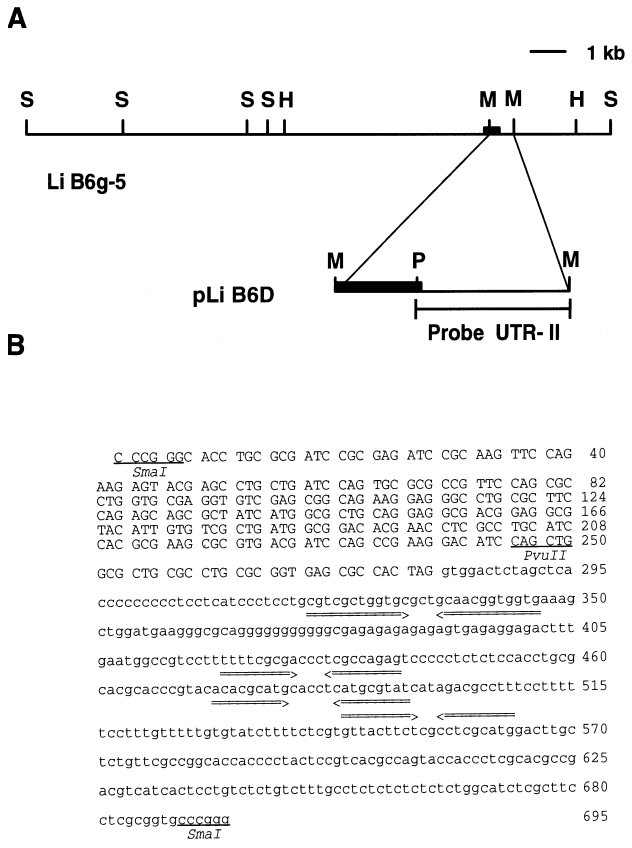


**Figure 2 Genomic organization and sequence analysis of the H3 gene locus in the LiB6g-5 clone**

(**A**) Restriction maps of the genomic recombinant clone LiB6g-5 and the subclone pLiB6D. The location of probe UTR-II is also indicated. Restriction sites: S, *Sal*I; H, *Hin*dIII; M, *Sma*I; P, *Pvu*II. (**B**) Nucleotide sequence of the LiB6D clone. The coding region is indicated by capital letters grouped in triplets. Double lines indicate the location of inverted repeats with potential to form stem–loop structures.

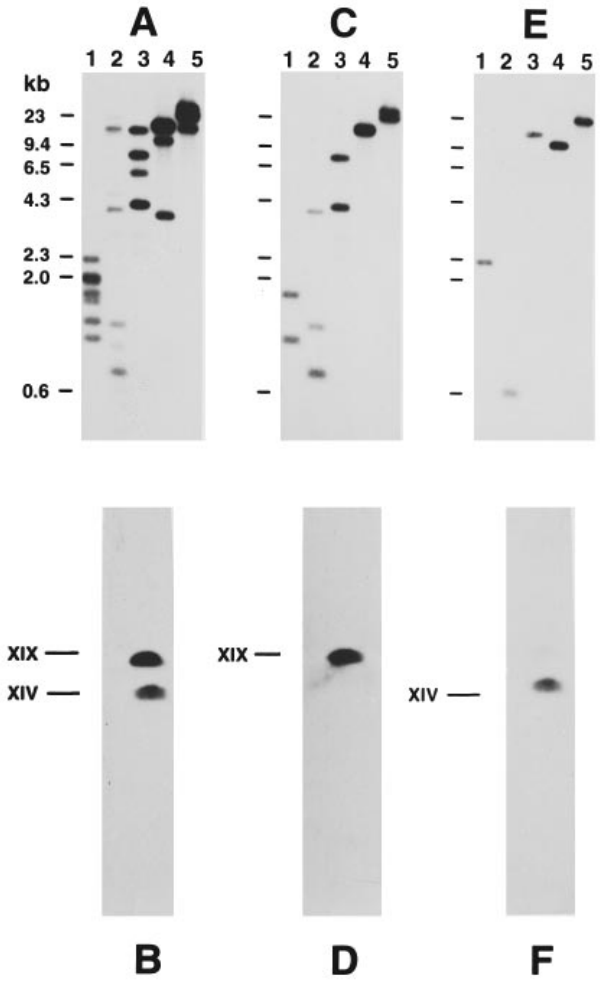


**Figure 3 Genomic arrangement of the histone H3 genes**

For the genomic Southern blot (**A**, **C** and **E**), 2 $\mu$g of total DNA from *L. infantum* promastigotes was digested with the restriction enzymes *Apa*LI (lane 1), *Sma*I (lane 2), *Sal*I (lane 3), *Cla*I (lane 4) and *Bam*HI (lane 5), and separated on a 0.8%-agarose gel. Numbers at the left indicate the size (in kb) and mobility of the restriction fragments from *Hin*dIII-digested $\lambda$ DNA. For PFGE Southern blots (**B**, **D** and **F**), intact *L. infantum* promastigote DNA was resolved by contour-clamped homogeneous electric field electrophoresis (CHEF) using a 65–90 s ramping pulse and 170 V. The chromosome numbering of the *L. infantum* karyotype has been described previously [24]. After blotting, the filters were hybridized with the probes LiB6 (**A** and **B**), UTR-I (**C** and **D**), and UTR-II (**E** and **F**).

screening of a $\lambda$-gt11 cDNA expression library with serum from a dog with visceral leishmaniasis. The nucleotide and the deduced amino acid sequences of the cDNA clone are shown in Figure 1. A genomic clone (Figure 2A) was also isolated after screening of an *L. infantum* EMBL-3 library using radiolabelled LiB6 cDNA as probe. From this clone, the 0.69 kb *Sma*I–*Sma*I DNA fragment was subcloned, clone pLiB6D, and sequenced (Figure 2B). Nucleotide sequence comparison between the LiB6 and LiB6D sequences indicated that the two clones share the same nucleotide sequence in the coding region, but that a sudden loss of sequence identity occurs one nucleotide down-stream of the TAG termination codon. These data suggest that at least two different H3 genes must be present in the *L. infantum* genome. Examination of the 3′-UTR nucleotide sequence of the LiB6 cDNA revealed the existence of inverted repeats, with the potential to form stem–loop structures that are located close to the poly(A)$^+$ tail (Figure 1B). Four elements with dyad symmetry were also detected within the putative 3′-UTR of LiB6D (Figure 2B). These stem–loop structures in the 3′-UTR of histone H3 mRNAs could provide a framework for interaction with regulatory elements.

To analyse further the genomic organization of the H3 genes, Southern blots of genomic DNA were probed with LiB6 cDNA (Figure 3A). The existence in the *L. infantum* genome of several H3 genes was indicated by the presence of multiple hybridization bands in all of the lanes containing DNA digested with various restriction enzymes. In order to determine the chromosomal location of the histone H3 genes, the same cDNA was used to probe a blot containing the *L. infantum* chromosomes separated by PFGE. As is shown in Figure 3B, hybridization signals were observed in the chromosomal bands XIX and XIV. Since these chromosomal bands do not represent a pair of homologues [24], we may conclude that, not only must different H3 genes exist in the *Leishmania* genome, but that they are not physically linked. Since the H2A genes also map in the chromosomal bands XIX and XIV [24], we suggest the existence of certain physical grouping of the genes coding for the different histones in the genome of *L. infantum*.

In order to analyse the genomic distribution of the histone H3 genes contained in the clones LiB6 and LiB6D, two specific

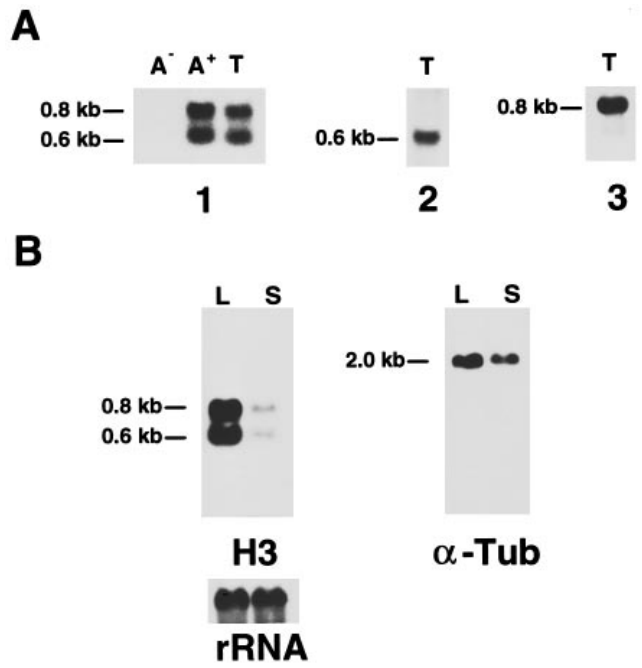


**Figure 4 Northern blot analysis of histone H3 mRNAs**

(**A**) Non-polyadenylated RNA (10 $\mu$g; lane A$^-$), 2 $\mu$g of polyadenylated RNA (lane A$^+$) and 5 $\mu$g of total RNA (lanes T) from *L. infantum* promastigotes were separated on 1%-agarose/formaldehyde gels. After blotting, the filters were hybridized with the probes LiB6 (panel 1), UTR-I (panel 2), and UTR-II (panel 3). (**B**) Total RNA (5 $\mu$g) from promastigotes, at either logarithmic growth phase (lane L) or stationary growth phase (lane S), were also fractionated in agarose/formaldehyde gels and transferred to nylon membranes. The same filter was hybridized with probe LiB6 (H3 panel), and after autoradiographic exposure and removal of the probe the filter was rehybridized with a *T. cruzi* $\alpha$-tubulin probe [40] ($\alpha$-Tub panel). After removal of the probe, the filter was hybridized using an *L. infantum* 24 S-$\alpha$ rDNA probe (positions 1868–2457, laboratory data; rRNA panel). The positions and sizes of hybridization bands (in kb) are indicated.

probes were designed. Probe UTR-I corresponds to the 3′-UTR, downstream of *Apa*LI, present in LiB6 (Figure 1A). Probe UTR-II corresponds to the *Pvu*II–*Sma*I 445 bp fragment of LiB6D (Figure 2B). When a Southern blot of *L. infantum*-digested DNA was hybridized with the UTR-I probe (Figure 3C) significant differences from the hybridization pattern of the entire LiB6 probe (Figure 3A) were observed. Since three hybridization *Sma*I bands were observed after hybridization with the UTR-I probe, and *Sma*I does not cut this DNA fragment, it must be concluded that at least three histone H3 genes with sequence similarity to the LiB6 3′-UTR must exist in chromosomal band XIX (Figure 3D). The results obtained after hybridization of genomic and PFGE Southern blots with the UTR-II probe (Figures 3E and 3F) are consistent, on the other hand, with the existence of a single histone H3 gene with sequence similarity to that in LiB6D (Figure 2B). The gene containing the UTR-II probe is located in chromosomal band XIV. A comparative analysis of the Southern blots shown in Figures 3A, 3C and 3E indicated that other H3 genes besides the two identified H3 gene classes must be present in the *L. infantum* genome, since several of the LiB6-labelled bands, such as the 6.6 kb *Sal*I, the 15 kb *Bam*HI and the 3.7 kb *Cla*I did not hybridize with either the UTR-I or UTR-II probe.

Hybridization with the UTR-II probe suggests that the complex pattern of hybridizing bands observed after probing blots containing *L. infantum* DNA digested by *Apa*LI or *Sma*I DNA (Figure 3A) with LiB6 is not due to partial digestion; in the case of partial digestion a single hybridizing band should never be observed (Figure 3E). Moreover, the possibility of partial digestions due to DNA methylation associated with the *Sma*I restriction site in certain H3 genes was addressed. Since the same hybridizing bands were observed when the *L. infantum* DNA, digested with either the *Hpa*II (methyl sensitive) or *Msp*I (methyl insensitive) enzymes, was probed with LiB6 (results not shown), we think that the *Sma*I bands represent different H3 genes.

### Expression of the *Leishmania* histone H3 coding genes

When Northern blots containing total RNA from *L. infantum* promastigotes were probed with the LiB6 probe, two transcripts of about 0.8 and 0.6 kb were detected (Figure 4A, panel 1). The same hybridization pattern was obtained when the poly(A)$^+$ RNA fraction was used. No hybridization signals were observed in the poly(A)$^+$ fraction. The conclusion from these data is that the H3 transcripts are polyadenylated in the same manner as the *Leishmania* H2A [11] and H2B transcripts [10]. In fact, a poly(A)$^+$ tail was observed after sequencing of the LiB6 cDNA (Figure 1B). Subsequent hybridization of the Northern blots with the UTR-I (Figure 4A, panel 2) and UTR-II (panel 3) probes revealed that the 0.6 kb transcript must be derived from the expression of LiB6-related genes, whereas the 0.8 kb transcript band was found to correspond to the expression of the LiB6D-related gene. The differences observed in the size of the transcripts is most likely explained by the differences in the length of the highly divergent 3′-UTRs. We do not know the entire length of the 3′-UTR of the histone H3 type-II gene since at present we do not have any H3 type-II cDNA.

In order to determine whether the steady-state level of the H3 mRNA correlates with the parasite growth phases, Northern blots containing equal amounts of RNA from logarithmic- and stationary-phase parasites were probed with LiB6 (Figure 4B, panel H3). It was observed that the steady-state level of the H3 mRNAs was significatively higher in the logarithmic phase of growth than in the stationary phase and that the two H3 transcripts decreased in a similar way. The steady-state level of the H3 transcripts must be controlled by a specific mechanism connected with the proliferation state of the parasites, since the level of the $\alpha$-tubulin transcripts was similar in both phases of growth (Figure 4B, panel $\alpha$-Tub). A similar down-regulation mechanism operating on the *L. infantum* histone H2A RNA abundance during the stationary phase of growth was also observed [11].

### H3 mRNA levels and DNA synthesis

Except for the basal histones, the steady-state level of the histone mRNAs of lower and higher eukaryotes is cell-cycle regulated. High levels of the transcripts accumulate during DNA synthesis (S phase) due to an increase in both histone gene transcription and histone mRNA stability, and a decrease occurs to much lower levels in the absence of DNA synthesis [32]. In fact, the inhibition of DNA synthesis by hydroxyurea or aphidicolin is followed by a rapid reduction in histone mRNA levels both in yeast and in higher eukaryotic cells [33,34]. Since a full suppression of DNA synthesis of the protozoan parasites of the Trypanosomatidae family can also be achieved by incubation with hydroxyurea [35], several authors have addressed the question of whether histone expression is linked to DNA synthesis. While it has been reported that the level of *T. cruzi* H2B mRNAs was transiently reduced by inhibition of DNA synthesis after aphidicolin treatment [16], aphidicolin or hydroxyurea treatment did not affect the H2B mRNA levels in *L.*

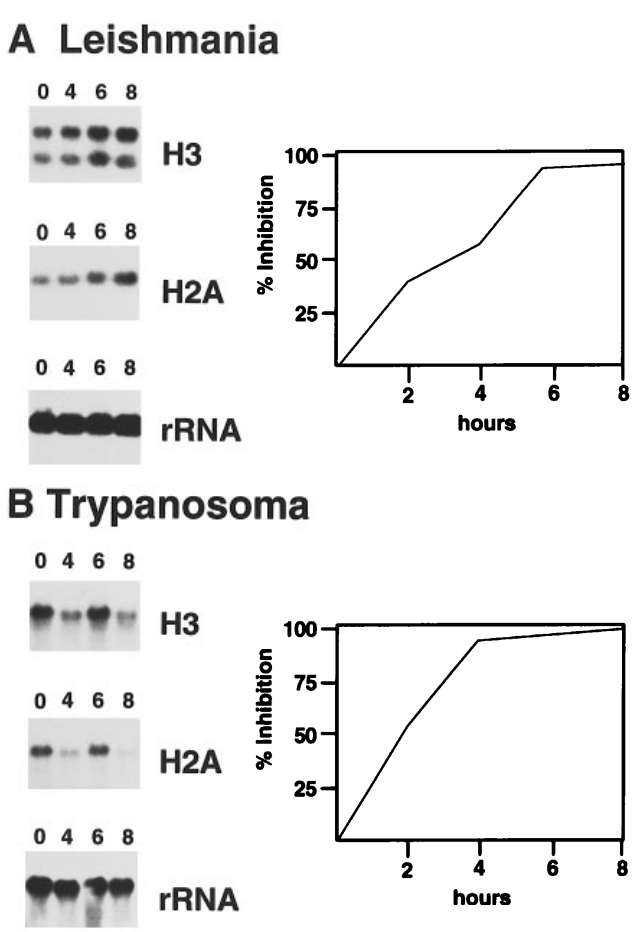


**Figure 5 Effect of DNA synthesis inhibition on H3 and H2A mRNA levels**

(**A**) *L. infantum* parasites were incubated with 5 mM hydroxyurea for 0, 4, 6 and 8 h. The kinetics of DNA synthesis inhibition is shown. Total *L. infantum* RNA (5 $\mu$g) from each time point was analysed by Northern blotting and probed with LiB6 (histone H3 gene, H3 panel), cL71 (histone H2A gene [11], H2A panel) and *L. infantum* 24 S-$\alpha$ rDNA (rRNA panel). (**B**) *T. cruzi* epimastigotes were incubated with 5 mM hydroxyurea for the same times as in (**A**). Total RNA was extracted and analysed by Northern blotting using the same probes as in (**A**).

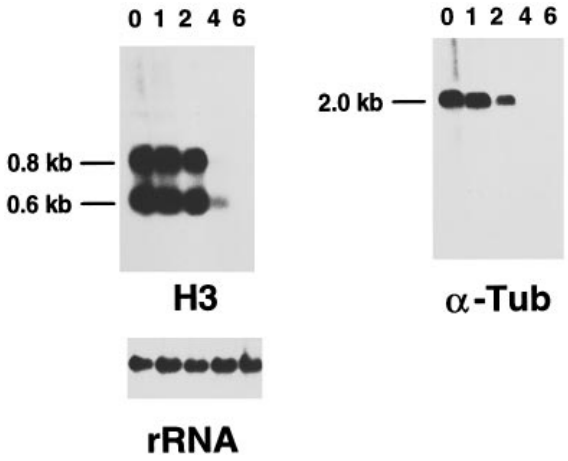


**Figure 6 Effect of RNA synthesis inhibition on H3 mRNA levels**

RNA from Actinomycin D-treated (10 $\mu$g/ml) logarithmically growing *L. infantum* promastigotes was isolated at the time intervals indicated (in hours). The samples of total RNA were analysed by Northern blotting and hybridized to probes LiB6 (H3 panel), $\alpha$-tubulin gene ($\alpha$-Tub panel) and 24 S-$\alpha$ rDNA (rRNA panel).

*enriettii* [10]. Thus, although at present little is known about the regulation of histone gene expression in kinetoplastids relative to DNA synthesis, and the few existing data can be considered as conflicting [23], it is likely that some differences in the regulation of histone expression in kinetoplastids must exist with respect to the rest of the eukaryotes.

To provide further data on the regulation of histone expression relative to DNA synthesis we analysed the steady-state level of the H3 and H2A transcripts in the presence of hydroxyurea. We observed that hydroxyurea is an effective inhibitor of DNA synthesis in *L. infantum*, since 6 h of incubation of logarithmically growing promastigotes in the presence of 5 mM hydroxyurea resulted in an 87% reduction in DNA synthesis as measured by [*methyl*-$^3$H]thymidine incorporation into acid-precipitable material (Figure 5A). When total cytoplasmic RNA isolated from aliquots of the parasite culture, taken at timed intervals, was probed with LiB6 in Northern blots (Figure 5A, panel H3), it was observed that the amount of histone H3 transcripts was the same, even after 8 h of DNA inhibition. In fact, a slight accumulation of mRNA was observed in the 8 h-period aliquot. The reprobing of the same filter with a DNA probe of the *L. infantum* H2A gene [11] yielded a similar pattern of the steady-state level of histone H2A mRNA at all times during the hydroxyurea treatment (Figure 5A, panel H2A). We concluded, thus, that the expression of the H3 and H2A genes in *L. infantum* should be independent of DNA synthesis, in agreement with the results reported for the H2B genes of *L. enriettii* [10]. To rule out the possibility that the failure to detect a decrease in the levels of H3 transcripts, in relation to the inhibition of DNA synthesis, is due to the high stability of the H3 transcripts, the abundance of those transcripts was analysed in cultures treated with actinomycin D. Figure 6 shows that after 4 h of treatment, the 0.8 kb histone H3 transcripts decrease to undetectable levels and only traces of the 0.6 kb transcripts were observed. The stability of the H3 transcripts seems to be similar to that of the $\alpha$-tubulin transcripts (Figure 6). It is most likely therefore that the levels of the H3 transcripts observed during inhibition of DNA synthesis is due to continuous expression of the gene and that it occurs independently of DNA synthesis.

As controversy exists with respect to the control of histone gene expression between *Leishmania* and *T. cruzi*, we have analysed the effects of DNA inhibition on the levels of *T. cruzi* H2A and H3 mRNAs under the same conditions as the hydroxyurea treatments. A 95% reduction in DNA synthesis was achieved after incubation of the parasites for 4 h with 5 mM of the drug, with inhibition levels being maintained for up to 8 h (Figure 5B). Northern blot analysis showed the existence of large variations in H2A and H3 mRNA levels during the course of treatment. A decrease was observed after the first 4 h of treatment, a recovery at the 6 h period, and a new decrease at the end of the experiment. The Northern blots of *T. cruzi* RNA were probed with the H2A and H3 genes of *L. infantum* due to their high nucleotide sequence similarity with the H2A and H3 genes of *T. cruzi* [15,17]. Reprobing of the blots with an *L. infantum* 24 S-$\alpha$ rDNA probe confirmed that equal amounts of total RNA were loaded in each lane of the Northern blot (Figure 5B, rRNA panel). The results agree with those reported for *T. cruzi* H2B [16] and H2A expression (M. C. Lopez, personal communi-

cation). Thus, we may conclude that the differences observed in histone mRNA abundance, after inhibition of DNA synthesis, between *T. cruzi* and *Leishmania* are not due to differences in drug treatments, but they reveal the existence of differences in the mechanisms of control of histone expression in these protozoa.

## DISCUSSION

All the histones, and particularly H3 and H4, have evolved unusually slowly in the evolutionary history of eukaryotes [18,36], probably due to the fundamental role played by these proteins within the cell. A common feature of the histone genes is that they are frequently grouped in the genome [37], and such organization could be related to the complex cell-cycle regulation of histone gene transcription [38]. Analysis of the organization of the histone H3 gene of *L. infantum*, described in this work, has revealed the existence of several of those genes distributed on the same chromosomal bands (XIV and XIX) as the *L. infantum* H2A histone genes [24]. The *L. enriettii* H2B genes have been mapped in two different chromosomal bands, although the size and numbering were not established [10]. It is likely therefore that also in *Leishmania* there is a certain chromosomal clustering of the histone genes, although further data are required to confirm this suggestion. The analysis of several overlapping EMBL-3 genomic clones containing H3 or H2A genes indicated, however, that the *L. infantum* H3 and H2A genes are not closely linked and differ in their organization from most eukaryotic organisms.

Analysis of the expression of the *L. infantum* histone H3 genes revealed the existence of two classes of transcripts of different size, each derived from genes located in each of the chromosomes. The sequence analysis of two H3 genes showed that the differences in size of the transcripts are probably due to the high sequence divergency found within the 3′-UTRs. In these regions, inverted repeats, with predicted potential to form stem–loop structures, were observed. Similar structures have been described in equivalent positions in the 3′-UTRs of both *L. enriettii* histone H2B genes [10] and *L. infantum* histone H2A genes [22], indicating that they may play a functional role in the regulation of histone mRNA abundance. It was also observed that the two H3 transcripts are polyadenylated, as are all the histone transcripts from all of the trypanosomatids described to date [23].

In order to shed further light on the control of histone gene expression we first analysed the abundance of the H3 transcripts in relation to the proliferative state of the *Leishmania* cultures. In most eukaryotes, histone gene expression seems to be regulated both during the cell cycle itself and during the transitions between proliferating and quiescent cells [21,32,39]. A clear down-regulation of the abundance of histone H3 transcripts was observed when the parasites reached the stationary phase of growth, similar to that detected for histone H2A mRNA [11], which indicates that there must exist a common regulatory mechanism of control. Genske et al. [10] reported that the level of *L. enriettii* H2B mRNA in cultures of promastigotes is several-fold higher than that present in intracellular amastigotes. They suggest that histone mRNAs could accumulate to a higher level in promastigotes because of a more rapid rate of cellular division than in the intracellular amastigotes. Thus, it seems that in *Leishmania*, histone abundance is growth-rate dependent.

On the other hand, in contrast with the data on the direct coupling of histone transcript abundance and DNA synthesis in eukaryotes [33,34], the results reported in this paper show that in *L. infantum* such coupling does not exist. The abundance of histone H3 and H2A mRNAs did not decrease upon inhibition of DNA synthesis, rather, a slight increase was observed after a long period of DNA inhibition. The abundance of the histone transcripts in the absence of DNA synthesis can only be explained by continuous transcription of the genes, since the stability of the transcripts is not sufficient to account for it. Genske et al. [10] also reported this lack of coupling with regard to the regulation of H2B of *L. enriettii*. Our data, on the other hand, favour the hypothesis that histone expression in *T. cruzi* is in some way linked to DNA synthesis, since during hydroxyurea treatment we observed transient decreases followed by increases in histone H2A and H3 mRNA levels. Similar data have been reported by García-Salcedo et al. [16] for *T. cruzi* H2B gene regulation. Thus, it is most likely that in spite of the phylogenetic similarities between *Trypanosoma* and *Leishmania*, differences in control of histone abundance must exist among them. Whether the presence of non-proliferating stages in the *T. cruzi* life cycle, absent in the *Leishmania* life cycle, is the requirement for a specific control mechanism for histone regulation, warrants investigation. In fact, in the non-replicative trypomastigote stage of *T. cruzi* there are no detectable levels of histone H2B mRNA [16].

This work was supported by grants CAM 160-9, I + D0020/94 and AE00422/95 from Comunidad Autónoma de Madrid, SAF93-0146 from CICYT, and in part by a CDTI grant to Laboratorios LETI. An institutional grant from Fundación Ramón Areces is also acknowledged.

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Received 15 February 1996/30 April 1996; accepted 10 May 1996