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 immuno-screwing 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

*To whom correspondence should be addressed.*

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 x 10^6 promastigotes ml^-1 and subsequently harvested for study at different points during their transition from the logarithmic (5 x 10^6-9 x 10^6 promastigotes ml^-1; days 2-3) to the stationary (4 x 10^5-6 x 10^7 promastigotes ml^-1; days 6-7) phase of growth. Epimastigotes of T. cruzi (G strain) were cultured in liver infusion tryptone 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 EcoRI site of the pUC18 plasmid. Probe UTR-I corresponds to the 64 nt DNA fragment obtained after ApaLI-EcoRI double digestion of the LiB6 cDNA. Also, an L. infantum genomic library constructed in EMBL-3 [25] was screened with ^32P-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 Smal-Smal 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 PvuII–Smal 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 x SSC (1 x SSC is 0.15 M NaCl/0.15 M sodium citrate, pH 7.0), 0.1% (v/v) SDS and 0.25 mg·ml^-1 herring sperm DNA at 42°C overnight. Final post-hybridization washes were performed in 0.1 x 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-^3H]thymidine incorporation into DNA
Inhibition of DNA synthesis by hydroxyurea was estimated by measuring [methyl-^3H]thymidine incorporation into DNA in hydroxyurea-treated cultures relative to untreated cultures. For this purpose, parasites in logarithmic phase of growth (5 x 10^6 parasites ml^-1) were incubated in the presence of 10 ìCi·ml^-1 [methyl-^3H]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 x 10^6 cells·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·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-

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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 ApaLI restriction site is underlined.
screening of a λ-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 Smal–Smal 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 downstream 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
of partial digestion a single hybridizing band should never be observed (Figure 3E). Moreover, the possibility of partial digests due to DNA methylation associated with the Smal restriction site in certain H3 genes was addressed. Since the same hybridizing bands were observed when the L. infantum DNA, digested with either the HpaII (methyl sensitive) or MspI (methyl insensitive) enzymes, was probed with LiB6 (results not shown), we think that the Smal 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 significantly 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 α-tubulin transcripts was similar in both phases of growth (Figure 4B, panel α-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. infantum.
same, even after 8 h of DNA inhibition. In fact, a slight was observed that the amount of histone H3 transcripts was the material (Figure 5A). When total cytoplasmic RNA isolated from parasites were incubated with 5 mM hydroxyurea for the same times as in (Figure 5A, panel H3). Total RNA was extracted and analysed by Northern blotting using the same probes as in (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-α 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 commu-

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 µg) 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-α 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).

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-3H]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 α-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-α 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 commu-
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 trypanomastigote stage of *T. cruzi* there are no detectable levels of histone H2B mRNA [16].

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


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