Histone synthesis in *Leishmania infantum* is tightly linked to DNA replication by a translational control

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We have analysed the regulation of histone synthesis in *Leishmania infantum* following inhibition of DNA replication. Run-on experiments indicated that transcription rates of the genes coding for the four core histones (H2A, H2B, H3 and H4) were not affected by the inhibition with hydroxyurea of DNA synthesis. However, a dramatic decrease was observed in the newly synthesized histones after inhibition of DNA synthesis. Furthermore, the synthesis of both the histones and DNA resumed in promastigotes after removal of hydroxyurea, indicating that inhibition was reversible. Unlike most eukaryotes, in

which the replication-dependent histone transcripts decrease upon a replication blockade, the levels of *L. infantum* histone mRNAs do not change under similar conditions. Thus the present data indicate that histone synthesis in *Leishmania* is tightly coupled to DNA replication by a mechanism operating at the translational level.

Key words: DNA synthesis, hydroxyurea, post-transcriptional regulation, protein synthesis.

INTRODUCTION

Pathogenic protozoan parasites from the genus *Leishmania* cause a group of diseases, collectively called leishmaniasis, that are responsible for substantial morbidity and mortality in humans and other vertebrates. These parasites, belonging to the order Kinetoplastida, are considered to be among the most primitive eukaryotes [1]. As a consequence of such ancient phylogenetic location, Kinetoplastida present exceptional and sometimes surprising biological features like polycistronic transcription, *trans*-splicing of precursor RNAs and transcriptional editing of mitochondrial RNAs [2]. It is also noticeable that the chromatin does not condense into chromosomes and that the nuclear envelope is conserved during cell division [3]. Thus the study of the structural features of histones has been considered of particular interest and, as a result, in recent years a great number of histone genes have been characterized in trypanosomatids [4].

Histones, mainly the four forming the nucleosomal core (H2A, H2B, H3 and H4), are extremely conserved proteins, reflecting their apparent universality of function. Since these proteins play an important role in the organization and function of DNA in the eukaryotic nucleus, it is not unexpected that histone biosynthesis is tightly linked to cellular DNA replication [5]. Most histone protein synthesis is restricted to the S-phase of the cell cycle, when they are required for assembly into nucleosomes with the newly replicated DNA. Histone gene expression is controlled at transcriptional and post-transcriptional levels; the post-transcriptional regulation involves pre-mRNA processing and histone mRNA stability. Moreover, histone mRNAs are destabilized selectively at the natural completion of the S phase and following inhibition of DNA synthesis [5–7]. Thus histone gene regulation constitutes an excellent model system for studying mechanisms of the regulation of gene expression in these particular organisms. In recent years, the regulation of histone gene expression has been studied in trypanosomatids, mainly in the genera *Trypanosoma* and *Leishmania*, providing evidence for the existence of different levels of regulation. In *T. cruzi*, evidence indicates that

the core histones are synthesized during the S phase of the cell cycle [8]. In addition, transcriptional regulation operating in the non-replicative trypomastigote stage of *T. cruzi* and post-transcriptional regulation in the amastigote and epimastigote forms [9] have been demonstrated. Furthermore, the use of inhibitors of DNA synthesis has shown that the histone mRNA levels are coupled to the rate of DNA synthesis [9–11]. In *T. brucei*, the analysis of histone RNA levels by fluorescence *in situ* hybridization indicates that histone mRNA presence peaks during the S phase and that it is not detectable during all the other stages of the cell cycle [12]. However, such a direct relationship between DNA synthesis and histone gene expression has not been demonstrated in *Leishmania* to date. Remarkably, the levels of histone mRNAs do not decrease following treatment of *Leishmania* promastigotes with inhibitors of DNA synthesis [11,13,14].

The aim of the present work was to analyse in detail the effect of hydroxyurea (HU) treatment on DNA synthesis in *Leishmania infantum* and to study how the expression of the histone genes is affected by the inhibition of DNA synthesis. We have found that the transcription rate of the genes coding for all the four core histones is not affected by the HU treatment but that, instead, the inhibition of DNA synthesis promoted a specific blocking of the translation of the histone mRNAs. Thus it can be concluded from our data that histone protein synthesis in *L. infantum* is indeed tightly coupled to DNA replication but that this coupling is mediated by post-transcriptional mechanisms operating at the level of translation.

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 fetal calf serum (ICN Pharmaceuticals, Basingstoke, Hants,

Abbreviation used: HU, hydroxyurea.

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U.K.). Experimental cultures were initiated at 1×10^6 promastigotes ml^{-1} and harvested for study in the logarithmic phase of growth $[(5-8) \times 10^6$ promastigotes $\text{ml}^{-1}]$.

Production of specific anti-histone antibodies

Anti-H2A antibodies were obtained from a rabbit immunized with the *L. infantum* histone H2A expressed as a recombinant protein. The recombinant protein was obtained after cloning a cDNA coding for the histone H2A in the pQE31 expression plasmid (Qiagen, Chatsworth, CA, U.S.A.). For that purpose the pMal-cl71 clone, containing the cDNA coding for the *L. infantum* histone H2A [15], was digested with *SacI* and *HindIII* restriction enzymes and the resulting DNA insert was subcloned into the corresponding restriction sites of pQE-31. After overexpression in *Escherichia coli*, purification of the recombinant protein was performed by affinity chromatography on Ni^{2+} -nitrilotriacetate resin columns under denaturing conditions according to the method provided by the supplier (Qiagen, Chatsworth, CA, U.S.A.). Finally, the H2A recombinant protein was dialysed against PBS. A New Zealand white rabbit was immunized subcutaneously three times, every two weeks, with 50 μg of the recombinant protein mixed with complete Freund's adjuvant (the first time) or with incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). The serum showed a 1:40 500 titre when assayed by ELISA against the H2A recombinant protein 15 days after the last immunization. The antibody fraction against the recombinant histone H2A was affinity purified from the polyclonal rabbit antiserum on an antigen column. For that purpose, 1 mg of the histone H2A was bound covalently to CNBr-activated Sepharose 4B (Pharmacia AB, Stockholm, Sweden) and packed into a column. Coupling and blocking were carried out according to the manufacturer's instructions. The rabbit serum (2 ml) was passed through the antigen column and, after washing, the specific anti-H2A antibodies were eluted from the column with 0.1 M glycine, pH 2.8. Finally, the antibody fraction was equilibrated to pH 7.5 with 1 M Tris/HCl and adjusted to the original volume (2 ml).

Sera reactive against the four core histones were obtained from a collection of dogs infected naturally with *L. infantum* from the Extremadura region of Spain (Departament of Parasitology, Veterinary School, Extremadura University, Cáceres, Spain). As reported elsewhere [16], anti-histone reactivity was demonstrated by immunoblotting and ELISA assays using the four core histones expressed as recombinant proteins.

Isolation of nuclei

Leishmania nuclei were isolated from 10 ml of promastigote culture in logarithmic growth phase (5×10^6 parasites ml^{-1}). Parasites were harvested, washed twice with ice-cold PBS and resuspended in 500 μl of ice-cold hypotonic buffer (0.25 M sucrose, 5 mM Hepes, pH 7.5, 1 mM spermidine, 0.1 mM PMSF, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol). After addition of Nonidet P40 and Triton X-100 at final concentrations of 0.5 % (v/v) each, the cells were lysed by vigorous vortexing for 30 s. Immediately, 1 ml of ice-cold $2 \times$ nuclei-washing buffer (40 mM Tris/HCl, pH 7.5, 0.64 M sucrose, 1 mM spermidine, 0.1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 60 mM KCl) was added and mixed by vortexing. Nuclei were pelleted (3000 g), washed again in the same buffer and stored. For run-on assays the nuclei were stored in 100 μl of nuclei-storage buffer [50 % (v/v) glycerol, 4 mM MnCl_2 , 0.1 mM EDTA, 50 mM Hepes, pH 7.5, and 5 mM dithiothreitol] at -70°C . For SDS/PAGE the nuclei were extracted in the same way with the sole modification that the protease inhibitor *N*-

tosyl-lysylchloromethyl ketone (1.5 mM) was added to the hypotonic and nuclei-washing buffers. After washing, the nuclei were lysed in $1 \times$ Laemmli's buffer [17] supplemented with 8 M urea, and sonicated before loading on to the gel.

Separation of nuclear and cytosolic fractions was performed basically according to Schreiber et al. [18]. Briefly, 2×10^7 promastigotes were pelleted, washed twice in ice-cold PBS, resuspended in 400 μl of prechilled buffer A (10 mM Hepes, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF and 1.5 mM *N*-tosyl-lysylchloromethyl ketone), and incubated on ice for 15 min. After incubation, Nonidet P40 was added to a final concentration of 0.6 % (v/v), and cells were lysed by vigorous vortexing for 10 s, and immediately pelleted in a Mikroliter high-speed centrifuge (Hettich, Tuttlinger, Germany). The supernatant containing the cytosolic fraction was mixed 1:1 in $2 \times$ Laemmli's buffer and the pelleted nuclei were resuspended in $1 \times$ Laemmli's buffer supplemented with 8 M urea.

Electrophoretic procedures and immunoblot analysis

Nuclear preparations were separated by electrophoresis on linear 10–14 % gradient SDS/PAGE gels at 10 mA for 12 h using the Hoefer Scientific Instrument protein system (Pharmacia AB). For the immunoblot analysis, the electrophoresed proteins were transferred on to nitrocellulose membranes (Amersham, Little Chalfont, Bucks, U.K.). The transfer was blocked with 5 % (w/v) non-fat dried milk powder in PBS and 0.5 % (v/v) Tween 20. The filters were probed sequentially with primary and secondary antisera in blocking solution. An anti-dog IgG or anti-rabbit IgG peroxidase immunoconjugate (Nordic Immunologic, Tilburg, The Netherlands) was used as secondary antibody and the specific binding was revealed with the Western blotting ECL[®] detection system (Amersham).

Nuclear run-on assays

The nuclei, prepared as indicated above and stored at -70°C , were thawed by mixing with a volume of $2 \times$ transcription buffer [0.1 M Hepes, pH 7.5, 0.2 M KCl, 8 mM dithiothreitol, 60 μM EDTA, 2 mM ATP, 1 mM CTP, 1 mM GTP, 17.6 mM creatine phosphate and 80 $\mu\text{g} \cdot \text{ml}^{-1}$ creatine kinase (Boehringer Mannheim, Mannheim, Germany)]. The run-on transcripts were labelled by addition of 100 μCi of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (3000 Ci $\cdot \text{mmol}^{-1}$; Amersham) for 10 min at 26°C . The reaction was stopped by addition of DNase I (RNase-free; Boehringer Mannheim) and MgCl_2 to final concentrations of 25 $\mu\text{g} \cdot \text{ml}^{-1}$ and 5 mM, respectively, and incubated at 37°C for 20 min. Subsequently, the reaction continued in the presence of 0.15 $\mu\text{g} \cdot \text{ml}^{-1}$ proteinase K, 0.5 % (w/v) SDS and 5 mM EDTA for 20 min at 37°C . The radiolabelled nascent RNA was extracted by phenol/chloroform, and the non-incorporated isotopes were separated from the labelled product on a Sephadex G-50 column.

Samples (5 μg) of each plasmid to be tested were linearized, denatured and applied on to Zeta-probe membranes (Bio-Rad, Hercules, CA, U.S.A.) using a vacuum slot-blot apparatus. The membrane was then subjected to hybridization with the purified labelled RNA $[(2-6) \times 10^6$ c.p.m. $\cdot \text{ml}^{-1}]$ in a solution containing 50 % (v/v) formamide, $6 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1 % (w/v) SDS and 0.25 $\text{mg} \cdot \text{ml}^{-1}$ herring sperm DNA at 42°C for 3 days. Filters were washed at room temperature for 15 min in $2 \times$ SSC, followed by a wash at 65°C for 30 min in $2 \times$ SSC, and a final wash at 37°C for 20 min in $2 \times$ SSC containing 10 $\mu\text{g} \cdot \text{ml}^{-1}$ RNase A.

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

Inhibition of DNA synthesis by HU was estimated by measuring [*methyl*-³H]thymidine incorporation into DNA. For this purpose, parasites in the logarithmic phase of growth (5×10^6 promastigotes $\cdot$ ml⁻¹) were incubated in the presence of 10μ Ci $\cdot$ ml⁻¹ [*methyl*-³H]thymidine (2.0 Ci $\cdot$ mmol⁻¹; Amersham) with 5 mM HU. An equivalent (control) culture was incubated under identical conditions except that HU was absent. At the indicated periods of time, aliquots of 100 μ l from each one of the cultures (treated and untreated) were removed and the thymidine incorporation was determined using the MultiScreen Assay System (Millipore, Molsheim, France) following the manufacturer's instructions.

The recovery of the DNA synthesis after removal of HU was estimated also by measuring [*methyl*-³H]thymidine incorporation into DNA. For this purpose, parasites in the logarithmic phase of growth (3×10^6 promastigotes $\cdot$ ml⁻¹) were treated with 5 mM HU during 24 h. Afterwards, parasites were pelleted, washed twice with PBS and resuspended in culture medium without the drug. At the indicated periods of time, 1-ml aliquots were taken and incubated in the presence of 10μ Ci of [*methyl*-³H]thymidine for 2 h. Thymidine incorporation into DNA was determined using the MultiScreen Assay System.

Protein labelling

Protein labelling was performed on *L. infantum* promastigote cultures growing logarithmically. Aliquots (10 ml) were pelleted, washed twice with PBS and resuspended in 100 μ l of Dulbecco's modified Eagle's medium lacking methionine and cysteine. For labelling, the Pro-mixTM ³⁵S *in vitro* cell-labelling mixture, containing L-[³⁵S]methionine and L-[³⁵S]cysteine (1 mCi $\cdot$ ml⁻¹; 1000 Ci $\cdot$ mmol⁻¹), was added and the culture was incubated for 60 min at 26 °C. For those aliquots incubated in the presence of HU, the labelling medium was also supplemented with 5 mM HU. After labelling, the parasites were pelleted, washed and treated as indicated above for nuclei extraction.

Northern blotting

L. infantum RNA was isolated as described previously [19]. Total RNA was separated according to size on 1% agarose/formaldehyde gels [20] and electrotransferred on to nylon membranes using an LKB system (Pharmacia AB). Hybridization conditions were as described elsewhere [11].

Quantitative analysis

The autoradiographs were scanned with a laser densitometer (Image QuantTM version 3.0; Molecular Dynamics). Measurements were performed under conditions in which a linear correlation existed between the amount of proteins or RNAs and the band intensity on the autoradiographs.

RESULTS

Effects of HU treatment on *Leishmania* DNA replication

The inhibition of DNA synthesis by drugs such as HU has been employed to analyse regulation of histone gene expression and to demonstrate that synthesis of most histones is tightly coupled to DNA synthesis, which by definition occurs in the S phase [21]. HU inhibits ribonucleotide reductase, leading to depletion of deoxyribonucleotide pools and, therefore, to inhibition of DNA

synthesis. However, although the drug has been used to inhibit DNA synthesis in several Kinetoplastids the results obtained have been markedly different among them. For example, the addition of moderate amounts (7.5μ g $\cdot$ ml⁻¹) of HU to *T. brucei* cultures did not inhibit DNA synthesis, but arrested the cells after duplicating both the kinetoplast and the nucleus [22]. In contrast, the treatment of *T. cruzi* cultures with 20 mM HU induced synchrony of the DNA synthetic cycle without adversely affecting the cells, as judged by oxygen consumption, RNA and protein content [23]. In both *L. major* and *L. donovani* promastigotes, DNA but not RNA synthesis is inhibited (50% inhibition) by 0.5 mM HU [24]. It was found, furthermore, that the inhibition of DNA synthesis by HU is reversible when the inhibitor is removed 2, 4 or 24 h after its addition. The recent cloning of the gene coding for the small subunit (M₂) of *Leishmania* ribonucleotide reductase has allowed, by functional analysis, the demonstration that this protein is the main target of HU in *Leishmania* [25]. To inhibit DNA synthesis, we and others used treatments of 5 mM HU, obtaining rates of DNA inhibition around 90% in *L. infantum* [11] and *L. enriettii* [13].

As a first step in the present work, we considered it of interest to test the possible toxicity of the HU treatments to the *L. infantum* promastigotes. As shown in Figure 1(A), after 24 h of treatment with 5 mM HU, the DNA synthesis in promastigotes was reduced to 12% of the level of DNA synthesis measured in the absence of the drug (control cultures). Remarkably, the promastigotes were capable of reactivating DNA synthesis after removal of the drug as an indication that the process was reversible and that the promastigotes can resume DNA synthesis after a blockade of up to 24 h with this inhibitor (Figure 1B). Thus our data validate the use of 5 mM HU to achieve an efficient inhibition of DNA synthesis in *L. infantum*, since this treatment did not adversely affect the parasite.

It was also interesting to note that after removal of the drug the rate of [*methyl*-³H]thymidine incorporation increased, reaching at 6 h a value 5-fold higher than that of the untreated control cultures (Figure 1B). The existence of a peak of DNA synthesis suggests that the drug induced a degree of synchrony. However, by 24 h synchrony had decayed and the population could not be distinguished from untreated cultures. Therefore, present data indicate that HU induced synchrony in *L. infantum* promastigotes, but this synchronization is not distinguished in the next cell cycle. It must be noted that a similar HU-induced synchrony has been observed in the related Kinetoplastid *T. cruzi* [23].

Effect of HU treatments on *Leishmania* histone gene transcription

Previous results indicated that the HU treatment of promastigote cultures of *L. enriettii* [13] and *L. infantum* [11,14] failed to reduce the levels of the core-histone mRNAs. Although a stabilization of the histone transcripts by this treatment cannot be excluded, the finding was taken as an indication that transcription of *Leishmania* histone genes is not affected by the inhibition of DNA synthesis. In order to analyse whether or not the histone gene transcription is coupled to DNA synthesis, run-on studies were carried out with nuclei isolated from promastigote cultures after HU treatment for 0, 1, 2, and 3 h (Figure 2). A quantitative analysis, shown in Table 1, indicated that the transcription rates of the four histone genes (H2A, H2B, H3 and H4) were similar either in the absence or in the presence of HU. Thus contrary to most organisms, both prokaryotic (having histone-like proteins) and eukaryotic [5,26], in *Leishmania* the blockade of DNA replication does not down-regulate the transcription of histone genes.

Figure 1 Inhibition of DNA synthesis by HU in *L. infantum* promastigotes

(A) Kinetics of DNA-synthesis inhibition by HU. The percentage of DNA synthesis inhibition was estimated by measuring [*methy*-³H]thymidine incorporation into DNA in HU-treated cultures relative to untreated cultures. The data points shown are the means $\pm$ S.D. of triplicate samples. (B) Recovery of DNA synthesis after HU treatment. After 24 h of HU treatment, and washout of the drug, incorporation of [*methy*-³H]thymidine into DNA ($\blacklozenge$) was measured over a period of 24 h. The vertical dashed line indicates the time of the drug elimination. In each point, the cell number ($\blacksquare$) was also determined. Data represent the means $\pm$ S.D. from three separate experiments.

Figure 2 Analysis of histone gene transcription by nuclear run-on assays

L. infantum promastigotes were cultured either in the absence (0) or in the presence of HU for 1, 2 or 3 h. Afterwards, run-on assays were performed and the nascent transcripts, labelled with [³²P]UTP, were hybridized to slot-blots containing 5 μ g of DNA from the following clones: cl72 (*L. infantum* histone H2A cDNA [33]), LiH2B (*L. infantum* histone H2B cDNA [16]), LiB6 (*L. infantum* histone H3 cDNA [34]), LiH4-1 (*L. infantum* histone H4 cDNA [14]), pTc α 3 (*T. cruzi* α -tubulin cDNA [35]), pRIB (*L. infantum* 24S α rRNA [36]) and pUC18 (control plasmid). All clones were linearized by a *Sal*I digestion except the pRIB clone, which was linearized with *Eco*RI.

Effect of HU treatments on *Leishmania* histone mRNA translation

Considering the role of histones in packaging DNA into chromatin, it seemed reasonable to assume that the synthesis of histones must be tightly linked to DNA synthesis. However, whereas in most eukaryotic cells an arrest of DNA synthesis by aphidicolin or HU results in a dramatic drop in histone mRNA levels

Table 1 Effect of HU treatment on transcription rates of histone genes

The transcription rates of the four core histone genes were quantified by densitometric scanning of autoradiographs of run-on assays carried out as indicated in Figure 2. The transcription levels for each gene were standardized to the transcription rate of the rRNA genes (taken arbitrarily as 1). The data are presented as means $\pm$ S.D. from three separate experiments.

Probe	HU treatment ...	Transcription rate			
		None	1 h	2 h	3 h
H2A		0.116 $\pm$ 0.081	0.091 $\pm$ 0.046	0.057 $\pm$ 0.042	0.050 $\pm$ 0.012
H2B		0.136 $\pm$ 0.084	0.103 $\pm$ 0.095	0.067 $\pm$ 0.047	0.061 $\pm$ 0.011
H3		0.117 $\pm$ 0.082	0.094 $\pm$ 0.047	0.057 $\pm$ 0.042	0.065 $\pm$ 0.011
H4		0.215 $\pm$ 0.106	0.194 $\pm$ 0.122	0.125 $\pm$ 0.064	0.123 $\pm$ 0.016
α -Tubulin		0.227 $\pm$ 0.111	0.392 $\pm$ 0.110	0.290 $\pm$ 0.098	0.250 $\pm$ 0.042
rRNA		1	1	1	1

Figure 3 Effect of HU treatment on histone synthesis

[³⁵S]-Labelled nuclear proteins (20 μ g) from either untreated *L. infantum* promastigotes (lanes 0) or treated with 5 mM HU for 1 h (lanes 1), 2 h (lanes 2) or 3 h (lanes 3), were electrophoresed on linear 10–14% gradient SDS/PAGE gels. (A) Coomassie Brilliant Blue staining of the gel. (B) Autoradiographic exposure of the gel. Arrows indicate the positions of *L. infantum* histones. Molecular-mass markers (M) are shown in kDa.

mediated by both transcriptional blockade and increased mRNA turnover, our results indicate that in *L. infantum* neither transcription of histone genes (Figure 2) nor the histone transcript amounts [11,14] decreased upon inhibition of DNA synthesis. Thus in that context we considered it of interest to analyse the effect of HU treatment on the rate of the synthesis of the histone proteins. For that purpose, *Leishmania* proteins were labelled by incubating promastigotes with L-[³⁵S]methionine and L-[³⁵S]cysteine during HU treatment. After labelling, preparations of nuclear proteins were analysed in linear 10–14% gradient SDS/PAGE gels (Figure 3). As revealed by the Coomassie Brilliant Blue staining of the gels (Figure 3A), similar patterns of proteins were present in either control or HU-treated cultures. Remarkably, the autoradiograph of the gel showed that along the HU treatment there was a decrease in the intensity of the radioactive label of the two prominent bands believed to correspond to histones (Figure 3B). Densitometric measurements indicated that the labelling of those bands decreased 4-fold after 3 h of HU treatment.

Figure 4 Immunological identification of *L. infantum* histones

(A) Nuclear proteins (20 μ g) from untreated *L. infantum* promastigotes (lanes 0) or treated with 5 mM HU for 1 h (lanes 1), 2 h (lanes 2) or 3 h (lanes 3), were electrophoresed on two linear 10–14% gradient SDS/PAGE gels and electrophoretically transferred on to nitrocellulose filters. Equivalent blots were incubated either with a mixture of viscerocutaneous leishmaniasis sera (VCL sera) or with an anti-H2A antibody purified by affinity chromatography from a polyclonal serum obtained from a rabbit immunized with the recombinant *L. infantum* histone H2A protein. Arrows indicate the positions of *L. infantum* histones. Molecular-mass markers (M) are shown in kDa. (B) Western-blot analysis of nuclear (lanes N) and cytoplasmic (lanes C) protein fractions from *L. infantum* promastigotes either untreated (lanes 0) or incubated with 5 mM HU for 3 h (lanes 3). The blot was incubated with the affinity-purified anti-histone H2A antibody.

Figure 6 Effect of HU removal on histone synthesis

35 S-labelled nuclear proteins (20 μ g) from *L. infantum* promastigotes treated with 5 mM HU (lanes 0) for 24 h or from promastigotes further grown, after removal of the drug, for 1 h (lanes 1), 2 h (lanes 2) and 3 h (lanes 3), were electrophoresed on linear 10–14% gradient SDS/PAGE gels. (A) Coomassie Brilliant Blue staining of the gel. (B) Autoradiographic exposure of the gel. Arrows indicate the positions of *L. infantum* histones. Molecular-mass markers (M) are shown in kDa.

Figure 5 Specificity of the anti-H2A antibody

Nuclear proteins (20 μ g per lane) from *L. infantum* promastigotes were electrophoresed on two linear 10–14% gradient SDS/PAGE gels and either stained with Coomassie Brilliant Blue (N) or transferred electrophoretically on to nitrocellulose filters (blots 1–3). The anti-H2A polyclonal serum (incubated with blot 1) was passed through a column (containing recombinant histone H2A bound to Sepharose 4B), the flow-through fraction was incubated with blot 2, and the bound fraction, after elution, was incubated with blot 3. See the Materials and methods section for a more detailed description of the purification process.

In order to demonstrate accurately that those two bands were corresponding to the core histones, a similar gel was electro-transferred on to a nitrocellulose membrane and incubated with a mixture of sera obtained from dogs naturally infected with *L. infantum*. It is known that during *L. infantum* infection, dogs elicit a strong humoral response against the four core histones from the parasite [16]. As expected, the canine antibodies reacted strongly with the same protein bands that showed a decreased

labelling during HU treatment (Figure 4A), thus demonstrating that these two bands represent the core histones of the parasite. In addition, a similar blot was incubated with an anti-H2A antibody purified by affinity chromatography from the serum of a rabbit inoculated with the *L. infantum* recombinant histone H2A. The specificity of the binding reaction of this antibody was demonstrated as illustrated in Figure 5. This antibody reacted specifically with the upper band, indicating that this band contained the parasite histone H2A (Figure 4A).

Given that a remarkable decrease of the newly synthesized nuclear histones was observed, we analysed the possibility that this decrease could be due to an accumulation of these proteins in the cytoplasm of HU-treated parasites. However, as shown in Figure 4(B), no cytoplasmic histone H2A was detected in either treated or untreated parasites. In this Figure, 3-h treatment with HU is shown, but we did not detect cytoplasmic histones with shorter (1 and 2 h) or longer (4, 6 and 8 h) HU-treatments (results not shown). Altogether, these results suggested the existence of a control mechanism in *Leishmania* that, after a blockade of DNA synthesis, interrupts the synthesis of histones, probably through a specific translational blocking of the existing histone mRNAs. However, as an alternative explanation, a rapid degradation of the newly synthesized histones cannot be completely discarded.

Effects of HU removal on the *Leishmania* histone synthesis

Given that after removal of HU a rapid DNA synthesis was detected (Figure 1B), we decided to analyse the effect on histone protein synthesis at that moment. For that purpose, a promastigote culture was treated during 24 h with 5 mM HU. Afterwards, one aliquot was taken and the parasite proteins were labelled metabolically for 1 h in the presence of the drug. The remaining culture was allowed to grow in the absence of HU for 1, 2 or 3 h. The labelling of the newly synthesized proteins was performed during the last hour of incubation. After nuclei were extracted, samples of similar c.p.m. values were loaded on to

linear 10–14% gradient SDS/PAGE gels (Figure 6A). The densitometric analysis of the corresponding autoradiograph of the gel (Figure 6B) showed an 8-fold increase in the labelling of the two bands corresponding to the parasite histones, indicating that after removal of the drug a rapid recovery of the histone synthesis occurs.

Finally, we also analysed whether or not this increase in histone synthesis observed after removal of HU is linked to an increase in the steady-state levels of histone mRNAs (Figure 7). A densitometric analysis indicated that the levels of histone transcripts did not increase after removal of HU (Table 2). In fact, a slight decrease (about 2-fold) in the mRNAs for the four histones was observed 1 h after drug removal relative to the levels found in the HU-treated parasites. In conclusion, these data demonstrated that the increase in histone synthesis observed after HU removal (Figure 6) is not linked to an increase in the steady-state levels of histone mRNAs. Therefore, the data reinforced the hypothesis that histone synthesis in *L. infantum* is coupled to DNA synthesis by a mechanism that operates at the translational level.

DISCUSSION

Histone biosynthesis in eukaryotes is one of the best-studied examples of cell-cycle-regulated genes, expression of which is linked tightly to cellular DNA replication. Multiple levels of control are involved in restricting the synthesis of histones to the S phase of the cell cycle, mainly operating on transcription initiation, processing of pre-mRNA, and degradation of mRNA [5–7]. Two general approaches have been taken to study histone expression during the cell cycle: (i) synchronization of the cell cycle, and (ii) use of inhibitors of DNA synthesis. In the present work, we have used HU as specific inhibitor of DNA synthesis in *Leishmania* [24]. Our data indicated that, in the presence of 5 mM HU, the incorporation of [*methyl*-³H]thymidine into DNA is reduced to 10%. Also, it was found that the inhibition of DNA synthesis by HU was reversible, indicating that this treatment did not adversely affect the *Leishmania* cells. In previous works, we and others reported that inhibition of DNA synthesis in *Leishmania* does not induce a decrease in the steady-state abundance of histone mRNAs [11,13,14]. In agreement with these findings, here we report that the transcriptional rates of the *L. infantum* histone genes are not affected by inhibition of DNA synthesis. However, considering the role of histone proteins in packaging DNA into chromatin, it is expected that histone synthesis might be linked tightly to DNA synthesis. Therefore, we addressed the possibility that histone synthesis could be coupled to DNA synthesis by a mechanism operating at the translational level. In fact, the presented data indicate that, after 1 h of HU treatment, the levels of newly synthesized histones decrease dramatically and that after 2 h only a basal expression of *de novo* synthesized histones could be observed. Interestingly, the removal of the drug induced a rapid recovery of the synthesis of the histones, concomitant with a reactivating of the DNA replication. Altogether, these data demonstrate that, when in *Leishmania* DNA synthesis is arrested artificially, a feedback mechanism acts at the level of translation to avoid overproduction of histone proteins from the pre-existing histone mRNAs.

Although translational control is not a usual regulatory pathway of histone gene expression, some exceptions to this rule have been described. Translational regulation is a mechanism typically associated with expression of maternal mRNAs, which are synthesized during oogenesis and stored in the oocyte in a non-translated state until they are activated at early embryonic

Figure 7 Effect of the DNA-synthesis recovery on histone H2A RNA level

Northern-blot analysis of total RNA samples (10 µg) from promastigotes treated with 5 mM HU for 24 h (lane 1) or from 24-h HU-treated promastigotes that were incubated, after removal of the drug, for a further period of time: 1 h (lanes 2), 2 h (lanes 3), and 3 h (lanes 4). The blot was hybridized sequentially with probes for the four classes of histone genes (H2A, H2B, H3 and H4; see Figure 1 for probe descriptions), and a *T. cruzi* α-tubulin gene probe [35]. The ethidium bromide staining of the corresponding gel is also shown (rRNA).

Table 2 Effect of HU removal on histone RNA levels

Steady-state levels of the histone mRNAs were quantified by densitometric scanning of the autoradiographs shown in Figure 7. The RNA levels for each histone were normalized against the α-tubulin RNA level.

Histone mRNA	Time after HU removal ...	RNA level			
		0 h	1 h	2 h	3 h
H2A		0.73	0.38	0.48	0.37
H2B		1.26	0.65	1.15	1.04
H3		1.40	0.83	1.22	1.20
H4		0.90	0.50	0.83	0.67

development (see [27] for a review). A known example of this type of regulation is histone expression in *Xenopus* oocytes: translationally inactive histone mRNAs are stored in the frog oocytes, and translation is activated at oocyte maturation [28]. Also, translation regulation of histone mRNA has been described in *Physarum polycephalum* [29,30]. In this plasmodium, although histone mRNA begins to accumulate 3 h before the S phase, histone synthesis is limited to the S phase. During the G2 phase, histone H4 mRNAs are transported to the cytoplasm where they are stored and stabilized as translationally inactive ribonucleoprotein particles. Another example of translational regulation of histone gene expression has been recently reported to occur in *Nicotiana tabacum* [31]. The inhibition of DNA synthesis in tobacco cells by aphidicolin, an inhibitor of DNA polymerase α, results in the accumulation of H3 and H4 histone mRNAs. Under these conditions, although the histone mRNA amount is high, the amount of newly synthesized histone protein does not increase.

To our knowledge, this is the first report in which a translational mechanism has been implicated in the regulation of gene expression in *Leishmania*; a mechanism that, however, is

within the general rule of gene expression in trypanosomes in which the control occurs primarily at the post-transcriptional level (reviewed in [32]). A regulatory model operating at the translational level would implicate that the non-translating mRNAs must be 'masked' from the general translational apparatus by the binding of specific proteins. Also, such a mechanism would involve the existence of *cis*-regulatory signals in the 5'- or 3'-untranslated regions of the mRNAs where specific RNA-binding proteins are bound [28]. Therefore, it would now be of great interest to identify the proteins associated with the histone mRNAs, and the specific sequences of histone mRNAs involved, to understand how the blockade of DNA synthesis is detected by *Leishmania* parasites and how they arrest the translation of histone transcripts.

This work was supported through grants from Comunidad Autónoma de Madrid (08.2/0021/1998) and Comisión Interministerial de Ciencia y Tecnología (98-0064). An institutional grant from Fundación Ramón Areces is also acknowledged.

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Received 12 July 1999/25 October 1999; accepted 25 November 1999