Leishmania infantum possesses a complex family of histone H2A genes: structural characterization and analysis of expression

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SUMMARY

We have studied the genomic organization and transcription of the histone H2A genes in the protozoan parasite *Leishmania infantum*. In the parasite genome 2 gene clusters exist, each containing 3 *H2A* gene copies. Sequence analyses† showed the existence of significant sequence divergence among the *H2A* genes, mainly in their 5′- and 3′-untranslated regions (UTRs). Also, the existence of allelic alternatives has been evidenced. Based on the divergence in the 3′UTR regions, we have defined 3 classes of *H2A* transcripts, which are present at different levels in *L. infantum* promastigotes. However, transcription of the 3 classes of *H2A* genes occurs at similar levels, as measured by nuclear run-on assays, indicating that their abundance is regulated post-transcriptionally. Also, differences in regulation were observed among the *H2A* transcripts: the levels of transcripts with 3′-UTR type I and type III are affected by growth phase whereas transcripts with 3′-UTR type II, that are barely detected, remain constant. It is likely that the complexity, in both gene organization and differential expression exhibited by the *L. infantum H2A* genes, is imposed by the nature of the post-transcriptional mechanisms of regulation operating in this parasite.

Key words: *Leishmania infantum*, multi-gene family, differential gene expression, post-transcriptional regulation, allelic polymorphism.

INTRODUCTION

Histones are structural proteins that play an important role in DNA organization and gene regulation in eukaryotes. As a reflection of their fundamental and universal roles in chromatin architecture, histones are among the most conserved proteins (Thatcher & Gorovsky, 1994). Nucleosomes, the basic structural units of chromatin, are constituted by DNA wrapped around a protein core containing 2 molecules of each of the 4 core histones (i.e. H2A, H2B, H3 and H4). Two classes of core histone genes are found in most eukaryotes. The most frequent class contains the replication-dependent histone genes whose expression is cell cycle-regulated, whereas a second, less abundant class includes the genes that encode the histone variants, whose expression occurs at a basal level throughout the cell cycle (Osley, 1991). Multiple mechanisms of control are involved in restricting the synthesis of histones to S phase of the cell cycle. The main mechanisms act at the levels of transcription, pre-mRNA processing, and mRNA stability and their combined effect is the regulation of the histone mRNA abundance (Osley, 1991; Stein *et al*. 1994).

Phylogenetic analyses suggest that trypanosomes diverged early from the eukaryotic lineage (Sogin, Elwood & Gunderson, 1986) and several novel molecular and biochemical features of trypanosomatids also appear to reflect this divergence (Donelson, Gardner & El-Sayed, 1999). In trypanosomatids, almost all protein-coding genes are transcribed as polycistrons, and *trans*-splicing leads to the addition of a common 39-nucleotide spliced leader sequence to the 5′ end of every mRNA. Consequently, most of the genes appear to be transcribed constitutively, suggesting the need for a significant regulation of mRNA and/or protein abundance at the post-transcriptional level (Stiles *et al*. 1999). Histone genes in trypanosomatids constitute excellent models for the study of regulatory pathways of gene expression in these unusual eukaryotes. During the last decade the sequences of genes coding for core histones from the genera *Trypanosoma* and *Leishmania* have been deciphered (Genske *et al*. 1991; Soto *et al*. 1992, 1994, 1997; García-Salcedo *et al*. 1994; Puerta *et al*. 1994; Bontempi *et al*. 1994; Lukes & Maslov, 2000), showing that this family of protozoa possesses the most divergent histones described to date (reviewed by Galanti *et al*. 1998). Analyses of histone gene expression point to the existence of similarities and differences between the two genera. With the exception of the lack of transcription of histone

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genes in the non-replicative forms of *T. cruzi*, in which a transcriptional regulation has been described (Marañón *et al*. 1998), the regulation in both the replicative forms of *T. cruzi* and *Leishmania* promastigotes is exerted at post-transcriptional levels. Nevertheless, while in *Trypanosoma* the abundance of histone mRNAs is temporally coupled to DNA synthesis, suggesting a cell-cycle regulation (García-Salcedo *et al*. 1994; Ersfeld *et al*. 1996; Marañón *et al*. 1998, 2000; García-Salcedo, Gijón & Pays, 1999; Recinos, Kirchhoff & Donelson, 2001), the *Leishmania* histone mRNAs do not decrease in abundance after treatments with inhibitors of DNA synthesis (Genske *et al*. 1991; Soto *et al*. 1996, 1997, 2000). Interestingly, the expression of histone genes seems to be linked to DNA replication at the translational level in *Leishmania* (Soto *et al*. 2000).

We realized that studies on the number and organization of genes coding for the core histones in *Leishmania* were incomplete when we were planning an extended analysis of the sequences responsible for regulation of histone expression. It was known that each one of the *Leishmania* histones, H2A (Soto *et al*. 1992), H2B (Genske *et al*. 1991), H3 (Soto *et al*. 1996) and H4 (Soto *et al*. 1997), are coded by multiple genes. Furthermore, genes for H2A, H2B and H3 have been found to be distributed in more than one chromosome (Soto *et al*. 1995; Wincker *et al*. 1996). In this study, we have carried out a complete characterization of the number and organization of genes coding for histone H2A in *L. infantum*. We also have determined the sequence, transcription levels and mRNA abundance for the different classes of *H2A* genes.

MATERIALS AND METHODS

Culture conditions

Promastigotes of *L. infantum* (MCAN/ES/96/BCN150) were grown at 26 °C in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% (v/v) heat-inactivated foetal calf serum (Flow Laboratories, UK). Experimental cultures were initiated at 1×10^6 promastigotes/ml and harvested for study in either the logarithmic phase of growth ($5–8 \times 10^6$ promastigotes/ml) or the stationary phase of growth (2×10^7 promastigotes/ml).

Isolation and sequence of H2A *genes*

A genomic library, constructed in the vector EMBL-3 by using partial *Sau*3AI-digested *L. infantum* DNA (Soto *et al*. 1993), was screened with clone cL71 (Soto *et al*. 1992). Three positive phages were isolated and named H2Ag2, H2Ag6 and H2Ag7. The *Sal*I-restriction fragments of 5·2 kb (from clone H2Ag2), 5·8 kb (from H2Ag6), and 9 kb (from H2Ag7) were cloned into the pBluescript plasmid, and the resulting clones were termed pSalI-5·2, pSalI-5·8 and pSalI-9, respectively.

Additional subcloning of the pSalI-9 clone was carried out by *Bam*HI + *Sal*I double digestions, resulting in the subclones pB5·4 (5·4 kb *Bam*HI fragment), pSB2·3 (a 2·3 kb *Sal*I–*Bam*HI fragment), and pBS1·4 (an 1·4 kb *Bam*HI–*Sal*I fragment). Clone pB5·4 was completely sequenced using additional subclones and synthetic oligonucleotides. DNA sequences were determined using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA).

The 2·3 kb *Sal*I–*Bam*HI and 0·5 kb *Hin*dIII–*Sal*I fragments from clone pSalI-5·8 were sequenced. Clone pSalI-5·2 was completely sequenced using subclones and synthetic oligonucleotides.

Probes used for detection of H2A *RNAs*

Clone LiH2A-Ct-Q (Soto *et al*. 1998), which codes for the carboxyl-terminal 67 amino acids of the *L. infantum* histone H2A, was used as an H2A coding probe (CDS). Probes specific for the 3 types of 3′UTRs were generated by PCR amplification. The 3′UTR-I was amplified from clone cL71 using the following primers: sense, 5′-CGGGATCCGT CCTCCGGCCT GACAGCGC-3′ (restriction sites included for cloning purposes are underlined), and antisense, 5′-CGGGATCCGT CCGAGCCTAC CTGACTC-3′. The amplification product was digested with *Bam*HI and cloned into pBluescript. The 3′UTR-II was amplified using the following oligonucleotides: sense, 5′-CCATCGATAG ATA-CCCTTTG GAAGGTTC-3′, and antisense, 5′ GCGTCGACGC ACGCGCACAC ACAGAG-GT-3′. The PCR product was digested with *Cla*I and *Sal*I, and cloned into pBluescript. Finally, the 3′UTR-III was amplified using the following oligonucleotides as primers: sense, 5′-CCATCGATAG AGCACACCTA CCCCTCTC-3′, and antisense, 5′-GCGTCGACAA CATCACTGAG ACAACG-AG-3′. The amplification product was digested with *Cla*I and *Sal*I, and cloned into pBluescript.

Nucleic acid isolation and analyses

L. infantum DNA and RNA were isolated as described previously (Chomczynsk & Sacchi, 1987; Requena *et al*. 1988). Total DNA was digested with different restriction enzymes, electrophoresed on 0·8% agarose gels and transferred to nylon membranes (Hybond-N, Amersham Corp.) by standard methods (Sambrook, Fritsch & Maniatis, 1989). Total RNA was size-separated on 1% agarose–formaldehyde gels (Lerach *et al*. 1977) and electrophoresed on to nylon membranes using an LKB system (Pharmacia Biotech Inc.).

DNA probes were labelled by nick translation (Sambrook *et al*. 1989). Hybridizations, either for

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DNA or RNA, were performed in 50% formamide, 6 × SSC, 0·1% SDS, and 0·25 mg/ml herring sperm DNA at 42 °C overnight. Final post-hybridization washes were performed in 0·1 × SSC, 0·2% SDS at 50 °C for 1 h. For re-use, blots were treated with 0·1% SDS for 15 min at 95 °C to remove the previously hybridized probes.

Nuclease S1 protection assays

The following oligonucleotides, complementary to specific regions of *H2A* mRNAs were used: no. CDS, 5′-ACGGTGCGCG GGTTCAGGCG-3′ (specific for a coding sequence common to all *H2A* mRNAs); no. 3′UTR-I, 5′-ACGGTGCGCG GGTTCAGGCG-3′ (specific sequence of the 3′UTR-I); no. 3′UTR-II, 5′-CGACTCCCCG TGCGACTCTG-3′ (specific sequence of the 3′UTR-II); and no. 3′UTR-III, 5′-CAGCAACGCA CACACTCCCC-3′ (specific sequence of the 3′UTR-III). Oligonucleotides were labelled at the 5′ end using T4 polynucleotide kinase and gamma-[^{32}P]ATP as described (Quijada *et al*. 1997*a*). The oligonucleotides, in molar excess (0·07 pmol), were mixed with 2 μg of total RNA from logarithmic phase promastigotes and nuclease S1 protection experiments were performed as described elsewhere (Soto *et al*. 1993). Protection products were analysed on 15% polyacrylamide–7 M urea gels. Autoradiographs were scanned with a densitometer (Image Quant^{TM} version 3.0; Molecular Dynamics), and the relative densities of the bands were determined.

Determination of steady-state levels of specific transcripts using probes generated by reverse transcription

Probes were generated by reverse transcription (RT) of 2 μg of poly-A(+) RNA. In the priming step, poly-A(+) RNA was mixed with 2 μg of oligo-dT primer (5′-CGGAATTCT$_{18}$-3′) in a total volume of 10 μl. The mixture was placed at 70 °C for 10 min and then chilled on ice. For elongation, the following components were added: 6 μl of 5 × First Strand Buffer (Life Technologies); 1 μl 0·1 M DTT; 1·5 μl of a mixture containing dATP, dGTP and dTTP at 20 mM each; 1·5 μl of Reverse Transcriptase (SuperScript^{TM} II RNase H^{-}, Life Technologies, Cat. no. 18064-022); 10 μl of α-^{32}P-dCTP (10 mCi/ml, 3000 Ci/mmol; Amersham Pharmacia Biotech). The mixture was incubated for 90 min at 37 °C. The radio-isotope labelled cDNA was separated from the unincorporated dNTPs by chromatography on a Sephadex G-50 column (Sambrook *et al*. 1989).

Samples (5 μg) of the plasmids to be tested were linearized, denatured and applied onto Zeta-probe membranes (Bio-Rad) using a vacuum slot-blot apparatus. The membrane was then subjected to hybridization with the RT probe, following the hybridization conditions indicated above. Analysis of intensity of hybridization to individual slot blots was performed using a Phosphorimager (Molecular Dynamics).

Nuclear run-on assays

Nuclei were isolated from promastigotes in the logarithmic growth phase and used to run-on transcription assays following methodologies described elsewhere (Quijada *et al*. 1997*b*).

Use of the PCR to map the 5′UTRs of H2A transcripts

Reverse transcription (RT) of total promastigote RNA was performed using the MuLV reverse transcriptase (Perkin Elmer) and the oligonucleotide 5′-CGGAATTCT$_{18}$-3′ designed to prime from the poly(A) tail. The RT product was amplified by PCR using Taq DNA polymerase (Roche Diagnostics) and the following oligonucleotides: forward primer, 5′-GCTATATAAG TATCAGTTTC TGTAC-3′ (specific sequence of the *Leishmania* spliced leader), and reverse primer, 5′-GCTCCGCCGT CAGGTACTC-3′ (reverse and complementary to a coding sequence common to all *H2A* mRNAs). The amplification product was cloned into vector pSTBlue1 using the Perfectly blunt cloning kit (Novagen).

RESULTS

Genomic organization of the L. infantum *genes coding for histone H2A*

In order to characterize the organization of the *L. infantum H2A* genes, a genomic library was screened with the cL71 probe, a cDNA coding for the *L. infantum* H2A histone (Soto *et al*. 1992). Three phages were isolated and after characterization by restriction and hybridization analyses, it was deduced that they derived from 2 different genetic loci (Fig. 1). Restriction fragments, which hybridized with the cL71 probe, were subcloned and completely sequenced. The restriction and sequence analyses demonstrated that phages H2Ag6 and H2Ag7 derived from the same locus (named locus 1) while the phage H2Ag2 should be derived from a different locus (named locus 2). Each locus was found to be composed of 3 histone H2A genes (Fig. 1). Following the genetic nomenclature for *Trypanosoma* and *Leishmania*, proposed by Clayton *et al*. (1998), the 6 genes were named: *H2A1*, *H2A2* and *H2A3* for those located in locus 1; and *H2A4*, *H2A5* and *H2A6* for those located in locus 2 (Fig. 1). The existence of some sequence differences between phages H2Ag7 and H2Ag6 (see below) was interpreted as allelic divergence and the corresponding genes considered as allelic copies were named, consequently, *H2A1-1* and *H2A1-2* (Fig. 1).

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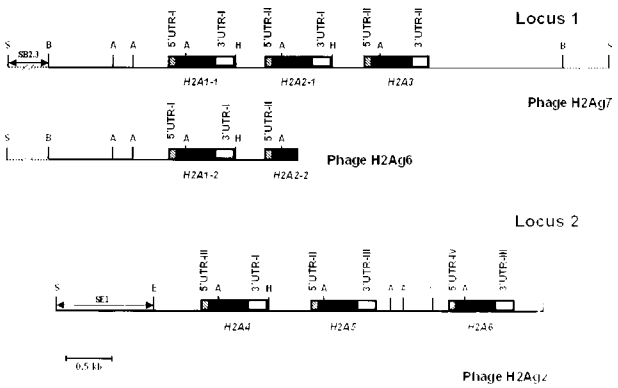

Fig. 1. Schematic representation of the *Leishmania infantum H2A* loci. The locations of the *H2A* genes are depicted as closed boxes, in which 5′-UTRs (cross-hatched areas), coding regions (black areas) and 3′-UTRs (blank areas) are indicated. The names of the genes are indicated below the map. The locations of the different 5′-UTRs (I–IV) and 3′-UTRs (I–III) are indicated on the physical maps. The restriction fragments used as probes in subsequent Southern blot analyses are shown above lines with open arrow-heads. Regions in which nucleotide sequences have not been completely determined are denoted with dotted lines. The restriction sites for *Sal*I (S), *Bam*HI (B), *Sac*II (A), *Hin*dIII (H) and *Sac*I (C) are shown.

The genomic organization of the *H2A* genes in *L. infantum* was analysed by Southern blot. Genomic DNA was digested with several restriction enzymes and hybridized with several probes (Fig. 2). Two *Sal*I bands (9·0 and 5·2 kb), 5 *Hin*dIII bands (19·8, 15·9, 8·7, 4·2, and 1·0 kb) and 2 *Eco*RI bands (19·9 and 12·7 kb) were detected by a probe containing exclusively protein-coding sequence (Fig. 2; panel CDS). Interestingly, the 2 *Sal*I restriction fragments (9·0 and 5·2 kb) were those contained in the genomic clones H2Ag7 and H2Ag2, respectively. This fact was taken as an indication that only 2 *H2A* loci exist in the genome of *L. infantum*. This was finally confirmed by the hybridization patterns obtained with other specific-probes. Thus, 2 probes derived from phage H2Ag7, named SB2.3 and 3′UTR-II (Fig. 2), showed that the 8·7 kb and 4·2 kb *Hin*dIII genomic fragments belong to the *H2A* locus 1. Since the 1·0 kb *Hin*dIII band was mapped in the insert of phage H2Ag7 (Fig. 1), the histone H2A locus 1 explained 3 out of the 5 *Hin*dIII genomic bands hybridizing with the protein-coding probe (Fig. 2). Both probes hybridized with the same 19·9 kb *Eco*RI and 9·0 kb *Sal*I bands (Fig. 2; panels SB2.3 and 3′UTR-II). In a similar way, the SE1 and 3′UTR-III probes, derived from phage H2Ag2 (Fig. 1), were used to define the genomic bands corresponding to the histone H2A locus 2 (Fig. 2). As expected, both probes hybridized with the same 5·2 kb *Sal*I genomic fragment. Also, in agreement with the existence of *Eco*RI and *Hin*dIII restriction sites separating both probes in phage H2Ag2, it was observed that each one of the probes hybridized with different *Eco*RI and *Hin*dIII bands. Thus, the SE1 probe hybridized with a 19·8 kb *Hin*dIII band and a 23·0 kb *Eco*RI band, and the 3′UTR-III probe hybridized with a 15·9 kb *Hin*dIII band and a 12·7 kb *Eco*RI band. In conclusion, the Southern blots shown in Fig. 2 confirmed that there are 2 histone H2A loci in the *L. infantum* genome. This finding is in agreement with the existence of 2 *H2A* gene-bearing chromosomes in *Leishmania* spp., i.e. Chr21 and Chr29 (Wincker *et al*. 1996).

Sequence analysis of the two histone H2A loci

Three open reading frames coding for histone H2A were found after sequencing the 5444 bp *Bam*HI DNA fragment that contained locus 1 (Fig. 1). A search for sequence homologies in the EMBL–EBI *Leishmania* Database showed a significant sequence identity with the following entries: AL161173, AQ850483, AQ846646, AL499623, AL446005 and AL596287. Interestingly, the sequences AL161173, AL446005 and AL596287 derive from cosmids mapping to the *L. major* Friedlin chromosome 21 (Ivens *et al*. 1998). In addition, the 5247 bp *Sal*I DNA fragment containing locus 2 (Fig. 1) was sequenced as well, showing another 3 *H2A* genes. Interestingly, this sequence was found to be highly homologous to the GenBank entry AC129009, a contig assigned to the *L. major* chromosome 29. Therefore, these data strongly suggest that the H2A locus 1 and locus 2 are located in the *Leishmania* chromosomes 21 and 29, respectively.

A sequence comparison between the 5444 bp *Bam*HI DNA fragment, which contains locus 1, and the 5247 bp *Sal*I DNA fragment, which contains locus 2, indicated that sequence homology is restricted to the histone H2A coding regions. The upstream and downstream sequences to the *H2A* gene clusters were found to be divergent between the two loci. Although the endonuclease-restriction pattern shown by the genomic clone H2Ag6 (Fig. 1) was coincident with that of the genomic region containing the histone H2A locus 1, after sequencing a 2434 bp DNA fragment from clone H2Ag6, it was found that the sequence identity with clone H2Ag7 was high (98·85%) but not complete. Since several lines of evidence suggest that *Leishmania* is mostly diploid (Ravel *et al*. 1998), we deduced from this finding that each genomic clone contains an allelic alternative.

The coding regions of the *H2A* genes were found to be extremely conserved (above 96·7%). However, 2 identical coding regions do not exist among the 7 deduced amino acid sequences (Fig. 3). It can be noticed that most of the amino acid changes occur in the carboxyl-terminal region of the protein.

Major sequence differences among the *L. infantum H2A* genes were found in the UTRs. In order to

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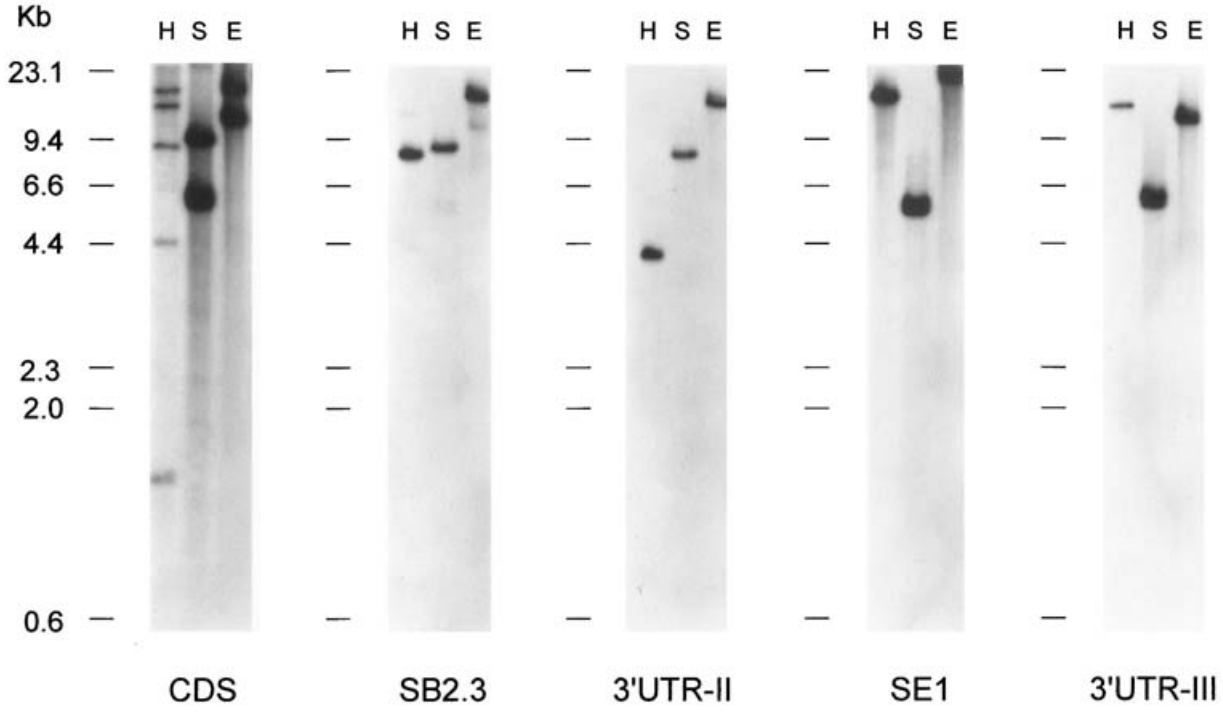

Fig. 2. Southern blot analysis of *Leishmania infantum* genomic DNA hybridized with *H2A*-specific probes. Two μg of total DNA were digested with the restriction enzymes *Hin*dIII (lanes H), *Sal*I (lanes S) and *Eco*RI (lanes E). As indicated below the blots, the following specific probes are used: CDS (H2A coding region), SB2.3, 3′UTR-II, SE1, and 3′UTR-III. The locations of the probes are shown in Fig. 1. Numbers at the left indicate the size (in kb) and mobility of the restriction fragments for *Hin*dIII-digested λ DNA.

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          1                                                   50
H2A1-1    MATPRSAKKA TRKSGSKSAK CGLIFPVGRV GGMMRRGQYA RRIGASGAVY
H2A1-2    MATPRSAKKA ARKSGSKSAK CGLIFPVGRV GGMMRRGQYA RRIGASGAVY
H2A2-1    MATPRSAKKA TRKSGSKSAK CGLIFPVGRV GGMMRRGQYA RRIGASGAVY
H2A3      MATPRSAKKA TRKSGSKSAK CGLIFPVGRV GGMMRRGQYA HRIGASGAVY
H2A4      MATPRSAKKA VRKSGSKSAK CGLIFPVGRV GGMMRRGQYA RRIGASGAVY
H2A5      MATPRSAKKA ARKSGSKSAK CGLIFPVGRV GGMMRRGQYA RRIGASGAVY
H2A6      MATPRSAKKA ARKSGSKSAK CGLIFPVGRV GGMMRRGQYA RRIGASGAVY
                     *                                   *
          51                                                 100
H2A1-1    LAAVLEYLTA ELLELSVKAA AQSGKKRCRL NPRTVMLAAR HDDDICMLLK
H2A1-2    LAAVLEYLTA ELLELSVKAA AQSGKKRCRL NPRTVMLAAR HDDDIGMLLK
H2A2-1    LAAVLEYLTA ELLELSVKAA AQSGKKRCRL NPRTVMLAAR HDDDICMLLK
H2A3      LAAVLEYLTA ELLELSVKAA AQSGKKRCRL NPRTVMLAAR HDDDICMLLK
H2A4      LAAVLEYLTA ELLELSVKAA AQSGKKRCRL NPRTVMLAAR HDDDIGTLLK
H2A5      LAAVLEYLTA ELLELSVKAA AQSGKKRCRL NPRTVMLAAR HDDDIGMLLK
H2A6      LAAVLEYLTA ELLELSVKAA AQSGKKRCRL NPRTVMLAAR HDDDIGTLLK
                                                        **
          101                                  132
H2A1-1    NVTLSRSGVV PSVSKAVAKK KGGKKGRATP SA
H2A1-2    NVTLSHSGVV PSVSKAVAKK KGGKKGRATP SA
H2A2-1    NVTLSRSGVV PSVSKAVAKK KGGKKGRATL SA
H2A3      NVTLSRSGVV PSASKAVAKK KGGKKGRATP SA
H2A4      NVTLSHSGVV PNISKAMAKK KGGKKGKATP SA
H2A5      NVTLSHSGVV PSVSKAMAKK KGGKKGKATP NA
H2A6      NVTLSHSGVV PNISKAMAKK KGGKKGKATP NA
               *      **   *          *  * *
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Fig. 3. Alignment of the amino acid sequences deduced from the different *Leishmania infantum H2A* genes. Amino acid positions that are non-identical in all proteins are indicated by asterisks.

define the 5′ ends of the *H2A* mRNAs, cDNA was synthesized using an oligo(dT) primer and amplified using a forward primer derived from the mini-exon sequence and a reverse primer complementary to a coding sequence common to all *H2A* genes (see 'Materials and Methods' section). Thus, after sequencing 12 clones, the splice acceptor sites could be experimentally defined for genes *H2A1-2*, *H2A2-2*

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Fig. 4. Comparison of the 5′-UTR sequences of the different *H2A* genes. Alignment for maximal identity was done using the CLUSTALW program, available at the European Bioinformatics Institute server (http://www.ebi.ac.uk/). The AG dinucleotide acceptor sites for spliced leader addition and the ATG initiation codons are underlined. Hyphens represent introduced gaps for the optimum alignment. Nucleotide residue numbering is shown at the end of each line. Conserved sequences in all the 5′-UTRs are indicated by asterisks.

and *H2A4*; the remaining were deduced from sequence comparison and theoretical considerations. From a multiple sequence alignment (Fig. 4), the existence of 4 types of 5′UTRs became evident. A 5′UTR type I (or 5′UTR-I) is present in both alleles of gene *H2A1*, a 5′UTR-II is shared by genes *H2A2* (both alleles), *H2A3* and *H2A5*, a 5′-UTR-III is found in the gene *H2A4*, and a 5′-UTR-IV is found in the gene *H2A6* (Fig. 1). The 5′UTRs types I and II are similar in sequence, but they are of a different size. Also, the 5′UTRs types III and IV are related, since they present a remarkable sequence conservation in a 33-nucleotide fragment located upstream from the ATG initiation codon (Fig. 4). A common feature for all 5′UTRs is the presence of polypyrimidine tracts.

In previous work, we isolated and sequenced 2 cDNAs clones (named cL71 and cL72) coding for *L. infantum* histone H2A (Soto *et al*. 1992). We noticed at that time that both sequences diverged in their 5′UTRs but were highly conserved in their coding and 3′UTRs sequences. Now, sequence comparison with the genomic clones allowed us to deduce that those cDNAs correspond to the mRNAs of genes *H2A1-1* (cDNA cL72) and *H2A4* (cDNA cL71). As previously reported, the 3′UTRs of the 2 cDNAs differ only in a single nucleotide, a difference also observed in the corresponding genomic sequences. For simplicity, we grouped these 3′UTR as type I (or 3′UTR-I). Aside from genes *H2A1-1* and *H2A4*, the 3′UTR-I was found to be present in the genes *H2A1-2* and *H2A2-1* (Fig. 1). At present, we have not defined the 3′ end of the 3′UTR for the remaining genes. Nevertheless, it was found that the sequences downstream from the termination codon in genes *H2A3*, *H2A5* and *H2A6* are absolutely divergent compared to the 3′UTR-I sequence. Immediately downstream from the termination codon, genes *H2A5* and *H2A6* share 99·1% of sequence identity in a 228 bp fragment, suggesting that this is the size of the 3′UTR for these genes. Thus, in summary, it can be concluded that, in addition to the 3′UTR-I found in genes *H2A1* (both alleles), *H2A2* and *H2A4*, two other types of 3′UTRs exist: a 3′UTR-II in the gene *H2A3* and a 3′-UTR-III in the genes *H2A5* and *H2A6* (Fig. 1).

Relative steady-state levels of the different L. infantum H2A *mRNAs*

As summarized in Fig. 1, the 6 *H2A* genes present in the *L. infantum* genome are heterogeneous in sequence, mainly within their 5′- and 3′-UTRs. In order to analyse whether the sequence divergences were associated with differences in the steady-state levels of *H2A* mRNAs, Northern blots containing *L. infantum* promastigote RNA were probed with the 3 different 3′-UTRs. Transcripts were detected with each of the probes; however, it was not possible to establish with this technique the relative abundance since the transcripts showed the same size. In order to quantify the relative levels of the different transcripts, we designed a nuclease S1 protection assay using specific oligonucleotides for each of the 3′-UTRs. Figure 5 A shows the result of a representative experiment in which poly-A^+ RNA extracted from parasites growing at logarithmic phase was used. Densitometric analyses of the autoradiographs from 3 independent experiments showed that transcripts with 3′UTR-I are 7- and 130-fold more abundant than those containing the 3′UTR-III and the 3′-UTR-II, respectively. As an alternative method to evaluate the relative levels of expression of the 3 types of *H2A* transcripts, poly-A^+ RNA was used as a template to prepare an α-^{32}P-labelled total cDNA probe that was then hybridized to a membrane containing the different 3′UTRs (Fig. 5B). After quantification of the hybridization signals, it was calculated that the 3′UTR-I containing transcripts are 7- and 200-fold more abundant than those containing the 3′UTR-III and the 3′UTR-II, respectively. In summary, these 2 independent approaches indicated that transcripts with 3′UTR-I account for the majority of the *H2A* transcripts in logarithmic-phase growing parasites, and that the steady-state level of the 3′UTR-II-containing transcripts is extremely low.

To determine whether the abundance of the different *H2A* transcripts is regulated at the transcriptional or post-transcriptional levels, we quantified the relative rates of transcription for the different *H2A* genes by nuclear run-on transcription analysis (Fig. 5C). The rate of transcription of each gene (or

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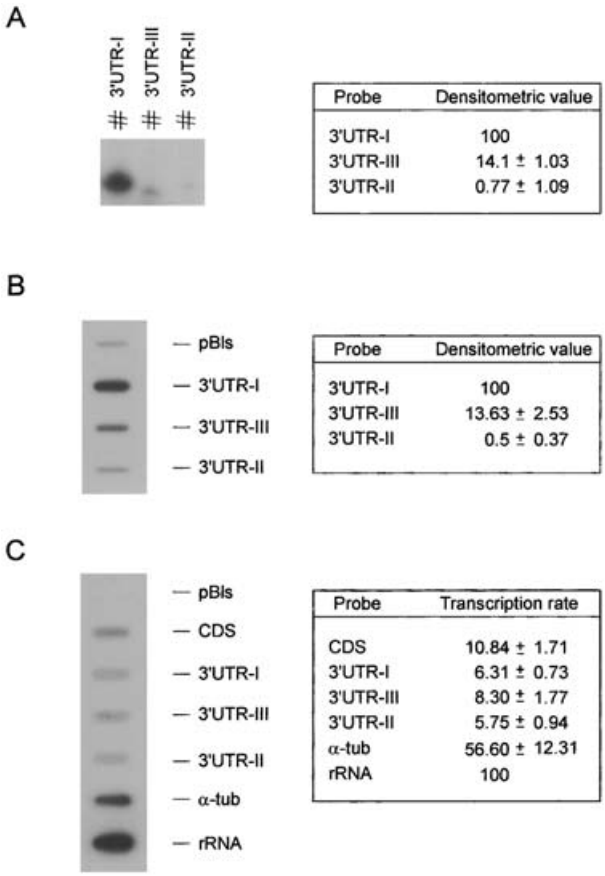

Fig. 5. Analysis of the expression levels of *Leishmania infantum H2A* RNAs. (A) The steady-state levels of the different *H2A* transcripts were determined by S1 protection analysis using 5′-labelled oligonucleotides, reverse and complementary to specific regions of the 3′UTR-I (no. 3′UTR-I), the 3′UTR-II (no. 3′ UTR-II), or the 3′UTR-III (no. 3′UTR-III). Oligonucleotides were hybridized in molar excess (0·07 pmol) with 2 μg of poly(A)$^+$ RNA from logarithmic promastigotes. After 3 h of hybridization, samples were incubated with 40 units of S1 nuclease for 15 min. The S1 products were analysed on 15% polyacrylamide–7 M urea gels. Three independent experiments were made and the autoradiographs were quantified by densitometric scanning (panel on the right). Densitometric measures were normalized to the protection signal values for the 3′UTR-I oligonucleotide (taken arbitrarily as 100). (B) *L. infantum* poly(A)$^+$ RNA was used as template to synthesize α-32P-labelled cDNA probe, which was hybridized to linearized plasmid DNA (5 μg/slot) immobilized on a membrane. Plasmids were pBluescript (pBls) containing the 3 types of *H2A* 3′UTRs: 3′UTR-I, 3′UTR-III and 3′UTR-II. Hybridization signals were quantitated using a Phosphorimager. After subtracting the unspecific hybridization value (pBls slot), the data were normalized to the hybridization value for the 3′UTR-I plasmid (set arbitrarily as 100). (C) Analysis of the *H2A* gene transcription by nuclear run-on assays. Run-on transcription was performed with nuclei from *L. infantum* logarithmic promastigotes. The run-on transcripts were hybridized to linearized plasmid DNA (5 μg/slot) immobilized on a nylon filter. Plasmids contain inserts for different regions of the *H2A* genes: the coding region (CDS), the 3′UTR-I, the 3′UTR-III and the 3′UTR-II. Also plasmids containing a *Trypanosoma cruzi* α-tubulin cDNA (Soares *et al*. 1989) (α-tub.) or a fragment of the *L. infantum 24Sα rDNA* gene (Quijada *et al*. 1997*b*) (rRNA) were used. The slot marked as pBls contains the Bluescript plasmid. The panel on the right shows the quantification of relative transcription rates for the different genes after densitometric analysis of the autoradiographs from 3 independent experiments. Data were normalized to the transcription rate of the rRNA genes (taken as 100).

group of genes) was normalized to the rate of rRNA transcription. The results of 3 independent experiments showed that transcription of the different *H2A* genes occurred at approximately the same rate. Therefore, the differences in the steady-state levels for the different *H2A* transcripts must be due to post-transcriptional processes.

The expression of H2A *genes is regulated by growth phase in* L. infantum *promastigotes*

In a previous report, we described that the levels of histone *H2A* mRNAs are affected by growth rate in *Leishmania* promastigotes (Soto *et al*. 1992). Indeed, as shown in Fig. 6 (panel CDS), the total amount of *H2A* mRNAs is significantly lower in stationary phase promastigotes than in logarithmic phase promastigotes. When Northern blots were probed with the different 3′-UTRs, it was observed that the steady-state levels of transcripts with either 3′UTR-I or 3′UTR-III were lower at the stationary phase of growth than at the logarithmic phase (Fig. 6). It was interesting to note that both types of *H2A* transcripts were down-regulated by a mechanism regulated by growth phase, despite the fact that they contain highly divergent UTR sequences. In contrast, the levels of the 3′UTR-II transcripts were found to be similar in both logarithmic or stationary promastigotes (Fig. 6, panel 3′UTR-II), as occurs with the α-tubulin transcripts (Fig. 6, panel α-tub). However, given the relative low level of this *H2A* transcript (Fig. 5), the physiological relevance of this finding remains unanswered.

Since there are numerous examples of *Leishmania* multicopy genes in which their differential expression is determined post-transcriptionally by sequences located in the 3′UTRs (Ramamoorthy *et al*. 1995; Charest, Zhang & Matlashewski, 1996; Coulson *et al*. 1996; Quijada *et al*. 2000; Boucher *et al*. 2002), we analysed whether the 3′UTRs could mediate the differential accumulation of the 3 types of *H2A* transcripts (Fig. 5). A series of constructs were prepared in which the different *H2A* 3′UTRs were cloned downstream of the *CAT* gene in plasmid pX63NEO. The constructs were electroporated into *L. infantum* promastigotes and stable transfectants were obtained. However, the levels of the *CAT*

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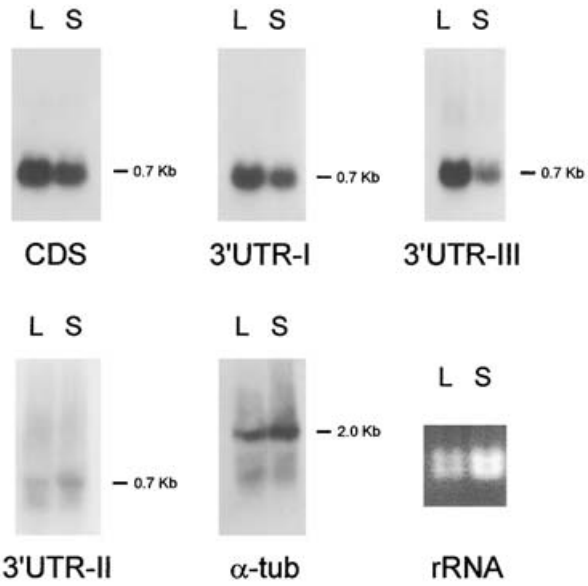

Fig. 6. Expression of the different *H2A* mRNAs in logarithmic and stationary phases of growth. Northern blots containing 5 μg of total RNA from *Leishmania infantum* promastigotes during logarithmic (L) or stationary (S) phase of growth were hybridized to specific probes for the *H2A* coding region (panel CDS), the 3′UTR-I (panel 3′UTR-I), the 3′UTR-III (panel 3′UTR-III), the 3′UTR-II (panel 3′UTR-II), or the *Trypanosoma cruzi* α-tubulin (panel α-tub.). The ethidium bromide staining of the agarose gel is shown (panel rRNA). The sizes of hybridization bands (in kb) are indicated.

RNAs were found to be similar in the transfectants (data not shown), indicating that sequences other than the 3′UTRs (either alone or acting in conjunction with the 3′UTRs) must be responsible for the differential expression levels shown by the different *H2A* genes (Fig. 5).

DISCUSSION

In this work we describe the organization and the steady-state expression levels of the *H2A* genes in *L. infantum*. The genome of this parasite contains 6 *H2A* genes distributed in 2 gene loci (each with 3 clustered genes), located on different chromosomes. Sequence analysis has indicated that 2 identical *H2A* genes do not exist. Major sequence differences are located in both 5′- and 3′-UTRs, although changes also occur in the deduced amino acid sequences (see below). The gene complexity could be even higher if allelic divergence is taken into account, since we have obtained evidence that gene alleles located in homologous chromosomes also accumulate sequence differences. Allelic differences in the 5′UTRs are significant, since they affect either the AG acceptor site (*H2A1* alleles) or the length of the 5′UTRs (*H2A2* alleles). This is not an unusual finding, several examples of allelic differences have been described in *Leishmania* and other trypanosomatids (Revelard, Lips & Pays, 1993; Bhatia *et al*. 1998; Göpfert *et al*. 1999; Landfear, 2001; Machado & Ayala, 2001). Interestingly, it has been shown in some studies that one of the alternative alleles is expressed at higher levels than the other allele, which is located on the homologous chromosome (Revelard *et al*. 1993; Bhatia *et al*. 1998). At present, experiments are being designed to analyse whether expression differences exist between the allelic alternatives of *L. infantum H2A* genes.

In this work, we have analysed differences in expression levels of *H2A* genes based on the sequence divergence of the 3′UTRs. Nuclear run-on experiments have indicated that all *H2A* genes are transcribed at similar rates, although the different *H2A* mRNAs accumulate at very different steady-state levels. Thus, transcripts with 3′UTR-I are the most abundant in logarithmic phase promastigotes, transcripts with 3′UTR-III have an intermediate level and transcripts with 3′UTR-II are expressed at a very low level. Also, it has been found that steady-state levels of transcripts with either 3′UTR-I or 3′UTR-III decrease when promastigotes reach the stationary phase of growth, whereas the low levels of transcripts with 3′UTR-II are similar in both growth phases. Based on studies from other *Leishmania* genes, in which it has been demonstrated that sequences located in the 3′UTRs are directly involved in post-transcriptional regulatory mechanisms (Ramamoorthy *et al*. 1995; Charest *et al*. 1996; Coulson *et al*. 1996; Quijada *et al*. 2000; Boucher *et al*. 2002), we analysed the effect of the 3 distinct *H2A* 3′UTRs on the transcript levels of a *CAT* reporter gene (data not shown). The results indicated that the different steady-state levels of the *H2A-3′UTR-I*, *H2A-3′UTR-II* and *3′UTR-III* transcripts in *Leishmania* promastigotes are not determined by the 3′UTR alone. It is worth mentioning that the work of García-Salcedo *et al*. (1999) that showed that the decrease of *H2B* mRNAs in *T. brucei* induced by hydroxyurea is not a direct effect of the 3′UTR.

Although major differences among the *Leishmania H2A* genes are located in the UTRs, it should be noted that the 7 deduced amino acid sequences from the *H2A* genes differ from each other in a few residues. Thus, it would be interesting to analyse whether these minor differences could have a functional role, taking into account that they are mainly located in the carboxyl-terminal region. The C-terminal domain of histone H2A is a flexible region of the protein postulated to be involved in the nucleosome disassembly and reassembly during the transcription process (Usachenko *et al*. 1994).

An interesting question arising from this work concerns the significance of such a gene organization. Two possibilities exist to explain why *Leishmania* possesses 6 markedly different *H2A* genes: (a) they are the result of stochastic gene duplications and

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genetic drift without functional meaning; (b) they are the result of an evolutionary pressure on these loci to ensure a variety of *cis*-acting sequences involved in the post-transcriptional regulation of the *H2A* transcripts in different development stages and in different metabolic conditions. Although studies are needed to ascertain the functional role of the different UTRs (both 5′ and 3′) in the regulation of *H2A* mRNAs, the fact that multi-copy genes with different UTRs are common in the *Leishmania* genome (reviewed by Stiles *et al*. 1999) favours the existence of a regulatory role for these sequences. Thus, this complex gene structure and organization could be envisaged as a strategy to regulate gene expression, designed by a parasite lacking transcriptional regulation mechanisms. Well-characterized examples, in which genes with different UTRs are expressed differentially, are the glucose transporter genes of *L. mexicana* (Burchmore & Landfear, 1998), the surface protease Gp63 genes in *L. chagasi* (Ramamoorthy *et al*. 1992), and the β-tubulin genes in *L. major* (Coulson *et al*. 1996).

In summary, the structural characterization of the *L. infantum H2A* genes and the analysis of their transcripts described here, constitute a reliable starting point to undertake the study of the regulatory mechanisms involved in the histone expression of trypanosomatids. The fact that the 3′UTRs *per se* did not show any effect on the transcripts derived from CAT-H2A 3′UTR chimeric genes points to the existence of a high complexity in the mechanisms regulating the translation of *Leishmania H2A* transcripts.

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