Analysis of Post-transcriptional Regulation Operating on Transcription Products of the Tandemly Linked Leishmania infantum hsp70 Genes*

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The genomic organization and expression of the hsp70 genes of Leishmania infantum were examined. In the cluster there are at least six copies of the hsp70 genes arranged in a head-to-tail tandem of 3.8-kilobase repetition units. The hsp70 gene copy (gene 6) located at the 3’ end of the tandem has a 3’-untranslated region highly divergent in sequence relative to the 3'-untranslated region of the rest of hsp70 gene copies (genes 1–5). Nuclease S1 protection assays indicated that the steady-state level of the mRNAs derived from gene 6 is about 50-fold more abundant than the transcript derived from genes 1–5. Nuclear run-on assays showed, however, that all hsp70 genes are transcribed at similar rates. Thus, it is likely that the differences in the steady-state levels of the transcripts from the hsp70 genes should be associated with variations in their processing or maturation rates. While the abundance of the mRNAs derived from hsp70 genes 1–5 is increased by heat shock, the hsp70 gene 6 mRNA level remains unaffected. Our data showed that ongoing protein synthesis is required for the maintenance of the heat inducement, depicting, thus, a post-transcriptional mechanism of positive regulation involving a labile protein factor that would be either induced or activated during heat shock.

Leishmania parasites experience a shift in temperature during their life cycle while being transferred from the sandflies, as flagellated promastigotes, to the vertebrate host where they enter into macrophages and transform to aflagellated amastigotes. This change in temperature is known to affect gene expression as well as stage differentiation (1). Although most of the eukaryotes respond to a heat shock by increasing the rate of transcription of specific genes to attain high levels of the heat-shock proteins (hsp), Leishmania and other trypanosomes do not induce the transcription of their hsp genes during a heat stress. Instead, the levels of hsp appear to be post-transcriptionally regulated. In fact, it has been shown by analysis of the expression of the hsp70 and hsp83 genes of Leishmania major and Leishmania donovani that there is no transcriptional activation of these genes when the parasites are exposed to a heat shock (2). Similarly, although no transcriptional activation of the Leishmania amazonensis hsp83 genes is induced upon a heat shock, hsp83 transcripts accumulate in this condition (3). The accumulation of the hsp83 transcripts results mainly from differences in stability since while the hsp83 mRNAs are rapidly degraded at the normal temperature they becomes stable at 35 °C (3). The regulation of Trypanosoma brucei hsp70 transcripts (4, 5) and the control of other stage-regulated genes seems to be also post-transcriptional (6–14).

Post-transcriptional regulation is probably a consequence of the clustering as tandem repeats of most Trypanosomatid genes and of the transcription of the genes from the cluster as polycistrone-transcriptionally processed by both 5’-trans- splicing of a capped leader RNA and polyadenylation (15). Therefore, regulation of the expression of individual genes within the cluster cannot occur at the level of transcription initiation. Particularly, the genes of the hsp70 and hsp83 families have been chosen as models for the study of the organization and expression of trypanosome genes. Hsp70-encoding genes have been found repeated and tandemly organized in T. brucei (16), Trypanosoma cruzi (17), L. major (18, 19), and L. amazonensis (20). Similarly, genes coding for hsp83 proteins have been found repeated and tandemly organized in T. cruzi (21), T. brucei (22), and L. mexicana amazonensis (23). In L. major, in addition to the four tandemly clustered hsp70 genes, a fifth hsp70 gene is located in a separate locus (18). Interestingly, while the expression levels of the tandemly linked hsp70 genes are increased after a heat-shock treatment at 37 °C the nonlinked gene is unaffected by a temperature shift (18).

In the present report we present the analysis of the regulation of the expression of the hsp70 genes from Leishmania infantum. As a first step in this study we examined the genomic organization of these genes. We found that the L. infantum hsp70 genes are located in a single cluster formed by six hsp70 units in a head-to-tail tandem array. We observed that when the parasites are grown at 26 °C the abundance of the steady-state transcripts derived from the gene located at the 3’ end of the cluster (gene 6) accumulates to higher levels than the transcripts derived from the rest of hsp70 genes (genes 1–5). Also, we detected that when the parasites are grown at 37 °C the levels of transcripts derived from the hsp70 genes 1–5 results increased by the heat treatment in contrast to the transcripts level from gene 6 that remains unaffected. Data from several experimental approaches indicate that the differential regulation of L. infantum hsp70 genes must be occurring at the post-transcriptional level by mechanisms involving specific sequences of the 3’-untranslated regions (UTR).
EXPERIMENTAL PROCEDURES

Parasites—Promastigotes of *L. infantum* (MHOM/FR/78/LEM 75) were cultured in vitro at 26 °C in RPMI 1640 medium (Life Technologies, Inc., Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, UK). In all the experiments logarithmic phase cultures (5 × 10⁶–10⁷ promastigotes ml⁻¹) were used.

Clones and Probes—Clone B2cDNA, a *L. infantum* hsp70 cDNA isolated from a large screening cDNA expression library, has been previously described (24). This cDNA was cloned into the EcoRI site of plasmid pUC18. A 330-bp DNA fragment, named B2 3’UTR-II, was obtained after SalI digestion of clone pB2cDNA (Fig. 1A). This fragment was cloned in the SalI site of pUC18 and the resulting clone named p3’UTR-II. It must be noted that this fragment exclusively contains 3’-UTR sequences of an *L. infantum* hsp70 gene (gene 6, see below).

The *L. infantum* EMBL-3 genomic library used in this work has been previously described (25). Screening was carried out by standard techniques (26) using the 32P-labeled B2cDNA as probe. Seven positive clones, B2g1 to B2g7, were isolated. Similarly, a screening was carried out on a *L. infantum* oligoT7-prime lambda-gt11 cDNA expression library (27) using the B2 3’UTR-II DNA fragment as probe. From this screening two cDNA clones, 70IA-1 and 70IA-2, were isolated.

Probe 3’UTR-I was obtained by PCR amplification from the 1.5-kb SalI-BamHI fragment (clone B2g6; Fig. 1A), subcloned in pUC18, using the oligonucleotide 70-I (5'-CACACCAAGTACACGTCAG-3'). The M13/pUC sequencing primer (Promega) was labeled with α-32PATP (6000 Ci/mmole; Amersham Corp.) using the T4-polydeoxyribonucleotide kinase kit (Boehringer Mannheim). The specific activity of the labeling was 10⁹ cpm/μg. A molar excess (0.07 pmol) of 32P-labeled oligonucleotide was hybridized with 2 μg of *L. infantum* poly(A⁺) RNA (purified by oligoT7-cellulose; Boehringer Mannheim) in a 2-μl reaction volume containing 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA. After heating for 15 min at 75 °C, hybridization was performed for 3 h at 52 °C. In parallel, as controls, hybridizations of the oligonucleotides to 4 μg of *Escherichia coli* rRNAs (Boehringer Mannheim) were carried out. After ice-curing of the hybridization reaction, 25 μl of 2 × 51 buffer (66 mM NaCl, pH 4.5, 10 mM Tris-HCl, 0.06 mM ZnSO₄, and 40 units of nuclease S1 (Boehringer Mannheim) were added. After 15 min of incubation at 37 °C the reaction was stopped by adding an equal volume of loading buffer (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). Finally, 6-μl samples were loaded on a 15% polyacrylamide, 7 M urea sequencing gel for 4 h at 60 watts. After drying the gel was exposed to a x-ray film at −70 °C for several hours. The autoradiographs were scanned with a laser densitometer (Image Quant 2.0), and the relative densities of the bands were determined.

Nuclear Run-on Assays—Promastigotes cultures in logarithmic phase growth (5 × 10⁶ parasites ml⁻¹) were preincubated at 37 °C during 0 (26 °C control), 10, 30, and 60 min. At the indicated times, 10-ml aliquots were harvested, and the parasites were suspended in 500 μl of ice-cold hypotonic buffer (0.25 M sucrose, 5 mM Hepes, pH 7.5, 1 mM spermidine, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol). Nonidet P-40 and Triton X-100 were added to a final concentration of 0.5% each, and the cells were lysed by vigorously vortexing for 30 s. Immediately, 2 volumes of ice-cold 2× nuclei washing buffer (40 mM Tris-HCl, pH 7.5, 0.84 M sucrose, 1 mM spermidine, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 60 mM KCl) were added and mixed by vortexing. The nuclei were pelleted (3000 × g), washed, and stored in 100 μl of nuclei storage buffer (50% glycerol, 4 mM MgCl₂, 1 mM MgCl₂, 0.1 mM EDTA, 50 mM Hepes, pH 7, 5, and 5 mM dithiothreitol) at −70 °C, until use. After thawing, 1 volume of 2× transcription buffer (1 mM Hepes, pH 7.5, 0.2 mM KCl, 8 mM dithiothreitol, 60 μM EDTA, 2 mM ATP, 1 mM CTP, 1 mM GTP, 17.6 mM creatine phosphate, and 80 μg ml⁻¹ creatine kinase (Boehringer Mannheim) was added. The run-on transcripts were labeled with adding 100 μCi of (α-32P)UTP (3000 Ci mmole⁻¹) (Amersham Corp.). Nuclei isolated from heat-shocked parasites were labeled during 10 min at 37 °C, while those from control parasites were labeled at 26 °C for the same time. The reaction was stopped by addition of DNase I (RNase-free) and MgCl₂ to final concentrations of 10 and 5 μM, respectively, and incubated for 20 min at 37 °C. Subsequently, the reaction continued in the presence of 0.15 mg ml⁻¹ protease K, 0.5% SDS, and 5 mM EDTA for 20 min at 37 °C. The radiolabeled nascent RNA was extracted by phenol/chloroform. Non-incorporated isotopes were separated from the labeled product on a Sephadex G-50 column.

A 5-μg sample of each plasmid to be tested was linearized, denatured, and applied onto Zeta-probe membranes (Bio-Rad) in a vacuum slot-blot apparatus according to the manufacturer's instructions. The membrane was then subjected to hybridization with the purified labeled RNA (2–6 × 10⁶ cpm ml⁻¹) in a solution containing 50% formamide, 6 × SSC, 0.1% SDS, and 0.25 mg ml⁻¹ herring sperm DNA for 3 days at 42 °C. Subsequently, the filters were washed at room temperature for 15 min in 2 × SSC, followed by a wash at 65 °C for 30 min in 2 × SSC, and a final wash at 37 °C for 20 min in 2 × SSC, 10 μg ml⁻¹ of RNase A.

RESULTS

Genomic Organization of the *L. infantum* hsp70 Genes

Recently, we reported the isolation of a cDNA clone for a fragment of the *L. infantum* hsp70 gene by immunoscreening of a cDNA expression library (24). The identified cDNA clone, named B2, contains the sequence coding for the carbonyl-terminal 20 amino acids and for 330 bp of the 3’-UTR. In order to analyse the genomic organization of the hsp70 gene, *L. infantum* DNA was digested with several restriction enzymes, transferred onto nylon membranes, and probed with clone B2 (Fig. 1). The presence of a 3.8-kb hybridization band in the lanes containing the DNA digested either with SalI or BamHI was taken as an indication of the presence of several copies of the hsp70 gene which are arranged in tandem and that these

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2 C. Alonso, unpublished data.
enzymes cut only once within the repeat. In addition, the fact that others restriction enzymes (HindIII and EcoRI) produce a single hybridizing band supported the fact that the hsp70 genes must be clustered in a single chromosomal locus.

To analyze in more detail the genomic organization of the hsp70 genes, a L. infantum genomic library was screened with the B2cDNA probe. Seven clones, named B2g1 to B2g7, were isolated and physically mapped (Fig. 1A). The restriction analysis of those clones confirmed that the hsp70 genes are tandemly arrayed. Southern blot analysis indicated that probe B2cDNA hybridizes more strongly with clones B2g1–5 and B2g7 than with clone B2g6 (data not shown), suggesting that the clone B2 must have the highest sequence homology with the 3' hsp70 gene copy of the cluster (Fig. 1A). This suggestion was further confirmed by hybridization of the 3' UTR present in the B2cDNA (probe B2 3'UTR-II) to the Southern blot shown in Fig. 1C. The probe hybridized only with the 5.7-kb SauI band, whereas it did not hybridize with the 3.8-kb BglII band, whereas it did not hybridize with the 3.8-kb SauI band.

Sequence Analysis of the L. infantum hsp70 Genes

To understand in more detail the organization of the hsp70 genes, we determined the nucleotide sequence of the more 5' copy of the hsp70 gene cluster (gene 1) and of the neighboring regions located at both sides of this gene. Thus, the 0.3-kb EcoRI-BamHI and the adjacent 3.8-kb BamHI fragment, which contains the first repetition unit present in clone B2g6 (Fig. 1A), were sequenced. A diagrammatic representation of the sequence is shown in Fig. 2A. Table I shows the comparison of the sequence obtained with the sequence of other Leishmania hsp70 genes. For this analysis, the hsp70 gene was subdivided into five regions.

Upstream Region (UPR)—This region extends 119 bp upstream from the BamHI restriction site and includes the putative spliced leader addition site. UPR regions should be present in all of the hsp70 copies of the cluster since the UPR region of gene 1 hybridizes to each one of the genomic phages from B2g1 to B2g7 (data not shown), indicating that the UPR of the hsp70 gene 1 and the UPR of the hsp70 gene 2 show total sequence identity (Fig. 2A). As shown in Table I the L. infantum hsp70 UPR is also conserved in the hsp70 genes from other Leishmania species. The main structural feature of the UPR is its polypyrimidine richness (79% of C + T content) catalogued in long pyrimidine runs. Polypyrimidine tracts have been described as a 5' essential sequence implicated in correct trans-splicing (33, 34). The spliced leader acceptor site of the L. infantum hsp70 genes was defined by sequence similarity with those of the L. major (18) and L. amazonensis (20) hsp70 genes.

5'-Untranslated Region (5'UTR)—The 5'-UTR is 161 bp long and extends from the AG spliced leader acceptor site to the ATG initiation codon. Comparison with the other Leishmania species indicated that this region is highly conserved (Table I).

Coding Region (CDR)—The CDR is 1959 bp long and extends just at the SauI restriction site (Fig. 2A). The derived protein product of the CDR region has been described elsewhere (24).

3'-UTR Type I (3'UTR-I)—The region is 1063 bp long and extends from the TAA termination codon to the putative polyadenylation site defined by sequence similarity with the L. amazonensis hsp70 gene La70c1/La70gA (20). This region is highly conserved among different Leishmania species (Table I). The nucleotide sequence differences are mainly due to length polymorphisms of microsatellites. In the L. infantum 3'-UTR runs of microsatellites with the sequence CA/GT are frequent. Another remarkable feature of the L. infantum 3'-UTR-I is a sequence with dyad symmetry (nucleotides 2948–3000) located next to the polyadenylation site with potential to form a stable stem-loop. Interestingly, this element is highly conserved in L. donovani and L. major hsp70 genes. Although divergent in sequence relative to the L. infantum 3'-UTR an equivalent stem-loop is found also in the 3'-UTR of L. amazonensis hsp70 (20).
Organization and Expression of L. infantum hsp70 Genes

**Fig. 2. Schematic representation of the sequenced regions of the hsp70 gene cluster.** A, sequence subregions of hsp70 gene 1. The nucleotide sequence of this gene was submitted to the EMBL data base with the accession number Y08020. B, upstream region; 5′-UTR, 5′-untranslated region; CDR, protein coding region; 3′-UTR-I, 3′-untranslated region, common to hsp70 genes 1–5; IIR, intergenic region; B, BamHI; S, SalI. B, diagrammatic representation of the sequenced region of hsp70 gene 6. This sequence is in the EMBL data base under the accession number Y08019. Seventeen nucleotides downstream from the coding region (CDR), the nucleotide sequence of the 3′-UTR of gene 6 (3′-UTR-II) was found different from the sequence of the 3′-UTR-I. Vertical arrows indicate the position of polyadenylation addition site.

### Table I
Sequence similarities among the hsp70 genes of different Leishmania species

|       | UPR | 5′-UTR | CDR | 3′-UTR-I | IR |
|-------|-----|--------|-----|----------|----|
| Li    |     |        |     |          |    |
| Ld    | 94.35<sup>a</sup> | 93.21 | 97.65 | 91.23 | 90.94 |
| Lm    | 96.52 | 94.78 | 93.36 | 93.93 | 89.5 | 91.17 | 89.8 | 90.62 |
| La    | 68.85 | 65.57 | 86.66 | 93.15 | 91.78 | 93.79 | 95.74 | 96.21 | 83.18 | 85.71 | 85.3 | 85.27 | 78.64 | 81.01 |

<sup>a</sup> The accession numbers of the nucleotide sequences employed are: L. amazonensis (La) L14601, L14604, and L14605; L. donovani (Ld) X60101, X60102, and X52314; L. major (Lm) X13441 and M36675.

<sup>b</sup> % similarity values were obtained with the gap program of the University of Wisconsin Genetics Computer Group.

**Intergenic Region (IR)—The polyadenylation site of hsp70 gene 1 is separated from the UPR of gene 2 by 312 bp. The L. infantum IR sequence is also conserved with respect to the corresponding regions of other Leishmania species (Table I). The IR size of the Leishmania hsp70 genes is in agreement with the estimated average size (383 bp) of the IRs from other Leishmania genes (35).**

As stated above, hybridization experiments (Fig. 1) indicated that the 3′-UTR of hsp70 gene 6 should be different to that present in the other hsp70 genes. The nucleotide sequence of this 3′-UTR, named 3′-UTR-II, was determined on the 5.72-kb SaII restriction fragment derived from clone B2g1 (Fig. 1A). A diagrammatic representation of the 3′-UTR-II is shown in Fig. 2B. The position of the polyadenylation site was determined by sequence analysis of two cDNAs (called 70IIA-1 and 70IIA-2) isolated after screening a oligo(dT)-primed cDNA library with probe B2 3′-UTR. The poly(A) site is placed 1059 bp downstream of the TAA termination codon (Fig. 2B). The poly(A) site resides in four adenosine residues. Interestingly, adenosine residues have been described as preferential polyadenylation sites, especially when repeated (36). Seventeen nucleotides beyond the termination codon, the nucleotide sequences of the two 3′-UTRs start to diverge and no sequence similarity is observed downstream. A comparative analysis between the L. infantum 3′-UTR-II and the hsp70 genes of other Leishmania species indicated that there is a high degree of sequence similarity (85%) with the 3′-UTR of the "orphan" hsp70 gene of L. major (18). Remarkably, the highest values of sequence similarity between both genes is found next to the poly(A) addition sites.

**Differential Expression of hsp70 Genes**

To evaluate the steady-state level of the RNA transcribed in the hsp70 gene cluster, total RNA extracted from logarithmic phase promastigote cultures at 26 °C (normal temperature) and at 37 °C (heat shock) and from stationary phase promastigote cultures was probed with 32P-labeled oligonucleotides complementary to specific regions of the 3′UTR-I and -II (see “Experimental Procedures” for more details). Densitometric analysis of the Northern blots showed that the levels of expression of genes 1 through 5 increased about 2–3-fold in the parasites grown at 37 °C when compared with those grown at 26 °C (Fig. 3). Parasites grown in stationary phase did not show increased levels of the genes 1–5 transcripts relative to those found in logarithmic phase parasites (Fig. 3A). On the other hand, the 3′UTR-II probe hybridized also with a 3.1-kb mRNA which was found to be constitutively transcribed. The levels of these transcripts did not change with a heat-shock treatment or in parasites from the stationary phase (Fig. 3B). Northern blots of poly(A)+ RNA from promastigotes in logarithmic (at 26 or 37 °C) and stationary phase probed with the different 3′-UTRs showed a similar pattern as blots of total RNA, indicating that both hsp70 RNAs contain poly(A) tails (not shown).

As judged by the exposure time required to have a similar autoradiographic signal in the Northern blots hybridized with each one of the probes, we deduced that the transcript derived from gene 6 is more abundant than the transcripts derived from genes 1–5. In order to quantify the abundance of the transcripts containing the 3′UTR-I (hsp70 genes 1–5) relative
heat-shock treatment promoted a 2.5-fold increase in the levels of hsp70 proteins.

In summary, the data show that the growth phase. This analysis indicated, moreover, that in transcripts from parasites growing at 26°C, independently of the temperature treatment had no effect on the abundance of the nascent transcripts containing the 3' UTR-II region. The results showed, moreover, that the transcript is regulated in a temperature-sensitive manner.

Post-Transcriptional Regulation of the hsp70 Gene Expression

To determine whether the abundance of the hsp70 transcripts derived from the two UTR-I regions was similar (Fig. 5) as would be expected if the cluster was transcribed as a polycistron starting at a single promoter site. The results showed, however, that the temperature treatment had no effect on the abundance of the nascent transcripts containing the 3' UTR-I region (Fig. 5) as an indication that the steady-state level of the transcripts derived from genes 1–5 must be regulated at the post-transcriptional level.

Temperature Control of hsp70 mRNA Stability

Since the differences in the steady-state level of the hsp70-3' UTR-I and hsp70-3' UTR-II transcripts seems to be due to post-transcriptional regulation, an analysis of the decay of those transcripts with time after inhibition of RNA synthesis by actinomycin D was done in Northern blots. The RNA extracted from actinomycin D-treated cells was hybridized sequentially with probes corresponding to the 3' UTR-I, the 3' UTR-II, the α-tubulin gene, and rDNA gene (Fig. 6). The results shown in Fig. 6 indicate that the transcripts derived from the genes containing the 3' UTR-I are more stable at 37°C than at 26°C. Densitometric analysis of the blots indicated that 2 h of incubation at 26°C in the presence of actinomycin D led to reduction of the 3' UTR-I mRNA to 5% of the transcripts present at 0 time, while after 2 h at 37°C the 3' UTR-I transcripts were 50% of those at the 0 time. In contrast, the transcripts derived from the gene containing the 3' UTR-II presented a temperature-independent decay. Instead, it seems that the decay of the 3' UTR-II transcripts and those of the α-tubulin is somewhat higher at 37°C than at 25°C. Also, Aly et al. (37) reported that the L. amazonensis α-tubulin mRNA was less stable at 35°C than at 26°C. Hybridization of the blots to the rDNA probe indicated that equivalent amounts of RNA were loaded in the different lanes (Fig. 6). The comparison of the decay rates of the 3' UTR-I and -II transcripts seems to indicate that while at 26°C the levels of 3' UTR-I transcripts decay faster than those containing the 3' UTR-II; at 37°C, the decay of both transcripts is similar. At a first view, the differences in the decay times of 3' UTR-I and 3' UTR-II transcripts at 26°C do not seem enough to explain the differences, about 50-fold, in the steady-state RNA levels (Fig. 4). However, we think that care should be taken in the interpretation of these data. A densitometric analysis of the...
3'UTR-II transcripts during actinomycin D treatment indicated that they do not show a first order decay curve; the 3'UTR-II transcripts decay slowly during the first 2 h and rapidly after that time. Thus, a secondary effect of actinomycin D on 3'UTR-II RNA levels cannot be excluded. Hence, alternative methods for mRNA half-time measurements are currently used in order to determine the stability of \textit{hsp70} 3'UTR-II transcripts.

\textbf{Ongoing Protein Synthesis Is Required for \textit{hsp70} 3'UTR-I mRNA Stabilization}

In order to test for potential mechanisms of mRNA stabilization, we examined whether the levels of decay were affected by inhibition of the protein synthesis. Thus, parallel cultures were incubated at 37 °C for different periods (0, 1, 2 and 4 h) either in the absence or the presence of cycloheximide A in conditions in which the synthesis of protein was inhibited to 92.6% (8). At the indicated times, culture aliquots were harvested for RNA extraction. Total RNA samples were blotted, and the filters were sequentially probed with the 3'UTR-I and 3'UTR-II probes. Densitometric analysis of the blots showed that in the absence of cycloheximide A the \textit{hsp70}-3'UTR-I transcripts accumulated with incubation time at 37 °C reaching a maximum after 2 h (Fig. 7A). Instead, when the same Northern blot was hybridized with probe 3'UTR-II (Fig. 7B), it was found that the \textit{hsp70}-3'UTR-II mRNA levels remained constant along the incubation time. However, at 37 °C and in the presence of cycloheximide A the \textit{hsp70}-3'UTR-I mRNAs did not accumulate (Fig. 7A). The presence of cycloheximide A did not influence the levels of the \textit{hsp70} 3'UTR-II transcripts along the heat-shock treatment (Fig. 7B). Thus, it can be concluded that the observed stabilization at 37 °C of the 3'UTR-I transcripts is dependent on on-going protein synthesis. Two interpretations to these data exist: (a) a labile regulator protein induced by heat shock should be involved in the 3'UTR-I RNA stabilization, or (b) the 3'UTR-I RNA needs to be actively translated in order to be stabilized by heat shock.

\textbf{DISCUSSION}

The heat-shock response is an evolutionary conserved mechanism that provides the cell with increased levels of a set of highly conserved proteins (hsps) which seem to be implicated in the adaptation and survival of the cell to heat and other stress conditions (38). In most of eukaryotes, \textit{hsp} expression is primarily controlled at the transcriptional level. This regulation is based on a highly conserved mechanism of DNA-protein interactions between a heat-shock transcription factor and a consensus DNA sequence known as the heat-shock element (39). Given that parasitic protozoa of the \textit{Trypanosoma} and \textit{Leishmania} genus are subjected to a heat shock when they are transferred from the temperature of their insect vector to the 37 °C temperature of their mammalian host, it has been suggested that the heat shock may be part of a differentiation mechanism (40). Thus, heat-shock genes have been chosen as a suitable system to study gene regulation in \textit{Trypanosomatids}. It has been shown that the heat-shock genes of the \textit{hsp70} and \textit{hsp83} families are transcribed constitutively at a high rate in different stages of the life cycle although the steady-state levels of the \textit{hsp} transcripts increase during heat shock (16, 18, 19, 22, 23, 41–43). However, in contrast to non-protozoan eukaryotes, the cellular concentration of the \textit{hsp} gene products in \textit{trypanosomes} is mainly regulated at a post-transcriptional level (2–5, 37, 44, 45). It appears, moreover, that post-transcriptional regulation is common to the expression of most \textit{trypanosome} genes (13).

Our data indicate that the \textit{L. infantum} \textit{hsp70} gene cluster contains, at least, six copies of the gene and that the units are arranged in tandem having conserved 5'UTRs and coding regions (Fig. 1A). However, the 3'UTR-I, common to genes 1–5, is divergent in sequence relative to the 3'UTR-II of gene 6. In \textit{L. major} four of the \textit{hsp70} gene copies are arranged in tandem (genes 1–4), whereas the fifth \textit{hsp70} orphan gene (gene 5) is located in a different locus (18). In the \textit{L. infantum} genome the \textit{hsp70} genes 1–5 with the 3'-UTR regions similar to those of the \textit{L. major} genes 1–4, and gene 6 with the 3'-UTR similar to that of the \textit{L. major} gene 5, are located in the same cluster. Remarkably, despite the different organization of the \textit{hsp70} genes between both \textit{Leishmania} species a parallelism in the pattern of gene expression is maintained. While the gene products of the \textit{L. major} \textit{hsp70} genes 1–4 (18) and those of the \textit{L. infantum} \textit{hsp70} genes 1–5 (this work) are increased after heat shock, the products of the \textit{L. major} \textit{hsp70} gene 5 and that of the \textit{L. infantum} \textit{hsp70} gene 6 are unaffected by temperature shifts.

Our data show also that during normal growth at 26 °C the steady-state level of the mRNAs derived from gene 6 is 50-fold higher than the level of the mRNAs derived from genes 1–5 and that only the expression of the genes 1–5 increased after a heat-shock treatment while the expression level of gene 6 remained unaffected. The results of the run-on assays showed that all genes are transcribed at similar rates before and after heat shock as it would be expected from the present of con-
erved 5'-UTRs and intergenic regions along the cluster. The results are also in agreement with a polycistronic transcription of all the genes of the cluster from an unknown promoter.

Our data also showed that at 37 °C the hsp70-3 UTR-I transcripts are more stable at 37 °C than at 26 °C but that the heat-shock treatment did not affect the hsp70-3 UTR-II mRNA stability. Thus, the mechanisms responsible for the preferential accumulation of L. infantum hsp70-3 UTR-I mRNAs upon temperature elevation must be related with the sequence divergence between the two types of 3'-UTRs. Remarkably, the 3' UTR-I of genes 1–5 contains, next to the putative polyadenylation site, an inverted repeat with potential to form a stable stem-loop structure that might be implicated in the stabilization of the hsp70-3 UTR-I transcripts. A potentially similar stable stem-loop structure is absent in the 3' UTR-II of gene 6. In other eukaryotes, in addition to transcriptional activation, post-transcriptional regulation of hsp70 genes has been also observed. For example, it has been reported that the Drosophila hsp70 mRNA is rapidly degraded at normal temperatures and stabilized by heat shock and that the regulatory mechanism operates through recognition of the 3'-UTR of the hsp70 mRNA (46). The question, however, of whether the Drosophila hsp70 mRNA is an inherently stable message that is selectively degraded at normal temperatures or it is an inherently unstable message that is stabilized by heat shock remains open (47). In humans cells, it was observed that the heat shock increases the HSP70 mRNA stability at least 10-fold and that the HSP70 mRNA is more stable in cells treated with protein synthesis inhibitors suggesting that a heat shock-sensitive labile protein regulates its turnover (48). The effect of the heat shock on the L. infantum hsp70-3 UTR-I mRNA levels is similar to the effect of the treatment on the L. amazonensis hsp83 mRNA levels (3). However, the mechanism responsible for the temperature-induced accumulation of the L. infantum hsp70 mRNAs seems to be different from the one responsible for the regulation of L. amazonensis hsp83 mRNAs. The degradation of hsp83 mRNAs in L. amazonensis depends on active protein synthesis suggesting the implication of a labile nuclease that is active mainly at 26 °C (3). Our results indicated, however, that in the presence of cycloheximide at 37 °C the levels of the hsp70 3' UTR-I transcripts remain constant while in the absence of the drug the levels of the transcripts increased as an indication that ongoing protein synthesis is required to attain their stability. A model which might explain this result would implicate the direct interaction of a labile protein factor with the 3'-UTR-I of the mRNA hindering the activity of the nucleases implicated in mRNA degradation pathways. An alternative model would invoke the presence of a labile protein factor which can promote a down-regulation of a specific nuclease for the hsp70-3 UTR-I transcripts. In both models the putative labile protein factor would be active mainly at 37 °C. At present, a search for this putative protein factor is underway.

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