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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 level 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),[1] *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 not 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 polycistrons co-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).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y08019 and Y08020.

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[1] The abbreviations used are: hsp, heat-shock protein; kb, kilobase(s); PCR, polymerase chain reaction; UTR, untranslated region; bp, base pair(s); UPR, upstream region; IR, intergenic region.

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 (5–9 × 10^6 promastigotes ml^{-1}) were used.

Clones and Probes—Clone B2cDNA, a *L. infantum hsp70* cDNA isolated by immunological screening of a λ-gt11 cDNA expression library, has been previously described (24). This cDNA was cloned into the *Eco*RI site of plasmid pUC18. A 330-bp DNA fragment, named B2 3′UTR-II, was obtained after *Sal*I digestion of clone pB2cDNA (Fig. 1*A*). This fragment was cloned in the *Sal*I site of pUC18 and the resulting clone named pU3′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 ^{32}P-labeled B2cDNA as probe. Seven positive clones, B2g1 to B2g7, were isolated. Similarly, a screening was carried out on a *L. infantum* oligo(dT)-primed lambda-gt11 cDNA expression library (27) using the B2 3′UTR-II DNA fragment as probe. From this screening two cDNA clones, 70IIA-1 and 70IIA-2, were isolated.

Probe 3′UTR-I was obtained by PCR amplification from the 1.5-kb *Sal*I-*Bam*HI fragment (clone B2g6; Fig. 1*A*), subcloned in pUC18, using the oligonucleotide 70-I (5′-CACACCCAAGTACACGTCAG-3′) and the M13/pUC sequencing primer (−20) 17-mer (New England BioLabs, Inc.). Standard PCR conditions (Perkin-Elmer) in the presence of 5% dimethyl sulfoxide were used. The cycle profile, repeated 30 times, was 1 min at 94 °C, 1 min at 55 °C, and 1 min 72 °C. The PCR product was digested with *Sal*I, and the resulting 146-bp fragment was purified over a spin bind column (FMC Bio Products) and cloned in a *Sal*I-*Sma*I digested pBlueScript SK(−) vector. The resulting clone was named pB3′UTR-I.

Clone pTcα3 contains the α-*tubulin* gene of *T. cruzi* (28). Clone pRIB contains a 590-bp *Sma*I-*Hin*dIII fragment of the *L. infantum* 24Sα rRNA gene.[2]

Sequence Determinations—DNA sequencing was conducted on double-stranded DNA by the dideoxy chain termination method (29), using the Sequenase™ kit (United States Biochemical Corp.). The nucleotide sequence of the cDNAs was determined in both strands using internal synthetic oligonucleotides.

The restriction map of the inserts of the B2g1 to B2g7 genomic clones was determined for a variety of restriction enzymes (Fig. 1*A*). The consecutive 0.3-kb *Eco*RI-*Bam*HI, 2.1-kb *Bam*HI-*Sal*I, and 1.5-kb *Sal*I-*Bam*HI genomic fragments, conforming the 5′ repetition unit of the *L. infantum hsp70* gene cluster, were subcloned in pBlueScript SK(−) vector (Stratagene). Also, the 5.72-kb *Sal*I fragment from clone B2g1, containing the 3′-UTR of the *hsp70* gene located at 3′ end of the cluster (see Fig. 1*A* and text for details), was subcloned in pBlueScript. In order to obtain the complete sequence of the 2.1-kb *Bam*HI-*Sal*I fragment exonuclease III/mung bean nuclease deletions were carried out as described elsewhere (24). Synthetic oligonucleotides were used for sequencing of the other fragments. The analysis of the nucleotide and amino acid sequences was done using the University of Wisconsin Genetics Computer Group programs (30) and by accessing the GenBank and EMBL data bases.

Southern and Northern Blot Analyses—*L. infantum* DNA and RNA were isolated as described previously (17, 31). Promastigote total DNA was digested with a variety of restriction endonucleases, electrophoresed in 0.8% agarose gels, and transferred to nylon membranes (Hybond-N, Amersham Corp.) by standard methods (26). Five μg per lane of total RNA was size separated on 1% agarose-formaldehyde gels (32) and electrophoresed to nylon membranes using a LKB system (Pharmacia Biotech Inc.). Hybridizations, either for DNA or RNA analysis, were performed in 50% formamide, 6 × SSC, 0.1% SDS, and 0.25 mg ml^{-1} herring sperm DNA at 42 °C overnight. Final posthybridization washes were performed in 0.1 × SSC, 0.2% SDS at 50 °C for 1 h. For reuse, blots were treated with 0.1% SDS for 30 min at 95 °C to remove the previously hybridized probes. Removal of the probes was verified by autoradiography.

Nuclease S1 Protection Assays—Two oligonucleotides derived from the divergent 3′-UTR sequences were synthesized (Isogen): 70-I, 5′-CACACCCAAGTACACGTCAG-3′ (reverse and complementary to nucleotides 2079–2099; Fig. 2*A*), and 70-II, 5′-GGGAAGCCCCACAGCGGAAAAGTGG-3′ (reverse and complementary to nucleotides 525–550; Fig. 2*B*). The oligonucleotides were labeled with 50 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Corp.) using the T4-polynucleotide kinase kit (Boehringer Mannheim). The specific activity of the labeling was 10^9 cpm/μg. A molar excess (0.07 pmol) of ^{32}P-labeled oligonucleotide was hybridized with 2 μg of *L. infantum* poly(A^+) RNA (purified by oligo(dT)-cellulose; Boehringer Mannheim) in a 25-μl final 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-cooling of the hybridization reaction, 25 μl of 2 × S1 buffer (66 mM NaAc, pH 4.5, 100 mM NaCl, and 0.06 mM $ZnS0_4$) 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% bromphenol 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—Promastigote cultures in logarithmic phase growth (5 × 10^6 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, 0.64 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 $MnCl_2$, 1 mM $MgCl_2$, 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 (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 μg ml^{-1} creatine kinase (Boehringer Mannheim)) was added. The run-on transcripts were labeled by adding 100 μCi of [α-^{32}P]UTP (3000 Ci $mmol^{-1}$) (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 $MgCl_2$ to final concentrations of 25 μg ml^{-1} and 5 mM, respectively, and incubation for 20 min at 37 °C. Subsequently, the reaction continued in the presence of 0.15 mg ml^{-1} proteinase 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 × 10^6 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 coding 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 carboxyl-terminal 20 amino acids and for 330 bp of the 3′-UTR. In order to analyze the genomic organization of the *hsp70* genes, *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 *Sal*I or *Bam*HI was taken as an indication of the presence of several copies of the *hsp70* gene which are arranged in tandem and that these

[2] C. Alonso, unpublished data.

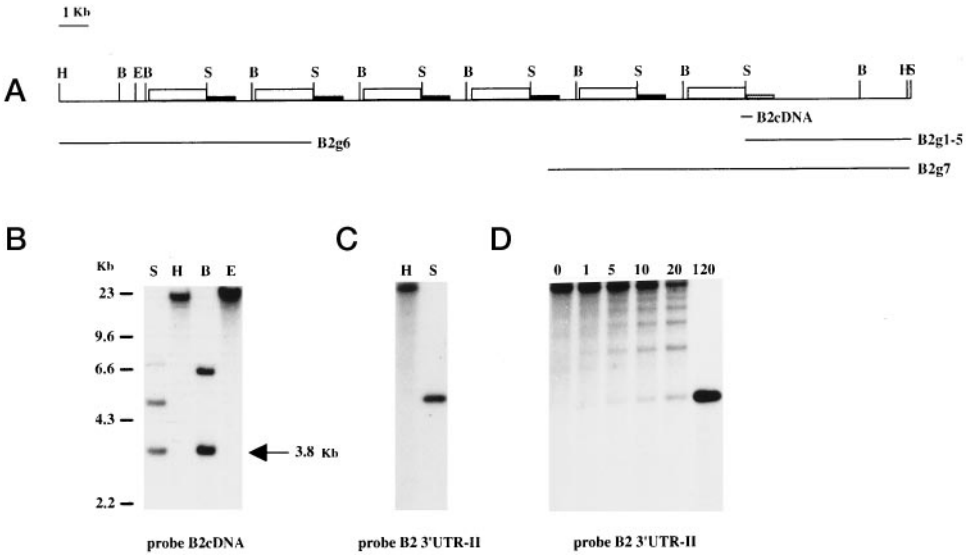

FIG. 1. **Genomic organization of *L.* infantum hsp70 locus.** *A,* schematic representation of the *hsp70* locus of *L. infantum*. The coding regions (*open boxes*) and 3′-UTRs (*filled boxes*) for the six *hsp70* genes are indicated. From *left* to *right*, *hsp70* genes were named 1 to 6. The location of the different clones is shown below the physical map. *H*, *Hin*dIII; *B*, *Bam*HI; *E*, *Eco*RI; *S*, *Sal*I. *B*, genomic organization analysis of *hsp70* genes. *L. infantum* DNA was digested, blotted, and probed with B2cDNA. *Lanes: S, Sal*I; *H*, *Hin*dIII; *B*, *Bam*HI; *E*, *Eco*RI. *C*, Southern blot analysis of genomic distribution of 3′UTR-II containing *hsp70* gene. *Lanes: H*, *Hin*dIII; *S*, *Sal*I. *D*, copy number determination of the *hsp70* genes. After complete *Hin*dIII digestion of *L. infantum* total DNA, it was partially digested with *Sal*I for 0, 1, 5, 10, 20, and 120 min. After electrophoresis and Southern blotting, blot was hybridized with probe B2 3′UTR-II.

enzymes cut only once within the repeat. In addition, the fact that others restriction enzymes (*Hin*dIII and *Eco*RI) 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. 1*A*). 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. 1*A*). 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. 1*C*. The probe hybridized only with the 5.7-kb *Sal*I band, whereas it did not hybridize with the 3.8-kb *Sal*I repetition unit. In order to determine the copy number of the *hsp70* genes, the genomic DNA was first digested at completion with *Hin*dIII and then partially digested with *Sal*I (Fig. 1*D*). As the Southern blot was hybridized with the 3′UTR-II fragment of B2cDNA, the first unit of the ladder would be 5.7 kb long and the length of the rest of the units would be increased by 3.8 kb. Thus, since six hybridization bands were clearly observed we concluded that at least six *hsp70* gene copies should be tandemly arranged in the *L. infantum hsp70* gene cluster (Fig. 1*A*).

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 *Eco*RI-*Bam*HI and the adjacent 3.8-kb *Bam*HI fragment, which contains the first repetition unit present in clone B2g6 (Fig. 1*A*), were sequenced. A diagrammatic representation of the sequence is shown in Fig. 2*A*. 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 *Bam*HI 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 indicated in Fig. 1*A*. The sequence analysis indicated, moreover, that the UPR of the *hsp70* gene 1 and the UPR of the *hsp70* gene 2 show total sequence identity (Fig. 2*A*). 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) arrayed 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 also highly conserved (Table I).

Coding Region (CDR)—The CDR is 1959 bp long and ends just at the *Sal*I restriction site (Fig. 2*A*). 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).

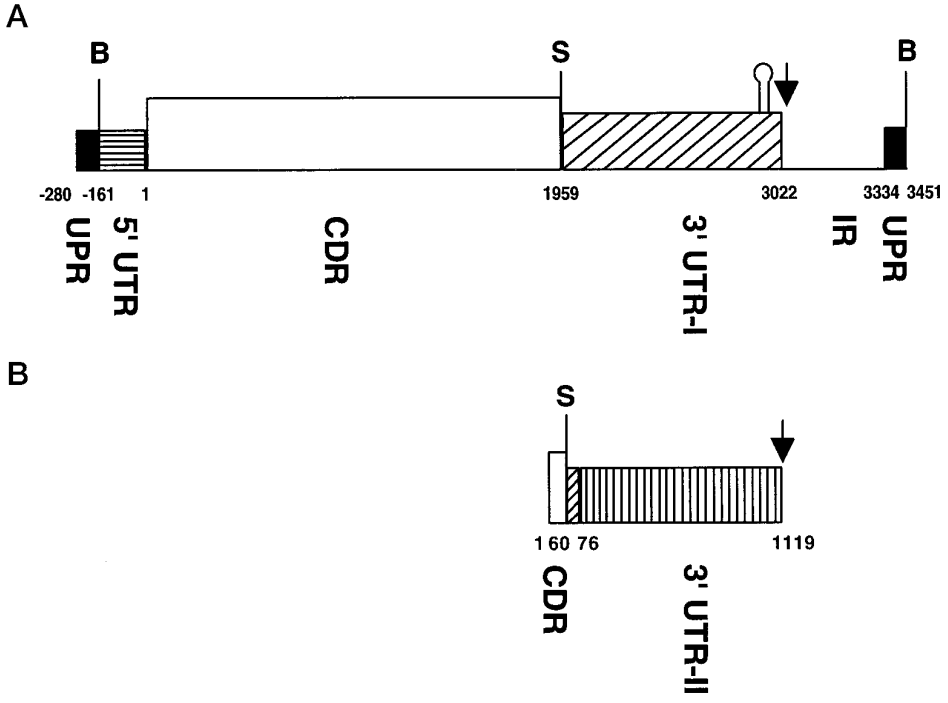

FIG. 2. **Schematic representation of the sequenced regions of the *hsp70* gene cluster.** *A*, sequence subregions of *hsp70* gene 1. The nucleotide sequence of this gene was submitted to the EMBL data base with the accession number Y08020. *UPR*, upstream region; *5′UTR*, 5′-untranslated region; *CDR*, protein coding region; *3′UTR-I*, 3′-untranslated region, common to *hsp70* genes 1–5; *IR*, intergenic region; *B*, *Bam*HI; *S*, *Sal*I. *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	Lm	Li	Ld	Lm	Li	Ld	Lm	Li	Ld	Lm	Li	Ld	Lm
Ld[a]	94.35[b]			93.21			97.65			91.23			90.94		
Lm	96.52	94.78		93.36	93.93		97.44	97.64		89.5	91.17		89.8	90.62	
La	68.85	65.57	86.66	93.15	91.78	93.79	95.74	96.42	96.21	83.18	85.71	85.3	85.27	78.64	81.01

[a] 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.
[b] % 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 *Sal*I restriction fragment derived from clone B2g1 (Fig. 1*A*). A diagrammatic representation of the 3′UTR-II is shown in Fig. 2*B*. 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-II. The poly(A) site is placed 1059 bp downstream of the TAA termination codon (Fig. 2*B*). 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 ^{32}P-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*A*). 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. 3*A*). 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. 3*B*). 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

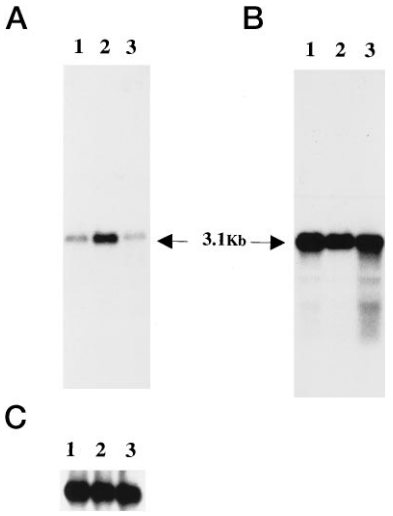

FIG. 3. **Northern blot analysis of transcripts from the *hsp70* gene cluster.** The Northern blot containing total RNA from logarithmic phase promastigotes incubated either at 26 °C (*lane 1*) or at 37 °C for 2 h (*lane 2*) and from stationary phase promastigotes (*lane 3*) was probed with oligonucleotides specific to the 3′UTR-I (70-I, *A*) and to the 3′UTR-II (70-II, *B*). Finally, the blot was probed with clone pRIB, containing part of the 24Sα rRNA gene (*C*).

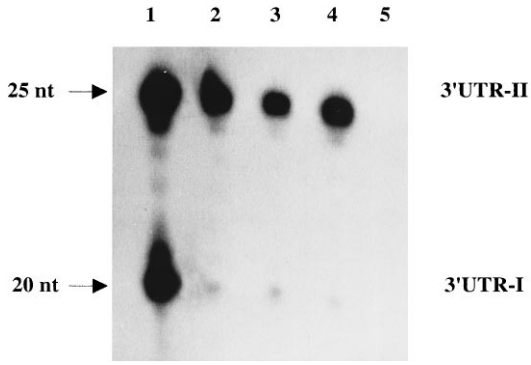

FIG. 4. **S1 protection analysis of the *hsp70* mRNAs.** 5′-Labeled synthetic oligonucleotides derived from the sequence of 3′UTR-I and 3′UTR-II regions were hybridized in molar excess (0.07 pmol) with 2 μg of poly(A)$^+$ RNA isolated from either logarithmic phase promastigotes incubated at 26 °C (*lane 2*), logarithmic phase promastigotes incubated at 37 °C for 2 h (*lane 3*), or stationary phase promastigotes incubated at 26 °C (*lane 4*). As control, the labeled oligonucleotides were hybridized with 4 μg of *E. coli* rRNAs (*lane 5*). After 3 h of hybridization, samples were incubated with 40 units of S1 nuclease for 15 min and loaded in a sequencing gel (see "Experimental Procedures"). As markers, 2 fmol of each one of the labeled oligonucleotides (*nt*) were loaded (*lane 1*).

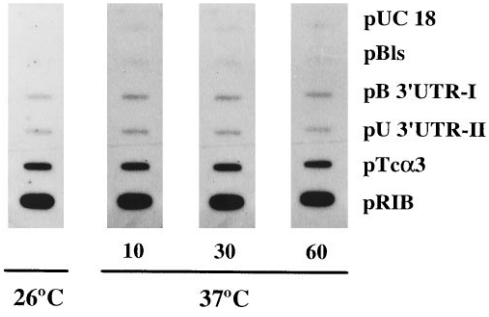

FIG. 5. **Nuclear run-on assays of transcription of *hsp70* gene cluster.** Promastigotes of *L. infantum* were grown at 26 °C, and parallel cultures were incubated at 37 °C during 10, 30, and 60 min. Run-on transcripts, labeled with [^{32}P]UTP, were hybridized to slot blot containing 5 μg of linearized plasmid DNA. The plasmid probes that were used included the *hsp70* 3′UTR-I (pB3′UTR-I), the *hsp70* 3′UTR-II (pU3′UTR-II), the *T. cruzi α-tubulin* gene (*pTcα3*), an rDNA clone from *L. infantum* (*pRIB*), and as control the plasmids pUC18 and pBlueScript (*pBls*).

to those containing the 3′UTR-II (*hsp70* gene 6), a nuclease S1 protection assay was carried out using UTR-I- or UTR-II-specific oligonucleotides (Fig. 4). The densitometric evaluation of the resulting bands showed that *hsp70* 3′UTR-II transcripts were about 50 times more abundant than the *hsp70* 3′UTR-I transcripts from parasites growing at 26 °C, independently of the growth phase. This analysis indicated, moreover, that in agreement with the Northern blot analysis shown in Fig. 3*A*, a heat-shock treatment promoted a 2.5-fold increase in the levels of 3′UTR-I transcripts. There were no changes in the level of the *hsp70*-3′UTR-II transcripts after a heat-shock treatment. In summary, the data show that the *hsp70* gene cluster of *L. infantum* contains a *hsp70* gene which is expressed constitutively and five additional *hsp70* genes whose expression product is regulated in a temperature-sensitive manner.

Post-Transcriptional Regulation of the hsp70 Gene Expression

To determine whether the abundance of the *hsp70* 3′UTR-II transcripts relative to those containing the 3′UTR-I are due to differential transcriptional activation, run-on transcription analysis was carried out. The results showed that the abundance of the nascent transcripts derived from both types of *hsp70* genes was similar (Fig. 5) as would be expected if the cluster was transcribed as a polycistron starting at a single promotor site. The results showed, moreover, 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 reduction 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

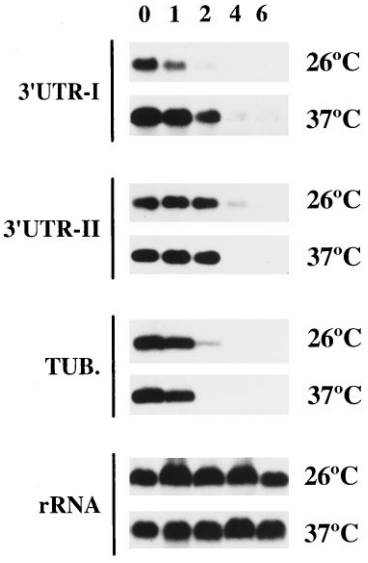

FIG. 6. **Effect of heat shock on the stability of the *hsp70* transcripts.** Promastigote cultures were incubated with actinomycin D either at 26 or 37 °C for 0, 1, 2, 4, and 6 h. Afterwards, total RNA was extracted, and RNA aliquots of 5 μg each were analyzed by Northern blotting. The blot was probed in succession with the 3′UTR-I (pB3′UTR-I), the 3′UTR-II (pU3′UTR-II), α-tubulin (*TUB.*) (pTcα3), and rRNA (pRIB).

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 *hsp70* 3′UTR-II transcripts.

Ongoing Protein Synthesis Is Required for 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 *hsp70*-3′UTR-I transcripts accumulated with incubation time at 37 °C reaching a maximum after 2 h (Fig. 7*A*). Instead, when the same Northern blot was hybridized with probe 3′UTR-II (Fig. 7*B*), it was found that the *hsp70*-3′UTR-II mRNA levels remained constant along the incubation time. However, at 37 °C and in the presence of cycloheximide A the *hsp70*-3′UTR-I mRNAs did not accumulate (Fig. 7*A*). The presence of cycloheximide A did not influence the levels of the *hsp70* 3′UTR-II transcripts along the heat-shock treatment (Fig. 7*B*). Thus, it can be concluded that the observed stabilization at 37 °C of the 3′UTR-I transcripts is dependent of 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.

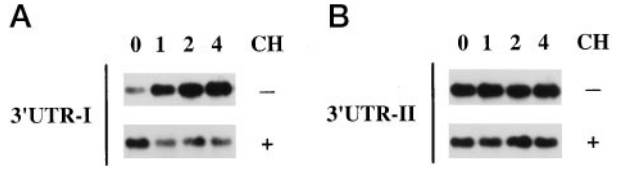

FIG. 7. **Effect of cycloheximide on the steady-state levels of *hsp70* mRNAs.** RNA was extracted from promastigote cultures grown at 37 °C for 0, 1, 2, and 4 h. Parallel cultures were incubated with cycloheximide (*CH*) at 37 °C for the same periods. The Northern blots were hybridized with a probe specific for the 3′UTR-I (*A*). The same blots were stripped and rehybridized with a probe specific for the 3′UTR-II (*B*).

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, 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 *Trypanosoma* and *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 Trypanosomatids. It has been shown that the heat-shock genes of the *hsp70* and *hsp83* families are transcribed constitutively at a high rate in different stages of the life cycle although the steady-state levels of the 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 *hsp* gene products in 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 trypanosome genes (13).

Our data indicate that the *L. infantum 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. 1*A*). However, the 3′UTR-I, common to genes 1–5, is divergent in sequence relative to the 3′UTR-II of gene 6. In *L. major* four of the *hsp70* gene copies are arranged in tandem (genes 1–4), whereas the fifth *hsp70* orphan gene (gene 5) is located in a different locus (18). In the *L. infantum* genome the *hsp70* genes 1–5 with the 3′-UTR regions similar to those of the *L. major* genes 1–4, and gene 6 with the 3′-UTR similar to that of the *L. major* gene 5, are located in the same cluster. Remarkably, despite the different organization of the *hsp70* genes between both *Leishmania* species a parallelism in the pattern of gene expression is maintained. While the gene products of the *L. major hsp70* genes 1–4 (18) and those of the *L. infantum hsp70* genes 1–5 (this work) are increased after heat shock, the products of the *L. major hsp70* gene 5 and that of the *L. infantum 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-

served 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 promotor.

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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REFERENCES

1. Zilberstein, D., and Shapira, M. (1994) *Annu. Rev. Microbiol.* **48,** 449–470
2. Brandau, D., Dresel, A., and Clos, J. (1995) *Biochem. J.* **310,** 225–232
3. Argaman, M., Aly, R., and Shapira, M. (1994) *Mol. Biochem. Parasitol.* **64,** 95–110
4. Häusler, T., and Clayton, C. (1996) *Mol. Biochem. Parasitol.* **76,** 57–71
5. Lee, M. G.-S. (1996) *Mol. Cell. Biol.* **16,** 1220–1230
6. Gibson, W. C., Swinkels, B. W., and Borst, P. (1988) *J. Mol. Biol.* **201,** 315–325
7. Pays, E., Coquelet, H., Pays, A., Tebabi, P., and Steinert, M. (1989) *Mol. Cell. Biol.* **9,** 4018–4021
8. Wilson, M. E., Paetz, K. E., Ramamoorthy, R., and Donelson, J. E. (1993) *J. Biol. Chem.* **268,** 15731–15736
9. Berberof, M., Vanhamme, L., Tebabi, P., Pays, A., Jefferies, D., Welburn, S., and Pays, E. (1995) *EMBO J.* **14,** 2925–2934
10. Teixeira, S. M. R., Kirchhoff, L. V., and Donelson, J. E. (1995) *J. Biol. Chem.* **270,** 22586–22594
11. Blattner, J., and Clayton, C. E. (1995) *Gene* (*Amst.*) **162,** 153–156
12. Hotz, H.-R., Lorenz, P., Fischer, R., Krieger, S., and Clayton, C. (1995) *Mol. Biochem. Parasitol.* **75,** 1–14
13. Graham, S. V. (1995) *Parasitol. Today* **11,** 217–223
14. Vanhamme, L., and Pays, E. (1995) *Microbiol. Rev.* **59,** 223–240
15. Agabian, N. (1990) *Cell* **61,** 1157–1160
16. Glass, D. J., Polvere, R. I., and Van der Ploeg, L. H. T. (1986) *Mol. Cell. Biol.* **6,** 4657–4666
17. Requena, J. M., Lopez, M. C., Jimenez-Ruiz, A., de la Torre, J. C., and Alonso, C. (1988) *Nucleic Acids Res.* **16,** 1393–1406
18. Lee, M. G.-S., Atkinson, B. L., Giannini, S. H., and Van der Ploeg, L. H. T. (1988) *Nucleic Acids Res.* **16,** 9567–9585
19. Searle, S., Campos, A. J. R., Coulson, R. M. R., Spithill, T. W., and Smith, D. F. (1989) *Nucleic Acids Res.* **17,** 5081–5095
20. Bock, J. H., and Langer, P. J. (1993) *Mol. Biochem. Parasitol.* **62,** 187–198
21. Dragon, E. A., Sias, S. R., Kato, E. A., and Gabe, J. D. (1987) *Mol. Cell. Biol.* **7,** 1271–1275
22. Mottram, J. C., Murphy, W. J., and Agabian, N. (1989) *Mol. Biochem. Parasitol.* **37,** 115–128
23. Shapira, M., and Pinelli, E. (1989) *Eur. J. Biochem.* **185,** 231–236
24. Quijada, L., Requena, J. M., Soto, M., and Alonso, C. (1996) *Parasitology* **112,** 277–284
25. Soto, M., Requena, J. M., Garcia, M., Gómez, L. C., Navarrete, I., and Alonso, C. (1993) *J. Biol. Chem.* **268,** 21835–21843
26. Maniatis, T., Fritsch, E. F., and Sambrook, J., (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Soto, M., Requena, J. M., Gomez, L. C., Navarrete, I., and Alonso, C. (1992) *Eur. J. Biochem.* **205,** 211–216
28. Soares, C. M. A., de Carvalho, E. F., Urményi, T. P., Carvalho, J. F. O., de Castro, F. T., and Rondinelli, E. (1989) *FEBS Lett.* **250,** 497–502
29. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
30. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395
31. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
32. Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977) *Biochemistry* **16,** 4743–4751
33. Curotto de Lafaille, M. A., Laban, A., and Wirth, D. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2703–2707
34. Huang, J., and Van der Ploeg, L. H. T. (1991) *EMBO J.* **10,** 3877–3885
35. LeBowitz, J. H., Smith, H. Q., Rusche, L., and Beverley, S. M. (1993) *Genes Dev.* **7,** 996–1007
36. Hug, M., Hotz, H.-R., Hartmann, C., and Clayton, C. (1994) *Mol. Cell. Biol.* **14,** 7428–7435
37. Aly, R., Argaman, M., Halman, S., and Shapira, M. (1994) *Nucleic Acids Res.* **22,** 2922–2929
38. Lindquist, S. (1986) *Annu. Rev. Biochem.* **55,** 1151–1191
39. Morimoto, R. I., Sarge, K. D., and Abravaya, K. (1992) *J. Biol. Chem.* **267,** 21987–21990
40. Van der Ploeg, L. H. T., Giannini, S. H., and Cantor, C. R. (1985) *Science* **228,** 1443–1446
41. MacFarlane, J., Blaxter, M. L., Bishop, R. P., and Kelly, J. M. (1990) *Eur. J. Biochem.* **190,** 377–384
42. Lee, M. G.-S., and Van der Ploeg, L. H. T. (1990) *Mol. Biochem. Parasitol.* **41,** 221–232
43. Requena, J. M., Jimenez-Ruiz, A., Soto, M., Assiego, R., Santarén, J. F., Lopez, M. C., Patarroyo, M. E., and Alonso, C. (1992) *Mol. Biochem. Parasitol.* **53,** 201–212
44. Aly, R., Argaman, M., Pinelli, E., and Shapira, M. (1993) *Gene* (*Amst.*) **127,** 155–163
45. Aly, R., Miriam, A., and Michal, S. (1995) *Exp. Parasitol.* **80,** 159–162
46. Petersen, R., and Lindquist, S. (1988) *Gene* (*Amst.*) **72,** 161–168
47. Yost, J., Petersen, R. B., and Lindquist, S. (1990) *Trends Genet.* **6,** 223–227
48. Theodorakis, N. G., and Morimoto, R. I. (1987) *Mol. Cell. Biol.* **7,** 4357–4368

Nucleic Acids, Protein Synthesis, and Molecular Genetics:

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

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