The Translational Efficiencies of the Two Leishmania infantum HSP70 mRNAs, Differing in Their 3′-Untranslated Regions, Are Affected by Shifts in the Temperature of Growth through Different Mechanisms*

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Exposure of Leishmania promastigotes to the temperature of their mammalian hosts induces a typical heat-shock response. In Leishmania infantum, HSP70 is encoded by two types of genes that differ in their 3′-untranslated regions (3′-UTRs). Previously, we have shown that specific transcripts for each gene are present in promastigotes growing at normal temperature (26 °C), but only transcripts with 3′-UTR-type I (3′-UTRI) accumulate in a temperature-dependent manner. Here, we have investigated the translational efficiencies of both types of HSP70 transcripts at the different temperatures that the parasite encounters in the insect (26 °C, normal temperature) or in the mammalian host (heat-shock temperatures). Interestingly, 3′-UTRI-bearing transcripts (HSP70-I) were found associated with ribosomes in promastigotes at normal and heat-shock temperatures, whereas the HSP70-II transcripts appear to be preferentially translated at heat-shock temperatures but not at 26 °C. We have analyzed the function of these UTRs in the translational control by use of plasmid constructs in which the CAT reporter gene was flanked by UTRs of the HSP70 genes. Unexpectedly, it was found that CAT transcripts with 3′-UTRII bind to ribosomes at 26 °C, and, indeed, the CAT protein is synthesized. A valid conclusion of these experiments was that both types of 3′-UTRs are essential for translation of HSP70 mRNAs at heat shock temperatures, although the 3′-UTRII is more efficient during severe heat shock (39 °C). In addition, these results suggest that sequence region other than the 3′-UTR of HSP70-II gene is involved in the translational silent state of HSP70-II transcripts at 26 °C. Finally, a null mutant has been created by targeted disruption of both HSP70-II alleles. Remarkably, the ΔHSP70 mutant synthesizes HSP70 at a lower rate than the wild-type parasites. Overall, our data suggest that the biological function of the HSP70-II gene is to top up HSP70 levels under conditions of stress.

Protozoan parasites of the genus Leishmania are causative agents of leishmaniasis, one of the major parasitic diseases in humans. Leishmaniasis affects ~12 million people worldwide, and 1.5–2 million new cases occur every year (1). Leishmania is a member of the family Trypanosomatidae and of the order Kinetoplastida. Kinetoplastid parasites are evolutionarily ancient organisms possessing very peculiar mechanisms to control gene expression (2). Thus, regulation of gene expression in Leishmania, and other kinetoplastid protozoa, occurs by mechanisms operating almost exclusively at post-transcriptional level (3, 4). It is likely that the mechanisms of gene expression are related to the unusual gene organization in these organisms. Sequencing of L. major chromosomes, recently completed (5), has revealed that most of the genes in Leishmania are organized into large clusters of genes located on the same DNA strand. Current data suggest that transcription by RNA polymerase II starts upstream of the most-5′-gene of each cluster, proceeding along the cluster and yielding polycistronic transcripts (6, 7). In these organisms, the mature nuclear mRNAs are generated from polycistronic transcripts by trans-splicing and polyadenylation (8). Given this constitutive production of transcripts, the regulation of gene expression must rely strongly on the stability of the mature transcript and on the efficiency with which is translated. However, how gene expression is regulated is one of the least understood aspects of these organisms. Leishmania parasites exhibit a two-phase life cycle, alternating between a bloodfeeding insect vector and a mammalian host. The parasites are exposed to growth conditions that differ significantly between vector and host in temperature, pH and nutrients. These environmental alterations act as differentiation signals, triggering changes in gene expression necessary to allow adaptation and differentiation. The temperature-induced gene expression, or heat shock response, is considered paramount to the differentiation process of Leishmania parasites (9, 10). The heat shock or stress response has been conserved in evolution from bacteria to man, and the genes encoding heat shock proteins (HSPs)2 are among the most conserved evolutionarily (11). Furthermore, the heat shock response is an ideal model to study inducible genetic responses, offering an appropriate system to understand how the cell recognizes and responds to environmental cues (12). Thus, the genes encoding HSPs in Leishmania are being extensively studied not only because of their importance for parasite differentiation but also as a prototypic gene model for deciphering the mechanisms of gene expression in this parasite.

Exposure of Leishmania promastigotes to temperatures typical of mammalian hosts leads to an accumulation of HSP70 and HSP83 transcripts. However, contrary to most eukaryotes, the transcription of HSP genes is not induced by heat shock in Leishmania (13, 14). Hence, it was concluded that the temperature-induced accumulation of these HSP transcripts is promoted through post-transcriptional mechanisms. The gene organization and expression of HSP70 and HSP83 have been thoroughly studied in L. infantum and L. amazonensis. The L. amazonensis HSP83 gene cluster contains 18 gene copies, apparently identical, that are organized in a head-to-tail tandem (15). A similar organization is

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2 The abbreviations used are: HSP, heat shock protein; UTR, untranslated region; UTRI, UTR-type I; CAT, chloramphenicol acetyltransferase; ORF, open reading frame.
Translational Regulation of Leishmania HSP70 Genes

observed in *L. infantum* (16). By use of plasmid constructs, it was demonstrated that the 3′-untranslated region (3′-UTR) of HSP83 contains the regulatory cis-elements responsible for the temperature-dependent accumulation of the corresponding transcripts (15). Also, the increased translation of HSP83 transcripts, occurring upon temperature elevation, is controlled through an unknown mechanism recognizing sequence elements located in their 3′-UTRs (15, 17). The organization and expression of HSP70 genes in *L. infantum* show peculiar features. There are six HSP70 genes arranged in a head-to-tail tandem and showing very limited sequence divergence (14). All the genes are conserved at their 5′-UTRs and coding regions; only the 3′- UTR of HSP70–6 gene is completely divergent relative to the 3′-UTR sequence shared by the other five genes. The most abundant HSP70 mRNAs correspond to HSP70–6 gene, but only mRNAs from genes 1 to 5 accumulate by heat shock (14). The 3′-UTRs together with downstream sequences of the HSP70 genes are necessary for a correct polyadenylation of both types of transcripts and are responsible for the differences in their steady-state levels (18). Also, it was found that mRNAs derived from genes 1 to 5 contain a cis-acting sequence which functions as a positive element that is responsible for the temperature-dependent accumulation of HSP70–1–5 mRNAs. From now on, and for simplicity, we will refer to genes HSP70–1 to HSP70–5 as HSP70-I gene, and to gene HSP70–6 as HSP70-II. Here we have analyzed the translational profile of both types of HSP70 mRNAs at both normal and heat-shock temperatures. We find that the abundant HSP70-II transcripts are translationally silent at normal growth conditions, whereas the heat-inducible HSP70-I transcripts are translated at both normal and heat shock temperatures. The results suggest that the HSP70-II transcripts are stored in *Leishmania* parasites with the biological function of providing de novo synthesis of HSP70 when parasites encounter stress conditions.

**EXPERIMENTAL PROCEDURES**

Parasites and Treatments—Promastigotes of *Leishmania infantum* (MCAN/ES/96/BCN150) were cultured *in vitro* at 26 °C in RPMI 1640 medium (Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), adding penicillin G (100 units/ml), and streptomycin (0.1 mg/ml). Logarithmic phase cultures (5–9 × 10^6 promastigotes/ml) were used in all the experiments described. For heat shock experiments, aliquots of cultured *L. infantum* promastigotes were incubated at different temperatures (26 °C, 37 °C, 39 °C, and 41 °C) for different periods of time. Immediately after heat shock, parasites were spun down and harvested for the analysis of steady-state mRNA levels or de novo protein synthesis.

**Plasmid Constructs**—The 5′-UTR, common to all *L. infantum* HSP70 genes, together with upstream sequences containing the appropriate signals for trans-splicing, were PCR-amplified using as template the pUCB2F clone. This clone is a pUC18 derivative that contains the 3.81-kb Sall DNA fragment from the genomic B2g6 clone (14). As primers, the following oligonucleotides were used: forward, 5′-TCTAGAAA-GATCTCGGAAGTCTGCTGAGAGCA-3′, and reverse, 5′-GATATCG-GATGCGCCCTGAATGTCAT-3′ (underlined are restriction sites included in the primers to facilitate cloning). After digesting the PCR product with XbaI plus EcoRV, the 1664-bp fragment was cloned into the pBluescript plasmid to generate CATC3′ clone. pBluescript is a plucksbeat derivative containing the coding region of *CAT* gene (17). To clone the 3′-UTR together with downstream sequences, the pUCB2F clone was also used as template for amplification with the following oligonucleotides: forward, 5′-AACCTTATCGCCCGAGTGGCAGGAA-3′, and reverse, 5′-CTCAGATCTGTGGCCGCTTCAATGTCAT-3′. The PCR product was digested with HindIII plus XhoI and cloned into CATC3′ to generate CATC3′C4 clone. Finally, CATC3′C4 was digested with BglII, and the chimeric CAT–HSP70-I gene was cloned into pX63NEO (19), a vector for transfecting *Leishmania*, to generate pXcat70-I.

To clone the 3′-UTR-II together with downstream sequences, PCR amplification with specific primers was performed using the pUCB2C clone as template. This clone is a pUC18-derivative containing the 5.72-kb Sall fragment from the genomic clone pB2g3 (14). The specific oligonucleotides were: forward, 5′-AACCTTATCGCCCGAGTGGCAGGAA-3′, and reverse, 5′-CTCAGACATCTGGAGGGTGATCGTGA-3′. The amplified DNA fragment was digested with HindIII plus Sall, and the 1685-bp fragment was cloned into CATC3′ to obtain CATC3′C6. This plasmid was digested with BglII, and the chimeric CAT–HSP70-II gene was cloned into pX63NEO to generate pXcat70-II. The authenticity of each of the constructs and the fidelity of the PCR-amplified fragments were verified by nucleotide sequencing. The construction of plasmid pXHisCAT3His, which contains the *CAT* gene flanked by UTRs derived from the *L. infantum* histone H2A gene, has been described elsewhere (17).

**Generation of the Δhsp70-II Null Mutant—**HSP70-II alleles were replaced in which the marker genes were flanked by specific regions located upstream and downstream of the ORF for HSP70-II gene. For this purpose, we chose the CATC3′C6 clone, which contains the *CAT* gene flanked by upstream plus 5′-UTR and 3′-UTR plus downstream sequences of the HSP70-II gene (see above). The *CAT* gene in CATC3′C6 was removed by BstBI plus HindIII double digestion and replaced by the appropriate cassettes containing either *NEO* or *HYG* genes. For the amplification of the *NEO* cassette, the pcDNA3.1 cloning vector (Invitrogen) was used as template and the following primers: forward, 5′-GGTGCAGAATGGAAAGGATGGTCCA-3′ (underlined is the BstBI restriction site); reverse, 5′-CCGAGGTTCTTCGAA-3′ (underlined is the HindIII restriction site). For the amplification of the *HYG* cassette, the pRES1hyg cloning vector (Clontech) was used as template and the following primers: forward, 5′-GGTGCAGAATGGAAAGGATGGTCCA-3′ (underlined is the BstBI restriction site); reverse, 5′-CCGAGGTTCTTCGAA-3′ (underlined is the HindIII restriction site). The resulting constructs were named Neo70-II and Hyg70-II, respectively. 2 μg of each of these constructs was cut with BglII, and the linearized DNAs were individually used for transfection to disrupt HSP70-II gene in *L. infantum*. After transfection and selection (see below), the resulting HSP70-II null mutant was designated as Δhsp70-II::NEO/Δhsp70-II::HYG, following the genetic nomenclature for *Trypanosoma* and *Leishmania* outlined by Clayton et al. (20).

**Transfections of Leishmania Promastigotes—**DNA for transfections was prepared using the Qiagen plasmid Maxi Kit (Qiagen, Hilden, Germany). Late logarithmic phase *L. infantum* promastigotes were harvested by centrifugation and suspended at 2 × 10^6 parasites/ml in CytoMix buffer (21), using 10^6 parasites per transfection. Electroporation was performed in a Bio-Rad GenePulser using the conditions recently described by Robinson and Beverley (22). For clonal selection, the transfected parasites were plated on blood-agar plates (23) supplemented with either 20 μg/ml G418 (Roche Applied Science) or 50 μg/ml hygromycin B (Sigma).

**Polysome Fractionation by Sucrose Gradients, RNA Purification, and Northern Blotting—**The analysis of polysomal distribution of HSP70 mRNAs by sucrose gradients was performed basically as described elsewhere (24), with modifications intending to minimize RNA degradation. Briefly, *L. infantum* parasites (2.5 × 10^9 promastigotes) were har-
Translational Regulation of Leishmania HSP70 Genes

vested, washed twice with pre-chilled phosphate-buffered saline, and resuspended in 1 ml of lysis buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1.5 mM MgCl₂, 0.6 mg/ml heparin, and 0.5% Nonidet P40), supplemented with 240 units of SUPERase-In (Ambion, Austin, TX). Lysis was favored by pipetting up and down ten times. After lysis, samples were centrifuged at 3,000 × g for 2 min at 4 °C to pellet the nuclei. The supernatant was supplemented with 150 μg/ml cycloheximide, 20 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 13,000 × g for 5 min at 4 °C to remove mitochondria and cellular debris. The supernatant was layered on 11 ml of sucrose gradient and centrifuged in an SW-41T rotor at 170,000 × g for 2 h at 4 °C. After centrifugation, the gradients were harvested from the top using a fraction collector. The A₂₆₄ nm was recorded throughout the harvest. Fifteen fractions of 800 μl were collected. SDS (1% final concentration), EDTA (pH 8.0; final concentration, 10 mM), and proteinase K (final concentration, 200 mg/ml) were added to each fraction and incubated for 30 min at 37 °C. After extraction with a mixture of phenol/chloroform/isoamyl alcohol (25/24/1), the RNA from the fractions was precipitated with ethanol at −20 °C. RNA samples purified from each fraction were separated on denaturing 1% agarose-formaldehyde gels and transferred onto nylon membranes. Aliquots of the RNA samples were also analyzed using the 2100 Bioanalyzer (Agilent Technologies).

RNA from *L. infantum* promastigotes was isolated using the Total Quick RNA Cells and Tissues kit (Talent, Trieste, Italy). Total RNA (4 μg/lane) was size-separated on 1% (w/v) agarose-formaldehyde gels and electrophoresed onto nylon membranes using the Transfer Power 2D System (Hoefer, San Francisco, CA). DNA probes were labeled with [α-³²P]dCTP by nick translation (25). Hybridizations were performed as reported early (14). For reuse, blots were treated with 0.1% SDS for 30 min at 95 °C to remove previously hybridized probes. The 3' UTR probe was obtained by BamHI digestion of clone pB3'UTRic, a pBlueScript derivative that contains the complete 3'-UTR-I from *L. infantum* HSP70 gene 1 (18). The 3'- UTR-II probe was obtained by HindIII plus SacI double digestion of clone pTC6, which is a pBluScript derivative, including the PCR amplification product of the 3'-UTR-II and downstream sequences (this product was used to generate the construct pXcat70-1 (see above)). The α-tubulin probe was obtained from clone pTcaα2 (26). The *Escherichia coli* CAT probe was obtained from clone pBICAT (17).

Metabolic Labeling, Western Blotting, and Immunoprecipitation—After appropriate treatment, 6 × 10⁷ parasites were collected and resuspended in 100 μl of Dulbecco’s modified Eagle’s medium without methionine and cysteine (Met-, Invitrogen), supplemented with 10% (v/v) heat-inactivated fetal calf serum. Proteins were labeled with 100 μCi of [³⁵S]methionine/cysteine protein labeling mix (Redivue Pro-mix [L-³⁵S], >1000 Ci/mmol, Amershams Biosciences). After labeling, cells were harvested, washed twice with phosphate-buffered saline, and lysed in SDS-polyacrylamide gel sample buffer. Protein samples were separated by SDS-PAGE (10% gels) in a Mini-protein system (Bio-Rad). For immunoblot analysis, the electrophoresed proteins were transferred onto nitrocellulose membranes (Amershams Biosciences). Membranes were blocked with 5% (w/v) nonfat dried milk powder in phosphate-buffered saline and 0.5% Tween 20. The filters were probed with anti-HSP70 serum obtained from a rabbit immunized with the recombinant *L. infantum* HSP70 (27), or with rabbit anti-CAT serum (Sigma). An anti-rabbit IgG-peroxidase immunconjugate (Nordic Immunologic, Tilburg, The Netherlands) was used as secondary antibody, and the specific binding was revealed with the ECL® Western blot detection system (Amershams Biosciences).

Analysis of the *de novo* HSP70 and CAT synthesis was performed on ³⁵S-labeled promastigotes by immunoprecipitation. After labeling (see above), cells were harvested by centrifugation, washed twice in pre-chilled phosphate-buffered saline, and incubated for 30 min with gentle shaking, in 100 μl of lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100) and a protease inhibitor mixture, containing 1 mM phenylmethylsulfonyl fluoride, 8 μg/ml leupeptin, 4 μg/ml aprotinin, and 4 μg/ml pepstatin. Lysates were sonicated for 10 min for clearance of the nucleic acids and centrifuged at 13,000 × g for 15 min at 4 °C. The soluble extract was mixed with 20 μl of rabbit antisera against *L. infantum* HSP70, or 5 μl of rabbit anti-CAT serum, and incubated on an orbital rotator for 15 h at 4 °C. Agarose beads (15 μl), conjugated with Protein A (Sigma), were equilibrated in 50 μl of lysis buffer and added to the *L. infantum* extract-HSP70/CAT antisera mixture. The mixture was incubated on a rotator for 2 h at 4 °C, the beads were collected by centrifugation, and washed three times with 0.5 ml of buffer B (10 mM Tris/HCl, pH 8.0, 30 mM NaCl, 2% Triton X-100), twice in 0.5 ml of buffer B (10 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.1% Triton X-100), and once in 0.5 ml of buffer C (10 mM Tris/HCl, pH 8.0, 0.05% Triton X-100). Finally, the beads were resuspended in 60 μl of 2 × Laemmli buffer. Immunoprecipitated proteins were resolved by SDS-PAGE (10%) and, after drying of the gel, analyzed by exposure to x-ray films. Total amount of immunoprecipitated HSP70 and CAT proteins was analyzed by Western blotting using either the anti-HSP70 or anti-CAT sera (see above).

**RESULTS**

Expression of the *L. infantum* HSP70 Genes—The *L. infantum* HSP70 locus contains six tandemly arranged genes (14) that are highly similar in sequence, and the main difference among them is found at the 3'-untranslated regions (3'-UTRs). As depicted in Fig. 1A, the sixth gene of the cluster has a divergent 3'-UTR (named 3'-UTRII) relative to the 3'-UTR (named 3'-UTRI) that is common to the other five genes. We will refer to genes HSP70-I to -5 as HSP70-type I (or HSP70-I) and to gene 6 as HSP70-type II (or HSP70-II). A BLAST search in the genome database of *Leishmania major* strain Friedlin (www.geneDB.org) identified on chromosome 28 a similar genomic organization for the two types of HSP70 genes in this *L. major* strain, although the number of HSP70-type I genes is lower in *L. major* than in *L. infantum*. Northern blots of *L. infantum* RNA samples, probed with the different 3'-UTRs, revealed that the abundance of HSP70 transcripts with 3'-UTRI increases in promastigotes incubated at 37 °C (Fig. 1B). In contrast, the levels of HSP70 mRNAs with 3'-UTRII remained approximately constant at the different temperatures. These results agree with our previously reported data, which demonstrated that only HSP70-3'-UTRII transcripts accumulate in a temperature-dependent manner (14, 17, 18).

To determine the effect of temperature on the synthesis of HSP70 protein, *L. infantum* promastigotes were labeled by metabolic incorporation of [³⁵S]methionine/cysteine at different temperatures, and HSP70 was immunoprecipitated with a specific serum. Fig. 1C shows an autoradiograph of the total promastigote proteins labeled at 26 °C, 37 °C, 39 °C, and 41 °C. Total protein synthesis was only slightly affected by incubation at 37 °C, indicating that 37 °C is not a severe heat-shock temperature for *Leishmania*. This is not surprising taking into account that the parasite grows at this temperature when transmitted to its mammalian hosts. However, the synthesis of proteins was clearly
impaired when promastigotes were incubated at either 39 °C or 41 °C, suggesting that these are severe heat shock temperatures for *Leishmania*. Nevertheless, an increase in the bands corresponding to HSP70 and HSP83 was observed after incubation of parasites at 37 °C or 39 °C (Fig. 1C). Despite this increased synthesis of HSPs, it must be noticed that incubation at the different temperatures did not result in any detectable change in the steady-state level of HSP70 (Fig. 1C, bottom panel). This result can be explained considering that *Leishmania* has high steady-state levels for these proteins: HSP70 and HSP83 make, respectively, up to 2.1% and 2.8% of the total protein in unstressed promastigotes (13).

To quantify the *de novo* synthesis of HSP70 at the different temperatures, immunoprecipitations of HSP70 from lysates of promastigotes were performed using an antiserum specific for *Leishmania* HSP70 (Fig. 1D). The rate of HSP70 synthesis was estimated to increase 4- to 5-fold at 37 °C relative to the level at 26 °C; a 2- to 3-fold increase was also observed at 39 °C, whereas the level of synthesis was undetectable at 41 °C (Fig. 1E).

**Analysis of the Polysomal Distribution of the Two Types of HSP70 mRNAs**—Based on the temperature-dependent patterns of mRNA accumulation (Fig. 1), our initial hypothesis was that the increase in the *de novo* HSP70 synthesis during heat shock would be linked to the concomitant increase in the mRNA abundance of the HSP70-I transcripts. To test this, we next investigated the polysomal distribution of the two types of HSP70 mRNAs in unstressed and stressed promastigotes by sucrose gradient centrifugation of cytosolic extracts and Northern blotting. According to the A254 profiles and the rRNA species distribution of the gradients (Fig. 2, A and B), fractions 1–4 were essentially free of assembled ribosomes. Equimolarity of the three large rRNAs (18S, 24S, and 245β), composing the ribosomes of kinetoplastids, was observed in fraction 6 and henceforward. A prominent peak corresponding to the 80 S ribosomes was observed, but individual peaks corresponding to polyribosomes could not be distinguished. First, we analyzed the polysomal distribution of HSP70-I transcripts in *L. infantum* promastigotes incubated at 26 °C, 37 °C, or 39 °C (Fig. 2, C and D). Transcripts were detected in both polysome and ribosome-free fractions at the three temperatures tested. These results were somewhat surprising, because they indicate that HSP70-I transcripts are translated at both normal and heat shock temperatures. Nevertheless, a certain increase in the ribosomal association of these transcripts was observed at 37 °C/39 °C relative to that observed at the control temperature (26 °C, Fig. 2, C and D). A very different profile was found when the polysomal distribution of HSP70-II transcripts was analyzed (Fig. 2, E and F): these transcripts were not bound to ribosomes at 26 °C, whereas the heat shock treatments (37 °C and 39 °C) caused a drastic increase in transcript association to polysomes. These results clearly show that the overall polysomal distribution of HSP70-II transcripts is dramatically affected by heat shock. As an experimental control, we analyzed the polysomal distribution of a non-heat shock gene, the α-tubulin (Fig. 2, G and H). As expected, the association of this transcript with ribosomes, occurring at 26 °C, was patently impaired at both 37 °C and 39 °C.

**Analysis of the Function of the UTRs of the HSP70 Genes in the Translational Efficiency of the Transcripts**—The results of ribosomal distribution of the two types of HSP70 transcripts pointed to the hypothesis that increased synthesis of HSP70 during heat shock is most probable caused by a higher
ribosome association of HSP70-II transcripts. For further analysis of this hypothesis, we prepared CAT reporter constructs (Fig. 3A) in which the CAT coding region was flanked by the UTRs of the two types of HSP70 genes. Also, upstream and downstream genomic regions were included to provide the wild-type signals for mRNA processing, i.e. splice acceptor and polyadenylation sites. As shown in Fig. 3B, the chimeric CAT-HSP70 tran-
scripts expressed by constructs pXcat70-I and pXcat70-II had the expected size. Also, it was found that transcripts derived from pXcat70-I construct accumulate in a temperature-dependent manner, whereas the levels of transcripts derived from pXcat70-II construct remained constant at the different temperatures. Because we previously have demonstrated that the 3' UTR of \textit{L. infantum} HSP70 genes contains a regulatory element responsible for the accumulation of the corresponding transcripts at 37 °C (18), this was an expected result.

Next, we analyzed the effect of the flanking, non-coding sequences of HSP70\textsuperscript{35177} transcripts on the synthesis of CAT in \textit{L. infantum} promastigotes transfected with either pXcat70-I or pXcat70-II constructs. For this purpose, the transfected parasites were incubated, and metabolically labeled, at different temperatures (26 °C, 37 °C, 39 °C, or 41 °C). Fig. 4A shows the \textit{de novo} protein synthesis pattern of pXcat70-I-transfected promastigotes. The bands corresponding to HSP70 and HSP83 increased in intensity at 37 °C and 39 °C in comparison to 26 °C, but an increase in the signal of other protein bands was not observed. The molecular mass for the CAT protein is 25 663.13 Da. When total CAT protein was determined by Western blotting (Fig. 4A, bottom panel), it was observed that the total amount of CAT does not change after 1 h incubation at the assayed temperatures. We performed immunoprecipitation experiments to quantify the temperature-dependent induction of the \textit{de novo} synthesis of CAT (Fig. 4B). Densitometric analysis of the results (Fig. 4C) determined that the rate of CAT synthesis is similar at 26 °C and 37 °C, whereas the incubation at higher temperatures leads to either an obvious decrease (39 °C) or a stop (41 °C) in protein synthesis. These results are in agreement with the experiments of polysomal distribution of HSP70-I transcripts (Fig. 2), that are associated with ribosomal fractions either at normal temperature or at heat shock temperatures (37 °C and 39 °C). Nonetheless, we wanted to analyze the ribosomal distribution of chimeric CAT-HSP70-I transcripts at 26 °C and at 39 °C. Indeed, it was observed that this transcript is associated with ribosomal fractions at both temperatures (Fig. 4D). The reason for the decreased synthesis of CAT at 39 °C compared with that at 26 °C is not apparent, because quantitative differences in the polysomal distribution were not observed between 26 °C and 39 °C. Furthermore, it should be noted the existence of qualitative differences between both profiles: at 39 °C the CAT-HSP70-I transcript is located mainly in the high density fractions, whereas at 26 °C the transcript is uniformly distributed along the ribosomal fractions. As a plausible explanation, it can be postulated that the rate of translational termination of the CAT-HSP70-I transcripts decrease at 39 °C. A slow termination step in the translation process would result in an increase in the number of ribosomes bound to a given mRNA (28). As controls, the filter was hybridized with a specific probe for endogenous \textit{HSP70-II} transcript, showing again that this transcript is poorly bound to ribosomes at normal temperature, but it binds efficiently at 39 °C (Fig. 4F). The hybridization of the filter with an α-tubulin probe demonstrated that the binding of α-tubulin mRNAs is strongly impairead at 39 °C (Fig. 4H).

Fig. 5 summarizes the studies on the translational efficiency of CAT protein in promastigotes transfected with pXcat70-II. From the analysis of the autoradiograph of labeled proteins (Fig. 5A), it became evident that this cell line synthesizes more CAT protein than the promastigotes transfected with pXcat70-I (Fig. 4A) either at normal or at heat-shock (37 °C and 39 °C) temperatures. To quantify the relative amounts of CAT that are being synthesized \textit{de novo} at the different temperatures, we used an anti-CAT antibody to immunoprecipitate the protein (Fig. 5B). Densitometric analysis of the data indicated that the CAT protein is synthesized with similar efficiency at 26 °C, 37 °C, and 39 °C (Fig. 5C). The observation of such an amount of \textit{de novo} CAT in promastigotes at 26 °C was somewhat disappointing, because we had hypothesized that the presence of the 3' UTRII in the CAT-HSP70-II transcript would...
FIGURE 4. Translational efficiency of the CAT-HSP70-I chimeric transcripts in pXcat70-I transfected promastigotes. A, promastigotes were metabolically labeled for 1 h with [35S]methionine/cysteine at the indicated temperatures (26 °C, 37 °C, 39 °C, or 41 °C). Protein labeling was analyzed by SDS-PAGE and autoradiography. In parallel, a Western blot analysis was performed with an anti-CAT antibody (bottom panel). B, the CAT protein was immunoprecipitated with a specific antibody, and the immunoprecipitates were analyzed by SDS-PAGE and either autoradiography (De novo panel) or Western blotting with anti-CAT antibodies (TP panel). C, plotting of the ratios of de novo synthesized CAT to total amount of CAT; the De novo/TP ratio at 26 °C was set as 1. D, F, and H, ribosomal distribution of CAT-HSP70-I, HSP70-II, and α-tubulin transcripts, respectively, in pXcat70-I-transfected promastigotes after incubation at 26 °C or 39 °C. The autoradiographs were analyzed by densitometry, and the results are presented as relative hybridization signal (panels on the right, E, G, and I).
FIGURE 5. Translational efficiency of the CAT-HSP70-II chimeric transcripts in pXcat70-II transfected promastigotes. A, promastigotes were metabolically labeled for 1 h with \([35S]\)methionine/cysteine at the indicated temperatures (26 °C, 37 °C, 39 °C, or 41 °C). Protein labeling was analyzed by SDS-PAGE and autoradiography. The putative CAT band is marked with an arrow. In parallel, a Western blot analysis was performed with an anti-CAT antibody (bottom panel). B, the CAT protein was immunoprecipitated with a specific antibody and the immunoprecipitates were analyzed by SDS-PAGE, and either autoradiography (De novo panel) or Western blotting with anti-CAT antibodies (TP panel). C, plotting of the ratios of de novo synthesized CAT to total amount of CAT; the De novo/TP ratio at 26 °C was set as 1. D, F, and H, ribosomal distribution of CAT-HSP70-II, HSP70-I and \(\alpha\)-tubulin transcripts, respectively, in pXcat70-II-transfected promastigotes after incubation at 26 °C and 39 °C. The autoradiographs were analyzed by densitometry, and the results are presented as relative hybridization signal (panels on the right, E, G, and I).
Translational Regulation of Leishmania HSP70 Genes

FIGURE 6. Targeted replacement of the HSP70-II gene. A, a map of the HSP70 locus showing the restriction sites relevant for Southern blot analysis. The resistance genes NEO and HYG used for the construction of gene deletion cassettes are also shown. B, genomic DNAs (1 μg per lane) from wild-type (+/+), heterozygous line after integration of the NEO gene (+/−), or Δhsp70-II::NEO/Δhsp70-II::HYG null mutant (−/−) were digested with BamHI or with SalI and hybridized with a radiolabeled probe containing the 3′-UTR of the HSP70-II gene. The positions and sizes of DNA standards are indicated. C, Northern blot analysis of total RNA isolated from wild-type (+/+), Δhsp70-II null promastigotes (−/−), previously incubated for 1 h either at 26 °C or 37 °C. As probe, the 3′-UTR of the HSP70-II gene was used. Ethidium bromide staining of the corresponding gel is also shown (RNA panel).

have an inhibitory effect on the translational activity of that transcript (see above). This result prompted us to analyze the polyosomal profile of the chimeric CAT-HSP70-II transcripts in promastigotes incubated at 26 °C and 39 °C. This assay gave an explanation to the paradoxical existence of a high rate of CAT synthesis at 26 °C in pXcat70-II transfected promastigotes: the CAT-HSP70-II transcripts are associated to ribosomes at a similar extent at both temperatures (Fig. 5D) in contrast to the HSP70-II transcripts that are not associated with ribosomes at 26 °C (Figs. 2C and 4F). These results indicate that the replacement of HSP70 ORF by CAT ORF affects the regulation of the transcripts. Therefore, it seems that the presence of the CAT sequence itself, or alternatively, the lack of the HSP70 coding sequence in the chimeric CAT-HSP70-II transcript allows these mRNAs to associate with ribosomes at 26 °C. As controls of the experiment, the filters were hybridized with probes for HSP70-I mRNAs (Fig. 5F) and for α-tubulin mRNAs (Fig. 5H), yielding the expected ribosomal profiles (Fig. 2).

An interesting result from the studies with the pXcat70-II line is the observation that the synthesis of CAT was as active at 39 °C as it was at 26 °C or 37 °C (Fig. 5B). Accordingly, the polyosomal profiles of the CAT-HSP70-II transcripts were essentially the same at 26 °C or 39 °C (Fig. 5D). Thus, it can be concluded that sequences in the 3′-UTR would be responsible for the translational efficiency of CAT-HSP70-II transcripts at 39 °C. This conclusion is based on the fact that the translational efficiency at 39 °C of CAT-HSP70-I transcripts in pXcat70-I transfected promastigotes was lower than that observed at either 26 °C or 37 °C (Fig. 4, B and C). It should be noted that CAT-HSP70-I and CAT-HSP70-II transcripts only differed in the 3′-UTR (Fig. 3A). The contribution of CAT coding sequences to the enhanced translation at 39 °C of the CAT-HSP70-II transcripts must be minimal, because CAT is not synthesized at 39 °C when the CAT coding region is flanked by the UTRs from a non-heat shock gene (17). Concretely, the analysis of the de novo synthesis of CAT in promastigotes transfected with the pX5HisCAT3His construct indicated that the protein is not produced at 39 °C. The pX5HisCAT3His construct bears the CAT ORF flanked by 5′-UTR and 3′-UTR derived from the L.h2A4 gene, one of the L. infantum genes coding for the histone H2A (29). For the present work, we analyzed the polyosomal distribution of the CAT-H2A transcripts in pX5HisCAT3His-transfected promastigotes incubated at 26 °C and 39 °C. The incubation of parasites at 39 °C led to a decrease in the level of CAT-H2A transcripts associated with high density fractions relative to that observed at 26 °C (data not shown). Thus, the temperature-induced changes in the polyosomal distribution of CAT-H2A transcripts are in agreement with the very low CAT synthesis at 39 °C in pX5HisCAT3His-transfected promastigotes (17).

Targeted Replacement of the HSP70-II Gene—Knock-out parasites for the HSP70-II gene were created by double-targeted gene replacement to gain a better understanding on the regulatory mechanisms of HSP70 gene expression in Leishmania (Fig. 6). A null-mutant clonal cell line of the HSP70-II locus, designated Δhsp70-II::NEO/Δhsp70-II::HYG, was examined by Southern blot analysis to demonstrate that the correct homologous integration had occurred (Fig. 6A). Probing the Southern blot with a 3′-UTRIII probe (specific for HSP70-II gene) revealed that one of the 7.7-kb BamHI bands containing the HSP70-II alleles was reduced in size to a predicted band of 6.32 kb in the Δhsp70-II::NEO/HSP70-II heterozygous line (+/−, Fig. 6B), whereas the 7.7-kb BamHI bands were reduced to two BamHI bands of 6.5- and 6.32-kb in the Δhsp70-II::NEO/Δhsp70-II::HYG knock-out line (−/−, Fig. 6B). In a different way, the wild-type 5.45-kb SalI bands were predicted to increase in size after replacement with NEO and HYG ORFs to 8 and 8.2 kb, respectively, given that the Sall restriction site located within the HSP70-II gene is lost after the replacements (Fig. 6A). Together, these results confirm that the correct homologous integrations had occurred and that the Δhsp70-II::NEO/Δhsp70-II::HYG knock-out line. The lack of HSP70-II genes in the mutant line was further demonstrated by Northern blot analysis (Fig. 6C). Northern blots hybridized with a 3′-UTRIII probe showed the different mRNAs expected for wild-type or null mutant promastigotes. Null mutant cells revealed two transcripts corresponding to the NEO (2 kb) and HYG (2.2 kb) transcripts.

The Δhsp70-II null mutant showed only a mild growth-rate defect in the logarithmic growth phase compared with wild-type parasites (Fig. 7). However, the null mutant reached a lower cell density in stationary phase. In fact, after 7 days, the number of Δhsp70-II parasites continuously decreased in the culture, whereas the number of wild-type parasites in the stationary phase remained constant. Thus, it can be postulated that the expression of the HSP70-II gene plays a vital role in the stationary phase. At present, we are performing more experiments to reveal other defects in this mutant cell line, e.g. macrophage invasion and promastigote-to-amastigote differentiation.
We analyzed the effect of deleting the HSP70-II gene on the expression of HSP70. Taking into account that HSP70-II transcripts are not seemingly translated in promastigotes at 26 °C, but highly translated at 37 °C and 39 °C, it could be expected that the Δhsp70-II mutant cell line must have a lower de novo synthesis of HSP70 that the wild-type cell line at heat shock temperatures. Metabolic labeling experiments were performed in both wild-type and Δhsp70-II::NEO/Δhsp70-II::HYG promastigotes (Fig. 8A). As expected, the total amount of HSP70 was similar in both cell lines (Fig. 8A, bottom panel). However, when the de novo synthesis of HSP70 was determined by immunoprecipitation experiments, it was observed that the mutant cell line has a lower rate of synthesis than the wild-type parasites (Fig. 8B). By densitometric analysis (Fig. 8C), it was determined that the HSP70 synthesis is 2-fold lower in the mutant cell line than in the wild-type parasites at 37 °C. Remarkably, the difference in the de novo synthesis of HSP70 is higher at 39 °C: the wild-type promastigotes synthesized 5-fold more HSP70 than the Δhsp70-II null promastigotes. In conclusion, these results reinforce the hypothesis that HSP70-II transcripts are translated actively at heat shock conditions, suggesting that these transcripts have been evolutionarily selected for providing newly synthesized HSP70 when Leishmania promastigotes are under stress conditions.

DISCUSSION

Post-transcriptional regulation of mRNAs is increasingly recognized as a central pathway controlling gene expression in eukaryotes, being particularly important in trypanosomes, where accumulated data provide little or no evidence for regulation of transcription initiation (reviewed in Ref. 2). A molecular basis for this lack of transcriptional regulation is found in the organization of genes in Trypanosomatids. Leishmania, as well as other members of the Trypanosomatidae family, presents a polycistronic transcription that initiates upstream of the most-5' gene of large clusters of genes, which have the same transcriptional orientation (6, 7). Thus, the number of RNA polymerase II initiation sites is believed to be very low compared with other eukaryotes, probably only a few per chromosome (7). Another peculiarity of gene organization in Leishmania is that relevant housekeeping genes are repeated, forming tandem arrays that shares total (or almost total) sequence conservation in the coding regions but showing untranslated regions with high sequence divergence (4, 30). Examples are genes coding for ribosomal proteins (31, 32), histones (29, 33), and phosphoglycerate kinase (34). Also, genes showing this peculiar type of genomic organization have been found on several L. major chromosomes (35, 36). An appealing hypothesis is that this gene display has a regulatory purpose related with gene expression in different environmental conditions and developmental stages. The Leishmania HSP70 gene locus constitutes an adequate model for testing this hypothesis. As indicated above and summarized in Fig. 1A, the HSP70 gene locus contains two types of genes differing in their 3'-UTRs. Transcripts from both genes are observed at both normal and heat shock temperatures, although only transcripts derived from HSP70-I gene shows a temperature-dependent accumulation (Fig. 1B (14)). The most remarkable finding in this work is that HSP70-II mRNAs are not bound to functional ribosomes at normal temperature of growth, but the situation changes when parasites are incubated at heat shock temperatures. In contrast, the HSP70-I mRNAs were observed associated with ribosomal fractions at both normal and heat shock temperatures. These results favor the idea that HSP70-II transcripts are stored in the cytoplasm of Leishmania promastigotes in a translational silent state until parasites encounter stress conditions, like a heat shock. Regulation at the translational level provides a means of rapid response in gene expression patterns to environmental cues. Therefore, the purpose of the HSP70-II transcripts may be to actively translate the protein when an extra supply of HSP70 is needed. Support for this hypothesis was obtained after analyzing the de novo synthesis of HSP70 in a Δhsp70-II null mutant (Fig. 8). It was observed that this mutant synthesizes 2- and 5-fold less HSP70 than wild-type promastigotes at 37 °C and 39 °C, respectively. However, this difference in the HSP70 synthesis at heat-shock temperatures had only a marginal effect on the growth curve at normal temperature (Fig. 7), indicating that the HSP70-II gene is not required to grow in axenic conditions. This is not unexpected, because the Δhsp70-II mutant still has a high abundance of HSP70, derived from the expression of HSP70-I genes. It has been reported in a recent work that deletion of the six HSP70 genes in Drosophila melanogaster yields HSP70-null flies that are viable and fertile (37); however, the authors announce that a detailed characterization of other phenotypes is forthcoming. At present, we are studying other biological features of the L. infantum Δhsp70-II null mutant, i.e. thermotolerance, metacyclogenesis, promastigote-to-amastigote differentiation, macrophage invasiveness, and infectivity.

Many examples in Leishmania species support the notion that developmental regulation of mRNA levels is determined post-transcriptionally by sequences located in the 3'-UTRs that usually control mRNA stability (see Ref. 38 and references therein). Indeed, the expression of the L. infantum HSP70 mRNAs is another example (18). In this work, we have found that 3'-UTRs are also involved in controlling the expression of Leishmania HSP70 genes at the translational level. Thus, the 3'-UTR of HSP70-I mRNAs must be considered as responsible for the translaional activity of these transcripts at both normal and heat shock temperatures (Fig. 2, C and D). Also, the 3'-UTR of HSP70-II mRNAs participates in the binding to ribosomes at heat shock temperatures (Fig. 2, E and F). However, our present data do not allow excluding that other gene regions than 3'-UTR are also involved. To address this question, we prepared Leishmania transfection constructs in which the CAT reporter gene was flanked by the UTRs of HSP70 genes. However, the CAT-HSP70-II chimeric transcripts showed an unpredicted behavior: contrary to that observed with HSP70-II transcripts at 26 °C, the chimeric transcripts are associated to ribosomes and, consequently, the synthesis of CAT occurs at this temperature. At least two non-excluding explanations are envisaged: (i) the CAT ORF contains sequences that target the transcripts to the ribosomes at 26 °C, and/or (ii) other sequences of the HSP70-II gene, in addition to those located in the 3'-UTR, are needed for the observed inhibition of HSP70-II mRNA binding to ribosomes at 26 °C. In fact, for some genes in Kinetoplastida,
elements located within the coding regions have been found to contribute to their regulation (39, 40). At present, we are preparing new constructs to discriminate between both possibilities. Nevertheless, the use of these constructs served to show that the 3’-UTRs of the *Leishmania HSP70* genes are involved in the translation of *HSP70* mRNAs at 39 °C, a severe heat shock condition for *Leishmania*. Furthermore, the 3’-UTR-II seems to be more effective than the 3’-UTRI to promote the translation of the corresponding transcript during heat shock (Figs. 4 and 5). This is reinforced by the observation that the rate of synthesis of *HSP70* is 5-fold lower in the Δ*hsp70-II* mutant than in wild-type promastigotes.

The knowledge about the functional significance of the *Leishmania HSP70* gene organization has clearly increased with data presented in this work. As a summary, we envisage the following model, depicting the involvement of both types of *HSP70* genes in the *Leishmania* cell cycle. As occurs for most genes in *Leishmania*, the transcription of both *HSP70* genes is not regulated and the two types of transcripts are detected at both normal and heat shock temperatures. At normal temperature, *HSP70-I* transcripts are translated, whereas the association of *HSP70-II* transcripts to ribosomes is hindered. Meanwhile, the *HSP70-II* transcripts are stored in a translational silent form, whose purpose is to be rapidly translated when parasites encounter a stress situation, probably when an extra amount of *HSP70* is required. Therefore, translational control emerges as an important regulatory mechanism operating on the expression of *HSP70* genes in *Leishmania*. However, we have previously shown that there exists another regulatory mechanism in *Leishmania HSP70* genes that operates through the control of mRNA stabilities (14). This mechanism operates on the *HSP70-I* genes, and it would be responsible for the enhanced synthesis of *HSP70* in the Δ*hsp70-II* mutant cell line at 37 °C compared with that observed at 26 °C (Fig. 8). In conclusion, the *HSP70* cluster constitutes an interesting model that illustrates how the gene organization in *Leishmania* has evolved to fit in multiple levels of post-transcriptional gene regulation. Another outstanding example is found in the organization and expression of the genes coding for the major surface protease of *Leishmania* (41).

The location of the regulatory sequence elements within genes in Kinetoplastida has turned out to be a hard task. With few exceptions (42, 43), most attempts to define cis-elements in genes from protozoa of the Trypanosomatidae family have led to involve large regions with a complexity of interacting elements (15, 18, 38, 44, 45). It is likely that this feature is related to the regulatory mechanisms controlling gene expression in these ancient eukaryotes. Changes in mRNA location is a regulatory mechanism in which the definition of the mRNA signals involved has been found to be very complex and difficult and are often composed of several motifs (see Refs. 46 and 47 for reviews). The reason may be found in the fact that the are many trans-acting factors interpreting the mRNA signals, and their combinations change during the life of an mRNA, perhaps allowing the selection of many sub-destinations in the cell. RNA localization is now known to be a widespread phenomenon that occurs in unicellular organisms, in animal and plant tissues, and in developing embryos from a variety of animal phyla (46). Furthermore, accumulated evidence has emerged linking the processes of RNA localization and translational regulation (46). Taken into account all these considerations, the idea that the *Leishmania HSP70-II* gene could be regulated by a mechanism based in changes in mRNA location in the cytoplasm is appealing. Because there are techniques to visualize mRNAs in living cells (46), the idea can be tested experimentally. On the other hand, the identification of trans-acting factors responsible for the translational silencing of *HSP70-II* mRNAs is another future challenge.

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The Translational Efficiencies of the Two *Leishmania infantum* HSP70 mRNAs, Differing in Their 3′-Untranslated Regions, Are Affected by Shifts in the Temperature of Growth through Different Mechanisms

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