# Genomic organization and expression of the *HSP70* locus in New and Old World *Leishmania* species

C. FOLGUEIRA[1], C. CAÑAVATE[2], C. CHICHARRO[2] *and* J. M. REQUENA[1]*

[1] *Centro de Biología Molecular «Severo Ochoa», Universidad Autónoma de Madrid, 28049 Madrid, Spain*
[2] *WHO Collaborating Centre for Leishmaniasis, Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Madrid, Spain*

(*Received 20 June 2006; revised 31 August 2006; accepted 31 August 2006; first published online 23 October 2006*)

SUMMARY

Heat shock is believed to be a developmental inductor of differentiation in *Leishmania*. Furthermore, heat shock genes are extensively studied as gene models to decipher mechanisms of gene regulation in kinetoplastids. Here, we describe the organization and expression of the *HSP70* loci in representative *Leishmania* species (*L. infantum*, *L. major*, *L. tropica*, *L. mexicana*, *L. amazonensis* and *L. braziliensis*). With the exception of *L. braziliensis*, the organization of the *HSP70* loci was found to be well conserved among the other *Leishmania* species. Two types of genes, *HSP70-I* and *HSP70-II*, were found to be present in these *Leishmania* species except for *L. braziliensis* that lacks *HSP70-II* gene. Polymorphisms in the *HSP70* locus allow the differentiation of the Old and New World species within the subgenus *Leishmania*. A notable discrepancy between our data and those of the *L. major* genome database in relation to the gene copy number composing the *L. major HSP70* locus was revealed. The temperature-dependent accumulation of the *HSP70-I* mRNAs is also conserved among the different *Leishmania* species with the exception of *L. braziliensis*. In spite of these differences, analysis of the HSP70 synthesis indicated that the HSP70 mRNAs are also preferentially translated during heat shock in *L. braziliensis*.

Key words: *Leishmania*, HSP70, genomic organization, 3′UTR, mRNA stability, *de novo* synthesis of proteins.

## INTRODUCTION

Protozoan parasites of the genus *Leishmania* are the aetiological agents of leishmaniasis, a group of diseases ranging in severity from cutaneous (CL) to mucocutaneous (MCL) and visceral (VL) forms (Murray *et al*. 2005). Worldwide, 2 million new cases of symptomatic disease occur each year (Desjeux, 2004). The different forms of the disease are produced by different species of *Leishmania*. CL is mainly caused by *L. tropica*, *L. major* and *L. aethiopica* in the Old World and by members of the *L. mexicana* (*L. mexicana* and *L. amazonensis*), *L. braziliensis* (*L. braziliensis* and *L. peruviana*), and *L. guyanensis* (*L. guyanensis* and *L. panamensis*) complexes in the New World (Anonymous, 1990). Cutaneous infection causes lesions that are normally resolved after a few months, although, depending on the causative agent, they can disseminate to mucosal surfaces. MCL is characterized by the dissemination of parasites from the skin to the naso-oropharyngeal mucosa, and is caused by infection with species of the *L. braziliensis* complex, especially *L. braziliensis*, but also by species of the *L. guyanensis* complex. VL is mainly caused by members of the *L. donovani* complex (*L. donovani* and *L. infantum* in the Old World, and *L. chagasi* in the New World). It is generally accepted that the clinical spectrum of leishmaniasis is governed by both parasite and host factors, but these remain poorly understood (Pearson and de Queiroz Sousa, 1996; Murray *et al*. 2005).

*Leishmania* parasites exhibit a digenetic life-cycle. In the sandfly, they exist as extracellular promastigotes that are transferred to the mammalian host when the sandfly takes a bloodmeal. The parasite is phagocytosed by macrophages and, inside the acidic phagolysosomes, promastigotes differentiate to amastigotes. The mechanisms implicated in this transformation are not completely understood, although it is known that environmental factors such as pH and temperature are triggering factors of this process (Garlapati *et al*. 1999; Zilberstein and Shapira, 1994). The heat shock response is considered to be of vital importance for the stage-specific differentiation of *Leishmania* parasites, and the genes encoding heat shock proteins (HSPs) in *Leishmania* have been extensively studied not only for their importance in the differentiation process, but also as a gene models to study gene expression in this parasite (Lee *et al*. 1988; Quijada *et al*. 1997; Clos and Krobitsch, 1999; Zilka *et al*. 2001).

Among HSPs, HSP70 is the most highly conserved in both sequence and function; it assists a

* Corresponding author: Centro de Biología Molecular ''Severo Ochoa'', Universidad Autónoma de Madrid, 28049 Madrid, Spain. Tel: +34 914978454. Fax: +34 914974799. E-mail: jmrequena@cbm.uam.es

 
doi:10.1017/S0031182006001570 Printed in the United Kingdom

wide range of cellular processes, such as folding of new synthesized proteins and refolding of misfolded and aggregated proteins (Hartl and Hayer-Hartl, 2002). Genes coding for HSP70 were among the first genes to be studied in *Leishmania*, in particular in *L. major* (Lee *et al*. 1988). In this species, 5 *HSP70* genes were identified: 4 of them tandemly arranged and a fifth located at a separate locus, but on the same chromosome. Restriction and sequence analyses indicated that the coding regions of the 5 genes must be highly conserved. However, 5 nucleotides beyond the TAA termination codon, the sequence of the fifth gene was found to be divergent from that found in the tandemly linked *HSP70* genes. In addition, it was observed that transcripts derived from the tandemly linked *HSP70* genes increased in response to a temperature shift from 25 °C to 37 °C, whereas the transcripts derived from the fifth gene, being very abundant, are unaffected by temperature shifts (Lee *et al*. 1988). In *L. donovani*, a preliminary study showed the existence of 12–15 *HSP70* genes tandemly arranged on a single chromosome (MacFarlane *et al*. 1990). In addition, the genomic organization of *HSP70* genes in *L. amazonensis* has been examined (Bock and Langer, 1993). In this species, the existence of 7 *HSP70* genes organized in tandem was deduced and an eighth *HSP70* gene located at a distant site but on the same chromosome. Sequence analysis of cDNAs suggested the existence of 2 types of *HSP70* differing in their 3′UTRs. Recently, in *L. braziliensis*, the sequence of the *HSP70* coding region has been determined and the *HSP70* genomic organization has been analysed (Zurita *et al*. 2003). The authors suggested the existence of a single gene, but they showed only preliminary data.

In *L. infantum*, the genomic organization and expression of *HSP70* genes have extensively studied by our group (Quijada *et al*. 1997, 2000; Folgueira *et al*. 2005). The *L. infantum* genome contains 6 *HSP70* genes that are located in a single locus and arranged tandemly in a head-to-tail manner (Quijada *et al*. 1997). All the genes are highly conserved in sequence with the exception of the 3′-UTR of gene 6 (located at the 3′-end of the cluster) that is absolutely divergent relative to the 3′-UTR shared by the other 5 genes. Hence, for simplicity, genes 1 to 5 are referred as *HSP70-I* genes and gene 6 as *HSP70-II* gene (Folgueira *et al*. 2005). Remarkably, transcripts derived from gene 6 are more abundant than transcripts derived from all the other *HSP70* genes put together (Quijada *et al*. 1997). However, only mRNAs derived from *HSP70-I* genes accumulate in response to heat shock treatments, while the *HSP70-II* mRNAs remain unaffected. Subsequently, it was shown that the temperature-dependent accumulation of *HSP70-I* transcripts is associated with the presence of a *cis*-acting sequence in their 3′-UTR (Quijada *et al*. 2000). In a recent work, we have reported that *HSP70-II* mRNAs are preferentially translated at heat shock temperatures, but not at 26 °C, whereas *HSP70-I* mRNAs are bound to polysomes at 26 and 37 °C (Folgueira *et al*. 2005).

Because a relationship between gene organization and expression seems to exist in *Leishmania HSP70* genes, we considered it of interest to determine the extent of similarities and variations of the *HSP70* loci within the spectrum of *Leishmania* species. Here we describe the genomic organization of the *HSP70* genes in 6 *Leishmania* species. Our results are discussed in the light of the recently completed sequence of the *L. major* genome (Ivens *et al*. 2005). We have demonstrated the existence of 2 types of *HSP70* genes, previously reported in *L. major* and *L. infantum*, in all the species tested with the exception of *L. braziliensis*. The study was completed by analysing the expression of *HSP70* genes in representative *Leishmania* species at the levels of mRNA and protein synthesis.

## MATERIALS AND METHODS

### *Parasites and treatments*

Promastigotes of *L. infantum* (MCAN/ES/96/BCN150), *L. tropica* (MHOM/SU/74/K-27), *L. mexicana* (MNYC/BZ/62/M-379), *L. amazonensis* (IFLA/BR/67/PH-8), *L. braziliensis* (MHOM/BR/75/M-2904) and *L. major* (MHOM/IL/80/Friedlin) were cultured *in vitro* at 26 °C in RPMI 1640 medium (Sigma), supplemented with 10% heat-inactivated foetal calf serum (Sigma). The *L. major* Friedlin strain (clone V1) was generously provided by Dr Davis Sacks (NIAID, National Institutes of Health, Bethesda, USA). Logarithmic phase cultures ($5–9 \times 10^6$ promastigotes/ml) were used in the experiments. For heat shock experiments, aliquots of cultured promastigotes were incubated at 37 °C for different periods of time. Immediately after heat shock, parasites were spun down and harvested for the analysis of the steady-state mRNA levels or *de novo* protein synthesis.

### *Southern blot and Northern blot analyses*

Genomic DNA was isolated as described previously (Requena *et al*. 1988). One $\mu$g of total DNA was digested with a variety of restriction endonucleases, electrophoresed on 0·8% agarose gels, and transferred to nylon membranes (Hybond-N, Amersham Corp.) by standard methods (Sambrook *et al*. 1989). RNA from promastigotes was isolated using the Total Quick RNA Cells and Tissues kit (Talent, Trieste, Italy). Four $\mu$g per line of total RNA were size separated on 1% (w/v) agarose-formaldehyde gels and electrophoresed to nylon membranes using the Transfer Power Lid System (Hoefer, San Francisco, CA). DNA probes, either for DNA and

https://doi.org/10.1017/S0031182006001570 Published online by Cambridge University Press

RNA analysis, were labelled with [$\alpha$-$^{32}$P]dCTP by nick translation (Sambrook *et al*. 1989). Hybridizations were performed as reported earlier (Quijada *et al*. 1997). For reuse, blots were treated with 0·1% SDS for 30 min at 95 °C to remove previously hybridized probes.

### *DNA probes*

The 3′-UTR I probe corresponds to the 3′-UTR of *L. infantum HSP70-I* genes. The DNA fragment containing this region was obtained by *Bam*HI cleavage of clone pB3′UTRIc (Folgueira *et al*. 2005). The 3′-UTR II probe corresponds to the 3′-UTR of *L. infantum HSP70-II* gene; the corresponding DNA fragment was obtained by *Hin*dIII + *Sac*I double digestion of clone pTC6 (Folgueira *et al*. 2005). The CDS probe contains a region of the coding region, common to both types of *L. infantum HSP70* genes. The DNA fragment was obtained by *Eco*RI cleavage of clone pLd70.2, which contains the *L. infantum HSP70* gene region corresponding to positions 1701-2138 of the EBI Data Bank entry with Accession number Y08020.

### *Metabolic labelling, Western blotting, and immunoprecipitation*

For labelling, $6 \times 10^7$ promastigotes were collected and resuspended in 100 $\mu$l of Dulbecco's modified Eagle's medium without methionine and cysteine (Met-, Invitrogen), but supplemented with 10% heat-inactivated foetal calf serum. Proteins were labelled with 100 $\mu$Ci of [$^{35}$S]methionine/cysteine protein labelling mix (Redivue Pro-mix [L-$^{35}$S], >1000 Ci/mol, Amersham Biosciences). After labelling, cells were harvested, washed twice in pre-chilled PBS, and incubated for 30 min with shaking in 100 $\mu$l of lysis buffer (50 mM Tris/HCl, pH 7·5, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 8 $\mu$g/ml leupeptin, 4 $\mu$g/ml aprotinin and 4 $\mu$g/ml pepstatin). Lysates were sonicated for 10 min and centrifuged at 13 000 ***g*** for 15 min at 4 °C. The soluble extract was mixed with 20 $\mu$l of rabbit anti-*L. infantum* HSP70 polyclonal serum (Rico *et al*. 1999), and incubated for 15 h at 4 °C. Agarose beads (15 $\mu$l), conjugated with Protein A (Sigma), were equilibrated in 50 $\mu$l of lysis buffer and added to the protein extract-HSP70 antiserum mixture. The mixture was incubated on a rotator for 2 h at 4 °C, after which the beads were collected by centrifugation, and washed 3 times with 0·5 ml of buffer A (10 mM Tris-HCl, pH 8·0, 30 mM NaCl, 2% (v/v) Triton X-100), twice in 0·5 ml of buffer B (10 mM Tris-HCl, pH 8·0, 50 mM NaCl, 0·1% (v/v) Triton X-100), and once in 0·5 ml of buffer C (10 mM Tris-HCl, pH 8·0, 0·05% (v/v) Triton X-100). Finally, the beads were resuspended in 60 $\mu$l of 2× Laemmli buffer. Immunoprecipitated proteins were resolved by SDS-PAGE (10%) and, after drying the gel, proteins were analysed by exposure to X-ray films. The total amount of immunoprecipitated HSP70 protein was analysed by Western blotting using the rabbit anti-*L. infantum* HSP70 serum (Rico *et al*. 1999).

### *Quantitative analysis*

Autoradiographs were scanned with the GS-710 calibrated imaging densitometer (Quantity One version 3.0, Bio-Rad). Measurements were performed under conditions in which a linear correlation existed between the amount of proteins, DNA or RNA and the intensities of the bands.

## RESULTS

### *Genomic organization of the* Leishmania *HSP70 genes*

On the basis of the well-known genomic organization of *HSP70* genes in *L. infantum*, we studied the genomic organization of the *HSP70* genes in other *Leishmania* species by Southern blot analysis (Fig. 1). Hybridizations were performed using specific probes for the *L. infantum HSP70-I* and *HSP70-II* genes (3′-UTR I and 3′-UTR II, respectively). A probe common to both types of genes (CDS probe) was also used. Thus, in order to determine whether both types of *HSP70* genes exist in other *Leishmania* species, Southern blot analyses were carried out. Genomic DNA of *L. infantum*, *L. major*, *L. amazonensis*, *L. tropica*, *L. mexicana* and *L. braziliensis* was digested with restriction enzymes, transferred onto nylon membranes, and hybridized with probes corresponding to the 3′-UTR I, 3′-UTR II, and the coding region of *L. infantum HSP70* genes (Fig. 1). The results showed that the genomic organization for *HSP70* genes in *L. major* (Fig. 1B) and *L. tropica* (Fig. 1C) genomes is very similar to that present in *L. infantum* (Fig. 1A). In these species, several *HSP70* genes exist arranged in a head-to-tail manner. The 3·8-kb hybridization band, present in lanes containing the DNA digested with *Sal*I or *Bam*HI, corresponds to the size of the repetition unit of the tandem. The repetition unit is observed after probing with either the coding region (CDS) or the 3′-UTR I, suggesting that most of the *HSP70* genes in *L. major* (Fig. 1B) and *L. tropica* (Fig. 1C) are *HSP70-I* type genes as occurs in *L. infantum* (Fig. 1A; Quijada *et al*. 1997). However, the 3′-UTR II probe hybridized with a 5·7-kb *Sal*I band, but not with the 3·8-kb repetition unit, indicating the existence of a single *HSP70-II* gene in these species, located at the 3′-end of the cluster. The densitometric analysis of the CDS-hybridization bands (in lanes with DNA digested with either *Bam*HI or *Sal*I), assuming that the largest bands

https://doi.org/10.1017/S0031182006001570 Published online by Cambridge University Press

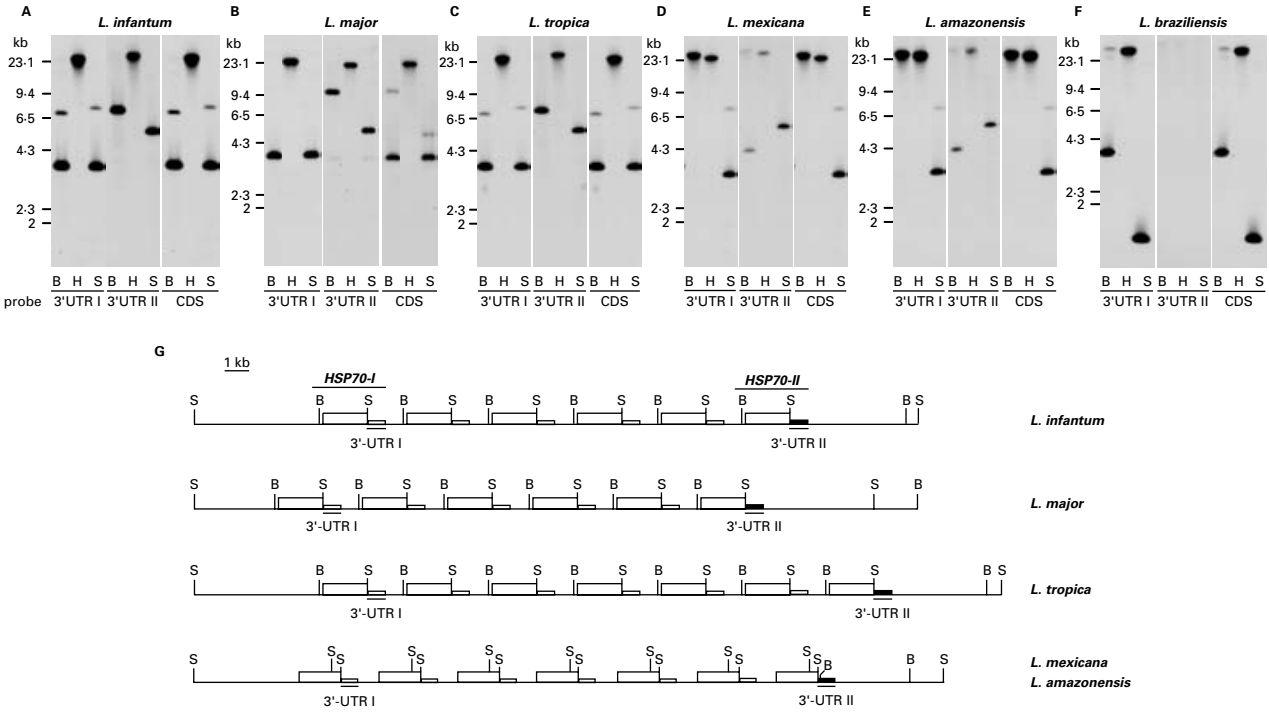


Fig. 1. Southern blot analyses of the *HSP70* gene loci in *Leishmania infantum* (A), *L. major* (B), *L. tropica* (C), *L. mexicana* (D), *L. amazonensis* (E), and *L. braziliensis* (F). Genomic DNA was cleaved with the restriction enzymes: B, *Bam*HI; H, *Hin*dIII; S, *Sal*I. The Southern blots were sequentially hybridized with the probes: 3′-UTR I, 3′-UTR of the *L. infantum HSP70-I* gene; 3′-UTR II, 3′-UTR of the *L. infantum HSP70-II* gene; and CDS, coding region of *L. infantum HSP70* genes (see Materials and Methods section for a detailed description of the sequences used as probes). (G) Deduced physical maps of the *HSP70* loci in *L. infantum*, *L. major*, *L. tropica*, *L. mexicana* and *L. amazonensis*.

contain the *HSP70* gene located at the 5′-end of the cluster, indicated that *L. major* haploid genome must contain 5 *HSP70-I* genes whereas *L. tropica* should have 6 *HSP70-I* genes. Densitometric analysis carried out in parallel with *L. infantum* DNA samples confirmed the existence of 5 *HSP70-I* genes per haploid genome in this species. Fig. 1G summarizes the genomic organization of the *HSP70* locus in *L. infantum*, *L. major*, and *L. tropica*.

The *HSP70* gene organization in *L. mexicana* and *L. amazonensis* was found to be identical (Fig. 1D, E) but different to that found in *L. infantum*, *L. major*, and *L. tropica* (Fig. 1A–C). The main difference was observed with the *Bam*HI cut site: this restriction enzyme did not cut within the *HSP70* locus in *L. amazonensis* and *L. mexicana* genomic DNA. However, the Southern blot analyses indicated the existence of a tandem array of *HSP70* genes in these *Leishmania* species also. The repetitive unit was of 3·4-kb, as defined by *Sal*I digestion. The 3·4-kb *Sal*I band was revealed after hybridizing with either CDS or 3′-UTR I probes, indicating that in *L. amazonensis* and *L. mexicana* also most of the genes are *HSP70-I* type. The 3′-UTR II probe hybridized with a 4·4-kb *Bam*HI band, indicating that the *Bam*HI enzyme cuts in the 3′-UTR of the *HSP70-II* gene, but not in the rest of the locus. This result agreed with the existence of a sole *HSP70-II* gene also in these *Leishmania* species. In contrast, the densitometric analyses, comparing the intensities of the 7·8-kb and 3·4-kb *Sal*I bands hybridizing with the CDS probe, revealed a copy number of 6–7 for the *HSP70-I* gene per haploid genome in *L. amazonensis* and *L. mexicana*. For these calculations, it was assumed that the 7·8-kb *Sal*I band contains a single gene. Our data are coincident with those of Bock and Langer (Bock and Langer, 1993), who described the existence in *L. amazonensis* of an *HSP70* tandem array composed of 7 genes. Fig. 1G summarizes the genomic organization of the *HSP70* locus in *L. amazonensis* and *L. mexicana*.

The most different *HSP70* genomic organization among the *Leishmania* species analysed in this work was found in *L. braziliensis* (Fig. 1F). Remarkably, the 3′-UTR II probe did not hybridized with any DNA band suggesting the absence of an *HSP70-II* gene in this species. By contrast, the 3′-UTR I and CDS probes hybridized with the same restriction bands suggesting that most of the *HSP70* genes in *L. braziliensis* are of the *HSP70-I* type. At present is not possible to deduce the number of *HSP70-I* genes and additional studies using genomic clones are needed to define both the *HSP70* copy number in the *L. braziliensis* genome and the probable existence of *HSP70* gene(s) with a divergent 3′-UTR (see below).

https://doi.org/10.1017/S0031182006001570 Published online by Cambridge University Press

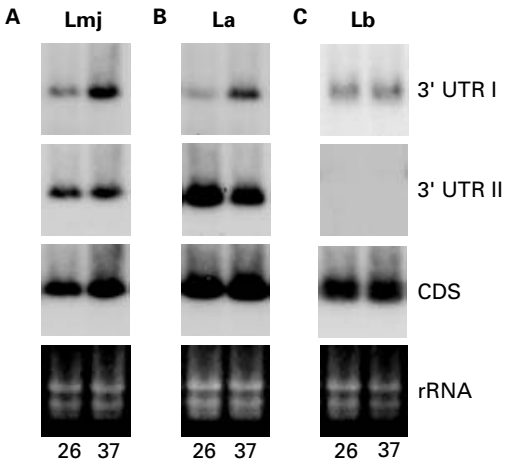


Fig. 2. Analysis of the expression of the *HSP70* genes in *Leishmania major* (A), *L. amazonensis* (B), and *L. braziliensis* (C). RNA samples were prepared from promastigotes grown at 26 °C (lanes 26) or incubated for 2 h at 37 °C (lanes 37), and analyzed by Northern blotting. The blots were hybridized with the probes 3′-UTR I, 3′-UTR II and CDS (see legend to Fig. 1). Bottom panel shows the ethidium bromide staining of the RNA samples loaded on agarose gel prior to transfer.

### *Expression of HSP70 genes in different* Leishmania *species*

In *L. infantum*, the abundance of transcripts derived from *HSP70-I* genes increases following heat shock, whereas the levels of *HSP70-II* mRNAs remain unaffected (Quijada *et al.* 1997; Folgueira *et al.* 2005). We considered it of interest to analyse whether the expression pattern of the 2 types of *HSP70* genes is conserved in other *Leishmania* species. According to the Southern blot analyses (Fig. 1), 3 different genomic organizations of the *HSP70* locus were found among the 6 *Leishmania* species analysed. So, we selected a representative species for each type of genomic organization in order to analyse the expression of the *HSP70* genes. Thus, for this study we chose *L. major*, *L. amazonensis*, and *L. braziliensis*. Total RNA was isolated from logarithmic phase promastigotes after incubation for 2 h at 26 °C or 37 °C, and analysed by Northern blotting. Fig. 2 shows the results after hybridizing with probes derived from the 3′-UTR I, 3′-UTR II and coding region (CDS) of *L. infantum HSP70* genes. For *L. major* and *L. amazonensis* promastigotes, the Northern blots revealed that *HSP70-I* transcripts accumulate when the parasites are incubated at elevated temperatures (Fig. 2A, B, panel 3′UTR I). Densitometric analysis of the blots indicated that the abundance of *HSP70-I* transcripts is 2 to 3-fold higher in promastigotes incubated at 37 °C than in promastigotes growing at normal temperature (26 °C). In contrast, the *HSP70-I* transcript levels in *L. braziliensis* promastigotes resulted unaffected upon heat shock treatment (Fig. 2C, panel 3′UTR I). On the other hand, the steady-state levels of *HSP70-II* transcripts in *L. major* and *L. amazonensis* did not change after heat treatment of the promastigotes (Fig. 2A, B, panel 3′UTR II). As expected, 3′-UTR II transcripts were not detected in the blots containing RNA from *L. braziliensis* promastigotes (Fig. 2C, panel 3′UTR II). When the Northern blots with *L. major* and *L. amazonensis* RNAs were hybridized with the CDS probe, the accumulation of *HSP70* transcripts was not evident, indicating that the *HSP70-II* mRNAs must be more abundant that the *HSP70-I* ones (Fig. 2A, B, panel CDS). These results agreed with previous studies in *L. major* (Lee *et al.* 1988). Noticeably, after probing the *L. braziliensis* blot with the *HSP70* CDS, 2 hybridization bands very close in size were seen (Fig. 2C, panel CDS). The lower band corresponds to that hybridizing with the 3′-UTR I probe. The other band may suggest the existence in *L. braziliensis* of a different type of *HSP70* gene(s) containing a 3′-UTR with low, or none, sequence homology to the 3′-UTR in *HSP70-I* or *HSP70-II* genes. However, the abundance of both types of *HSP70* transcripts in *L. braziliensis* was unaffected after temperature treatment of the promastigotes.

### *Analysis of HSP70 synthesis during heat shock*

It has been estimated that HSP70 accounts for around 2·1% of the total protein in unstressed *Leishmania* promastigotes (Brandau *et al.* 1995), making it one of the most abundant proteins. Due to its abundance, no substantial increase is observed after a heat shock treatment. However, by a combination of metabolic labelling and immunoprecipitation, it has been demonstrated that the rate of HSP70 synthesis is increased during heat shock. Thus, a heat stress at 37 °C of *L. donovani* promastigotes results in a 6-fold increase in the synthesis rate of HSP70 (Brandau *et al.* 1995). Similarly, in *L. infantum* promastigotes the rate of HSP70 synthesis was estimated to increase 4 to 5-fold at 37 °C relative to the rate at 26 °C (Folgueira *et al.* 2005). For this comparative study, we wanted to analyse the *de novo* synthesis of the HSP70 at both 26 °C and 37 °C in promastigotes of *L. major*, *L. amazonensis* and *L. braziliensis*, species in which this analysis has not been previously reported and that show a different genomic organization of the *HSP70* locus (Fig. 1). Fig. 3A shows an autoradiograph of the metabolically labelled proteins at 26 °C and at 37 °C in *L. amazonensis*, *L. braziliensis* and *L. major* promastigotes. Remarkably, total protein synthesis was drastically affected in *L. amazonensis* by incubation at 37 °C, with a global decrease of 41% relative to the protein synthesis at 26 °C. This finding indicates that 37 °C induces a severe heat shock in this species. *Leishmania* species differ in the temperature range which they resist, a feature that may be related to

https://doi.org/10.1017/S0031182006001570 Published online by Cambridge University Press

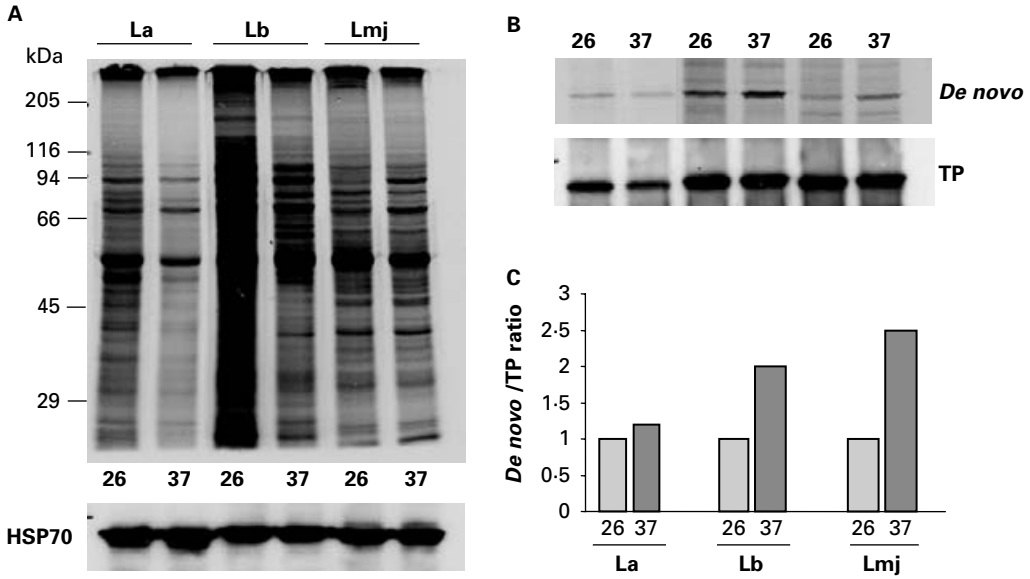


Fig. 3. Analysis of the *de novo* synthesis of HSP70 in *Leishmania* species. (A) Promastigotes of *L. amazonensis* (La), *L. braziliensis* (Lb), and *L. major* (Lmj) were metabolically labelled for 1 h at either 26 °C (lanes 26) or 37 °C (lanes 37), and the proteins were analysed by SDS-PAGE and autoradiography. Also, the proteins samples were analysed by Western blotting using an anti-HSP70 antibody (bottom panel). (B) The HSP70 of new synthesis was determined by immunoprecipitation of the HSP70 in the proteins extracts of these *Leishmania* species. The immunoprecipitates were analysed by SDS-PAGE and either autoradiography (*de novo* panel) or Western blotting with the anti-HSP70 antibody (TP panel). (C) Plot of the ratios of *de novo* synthesized HSP70 compared to total amount of HSP70; the *de novo*/TP ratio at 26 °C was set as 1.

their ability to establish infection at different sites in the mammalian host (Zilberstein and Shapira, 1994). In *L. braziliensis* and *L. major* the overall protein synthesis was not substantially modified when promastigotes were incubated at 37 °C indicating that in these species, although they cause cutaneous infections as *L. amazonensis*, the protein translation is more resistant to temperature increase than in *L. amazonensis* promastigotes.

Finally, we determined the rate of *de novo* HSP70 synthesis at both temperatures in these three *Leishmania* species. An anticipated but not yet demonstrated observation of this study was that the steady state level of HSP70 does not substantially change after heat stress in *L. amazonensis*, *L. braziliensis* and *L. major* promastigotes (Fig. 3A, panel HSP70). Therefore, it was necessary to perform a combination of metabolic labelling and immunoprecipitation to determine the rate of HSP70 synthesis in parasites incubated at 37 °C (Fig. 3B, C). It was found that there was a preferential translation of HSP70 at 37 °C in *L. braziliensis* and *L. major*, accounting for a 2-fold increase in the *de novo* HSP70 synthesis at 37 °C relative to the synthesis detected at 26 °C. In contrast, in *L. amazonensis* the rate of HSP70 synthesis was similar at both 26 °C and 37 °C. Nevertheless, taking into account the decrease in the global protein synthesis observed at 37 °C, it is concluded that the *HSP70* mRNA translation in *L. amazonensis* is resistant to the partial blockage of the global translational process observed at 37 °C.

## DISCUSSION

Given that the genome of *L. major* has been sequenced (Ivens *et al.* 2005) and this information is available at the GeneDB website (http://www.genedb.org/; Aslett *et al.* 2005), we performed a bioinformatics study of the *HSP70* gene locus in the *L. major* genome database. According to the *L. major* database, the *HSP70* locus is located in chromosome 28 and consists of 2 *HSP70* genes. The genomic organization is similar to that determined by Southern blotting in this work, i.e. the locus is composed of 2 types of genes (*HSP70-I* and *HSP70-II*) and the relative position of the genes is identical. The *HSP70-I* and *HSP70-II* genes correspond to the *L. major* database entries with systematic names LmjF28.2780 and LmjF28.2770, respectively. However, a clear difference exists between the *L. major* genome database and our results: there is no coincidence in the number of *HSP70-I* genes. Our analysis supports the existence of five *HSP70-I* genes in *L. major*, in close agreement with a previous report that described the existence in the *L. major* genome of a tandem array consisting of 4 *HSP70* genes (Lee *et al.* 1988). Sequence analysis of these genes indicated that they possess a 3′-UTR homologous to the *L. infantum HSP70-I* gene (Quijada *et al.* 1997). Furthermore, the discrepancy between our results and those of the *L. major* genome Project cannot be due to the use of a different *L. major* strain, since in both studies the strain Friedlin has

https://doi.org/10.1017/S0031182006001570 Published online by Cambridge University Press

been used. A plausible explanation is that the copy number of *HSP70-I* genes in the *L. major* genome has been underestimated because of the collapse of multiple tandem repeats into fewer copies during assembly of sequence data, a frequent problem associated with reiterated genes (El-Sayed *et al*. 2005). It is likely that similar discrepancies will emerge when other tandemly linked, repeated genes are analysed in the *L. major* database.

The Southern blot analyses reported here indicate that the *HSP70* gene locus is highly conserved in the different species of *Leishmania*, excepting *L. braziliensis*. For most species, the locus is composed of 2 types of *HSP70* genes: 5-6 *HSP70-I* genes and an *HSP70-II* gene, the latter always located at the 3′-end of the gene cluster. Nevertheless, restriction polymorphisms differentiate the *HSP70* locus from Old and New World *Leishmania* species. Thus, the *HSP70* loci in *L. infantum*, *L. major* and *L. tropica* have essentially the same structure and the repetition units of the tandem are defined by either *Bam*HI or *Sal*I restriction enzymes. In contrast, the *HSP70* locus in the New World species *L. mexicana* and *L. amazonensis* lacks the *Bam*HI restriction site but conserves the *Sal*I site. For *L. braziliensis*, our present data and those previously published (Zurita *et al*. 2003) are insufficient to determine the genomic organization of the *HSP70* genes. However, our Southern blot analysis demonstrates that this species lacks *HSP70-II* genes and, therefore, that the *L. braziliensis HSP70* locus is structurally different to the *HSP70* loci in the other *Leishmania* species. According to the genomic structure of the *HSP70* locus, the *Leishmania* species analyzed in this study may be clustered in 3 groups. The first cluster includes the Old World species *L. infantum*, *L. major* and *L. tropica*. The second cluster includes the New World species of the subgenus *Leishmania* (*L. mexicana* and *L. amazonensis*). Finally, *L. braziliensis*, New World species of the subgenus *Viannia*, possesses the most divergent *HSP70* locus. It should be necessary to analyse other species of the subgenus *Viannia* to see whether the *L. braziliensis HSP70* gene organization is conserved among species of this subgenus. Nevertheless, the clustering denoted by the organization of the *HSP70* locus is similar to that obtained after analysing in different isolates of *Leishmania* the mini-exon gene loci (Fernandes *et al*. 1994), the sequence of DNA and RNA polymerase genes (Croan *et al*. 1997), and the chromosomal karyotype (Britto *et al*. 1998). In those studies, the *L.* (*V.*) *braziliensis* complex represents the earliest divergent species of the genus *Leishmania*, a fact that agrees with the current beliefs on the origin and evolution of the genus *Leishmania* (Momen and Cupolillo, 2000). As a conclusion of our study, we think that the *HSP70* locus may be a useful genetic marker aiding for *Leishmania* species identification.

At the level of gene expression, differences were also found when comparing the expression pattern in species of the subgenus *Leishmania* with the expression in *L. braziliensis*, pointing to differences in the regulatory mechanisms. It is well established that transcripts derived from *HSP70-I* genes accumulated in response to heat treatment in both *L. major* (Lee *et al*. 1988; this work) and *L. infantum* (Quijada *et al*. 1997). Here, we found that this is also the case for *HSP70-I* mRNAs in *L. amazonensis* but not for those of *L. braziliensis*. As occurs for most genes in kinetoplastids (Clayton, 2002), the regulation of *HSP70* genes in *Leishmania* occurs at the post-transcriptional level (Brandau *et al*. 1995; Quijada *et al*. 1997). Taking into account this feature, it must be concluded that *L. braziliensis* lacks the regulatory mechanisms responsible of the temperature-dependent stabilization of the *HSP70-I* transcripts observed in the species of the subgenus *Leishmania*. However, when the rate of HSP70 synthesis was analysed, it became evident that *L. braziliensis* promastigotes have the capacity to translate preferentially the *HSP70* transcripts during heat stress, as do other *Leishmania* species. Further studies are needed to establish the genomic structure of the *HSP70* locus in *L. braziliensis*, the genome sequencing project is ongoing (Laurentino *et al*. 2004) and, more importantly, to determine the mechanisms of gene expression.

We gratefully acknowledge financial support from the Ministerio de Ciencia y Tecnología (BFU2005-08432-C02-01) and grants from the Fondo de Investigaciones Sanitarias (C03/04 and PI050973). Also, an institutional grant from Fundación Ramón Areces is acknowledged.

## REFERENCES

**Anonymous** (1990). Control of the leishmaniasis: Report of a WHO Expert Committee. *World Health Organization Technical Report Series*, No. **793**.

**Aslett, M., Mooney, P., Adlem, E., Berriman, M., Berry, A., Hertz-Fowler, C., Ivens, A. C., Kerhornou, A., Parkhill, J., Peacock, C. S., Wood, V., Rajandream, M. A., Barrell, B. and Tivey, A.** (2005). Integration of tools and resources for display and analysis of genomic data for protozoan parasites. *International Journal for Parasitology* **35**, 481–493.

**Bock, J. H. and Langer, P. J.** (1993). Sequence and genomic organization of the *hsp70* genes of *Leishmania amazonensis*. *Molecular and Biochemical Parasitology* **62**, 187–197.

**Brandau, S., Dresel, A. and Clos, J.** (1995). High constitutive levels of heat-shock proteins in human-pathogenic parasites of the genus *Leishmania*. *The Biochemical Journal* **310**, 225–232.

**Britto, C., Ravel, C., Bastien, P., Blaineau, C., Pages, M., Dedet, J. P. and Wincker, P.** (1998). Conserved linkage groups associated with large-scale chromosomal rearrangements between Old World and New World *Leishmania* genomes. *Gene* **222**, 107–117.

**Clayton, C. E.** (2002). Life without transcriptional control? From fly to man and back again. *The EMBO Journal* **21**, 1881–1888.

**Clos, J. and Krobitsch, S.** (1999). Heat shock as a regular feature of the life cycle of *Leishmania* parasites. *American Zoologist* **39**, 848–856.

**Croan, D. G., Morrison, D. A. and Ellis, J. T.** (1997). Evolution of the genus *Leishmania* revealed by comparison of DNA and RNA polymerase gene sequences. *Molecular and Biochemical Parasitology* **89**, 149–159.

**Desjeux, P.** (2004). Leishmaniasis: current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases* **27**, 305–318.

**El-Sayed, N. M., Myler, P. J., Bartholomeu, D. C., Nilsson, D., Aggarwal, G., Tran, A. N., Ghedin, E., Worthey, E. A., Delcher, A. L., Blandin, G., Westenberger, S. J., Caler, E., Cerqueira, G. C., Branche, C., Haas, B., Anupama, A., Arner, E., Aslund, L., Attipoe, P., Bontempi, E., Bringaud, F., Burton, P., Cadag, E., Campbell, D. A., Carrington, M., Crabtree, J., Darban, H., da Silveira, J. F., de Jong, P., Edwards, K., Englund, P. T., Fazelina, G., Feldblyum, T., Ferella, M., Frasch, A. C., Gull, K., Horn, D., Hou, L., Huang, Y., Kindlund, E., Klingbeil, M., Kluge, S., Koo, H., Lacerda, D., Levin, M. J., Lorenzi, H., Louie, T., Machado, C. R., McCulloch, R., McKenna, A., Mizuno, Y., Mottram, J. C., Nelson, S., Ochaya, S., Osoegawa, K., Pai, G., Parsons, M., Pentony, M., Pettersson, U., Pop, M., Ramirez, J. L., Rinta, J., Robertson, L., Salzberg, S. L., Sanchez, D. O., Seyler, A., Sharma, R., Shetty, J., Simpson, A. J., Sisk, E., Tammi, M. T., Tarleton, R., Teixeira, S., Van Aken, S., Vogt, C., Ward, P. N., Wickstead, B., Wortman, J., White, O., Fraser, C. M., Stuart, K. D. and Andersson, B.** (2005). The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science* **309**, 409–415.

**Fernandes, O., Murthy, V. K., Kurath, U., Degrave, W. M. and Campbell, D. A.** (1994). Mini-exon gene variation in human pathogenic *Leishmania* species. *Molecular and Biochemical Parasitology* **66**, 261–271.

**Folgueira, C., Quijada, L., Soto, M., Abanades, D. R., Alonso, C. and Requena, J. M.** (2005). 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. *The Journal of Biological Chemistry* **280**, 35172–35183.

**Garlapati, S., Dahan, E. and Shapira, M.** (1999). Effect of acidic pH on heat shock gene expression in *Leishmania*. *Molecular and Biochemical Parasitology* **100**, 95–101.

**Hartl, F. U. and Hayer-Hartl, M.** (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**, 1852–1858.

**Ivens, A. C., Peacock, C. S., Worthey, E. A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M. A., Adlem, E., Aert, R., Anupama, A., Apostolou, Z., Attipoe, P., Bason, N., Bauser, C., Beck, A., Beverley, S. M., Bianchettin, G., Borzym, K., Bothe, G., Bruschi, C. V., Collins, M., Cadag, E., Ciarloni, L., Clayton, C., Coulson, R. M., Cronin, A., Cruz, A. K., Davies, R. M., De Gaudenzi, J., Dobson, D. E., Duesterhoeft, A., Fazelina, G., Fosker, N., Frasch, A. C., Fraser, A., Fuchs, M., Gabel, C., Goble, A., Goffeau, A., Harris, D., Hertz-Fowler, C., Hilbert, H., Horn, D., Huang, Y., Klages, S., Knights, A., Kube, M., Larke, N., Litvin, L., Lord, A., Louie, T., Marra, M., Masuy, D., Matthews, K., Michaeli, S., Mottram, J. C., Muller-Auer, S., Munden, H., Nelson, S., Norbertczak, H., Oliver, K., O'Neil, S., Pentony, M., Pohl, T. M., Price, C., Purnelle, B., Quail, M. A., Rabbinowitsch, E., Reinhardt, R., Rieger, M., Rinta, J., Robben, J., Robertson, L., Ruiz, J. C., Rutter, S., Saunders, D., Schafer, M., Schein, J., Schwartz, D. C., Seeger, K., Seyler, A., Sharp, S., Shin, H., Sivam, D., Squares, R., Squares, S., Tosato, V., Vogt, C., Volckaert, G., Wambutt, R., Warren, T., Wedler, H., Woodward, J., Zhou, S., Zimmermann, W., Smith, D. F., Blackwell, J. M., Stuart, K. D., Barrell, B. and Myler, P. J.** (2005). The Genome of the Kinetoplastid Parasite, *Leishmania major*. *Science* **309**, 436–442.

**Laurentino, E. C., Ruiz, J. C., Fazelinia, G., Myler, P. J., Degrave, W., Alves-Ferreira, M., Ribeiro, J. M. C. and Cruz, A. K.** (2004). A survey of *Leishmania braziliensis* genome by shotgun sequencing. *Molecular and Biochemical Parasitology* **137**, 81–86.

**Lee, M. G.-S., Atkinson, B. L., Giannini, S. H. and Van der Ploeg, L. H. T.** (1988). Structure and expression of the *hsp70* gene family of *Leishmania major*. *Nucleic Acids Research* **16**, 9567–9585.

**MacFarlane, J., Blaxter, M. L., Bishop, R. P. and Kelly, J. M.** (1990). Identification and characterisation of a *Leishmania donovani* antigen belonging to the 70-kDa heat-shock protein family. *European Journal of Biochemistry* **190**, 377–384.

**Momen, H. and Cupolillo, E.** (2000). Speculations on the origin and evolution of the genus *Leishmania*. *Memórias do Instituto Oswaldo Cruz* **95**, 583–588.

**Murray, H. W., Berman, J. D., Davies, C. R. and Saravia, N. G.** (2005). Advances in leishmaniasis. *Lancet* **366**, 1561–1577.

**Pearson, R. D. and de Queiroz Sousa, A.** (1996). Clinical spectrum of Leishmaniasis. *Clinical Infectious Diseases* **22**, 1–13.

**Quijada, L., Soto, M., Alonso, C. and Requena, J. M.** (1997). Analysis of post-transcriptional regulation operating on transcription products of the tandemly linked *Leishmania infantum hsp70* genes. *The Journal of Biological Chemistry* **272**, 4493–4499.

**Quijada, L., Soto, M., Alonso, C. and Requena, J. M.** (2000). Identification of a putative regulatory element in the 3′-untranslated region that controls expression of HSP70 in *Leishmania infantum*. *Molecular and Biochemical Parasitology* **110**, 79–91.

**Requena, J. M., Lopez, M. C., Jimenez-Ruiz, A., de la Torre, J. C. and Alonso, C.** (1988). A head-to-tail tandem organization of *hsp70* genes in *Trypanosoma cruzi*. *Nucleic Acids Research* **16**, 1393–1406.

**Rico, A. I., Angel, S. O., Alonso, C. and Requena, J. M.** (1999). Immunostimulatory properties of the *Leishmania infantum* heat shock proteins HSP70 and HSP83. *Molecular Immunology* **36**, 1131–1139.

**Sambrook, J., Fritsch, E. F. and Maniatis, T.** (1989). *Molecular Cloning: A Laboratory Manual*. 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

**Zilberstein, D. and Shapira, M.** (1994). The role of pH and temperature in the development of *Leishmania* parasites. *Annual Reviews of Microbiology* **48**, 449–470.

**Zilka, A., Garlapati, S., Dahan, E., Yaolsky, V. and Shapira, M.** (2001). Developmental Regulation of Heat Shock Protein 83 in Leishmania. 3′ processing and mRNA stability control transcript abundance, and translation is directed by a determinant in the 3′-untranslated region. *The Journal of Biological Chemistry* **276**, 47922–47929.

**Zurita, A. I., Rodriguez, J., Piñero, J. E., Pacheco, R., Carmelo, E., del Castllo, A. and Valladares, B.** (2003). Cloning and characterization of the Leishmania (Viannia) braziliensis Hsp70 gene. Diagnostic use of the C-terminal fragment rLb70(513–663). *The Journal of Parasitology* **89**, 372–378.

https://doi.org/10.1017/S0031182006001570 Published online by Cambridge University Press