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A family of heat shock protein 70-related genes are expressed in the promastigotes of *Leishmania major*

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ABSTRACT

We describe the isolation and characterisation of two novel genes of the parasitic protozoan *Leishmania major* that are related by nucleotide sequence homology to eukaryotic genes encoding 70 Kd. heat shock proteins. The transcription of neither gene is heat-inducible but both are constituitively-expressed throughout the promastigote stage of the parasite life cycle. A third gene shows differential expression between non-infective and infective promastigote stages in the absence of any temperature change. These genes are related by sequence homology to the tandemly-repeated hsp70 genes of trypanosomatids, but are located on different, dispersed chromosomes within the genome of *L. major*. The open reading frame for translation derived from one of these sequences contains a putative mitochondrial signal peptide at its amino-terminus.

INTRODUCTION

Parasitic protozoa of the genus *Leishmania* exist as extracellular flagellated promastigotes within the gut of their sandfly vectors and as intracellular aflagellated amastigotes within the phagolysosomes of mammalian host macrophages. Therefore, together with other parasitic trypanosomatids, these unicellular organisms must be able to adapt to the considerable changes in ambient temperature associated with passage from a cold-blooded insect to a warm-blooded mammal. In all eukaryotes, sudden increases in temperature induce the expression of heat shock proteins (hsps) which are believed to be essential both for the protection of precursor proteins and the disaggregation of protein complexes formed during the period of stress [1]. In a classical heat shock response, one or more members of the hsp 70 gene family is expressed at higher than constitutive levels [2]. In *Leishmania*, where it has been shown that experimental heat shock induces the production of hsps [3,4], the hypothesis has been proposed that such proteins could be involved in triggering cell transformation from the promastigote to the amastigote stage [5]. As yet, however, no biochemical pathway has been proposed for such an hypothesis, and recent evidence strongly suggests that differential expression of hsp70 genes is merely concomitant with this transformation event [6]. Indeed, it should not be assumed that *Leishmania* displays a classic heat shock response when entering the mammalian host, because the elevation of temperature is a long-term and obligatory event in the life cycle, rather than an accidental and transient exposure to extreme conditions.

How the hsp70 genes of *Leishmania* are regulated during development is, therefore, of considerable interest in itself, but, in addition, there is a real need to understand differential expression of this gene family because of the high immunogenicity of some of the hsp70 proteins in both *Leishmania* and other parasitic species [7]. The mechanism whereby the mammalian host cell differentiates between self and invading stress proteins

is not understood. One important step in understanding the expression of parasite hsp70 genes must be to identify all members of the family, not all of which are normally heat-inducible in most eukaryotes [1]. In this report, we demonstrate that stationary phase (infective) promastigotes of *Leishmania major* [8,9] show increased levels of hsp70 messenger (m) RNA compared with logarithmic phase (non-infective) promastigotes when parasites are grown *in vitro* at a constant 25°C. This expression is not inducible following the transformation from promastigote to amastigote. The genes encoding this mRNA are members of a family of related sequences, which show different patterns of expression and whose products may have a variety of functions within the developing parasite.

MATERIALS AND METHODS

Parasites. The Friedlin strain of *L. major* (MHOM/IL/81/Friedlin), used in most experiments, was cultured and maintained as previously described [10]. Other *Leishmania* strains and species (*L. major* MRHO/SU/59/P, MHOM/TN/84/ISS108-TN2, MHOM/IL/67/Jericho II (V121), MHOM/IL/81/Friedlin/A1; *Leishmania tropica* MHOM/SU/74/K27; *Leishmania infantum* MHOM/IT/85/ISS177) were used in genomic DNA blots and pulsed field gradient gel electrophoresis. In RNA isolation experiments, the following nomenclature is used: log. phase cultures = 3 day cultures, 0% infective forms; stationary phase cultures = 10 day cultures, 60−80% infective forms. Cultures at time points between these two extremes show intermediate numbers of infective forms [10]. Promastigotes were heat-shocked by incubation for 20 hr. at 37°C. During this time, the parasites changed morphology and became rounded, as has been observed by other workers [5].

Extraction of nucleic acids. Total genomic DNA was isolated by a modification of the method of Cowman *et al.* [11]. Pelleted, washed parasites were lysed in 5M guanidinium isothiocyanate and the RNA isolated by density gradient centrifugation [12].

Construction and screening of recombinant DNA libraries. A genomic library of *L. major* DNA was constructed by the insertion of partial *Sau 3A* fragments into the *Bam H1* sites of the λ vector EMBL 4, followed by *in vitro* packaging and transfection into the host *E. coli* strain *Q 359* [13]. Screening was carried out using standard techniques [13].

DNA probes. The human hsp 70 probe used was the full-length cDNA clone described by Hunt and Morimoto [14]. The actin probe was a 3.0 Kb *Eco R1/ Sal 1* fragment (derived from the same *L. major* genomic library) containing a *Leishmania* actin gene (Searle and Smith, unpublished). All probes were radiolabelled by the random priming method [15].

Blotting and hybridisation. RNAs were denatured with glyoxal and dimethylsulphoxide before size-separation and blotting, as previously described [10]. Genomic and recombinant DNAs were analysed by blotting on to nylon membranes (Gene Screen Plus, Dupont), followed by hybridisation with radiolabelled probes, using standard techniques [12]. Post-hybridisational washes were at high stringency (0.2×SSC, 0.5% SDS, 65°C) except where indicated, and autoradiographic exposures were at −70°C.

Sub-cloning and DNA sequencing. DNA fragments from the hsp 70 genes of *L. major* were subcloned into the plasmid vectors pEMBL 8+ [16] or pAT 153 [17], or into the M13 vectors, TG131 and 132 (Amersham). Dideoxy-sequencing was carried out by the method of Sanger [18]. Sequences were assembled and analysed using the MICROGENIE (Beckman Instruments) computer program.

Pulsed field gradient gel electrophoresis. Pulsed field gradient (PFG) gel electrophoresis of chromosome-size DNA from promastigotes of *L. major* V121 and *L. major* Friedlin

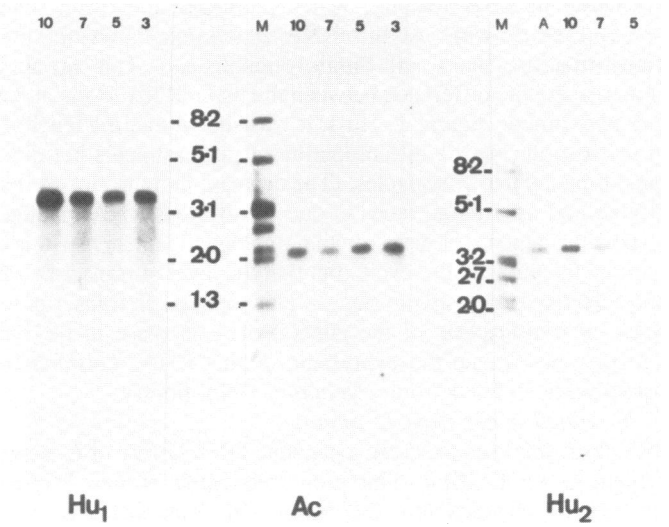

Figure 1. *L. major* transcripts with homology to the human hsp70 gene. Total RNA extracted from promastigotes at different stages in the growth cycle, and from amastigotes, was blotted and probed with either a human hsp70 cDNA or an actin probe from *L. major*. Hu_1: 10μg RNA per lane from 3, 5, 7 and 10 day promastigote cultures, probed with the human hsp70 probe (2 day exposure). Ac: the same blot as Hu_1, stripped and reprobed with the actin probe (2 day exposure). Hu_2: 5μg of RNA per lane from 5, 7, 10 promastigote cultures and 2.5μg of RNA from amastigotes (A), probed with the human hsp70 probe (overnight exposure). M: adenovirus-2/*Hind III* cut DNA markers.

A1 was performed using a CHEF apparatus [19]. The electrodes were 26 cm. apart. The procedures for the preparation of agarose blocks of chromosomal DNA and blotting on to nylon filters have been described [20]. DNA was fractionated in 1% agarose gels containing 0.5×TBE buffer at 12°C. Pulse times ranged from 80 sec. to 30 min. at 2.7−6.1 V/cm with run times of 1 to 4 days.

RESULTS

Differentially-expressed genes of L. major promastigotes.

As part of a more general project aimed at the identification of genes expressed in infective promastigotes [8,9,10; Coulson and Smith, submitted], heterologous DNA probes encoding evolutionarily-conserved proteins were used to investigate stage-specific gene expression. RNAs were isolated from amastigotes and promastigotes at different stages in their growth cycle, size fractionated and blotted on to nylon membranes. Following hybridisation with a radiolabelled human hsp70 cDNA clone, the filters were washed to high stringency and exposed for autoradiography. The human probe hybridised to a 3.4 Kb transcript in both promastigotes and amastigotes of *L. major* (Figure 1), which was polyadenylated (data not shown). This transcript was present in non-infective organisms (3 day cultures) at a level approximately equivalent to that of actin mRNA (which represents $<5\%$ of total mRNA). The amount of the 3.4 Kb transcript increased as the promastigotes transformed into infective forms, to a level at least 2-fold higher after 10 days in culture (Figure 1, Hu_1). Hybridisation of the same filter with a homologous actin probe (Figure 1, Ac)

showed some variation in RNA loading, which paralleled the result obtained with a ribosomal probe (data not shown). (Actin mRNA expression shows no variation during promastigote transformation; Searle and Smith, unpublished). This variation in loading has the effect of increasing the difference between the hsp mRNA levels at 3 and 10 days. In an independent experiment (Figure 1, Hu_2), it was shown that the level of hsp mRNA only showed an increase during the differentiation of promastigotes but did not increase following the transformation into amastigotes. (The decrease in transcript level seen between 10 day promastigote and amastigote lanes is due to different RNA loadings).

These results show that a gene, or genes, with homology to human hsp70 is constitutively expressed throughout the parasite life cycle, but that the level of transcript varies between non-infective and infective promastigote stages. This increase in transcript level could be due to an increase in transcription of the gene(s) or a decrease in the half-life of the mRNA(s). To further investigate the expression of this RNA, *Leishmania* genes with homology to hsp70 were isolated from a genomic DNA library.

Cloning of hsp 70-related genes from L. major.

The human cDNA clone was used to screen a genomic DNA library of *L. major* constructed in the λ replacement vector EMBL4. This probe recognises at least 3 related genes, of different copy number, in genomic DNA of *L. major* [10]. A number of clones were isolated and physically characterised using standard techniques. Restriction maps of three genomic regions with homology to human hsp70 are shown in Figure 2. None of these regions are overlapping and, as shown below, they are located on different chromosomes.

Cross-hybridisation experiments between these genomic clones and the human hsp70 probe showed that λLm hsp70.4 has the highest level of homology with the human gene (data not shown).

Transcripts recognised by the hsp70 genes

The DNA with homology to hsp70 within the clones described in Figure 2 may represent expressed or non-expressed genes. To determine which of these sequences are transcribed, fragments of each recombinant were used to probe RNA blots, under conditions which minimised cross-hybridisation between related sequences (high stringency, short exposure time). In particular, 3′ probes were chosen for use, given that the related hsp70 genes of other organisms show least homology to each other at their 3′ ends [2]. RNA samples were extracted from promastigotes in both log. and stationary phase of growth, and organisms from each stage were also exposed to heat shock prior to RNA isolation. The results obtained with probes from λLm 70.1 are shown in Figure 3. Initial probing of this blot with a 3′ probe (fragment 1.2, see Figure 2) identified 2 weak transcripts (following a 3 day autoradiographic exposure) of 3.4 and 5.5 Kb (positions arrowed in Figure 3). The intensity of these bands was similar in all lanes, suggesting that the gene contained within λLm hsp70.1 is constitutively expressed at low levels in both log. and stationary phase cultures. Heat shocking the promastigotes had no effect on the level of transcript seen. When the same blot was reprobed with a fragment containing 80% of the coding region homologous to human hsp70 (fragment 1.1, see Figure 2), a pattern of much stronger transcripts was seen (Figure 3). In addition to the bands recognised by the 3′ probe (fragment 1.2), which were much more intense, 2 other bands, of 3.2 and 4.5 Kb, were observed in each lane. While none of the transcripts showed increased amounts between log. and stationary phase RNA, the 3.2 Kb transcript was clearly up-regulated by heat shock in both 4 and 11 day RNA samples. Thus the 1.1 probe of λLm hsp70.1 has homology to a gene that is inducible by heat shock. Given the results obtained with the 3′ probe (fragment

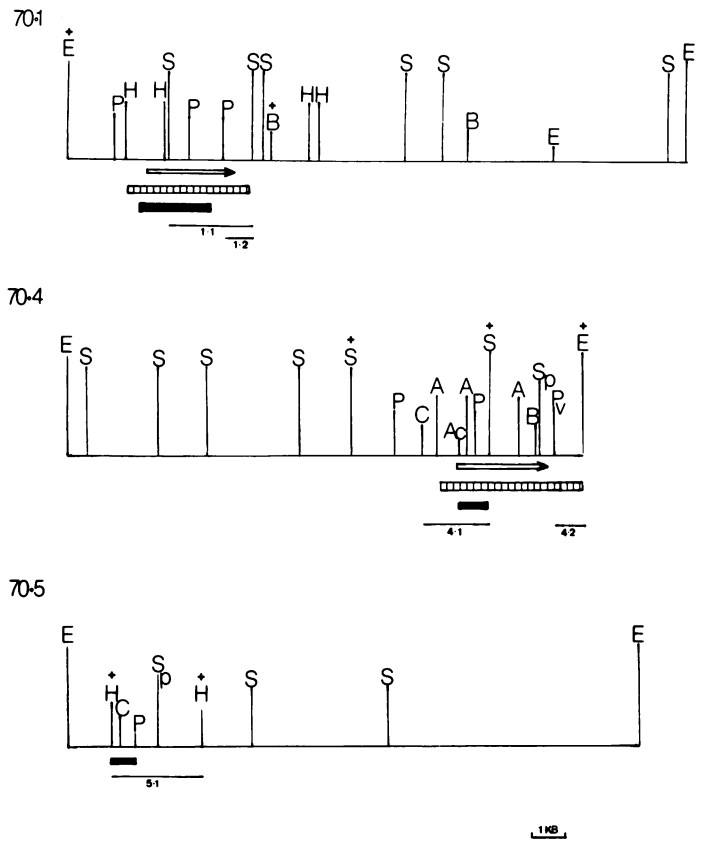

Figure 2. Restriction maps of hsp70-related genes of *L. major*. λLm hsp70.1 and λLm hsp70.5 represent the insertions of single genomic clones containing homology to human hsp70. λLm hsp 70.4 represents the linear map derived from 2 overlapping genomic clones. The homologous regions of each recombinant have been subcloned: adjacent restriction enzyme sites marked + indicate these subcloned fragments, which have been fine-scale mapped. The whole insertions have been mapped only with the following enzymes: B, E, H and S. Arrows indicate the regions with human hsp 70 homology (either by sequence or hybridisation analyses) and the direction of transcription (confirmed by probing RNA blots with single-stranded probes; data not shown). Hatched bars indicate the maximum length of DNA fragments showing hybridisation to the predominant 3.4/3.2 Kb RNAs on RNA blots (see text). Solid bars indicate the regions sequenced. The fragments used as probes in later experiments are shown by single lines and numbers (e.g. 1.1, 1.2 etc.).
A: *Ava 1*; Ac: *Acc1*; B: *Bam H1*; C: *Cla 1*; E: *Eco R1*;
H: *Hind 111*; P: *Pst 1*; Pv: *Pvu 1*; S: *Sal 1*; Sp: *Sph 1*.

1.2) above, we suggest that λLm hsp70.1 itself is not heat-inducible, but that it is related by sequence homology to a gene or genes that are.

The results of similar experiments carried out with 5′ and 3′ probes from λLm hsp70.4 are shown in Figure 4. The 5′ probe (fragment 4.1, see Figure 2) recognised a single transcript of 3.4 Kb which showed constituitive, non-heat inducible expression in both log. and stationary phase organisms. The slight differences in signal were due to un-equal loadings, as shown by the subsequent hybridisation of the same blot with an actin probe,

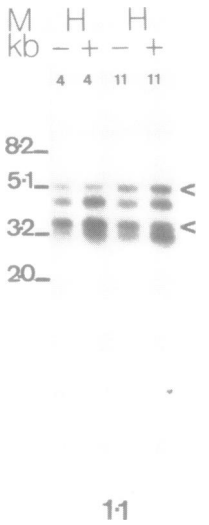

Figure 3. Transcripts with homology to λLm hsp70.1. 10μg samples of total RNA from 4 and 11 day promastigote cultures, heat-shocked (H+) or non heat-shocked (H−), were size-separated, blotted and probed sequentially with the 3′ specific 1.2 fragment and the 1.1 fragment (see Fig. 2). Arrows indicate the positions of the transcripts specifically recognised by the 1.2 fragment, which were just visible after a 3 day exposure (data not shown). The blot shown was exposed for 3 days, following hybridisation with the 1.1 probe. M: adenovirus-2/*Hind III* cut DNA markers.

which recognised a 2.2 Kb transcript (arrowed). After stripping both probes from the blot, it was rehybridised with a 3′ probe (fragment 4.2, see Figure 2), which gave an identical result to that obtained with the 5′ probe. We conclude that λLm hsp70.4 encodes a non-heat inducible 3.4 Kb transcript which is expressed, at a level similar to actin mRNA, throughout promastigote transformation.

RNA blots probed with the 5.1 fragment of λLm hsp70.5 (see Figure 2) also showed a constituitive 3.4 Kb transcript (data not shown) but subsequent sequence data suggest that the area of homology to hsp70 in this clone is small, perhaps indicative of an evolutionary remnant. The transcript was, therefore, probably recognised by cross-homology and is not encoded by λLm hsp70.5.

None of the transcripts described above showed increased accumulation as promastigotes differentiated into infective forms, as observed with the heterologous human hsp70 probe. In a separate, differential screen of a cDNA library, intended to identify genes showing up-regulation in infective stage organisms, a clone with hsp70 sequence homology was also isolated, which hybridised to several transcripts in both parasite stages [Coulson and Smith, submitted]. The principal 3.2 Kb transcript showed at least a 10-fold increase in stationary phase promastigotes, but little subsequent elevation in amastigotes. This expression pattern is the same as that seen with the heterologous human probe. However, the complexity in the number and pattern of expression of hsp 70-related transcripts reported in this paper suggests that the band originally detected by the human probe represents not a single transcript but a number of different transcripts (of 3.2−3.4 Kb), whose expression may be regulated by different factors.

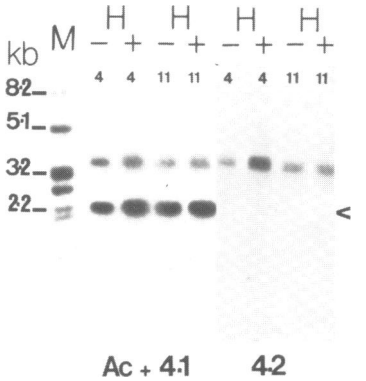

Figure 4. Transcripts with homology to λLm hsp70.4. 10μg samples of total RNA from 4 and 11 day promastigote cultures, heat-shocked (H+) or non heat-shocked (H−), were size-separated, blotted and probed sequentially with probes of the 4.1 and 4.2 fragments (see Fig. 2) and actin (position of single transcript arrowed). The blot was exposed for 2 days. M: adenovirus-2/*Hind 111* cut DNA markers.

Genomic organisation of the hsp70 genes of L. major.

In order to determine the copy number of the genes encoded within λLm hsp70.1 and λLm hsp70.4, probes from each recombinant were hybridised to genomic DNA blots. A *Sal 1* probe containing most of the coding region of λLm hsp70.1 (fragment 1.1, see Figure 2) hybridised to 4 genomic *Sal 1* fragments of 5.1, 4.0, 3.9 and 2.2 Kb, only the last of which corresponded to the 2.2 Kb fragment contained within the clone (Figure 5, lane 4). This suggested that λLm hsp70.1 is related to other highly homologous genes within the genome of *L. major*, and that these genes may be of different copy numbers. In particular, the 2.2 and 3.9 Kb fragments were present at higher intensity than the 4.0 and 5.1 Kb fragments. Cross-hybridisation between λLm hsp70.1 and λLm hsp70.4 might be expected, given their shared homology to the human hsp70 probe, but this did not occur under the hybridisation conditions used here, as shown by the lack of any large *Hind 111* fragments hybridising in Figure 5, lane 3 (λLm hsp70.4 resides on a large *Hind 111* fragment within the genome; see Figure 6). Instead, 3 bands of 7.0, 4.0 and 2.4 Kb were seen, of which only that of 4.0 Kb is accounted for in the parent phage (Figure 5, lane 3). Digests with *Pst 1* gave a much more complex hybridisation pattern of 7 bands, only 3 of which (of 2.6, 1.6 and 1.0 Kb) are derived from λLm hsp70.1 (Figure 5, lane 2). Double digests with both *Pst 1* and *Hind 111* showed a reduced pattern of hybridisation, of only 4 bands of 1.8, 1.6 (weak), 1.0 and 0.6 Kb (Figure 5, lane 1). Three of these can be accounted for by fragments within λLm hsp70.1. The 1.6 Kb 3′ fragment (which overlaps the end of the sub-cloned region of this recombinant; Figure 2) showed weaker signal in the double digest, indicative of a single hybridising band rather than the doublet presumed to be present in the single digest. The strong signal shown with the 1 Kb band in both single and double digests suggests that this fragment forms part of a repeat unit found elsewhere in the genome. The tandemly repeated hsp70-like genes recently reported by Lee *et al.* [6] do have a 1 Kb *Pst 1* repeat within them, but λLm hsp70.1 does not hybridise to the samechromosomal location as this sequence (see below).

To look for conservation of the gene encoded by λLm hsp70.1, the 1.1 fragment was hybridised to genomic DNA from different strains and species, digested with *Sal 1* (Figure

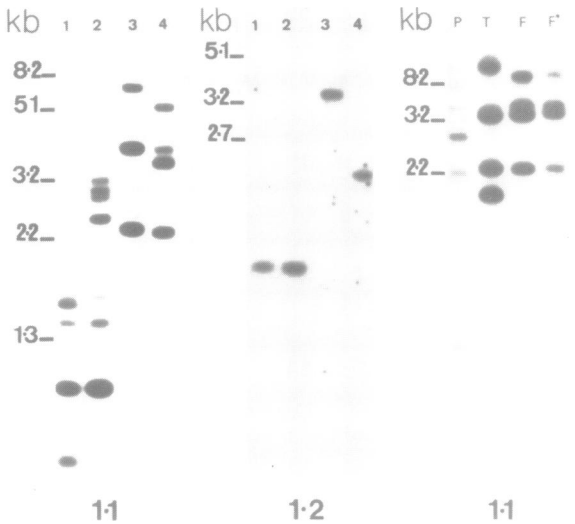

Figure 5. Genomic organisation of λLm hsp70.1. 3μg samples of genomic DNA were digested, blotted and probed with fragment 1.1 or 1.2 (see Fig. 2). Digests on the left-hand and centre blots (all of Friedlin strain *L. major*) are: 1. *Pst 1/Hind III*; 2. *Pst 1*; 3. *Hind III*; 4. *Sal 1*. The right-hand blot shows *Sal 1* digests of DNA from the following: P. *L. major*, P strain; F. *L. major*, Friedlin strain (F* = 1μg loading); T. *L. tropica*. M: adenovirus-2/*Hind III* cut DNA markers.

5, right hand blot). Strong signal from multiple bands was seen in all tracks, with the 2.2 Kb fragment showing conservation between all samples. It is interesting to note, however, that the 2 strains of *L. major* (P, F) showed differences in the sizes of the hybridising bands (presumably reflecting restriction enzyme site polymorphisms) while *L. tropica* showed some conservation, both in band size and number (Figure 5).

The genomic organisation of λLm hsp70.4 was rather easier to interpret (Figure 6). When a 5′ specific fragment (4.1, Figure 2) was used to probe a genomic blot, it hybridised to just one *Sal 1* fragment of 3.7 Kb, the size of which corresponded to the cloned fragment containing the probe (Figure 6, centre panel, lane 4). Two *Hind III* fragments hybridised to this probe (Figure 6, centre panel, lane 3) of which only the smaller (of 10Kb) could be accounted for within the mapped recombinant. Similarly, digestion with *Pst 1* yielded 3 hybridising bands (Figure 6, centre panel, lane 2) of which only the 5.1 and the 2.2 Kb fragments are derived from within the cloned region. Both the larger (>15 Kb) *Hind III* and the 1.6 Kb *Pst 1* fragments, which did not map within λLm hsp70.4, hybridised at a much lower level than the other fragments. This suggests that they were derived from other related sequences within the genome, which were cross-hybridising with the 5′ probe. Such a result might be expected as hsp70 genes have been shown to be most homologous at their 5′ ends [2]. Similar results were obtained after double digestion with *Pst 1* and *Hind III*: of the 4 hybridising fragments, only the 4.2 and 2.2 Kb bands were derived from the cloned region (Figure 6, centre panel, lane 1) . (The low hybridisation of the 4.2 Kb band was due to only a small portion of it being represented in the probe).

When a 3′ specific fragment of λLm hsp70.4 (4.2, Figure 2) was hybridised to a genomic blot of the same digests, only one fragment was detected in each lane and these could

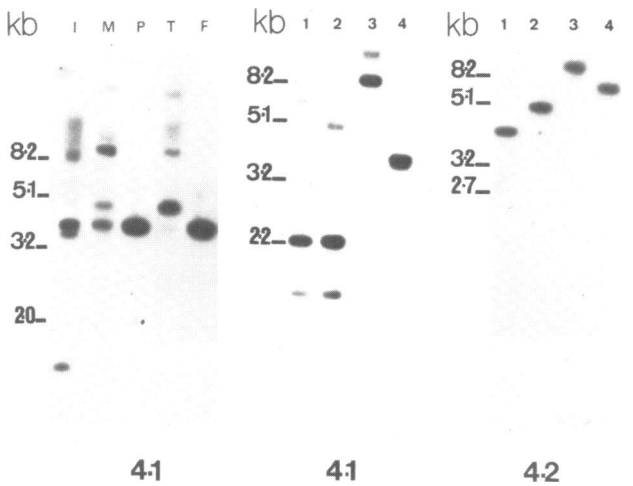

Figure 6. Genomic organisation of λLm hsp70.4. 3μg samples of genomic DNA were digested, blotted and probed with probe 4.1 or 4.2 (see Fig. 2). Digests on the right-hand and centre blots (all of Friedlin strain *L. major*) are: 1. *Pst 1/Hind 111*; 2. *Pst 1*; 3. *Hind 111*: 4. *Sal 1*. The left-hand blot shows *Sal 1* digests of DNA from the following: I. *L. infantum*; M. *L. major*, TN_2 strain; P. *L. major*, P strain; T. *L. tropica*; F. *L. major*, Friedlin strain. Markers are adenovirus-2/*Hind 111* cut DNA.

be accounted for at the 3′ end of λLm hsp70.4 (Figure 6, right-hand panel). Thus, a 7.3 Kb *Sal 1* fragment extends 3′ to the gene, as does a 5.1 Kb *Pst 1* fragment, which is cleaved by *Hind 111* to yield a 4.2 Kb band. This places the *Hind 111* site 3′ to the gene, which is at the end of the 10 Kb fragment recognised by both 3′ and 5′ probes. It can therefore be concluded that λLm hsp70.4 contains a single copy gene, whose 5′ end shows sequence homology to other hsp70 related genes in *L. major*.

To look at the conservation of the gene encoded by λLm hsp70.4, the 4.1 fragment was hybridised to genomic DNA from different strains and species, digested with *Sal 1* (Figure 6, left hand panel). The 3.7 Kb *Sal 1* fragment was the single hybridising band in two strains of *L. major* (P, F) but not in the third (M). No other cross-hybridising bands were detected following a short exposure and high stringency washing. A band of a similar size was one of several hybridising fragments in genomic DNA from both *L. infantum* (I) and *L. tropica* (T).

Chromosomal organisation of the hsp70 genes of L. major.

In order to map the chromosomal location of each hsp70 gene, chromosome-size DNA of *L. major* was fractionated by pulsed field gradient (PFG) gel electrophoresis. We have previously shown that *L. major* V121 exhibits 24 chromosome-size DNA bands separable by this technique and have mapped the location of several genes [21,22]. In particular, a *Drosophila* hsp70 probe (aDm 2.51) identified a hsp70-related sequence on chromosome band 17 of *L. major* V121 [21]. As shown in Figure 7A, the λLm hsp70.5 probe hybridised to chromosome band 15 (lane 5), the λLm hsp70.4 probe to chromosome band 16 (lane 16) whereas the λLm hsp70.1 probe hybridised to chromosomal DNA which was not resolved in this gel (lane 7). Each of these probes was clearly distinguished from the hsp70-related sequence on chromosome band 17 detected by the *Drosophila* aDm 2.51

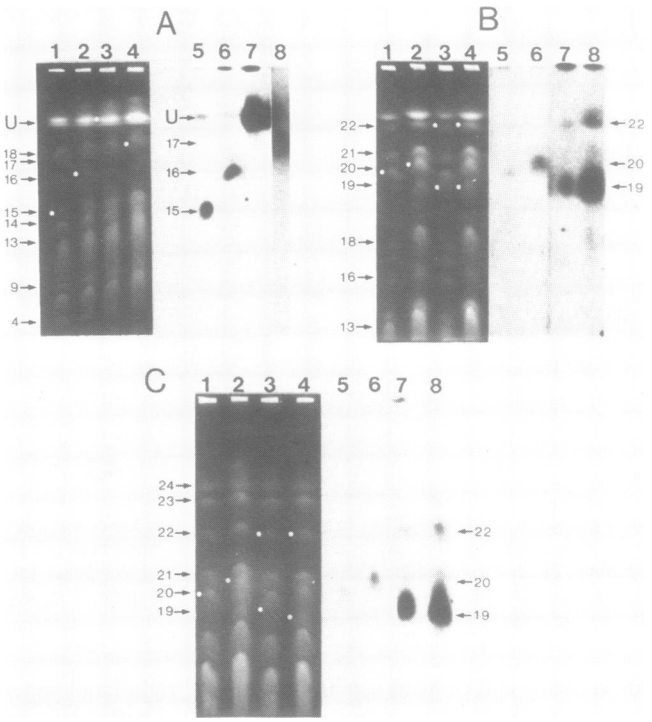

Figure 7. Mapping of hsp 70 genes to chromosomal loci of *L. major*. Chromosome-size DNA from *L. major* was fractionated by pulsed field gradient gel electrophoresis using a CHEF apparatus. **Panel A.** Fractionation of DNA of *L. major* V121: 80 sec/160 volts/1 day then 130 sec/160 volts/1 day. DNA in lanes 1−4 was blotted and hybridised to sub-cloned fragments (see Figure 2) of λLm hsp70.5 (lane 5), λLm hsp70.4 (5′ probe; lane 6), λLm hsp70.1 (lane 7) and the *Drosophila* hsp70 clone aDM 2.51 (lane 8). **Panel B.** Fractionation of DNA of *L. major* V121 (lanes 2,4) or *L. major* Friedlin A1 (lanes 1,3): 130 sec/160 volts/1 day then 270 sec/120 volts/2 days. DNA in lanes 1−4 was blotted and hybridised to probes for P100/11E reductase (lanes 5,6) or λLm hsp70.1 (lanes 7,8). **Panel C.** Fractionation of DNA of *L. major* V121 (lanes 2,4) or *L. major* Friedlin A1 (lanes 1,3): 270 sec/120 volts/2 days then 30 min/70 volts/2 days. DNA in lanes 1−4 was blotted and hybridised to probes for P100/11E reductase (lanes 5,6) or λLm hsp70.1 (lanes 7,8). The arrows to the left of the lanes indicate the positions of the chromosomal bands using the numerical system previously described [20,23]. U indicates a region of unresolved chromosomal DNA (bands 19−24) in Panel A. The identity of the chromosomal bands homologous to each probe is indicated by a white dot on the gel and by arrows on the autoradiograph. All filters were washed in 0.2×SSC, 0.1% SDS at 65°C except Panel A, lane 8 which was washed in 2×SSC, 0.1% SDS at 65°C.

probe (Figure 7A, lane 8). The location of the λLm hsp70.1 sequence was identified by separation of chromosome bands 19−24 at longer pulse times. As shown in Figures 7B and 7C, the λLm hsp70.1 probe hybridised strongly to chromosome band 19 and more weakly to chromosome band 22 (lanes 7,8). The identity of these bands was confirmed by hybridisation of the P100/11E reductase probe to chromosome band 20 (Figure 7B,C; lanes 5,6) as reported previously [22]. The location of the λLm hsp70.1 and P100/11E genes is conserved in the two strains of *L. major* examined although chromosome bands 19 and 20 show minor size polymorphism between the strains. These data extend our

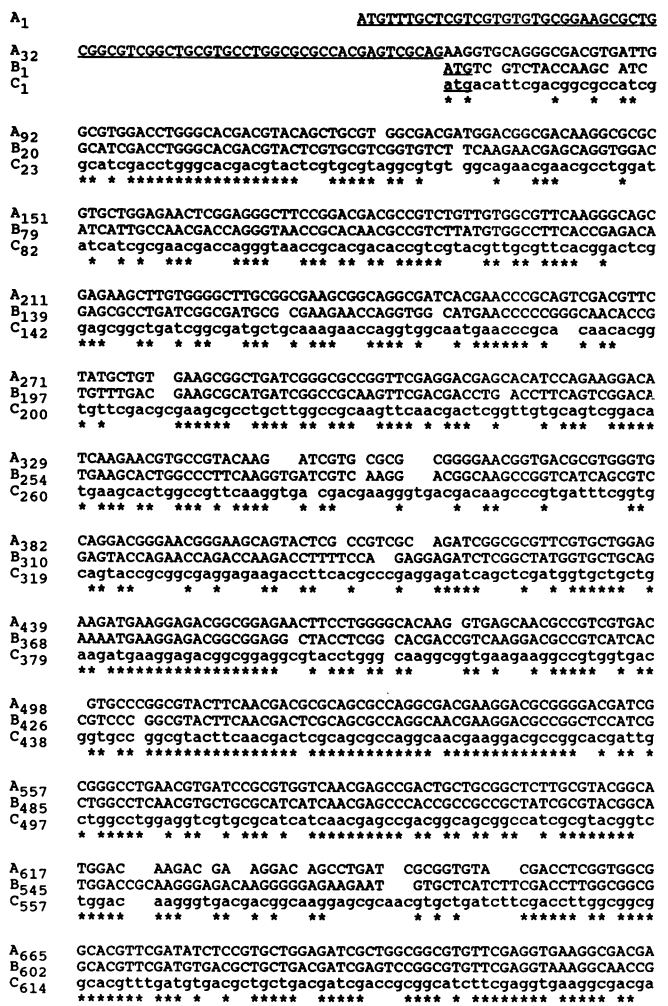

Figure 8. DNA sequence comparisons of the 5′ ends of the hsp70 genes of *L. major*. The sequences shown are from **A**: λLm hsp70.1 (this paper); **B**: λLm hsp70.4 (this paper); **C**: L hsp70 I [6]. Each sequence is numbered from the A of the ATG believed to be the start codon for translation. * indicate nucleotides which are shared between all three genes; the sequences have been aligned to show the best nucleotide match.

prediction that λLm hsp70.1 encodes a gene showing high homology to another repeated sequence within the genome; this would explain the hybridisation of different intensity to two chromosomal sites with this probe.

Cumulatively, these results show that the genome of *L. major* contains at least 4 different hsp 70-related sequences located at 5 separate chromosomal loci. The sequence on chromosome band 17 identified with the *Drosophila* aDm 2.51 probe is tandemly-repeated in *L. major* (Spithill, unpublished) and is probably identical to the tandemly-repeated hsp 70 sequence of *L. major* described by Lee *et al.* [6].

Table 1. DNA sequence homologies between trypanosomatid and human hsp70 genes. Sequence comparisons were made between [a] λLm hsp70.1; [b] λLm hsp70.4; [c] the tandemly repeated hsp70 genes of *L. major* [6] [d] the tandemly repeated hsp70 genes of *T. brucei* [28] [e] the tandemly repeated hsp70 genes of *T. cruzi* [29]. [f] the human hsp70 gene [16]. Figures shown are % homology between pairs of sequences. * indicates genes for which the available sequences do not overlap sufficiently to justify homology search.

	L. major			*T.brucei*	*T. cruzi*	Human
	70.1 [a]	70.4 [b]	70* [c]	[d]	[e]	[f]
L. major 70.1 [a]	–	67.5	65.5	64.3	67.2	67.2
70.4 [b]	67.5	–	78.9	76.4	*	77.9
70* [c]	65.5	78.9	–	82.2	80.0	75.4
T.brucei [d]	64.3	76.4	82.2	–	74.9	71.5
T.cruzi [e]	67.2	*	80.0	74.9	–	70.7
Human [f]	67.2	77.9	75.4	71.5	70.7	–

Sequence comparison between λLm hsp70.1 and λLm hsp70.4.

To determine the degree of relatedness between the genes encoded by λLm hsp70.1 and λLm hsp70.4, the sequences of the 5′ ends of each gene were determined. These are shown in Figure 8, and are compared to the sequence of the tandemly-repeated hsp70 gene sequenced by Lee *et al.*[6]. There is high sequence homology between all 3 genes (as shown in Table 1); the shared nucleotides are indicated (*). Assuming that the first methionine codon (ATG) upstream of the region of homology is that used to initiate translation, open reading frames can be derived from each sequence. Of particular interest is the 69bp extension in λLm hsp70.1, which is upstream of the first ATG used in the tandemly-repeated gene (underlined in Figure 8). If translated, this sequence would give the λLm hsp70.1 gene product an extra 23 amino acids at its amino terminus, of 56% hydrophobicity but lacking in acidic residues. Analysis of this putative peptide, using the rules reviewed by Verner and Schatz [23], allows the prediction that λLm hsp70.1 encodes a protein carrying a mitochondrial leader peptide which is presumably required for the transport of this protein into the mitochondrion.

The degree of homology shown between the hsp70 genes of *L. major* and those of the other trypanosomatids, and the human hsp70 gene, was determined by computer analysis and is shown in Table 1. It is clear that λLm hsp70.4 shows the highest level of homology to the human hsp70 gene, although the tandemly repeated *L. major* gene is also highly related. Interestingly, the tandemly repeated genes of the three trypanosomatid species show the highest homology to one another, perhaps suggesting a common ancestral gene. λLm hsp70.1 consistently exhibits lowest homology to the other genes, which further supports our hypothesis that it is a functionally distinct sequence.

DISCUSSION

This paper describes the isolation and characterisation of 2 novel hsp70 genes of *Leishmania*. Both are constitutively expressed in promastigotes, one (λLm hsp70.4) at a level comparable

to actin mRNA and the other (λLm hsp70.1) at a lower level. They are related, by sequence homology, to the hsp70 genes described by Lee *et al.*[6] (which are located on chromosome 17) but are found on different chromosomes within the *Leishmania* genome: λLm hsp70.4 is located on chromosome 16, while λLm hsp70.1 hybridises strongly to chromosome 19 and weakly to chromosome 22. Thus, the predominant transcripts recognised by heterologous hsp70 probes represent the products of a number of different genes and, for the first time, these have been resolved by size difference, as shown in Figure 3. It is clear that, while the transcripts arising from the tandemly-repeated hsp70 1 gene [6] are heat-inducible, the transcripts from our genes λLm hsp70.1 and λLm hsp70.4 are not. We do not yet know what triggers may affect the expression of these genes. Increases in intracellular glucose concentration, which modulate the production of the mammalian grp78 protein (an hsp70-related molecule [24]), do reduce the rate at which promastigotes transform to infective forms in culture [10], but this has no affect on the expression of any of the genes reported above [Searle and Smith, unpublished].

The DNA sequences at the 5′ ends of the hsp70 genes from *L. major* show high levels of homology and this is consistent with other eukaryotic hsp70-related genes, which generally show 5′ conservation and 3′ divergence of sequence [2]. The trypanosomatid hsp70 genes characterised to date all share homology, to each other and to human hsp70 (Table 1). There is some variation in the degree of homology, however, and this could be due to evolutionary divergence or, more interestingly, could relate to functional differences between the different gene products. Support for the latter conclusion comes from the sequence of λLm hsp70.1, in which the first ATG that could be used in translation is 69bp upstream from the corresponding ATG of the tandemly repeated hsp70 gene [6]. If the predicted reading frames are correct, then λLm hsp70.1 has a 23 amino acid extension at the amino-terminus which is not present in two of the other hsp70 genes. We predict that this peptide may represent a mitochondrial signal sequence. Work in progress, using the expressed products of the λLm hsp70.1 and 70.4 genes to raise antibodies specific for these stress proteins of *L. major*, will provide the tools with which to study the location and function of these proteins. An antibody to *Drosophila* hsp70 (#7.10; the gift of S. Lindquist) which recognises all eukaryotic hsp70s so far examined, recognises at least 3 molecules of 70−75 Kd on Western blots of total promastigote proteins from *L. major* (Searle and Smith, data not shown).

A non heat-inducible, hsp70-related antigen has recently been shown to be located within the kinetoplast (a component of the mitochondrion) of *T. cruzi* (Engman, D. and Donelson, J., submitted). The gene encoding this protein shares extensive sequence homology with the λLm hsp70.1 gene described in this paper, and this homology includes the 5′ terminal extension, which has the features of a mitochondrial leader-like peptide. Preliminary data suggests that these two trypanosomatid genes encode the same protein.

The role of the hsp70 proteins in eukaryotic cells is only now being elucidated. It has been proposed, for both yeast and mammalian cells, that the related hsp70 genes encode proteins of (perhaps subtly) different functions within the cell. Thus, a sub-set of 70 Kd stress proteins in yeast are involved in the post-translational import of precursor polypeptides into both mitochondria and the lumen of the endoplasmic reticulum [25] while the glucose-inducible grp78 protein acts as an immunoglobulin heavy chain binding protein in mammalian cells [24]. Another member of the family catalyses the ATP-dependent depolymerisation of clathrin from coated vesicles [26], while the major heat-inducible hsp70 protein relocates within the nucleus after heat shock and binds tightly to the nucleolus (in

an ATP-dependent reaction) [27]. It has been suggested that the general shared property of these proteins is to facilitate the transfer of precursor proteins across target membranes [1,25]. Such a function would involve a role in the folding and unfolding of proteins and, additionally, the protection of precursor molecules under adverse conditions of stress. The promastigotes of *Leishmania*, on entering the host cell macrophage, are undoubtedly subjected to multiple cellular stresses. We are interested in determining the function of the different stress proteins encoded by the multiple hsp70 genes and their role in the infection process.

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