Structure and expression of the hsp 70 gene family of *Leishmania major*

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ABSTRACT

The parasitic protozoan *Leishmania major* differentiates *in vitro*, from the insect-adapted promastigote to the mammalian infective amastigote, in response to a temperature shift from 25°C to 37°C. We studied the genes encoding 70 kilodalton heat shock proteins (hsp 70 genes) in *Leishmania* substocks, which vary in their capability to differentiate. In total, four hsp 70 genes are arranged in tandem with intergenic regions of about 380 bp. These hsp 70 genes are 89% conserved at the amino acid level when compared to the *T. brucei* hsp 70 genes. The expression of these four hsp 70 genes is increased, *in vitro* and *in vivo*, in response to a temperature shift from 25°C to 37°C. The parasite thus indeed responds to the transfer between hosts like it responds to a heat shock. In contrast, the high rate of transcription of a fifth identical hsp 70 gene, located at a separate locus, is unaffected by temperature shifts. The hsp 70 mRNAs have mini-exons trans-spliced onto their 5' ends and share unusually long (1000 nt) 3' untranslated extensions containing repetitive sequences. It is unclear whether or not the intergenic regions of the *L. major* hsp 70 genes function in transcription initiation and/or whether transcription results in the generation of polycistronic pre-mRNAs. Since each of the hsp 70 genes that we identified is expressed normally in an *L. major* substock that lost the capability to differentiate in response to an *in vitro* temperature shift, the inability to differentiate does not result from a general defect in the temperature-dependent control of transcription.

INTRODUCTION

The synthesis of heat shock proteins (hsps) is induced by a variety of stressful environmental stimuli in all organisms analyzed to date (21, 25). In some cases, the induction of heat shock gene expression can also occur as part of the developmental pathway, for example during embryogenesis in mice (2), sporulation of yeast (14), differentiation of *Naegleria gruberi* (34), *Histoplasma capsulatum* (15), Trypanosoma and

Leishmania (32). This process is primarily regulated at the transcriptional level (18, 21).

Leishmania major causes cutaneous leishmaniasis in humans. The parasite is transmitted by the sandfly between mammalian hosts, where it lives intracellularly in macrophages (12). Since the insect and mammalian hosts have different body temperatures, we assume that in the transfer of Leishmania major from its insect vector (25°C) to the mammalian host (37°C), the parasite thus responds to the transfer as if it is subjected to a heat shock leading to the observed increased and constitutive expression of its major hsp genes (6, 16, 19, 32, 35). The importance of the temperature shift in differentiation is also illustrated by the fact that an in vitro temperature shift is sufficient to induce differentiation from the promastigote (insect adapted stage) to the amastigote (mammalian adapted stage; 6, 16, 19, 32, 35). Differentiation of the insect-adapted promastigote to the mammalian-adapted amastigote is required for the multiplication of amastigotes in macrophages and is crucial for their pathogenicity. We therefore proposed that the genetic program leading to differentiation of this parasitic protozoan may be under temperature control, as is the case for the transcription of heat shock genes (32).

In order to understand the mechanism of temperature induced transcription and its relationship to parasite development, we analyzed the genomic organization and expression of hsp 70 genes in L. major and compared them to those of the previously described hsp 70 genes of T. brucei (10, 32). Several unusual features distinguish transcriptional control and pre-mRNA processing in Leishmania and related Kinetoplastidae, from those in other eukaryotes (for review 5, 33). First, several genes have been shown to be arranged in tandem and it has been proven that some of these genes are actually transcribed into polycistronic pre-mRNAs (11, 20, 27). Second, pre-mRNAs mature in a manner which drastically differs from that seen in other eukaryotes, since every mRNA consists of two exons that are originally transcribed from separate genes. These exons are joined post-transcriptionally by trans-splicing of the individual pre-RNAs (for review see 5, 33). RNA polymerase II

promoters of protein coding genes in *Leishmania* and related kinetoplastidae have thus far remained unidentified. The cause of this could be in the fact that the organization of a genome into polycistronic transcription units reflects a lack of transcription initiation sites located directly upstream of protein coding genes. The transcriptional control of hsp genes is based on highly conserved regulatory elements. We therefore initiated the analysis of hsp genes in *L.major* and *T.brucei* with the assumption that promoter sequence conservation will facilitate promoter identification. In addition, the peculiar control of heat shock gene transcription in organisms with life-cycles in host with different body temperatures may unravel functions for hsps, as well as a unique developmental program underlying parasite differentiation.

MATERIALS AND METHODS

Description of strains. *L.major* stocks D-1 and WR300 (9) were cloned by limiting dilution. Stocks were cryo-preserved in liquid nitrogen. Promastigotes were cultivated at either 25°C or 37°C in RPMI medium supplemented with 10% fetal calf serum (8).

DNA isolation and Southern blotting. *L.major* was grown as described (8) and nuclear DNA was isolated as previously published by Van der Ploeg et al. (30), digested with restriction enzymes, after which the products were resolved on 0.8% agarose gels and transferred to nitrocellulose filters. Southern blots were hybridized with ³²P labeled probes as described previously (13, 22). After the hybridizations filters were washed to a final stringency of 0.1 x SSC and 0.1% SDS at 65°C.

Construction and screening of genomic libraries. Nuclear DNA of cultured promastigotes of strain *L.major* WR 300 were partially and completely digested with Pst I, respectively. DNA was ligated into the plasmid pUC18 and the ligation mixtures were used to transform *E.coli* HB101. About 2×10^4 transformants were screened for each library with ³²P labeled probes. The clones were purified to homogeneity and plasmids were grown as described (3).

RNA preparation and analysis. *L.major* WR 300 promastigotes were grown in RPMI medium at 25°C or 37°C as described (8). Total RNA was isolated by LiCl Urea precipitation as described by Auffray and Rougeon (1). Northern analysis of RNA was carried out in 1% agarose gels containing 2.2M formaldehyde (4). Following hybridization filters were washed to a final stringency of 0.1 x SSC and 0.1% SDS at 65°C.

S1 protection analysis and primer extension. S1 mapping was performed essentially as described by Dudler and Travers on procyclic (25°C) RNA (7). Primer extensions were performed

essentially as described by Shea et al. (27). In the RNA sequencing, 50 ng of end-labeled primer was used in each reaction. One reaction was performed with 1mM of dATP, dCTP, dGTP and dTTP. The other four reactions were performed with dideoxy-: deoxy-nucleoside triphosphate mixtures containing ddA:dA, 30 uM:12 uM; ddC:dC, 4 uM:18 uM; ddT:dT, 117 uM:36 uM; ddG:dG, 66 uM:16 uM. Reverse transcriptions were carried out as described (27) in the presence of 200 ug actinomycin D per ml and 40 units of RNasin (Promega Biotec) with 54 units of AMV reverse transcriptase in a final volume of 100 ul at 40°C for 1 hour. The reaction products were analyzed on a 6% denaturing polyacrylamide gel. The gel was subsequently dried and put up for autoradiography at -90°C.

DNA sequence analysis. DNA sequence analysis was performed by the di-deoxy chain termination method (23). Two approaches were used to generate suitable DNA fragments for subcloning into the replicative form of bacteriophages M13 mp18 and M13 mp19. In the first method, DNA was cleaved with restriction enzymes and ligated into the appropriate M13 vectors. In the second method, DNA subclones were treated with Bal 31 exonuclease for various periods of time at room temperature to generate a series of overlapping clones. They were subsequently ligated into M13 vectors.

Pulsed field gel electrophoresis. The electrophoresis units and DNA samples were as described by Schwartz and Cantor (26) and Van der Ploeg et al. (31). Agarose blocks were made as described by Giannini et al. (9) and placed in the wells of 1% agarose gels. DNA was electrophoresed at a pulse frequency of 90 seconds at 200 volts/cm at 20°C. After electrophoresis the DNA was stained with ethidium bromide and transferred to nitrocellulose.

Estimation of leishmanial infectivity. First we constructed a standard curve relating inoculum size of infective promastigotes to the time of appearance of lesions in BALB/cj female mice, which are exquisitely susceptible to infection with *L.major*. Groups of 5 mice (aged 12-14 weeks, from Jackson Laboratories, Bar Harbor, ME) were injected intradermally with 10^2 , 10^3 or 10^4 stationary phase promastigotes cultivated as described previously (13). Lesions were first detected in the mice of each group at 6, 5 or 4 weeks, respectively. Therefore each tenfold increase in inoculum size resulted in one week acceleration of lesion appearance.

Lesions were first detected at 4 weeks in mice injected intradermally with 10^3 organisms of culture amastigotes. This was one week earlier than in mice injected at the same time with 10^3 promastigotes cultivated at 25°C, indicating an tenfold increase in infectivity. Mice infected with 10^6 promastigotes from a subline that had been passaged 186 times did not develop any lesions within an 8 week observation period. This indicates a loss of infectivity in excess of two logs (>>99%).

RESULTS

Physical organization of hsp 70 genes in *L.major*.

Coding sequences of hsp 70 genes in diverged organisms are highly conserved. We were thus able to isolate and determine

the organization of hsp 70 genes from *L.major* using the hsp 70 genes from the related protozoan, *T.brucei* as probes (10). From a library made with partial Pst-I digested *L.major* nuclear DNA, two sets of genomic clones were isolated that encompass two apparently separate hsp 70 loci. The physical map and organization of the hsp 70 genes in these recombinant clones revealed a tandem array of four hsp 70 genes in locus I (Figure 1). In addition, a fifth hsp 70 gene was isolated which did not belong to the same array. To address the potential physical linkage of genes 1 through 4 and gene 5, we hybridized genomic blots containing DNA that was partially digested with the restriction enzyme Pst I, with gene specific probes. The hybridization of partially digested nuclear DNA in figure 2 shows that probe B (specific for gene 5) detects only two

Lhsp70-I

Lhsp 70-II

Figure 1.

Physical map of the hsp 70 loci in *L.major*. The open blocks represent the transcribed regions of the hsp 70 genes. The numbers 1 through 5 refer to hsp 70 genes as discussed in the text. DNA fragments used for Southern and northern blot analysis are indicated A, B and C. The location of the translation initiation site is indicated ATG and the termination codon is indicated TAA. The lines and numbers below the physical map represent genomic clones and their location relative to the physical map. Only the 2/3 carboxy terminal coding region of the hsp 70 gene in locus II and its 3' untranslated extension was obtained in clone. The position of the ATG initiation codon and 5' end of this gene is based on comparison with genes 1 through 4 and the location of the 3' end of the mRNA of gene 5. Abbreviations of restriction enzyme sites are: P, Pst I; B, Bst EII; C, Cla I.

Figure 2.

Analysis of the hsp 70 loci in *L. major*. Genomic DNA from *L. major* WR 300 promastigotes was digested either completely (lanes 3) or partially (lanes 1, 2 and 4-6) with the restriction enzymes Pst I. The DNA samples were sized fractionated on 0.8% agarose gels and transferred to nitrocellulose filters. The filters were hybridized with ^{32}P labelled probes derived from the untranslated 3' extension of hsp 70 genes in locus I (probe A); the 3' untranslated extension and gene flanking sequences of hsp 70 gene 5 in locus II (probe B) or a hsp 70 gene internal fragments which hybridizes to all hsp 70 genes (probe C). Asterisks indicate the bands in the complete digestion of the panel hybridized with probe C that were used to quantitate the number of hsp 70 genes, by scanning of the autoradiogram. The location of the different probes is also indicated in the physical map of the hsp 70 gene in Figure 1. Following the hybridization, filters were washed to a final stringency of 0.1 x SSC and 0.1% SDS at 65°C.

strongly hybridizing restriction fragments in the partial Pst I digest, while probe A (specific for genes 1 through 4) hybridizes to the expected Pst I fragments generated from the array of hsp 70 genes. Because the partial digestion products that hybridize with probe B do not comigrate with those hybridizing to probe A, these hsp 70 genes are not obviously linked and may be located at distinct loci. The actual distance between these loci has not been further determined.

Figure 3.

Chromosomal location of the hsp 70 loci in *L. major*. Agarose blocks containing chromosome sized DNA of *L. major* strain D-1 (uncloned stock), and clones 3-1, 3-2, 3-3, 4-1 and 4-2 were electrophoresed and analyzed as described in Materials and Methods. DNA fragments used as probes (as indicated in Figure 1) are indicated on top of the panels. The molecular weights of different bands were determined in separate experiments by co-migration of *L. major* chromosome-sized DNA with phage lambda multimers and *Saccharomyces cerevisiae* chromosomes as size-standards. The post hybridizational wash of the hybridization with probe A was performed at 65°C and 1 x SSC.

A probe that hybridizes with equal efficiency to each of the genes located in locus I and II (Probe C) allows a more accurate quantitation of the total number of hsp 70 genes. Scanning of autoradiograms hybridized with probe C (Figure 2), enabled us to determine the relative ratio of the two hybridizing Pst I fragments to be 1:4 (bands indicated with asterisk in figure 2). Thus, assuming the 5th hsp 70 gene to be single copy (since the ploidy of *L. major* is not known) 5 hsp 70 genes are present in the *L. major* genome.

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Figure 4.
A) Sequence analysis of the hsp 70 genes at locus I and II. One entire repeat unit from an hsp 70 gene located at locus I (clone 19a.2 in figure 1) and the 3' part of gene 5 located at locus II were sequenced by the dideoxy chain termination method (23). The sequence is presented starting at a Pst I restriction enzyme site in the 3' end of untranslated extension of the preceding hsp 70 gene and ending at the next Pst I restriction enzyme site in the 3' end of untranslated extension of the preceding hsp 70 gene. Starting at ATG initiation codon the comparison of the L.major and T.brucei nucleotide sequences are presented. Only those residues in the T.brucei sequence that differ from the L.major nucleotide sequence are printed. 5 nucleotides beyond the 3' TAA termination codon the sequences in the 3' untranslated extension (abbreviated UT) of hsp 70 genes at locus I and locus II start to diverge. These sequences are therefore presented separately and they are labeled; 3' UT GENE 1-4 and 3' UT GENE 5. The 3' end of the hsp 70 mRNA of gene 5 was accurately determined by comparison of genomic and cDNA sequences. The location of the 3' end is indicated by the horizontal line in the sequence. The homologous sequences in the 3' untranslated extensions are located between the black arrowhead and the poly A addition site. The hyphens in the sequence indicate gaps that were introduced to optimize alignment. The boxed sequence in the 5' untranslated extension represents the oligonucleotide that was used to determine the nucleotide sequence of the mini-exon at the 5' end of the mRNA. The 3' splice site and intergenic regions are highlighted between the boxed area. The 14 nt boxed sequences represent the HSE related sequences. The mini-exon addition site, AG is underlined. B is the unique Bst EII site of the repeat unit. The region which is 50% homologous in the 3' untranslated extension starts from the arrow heads and extends to the poly A addition site.

B) Comparison of the intergenic regions of T.brucei and L.major hsp 70 genes. Asterisks indicate regions of homology between the L.major and T.brucei intergenic regions and 5' untranslated extension of the mRNA. The AG di-nucleotide at the 3' splice site is underlined.

stringency not shown) hybridize with the same unique chromosome band at about a 1000 kb. This result indicates that genes 1 through 4 and gene 5 are located on the same chromosome. Probe C which detects all hsp 70 genes gives the same hybridization pattern (data not shown) confirming that the hsp 70 genes are linked at the same chromosome. The hybridization at the well of the PFG gel most likely results from trapping of high molecular weight DNA at this location or it may be derived from the presence of identical alleles of the hsp 70 genes in chromosomes of a different size.

DNA sequence of the *L.major* hsp 70 genes.

To determine the extent of the intergenic region of the hsp 70 genes and compare its DNA sequence with those of the *T.brucei* hsp 70 intergenic regions, we determined the nucleotide sequence of a complete hsp 70 repeat unit of 3.6 kb (Figure 4; see material and methods for details). The *L.major* hsp 70 coding sequence (gene 2-3) has an open reading frame of 658 amino acids. The coding region of this gene is 80% conserved at the nucleotide level when compared to the *T.brucei* hsp 70 genes. Because 72% of the nucleotide changes are at the third base position of the codons, at the amino-acid level, the *T.brucei* and *L.major* hsp 70 genes are 89% conserved. 66% of the nucleotide changes are G/C to A/T transversions or transitions and 25.5% are G to C or C to G transversion (*L.major* versus *T.brucei*). The codon usage of the *L.major* hsp 70 genes therefore favors G/C bases. The extreme C-terminal coding regions of the hsp 70 genes are more diverged at both the nucleotide and amino-acid level.

We also determined two thirds of the coding sequence of *L.major* hsp 70 gene 5 at locus II. This C-terminal sequence is completely conserved when compared to the corresponding region of hsp 70 genes 2 and 3 at locus I. However, we cannot exclude that the sequence of the N-terminal portion of gene 5 is diverged from the genes found at locus I. Five nucleotides beyond the TAA termination codon, the nucleotide sequences of the hsp genes at both loci start to diverge.

Hsp 70 mRNA analysis.

We performed S1 nuclease protection analysis of the hsp 70

Figure 5.

Analysis of the 5' end of the hsp 70 mRNA. In the left hand panel the S1 protection analysis is indicated. A 5' end labeled DNA fragment (32P Bst EII - Cla I) which spans the 5' end of the hsp 70 genes in locus I (figure 1) was used to anneal with total RNA from *L.major* promastigotes. Following digestion of the DNA-RNA hybrids with S1 nuclease, the reaction products were size-separated in a 6% denaturing polyacrylamide gel. Lanes 1 and 2 are samples that were treated with 100 units per ml and 200 units per ml of S1 nuclease, respectively. Lane 3 is a control without adding RNA. Lane 4 is the input DNA fragment (674 bp). Lane 5 contains a Bst EII - Bam HI end labeled restriction fragment of 272 bp. Lanes labeled TCGA contain a di-deoxy size standard sequencing ladder.

The right hand panel contains the primer extension and direct dideoxy nucleotide sequence analysis of hsp 70 mRNA. The 32P end labeled oligonucleotide (boxed in figure 1) was used as a primer for the reverse transcription of total hsp 70 mRNA. The reaction products were resolved in a 6% denaturing polyacrylamide gel. Lane A, G, C and T are reactions in the presence of dideoxy-A, -G, -C, and -T respectively. Lane O is the reaction in the absence of any dideoxy-nucleotides. The sequence in the left hand side is the mini-exon sequence of *L.enrietti*. Dots in the sequence match the *L.enrietti* mini-exon sequence. Only the sequence indicated in the boxed regions can be read unambiguously.

genes to determine the exact location of the 5' splice acceptor site for mini-exon addition of the hsp 70 pre-mRNA. A 5' end-labeled 674 bp restriction fragment spanning the 5' end of the hsp 70 gene 1 (³²P Bst EII - Cla I, see Figure 1 for probe location) was hybridized with promastigote-derived total RNA. Following the S1 nuclease digestion, a protected fragment of 272 bp could be detected, thereby indicating that the 5' end of the hsp 70 mRNA is located at 172 bp upstream of the ATG initiation codon (figure 4 and figure 5A). As is the case with all other mRNAs in kinetoplastidae, the 5' end of the mRNA is presumably the site of mini-exon addition, and indeed the 5' end of the S1 protected fragment is located at a sequence containing the AG consensus nucleotides of a 3' splice site. In order to confirm the presence of a mini-exon at the 5' end of the hsp 70 mRNA, we performed direct dideoxy nucleotide sequencing of the hsp 70 mRNA. A synthetic 21-mer oligonucleotide complementary to the sequence located from + 3 to + 24 nucleotides (figure 4, boxed sequence) was hybridized to total L.major RNA and subsequently extended with reverse transcriptase. The resulting cDNA in fact extends for another 35 nt beyond the 3' splice site of the L.major hsp 70 gene. The direct dideoxy nucleotide sequence of this extended product is identical to the previously published mini-exon sequence of L.enrietti (Figure 5B; sequence highlighted with black dots). The sequence in the boxed areas can be read unambiguously, in the other regions, the mini-exon nucleotide sequence of L.major appears to be identical to the L.enrietti sequence, but the presence of additional bands makes the interpretation of the sequence less straight forward.

The S1 protection and primer extension analysis were performed with procyclic (25°C) RNA, using a gene 1 derived DNA fragment as probe. In the next sections we show that genes 1 through 4 as well as gene 5 are transcribed in these cells. The 5' splice acceptor site for mini-exon addition of the hsp pre-mRNA that we mapped is, therefore, that of any, or all of the genes 1 through 4 and/or gene 5.

The location of the 3' end of the hsp 70 mRNA was determined by aligning the nucleotide sequence of cDNA clones containing poly A tails at the 3' end with the hsp 70 genomic sequences.

We obtained such cDNA clones only for hsp 70 gene 5 (locus II). The 3' end of the hsp 70 mRNA of gene 5 is therefore located at 1088 nt downstream of the TAA termination codon. The polyadenylation signal, AAUAAA of higher eukaryotes, is absent, as in other T.brucei genes. We have not directly determined the location of the 3' ends of the hsp 70 genes found in the tandem array at locus I (genes 1 through 4). However, because the mRNAs derived from gene 5 and genes 1 through 4 are of identical size, the approximate location of the 3' end of the mRNA demarcates intergenic regions of roughly 380 bp.

In these short intergenic regions, two palindromic heat shock element (HSE) like sequences can be found (Figure 4). One of them (TTTCAATCTTGAGT) has only 4 out of 8 nt in common with the HSE consensus sequence (CNNGAANN TTCNNG), but has a perfect palindrome. Another (GCTGACTGTTCTGTC) shares 5 out of 8 nt homology (boxed sequences in Figure 4). Two similar HSE related sequences have also been found in the intergenic regions of hsp 70 genes of T.brucei. Our unpublished data of the T.brucei hsp 70 locus revealed three HSE elements with a 6 out of 8 nt match located directly upstream of the first gene of the hsp 70 array (Lee and Van der Ploeg, unpublished). In higher eukaryotes, heat shock genes containing HSEs having only a 5/8 nt match still allow foot printing of the HSE binding protein. It is therefore possible that these elements are functional.

The 3' untranslated extensions of hsp 70 mRNAs from genes in loci I and II are unusually long, about 1000 bp each. Using the untranslated extensions of these hsp 70 genes as probes, we found that the sequences in the 3' untranslated extensions are highly repetitive in the genome (Figure 3; probe A, most right hand panel). The hybridization in the low molecular weight range in lane D1 in figure 3 is due to slight degradation of the DNA. Hybridization of Northern blots of L.major RNA showed in addition that these repeats are transcribed (data not shown). We are currently testing whether or not the repeats have a function in mRNA maturation or turn-over.

Differential regulation of hsp 70 gene transcription in L.major.

Hsp 70 genes in T.brucei and L.major are developmentally regulated (32). An *in vitro* temperature shift of L.major from 25°C to 37°C affects transcription of hsp 70 genes, and induces

Figure 6.

Northern analysis of RNA derived from the hsp 70 genes of *L. major*. Total RNA was isolated from two stocks of *L. major* WR 300 promastigotes. Stock P3 (lanes 1 and 2, passaged three times in culture) contains RNA from cells that still respond normally to an *in vitro* temperature shift, and stock P186 (lanes 3 and 4, passaged 186 times in culture), containing RNA from cells that can no longer differentiate due to an *in vitro* temperature shift. Lanes 2 and 3 contain RNA from cells grown at 25°C, lanes 1 and 4 contain RNA from cells grown at 37°C for 24 hours. RNA was resolved in a 1% formaldehyde agarose gel and transferred to nitrocellulose. Following hybridization with the probes indicated (probe A, B and C as labeled in Figure 1) filters were washed to a final stringency of 0.1 x SSC, 0.1% SDS at 65°C. The small panel on the left hand side shows the ethidium staining pattern of the lanes with RNA of this northern to indicated that equal amounts of RNA were loaded in each lane.

differentiation of promastigotes into amastigotes. After the temperature induction, the infectivity of culture form amastigotes (derived by cultivating promastigotes at 37°C for 5 days; 8) was about tenfold greater than that of non heat-shocked promastigotes from which they were derived (see Materials and Methods for details). Using the probes specific for genes 1 through 4 (probe A, the 3' untranslated extension of gene 1-4)

and gene 5 (probe B, the 3' untranslated extension of gene 5) we analyzed whether or not increased infectivity was preceded by enhanced transcription of hsp 70 genes. The northern analysis of RNA in figure 6 shows that the level of expression of genes 1 through 4 is about tenfold increased in cells grown at 37°C when compared with those at 25°C (Figure 6, lanes 1 and 2, respectively). However, transcription of gene 5 at locus II appears to be regulated by a different mechanism, since probe B, specific for gene 5, reveals that this hsp 70 gene is constitutively transcribed (Figure 6, probe B, panel B). Panel C of figure 6 shows the control hybridization, with a probe that detects all five hsp 70 genes, on the same Northern panel. In summary, the genome of L.major encodes a conserved hsp 70 gene, which is expressed constitutively, and four additional hsp 70 genes which are transcribed in a temperature sensitive manner.

After prolonged passage in culture at 25°C, L.major clones that have lost the ability to differentiate in vitro in response to a temperature shift can be isolated. In addition these clones have virtually lost their ability to infect mice. One such substock of L.major, which was passed 186 times at 25°C and which had lost 99% of its ability to infect mice, was tested in its response to a temperature shift. The northern in figure 6 shows that hsp 70 genes of this substock still respond normally to the in vitro temperature shift. Thus, the failure to differentiate in vitro does not result from a general defect in temperature sensitive induction of transcription of protein coding genes. The defect may therefore be in other heat-induced genes or their products.

DISCUSSION

Our main goal in the analysis of hsp 70 genes of L.major was to perform a comparison between these genes and the hsp 70 genes of the related protozoan T.brucei, and to study their transcriptional control and differentiation. The hsp 70 genes of L.major and T.brucei are both arranged in tandem arrays with relatively short intergenic regions. The nucleotide sequence of the intergenic regions up to 200 bp upstream of the 3' splice site is 50% conserved when compared between T.brucei and L.major

Figure 7.

Complementarity between the medRNA and sequences flanking the 3' splice site (boxed AG splice acceptor site) of L.major hsp 70 genes. The top line is the DNA sequence spanning the 3' splice site of hsp 70 genes in L.major. The lower line is the medRNA sequence of L.major, which is homologous to the medRNA sequence of L.enrietti. The underlined sequence highlights the mini-exon sequence.

(Figure 4B). The nucleotide sequence conservation at the intergenic regions is too limited to assign sequences a specific role in transcription initiation. The only obvious conserved elements are two HSE like sequences with limited homology to the HSE consensus, like those found in the T.brucei intergenic regions (Figure 4). However, we have recently determined that the tandem array of T.brucei hsp 70 genes is transcribed polycistronically (Lee and Van der Ploeg, unpublished). Therefore we favor a model of transcription at the hsp 70 loci in these species, in which transcription initiates in front of each of the hsp 70 genes leading to read through into the downstream hsp 70 genes. This can also result in the generation of polycistronic pre-mRNAs. However, the evidence for this model is weak; it is also possible that transcription initiation of the hsp 70 genes in these organisms results from the presence of one or a few promoters located further upstream of the hsp 70 genes. Post-transcriptional modification of mRNA stability may then be an important factor in the regulation of mRNA abundance.

Since all hsp 70 mRNAs have a mini-exon trans-spliced onto their 5' ends it is obvious that the intergenic regions must encode the splice acceptor signals involved in trans-splicing. The conserved sequence elements that are shared between the intergenic regions of the hsp 70 genes of T.brucei and L.major and other protein coding genes of T.brucei (tubulin, 24; calmodulin, 29) are restricted to a polypyrimidine stretch

located immediately (about -30 nt) upstream of the 3' splice site. In addition limited sequence complementarity between the L.major medRNA and the L.major hsp 70 3' splice site may allow base pairing of medRNA with the 3' splice site, as proposed by a model to explain trans-splicing (Layden and Eisen; 17; Figure 7).

The L.major hsp 70 mRNA is drastically different from the T.brucei hsp 70 mRNA in that it has an unusually long 3' untranslated extension of about 1000 nt. Physical mapping and hybridization of cloned hsp 70 DNA indicates that the 3' untranslated extensions of hsp 70 genes 1 through 4 are fully conserved. In addition, the 3' untranslated extensions when compared with gene 5 are 50% conserved in the terminal 800 nt of the mRNA. The only large blocks of sequence homology are in arrays of the dinucleotide GT (underlined sequences). Interestingly, the previously identified hsp 83 mRNA of L.major (8) is also about 1000 nt larger than the T.brucei hsp 83 mRNA on the northern blot analysis. Since the 3' untranslated extensions of L.major are repetitive and are also transcribed (data not shown), it is possible that in Leishmania many mRNAs share the same sequences at their 3' ends. A possible function of repetitive untranslated extensions could be in the determination of mRNA half-life.

As is common in other eukaryotes, heat shock genes in L.major, are transcribed in response to environmental stress signals. We have now identified an L.major hsp 70 gene, the transcription of which is constitutive and thus regulated differently from the other temperature-inducible L.major hsp 70 genes. The function of constitutive transcription of hsp 70 genes is unclear. That cognate-like as well as temperature sensitive hsp 70 genes are present in Leishmania may indicate that the protozoan L.major responds to signals other than temperature to induce the transcription of hsp 70 genes.

Our analysis of the temperature-induced transcription of L.major promastigotes and L.major culture form amastigotes and mammalian derived amastigotes (32) again indicates that the parasite responds to the temperature difference between its two hosts like it does to a heat shock, by increasing the

transcription of its major hsp 70 genes. An L.major substock that had lost the ability to differentiate in response to this temperature shift was not defective in its temperature- induced transcription of hsp 70 genes. We are currently analyzing additional mutants in order to test the importance of temperature in the process of differentiation in L.major.

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