We recently characterized a large developmentally regulated gene family in Leishmania encoding the amastin surface proteins. While studying the regulation of these genes, we identified a region of 770 nt within the 2055 nt 3'-untranslated region (3'UTR) that regulates stage-specific gene expression at the level of translation. An intriguing feature of this 3'UTR regulatory region is the presence of a ~450 nt element that is highly conserved among several Leishmania mRNAs. Here we show, using a luciferase reporter system and polysome profiling experiments, that the 450 nt element stimulates translation initiation of the amastin mRNA in response to heat shock, which is the main environmental change that the parasite encounters upon its entry into the mammalian host. Deletional analyses depicted a second region of ~100 nucleotides located at the 3'-end of several amastin transcripts which also activates translation in response to elevated temperature. Both 3'UTR regulatory elements act in an additive manner to stimulate amastin mRNA translation. In addition, we show that acidic pH encountered in the phagolysosomes of macrophages, the location of parasitic differentiation, triggers the accumulation of amastin transcripts by a distinct mechanism that is independent of the 450 nt and 100 nt elements. Overall, these important findings support that stage-specific posttranscriptional regulation of the amastin mRNAs in Leishmania is complex and involves the coordination of distinct mechanisms controlling mRNA stability and translation that are independently triggered by key environmental signals inducing parasite’s differentiation within macrophages.

The protozoan parasite Leishmania constitutes a major health problem in several endemic tropical and sub-tropical regions around the world, threatening over 350 million people of which more than 15 million are infected (1,2). At least 20 different Leishmania species are responsible for the various clinical manifestations of leishmaniasis, ranging from chronic skin ulcers (L. major, L. tropica, L. mexicana) to more severe naso-pharynx mucosal destruction (L. braziliensis) or life-threatening visceral diseases (L. donovani, L. infantum, L. chagasi) (3). No effective vaccine is currently available against Leishmania infections and resistance to the main anti-leishmanial agents is dramatically increasing in several endemic areas (4). These facts have underscored the urgency of identification of new drug targets for chemotherapy and/or vaccine development.

The life cycle of Leishmania includes two developmental stages: the extracellular promastigote form, transmitted to the mammalian host by the sand fly vector, and the amastigote form, adapted to resist and replicate within the threatening environment of the phagolysosomes. This adaptation requires a dynamic process implicating morphological and physiological changes within the parasite (5-9) that are mainly orchestrated by the differential
expression of a variety of genes. To date, several amastigote-specific genes have been characterized in Leishmania (reviewed in (10)). Due to the absence of transcriptional control in Leishmania, stage-specific regulation of gene expression occurs exclusively at the posttranscriptional level (reviewed in (11)) and involves mainly sequences within the 3'UTR of mRNAs that determine mRNA abundance by modulating RNA stability (12-14) or translational efficiency (15-17). However, the molecular mechanisms underlying stage-specific regulation of gene expression in this parasite are not yet understood. Recent microarray analyses suggested that only ~1-2% of L. major genes show significant changes in their mRNA levels (2-fold or more) throughout the parasite's life cycle (18,19). Similar data were obtained with the related parasite Trypanosoma brucei, where only ~2% of mRNAs showed more than 2-fold differences in expression (20). However, recent proteomic analyses revealed that ~9% of the Leishmania proteins are differentially expressed in the amastigote stage ((21,22), McNicoll et al., in preparation). This difference between large-scale transcriptomic and proteomic studies further outlines the importance of translational and posttranslational control in regulating gene expression in this organism.

We previously identified a developmentally regulated gene family in Leishmania encoding the amastin surface proteins (14) that share homology with the T. cruzi amastin proteins (23). We recently characterized this large gene family in Leishmania and showed that it comprises up to 45 members, the majority of which are specifically expressed in the intracellular amastigote stage of the parasite (24). Developmental regulation of the amastin transcripts in Leishmania (14,15) as well as in T. cruzi (25) is mediated by defined regions within the 3'UTR that are different in size and in sequence composition. While studying one of the L. infantum amastin gene homologs, we delimited a region of 770 nt within the 3'UTR that regulates stage-specific gene expression most likely at the level of translation (15). An intriguing feature of this 3'UTR regulatory region is the presence of a ~450 nt element that is highly conserved not only among the majority of the amastin transcripts (24) but also among several other Leishmania mRNAs, including known amastigote-specific mRNAs (15). Here, we show that the 450 nt element stimulates translation of the amastin mRNAs in response to heat shock, which is the major environmental change that the parasite encounters upon its transmission from the sandfly to the mammalian host. Furthermore, we show that a second region of ~100 nucleotides located at the 3'-end of several amastin transcripts activates also amastin translation in response to elevated temperature. Importantly, we describe here that acidic pH encountered in the phagolysosomes, the location of parasite's differentiation, has no effect on amastin translational regulation but it triggers the accumulation of amastin transcripts specifically in the amastigote stage by a distinct mechanism that is independent of the 450 nt- and 100 nt-mediated translational control. Overall, our findings support that stage-specific posttranscriptional regulation of the amastin transcripts in Leishmania is complex and involves the coordination of different mechanisms that are independently triggered by key environmental signals inducing promastigote to amastigote differentiation within macrophages.

EXPERIMENTAL PROCEDURES

Cell Lines, Leishmania Culture, and Macrophage Infections In Vitro- The Leishmania infantum MHOM/MA/67/ITMAP-263 strain used in this study was described previously (26). Promastigotes were cultured at pH 7.0 and 25°C in SDM-79 medium supplemented with 10% heat-inactivated fetal calf serum (Multicell, Wisent Inc.) and 5 µg/ml hemin. L. infantum promastigote to amastigote differentiation in a host-free culture and the maintenance of axenic amastigotes were performed as previously described (22,27). Briefly, late stationary phase promastigotes (in average day-6 promastigotes) were inoculated in MAA/20 medium in 25-cm² ventilated flasks and grown at 37°C and pH 5.8 with 5% CO₂ for
~5 days until they fully differentiated to amastigote-like forms. Axenic amastigotes remained stable in culture and after 2-3 passages they were recycled back to promastigotes in order to maintain their pathogenic potential. Axenic amastigotes grown under these conditions show morphological, biochemical, and biological characteristics similar to those of in vivo isolated amastigotes and are capable of infecting macrophages (28). In vitro infections of the THP-1 human leukemia monocyte cell line with *Leishmania* stationary promastigotes were carried out as previously reported (14,29).

**DNA Constructs and Transfections**

Expression vectors LUC and LUC-770 previously referred to as pSPYNEOαLUC-IR and pSPYNEOαLUC-770-IR were described elsewhere (14,15). The remaining vectors expressing the *LUC*-chimeric transcripts listed in Fig. 1B were made as follows. Briefly, various parts of the 770 nt 3'UTR regulatory region of the *L. infantum* LinJ34.0840 amastin gene homolog (14,15) were amplified by PCR using Taq DNA polymerase (Qiagen). PCR fragments were digested with *Bam*HI and cloned into the *Bam*HI site of vector pSPYNEOαLUC-IR downstream of the firefly luciferase (*LUC*) open reading frame. In plasmid LUC-550, a region of 550 nucleotides containing the conserved 450 nt element flanked by approximately 40 and 60 nucleotides on either side was amplified using primers P5'-550 (5' - CGGGATCCCGCCCTCGGCCCCCTGGC-3') and P3' - 5' - CGGGATCCCGGCCAGGAACGGAGACAA-3'). In LUC-200R, the last 200 nucleotides of the amastin 3'UTR were amplified using primers P 5' - 2 0 0 R (5' - CGGGATCCCGTGATACACTACATATGT-3') and P3'-300. Primers P5'-300 and P3'-2 0 0 L (5' - CGGGATCCCGCCGCAGGTAAAG-3') were used to amplify the first 200 nucleotides of the 300 nt region (LUC-200L). The vector 5'UTR-LUC-770 was made by a three-step cloning. First, vector pGEM3-NcoI (31) was digested with *Sac*I and *Sma*I and ligated to the LUC-770 *Pst*I-*Pvu*II fragment containing the *LUC* gene fused to the 770 nt region of the amastin 3'UTR. The plasmid copy number in each transfectant was evaluated by Southern blot and PhosphorImager analysis and it was found to be similar for the different LUC-containing constructs (data not shown). The BT1-LUC, BT1-LUC-770 and BT1-LUC-300 constructs (see Fig. 3A) were made as follows. Vectors LUC-770, LUC-300 and LUC control (see Fig. 1B) were digested with *Hpa*I and *Hind*III, filled in with Klenow and subcloned into the unique BgII site (also filled in with Klenow) of vector pSP-BT1 that contains the open reading frame of the biopterin transporter 1 gene (*BT1*) (32).

For genomic integration into the *BT1* locus, ~2.5 µg of *Hpa*I-HindIII digests (these enzymes cut on either side of *BT1*) containing the different targeting cassettes were transfected by electroporation into *L. infantum* as described elsewhere (31). Approximately 10-20 µg of purified plasmid DNA (Qiagen) was used for transfections. Transfected cells were plated on SDM-79 (2X) medium with 1.5% agar and 0.01 mg/ml of G418 (Sigma), and individual clones were obtained after 2-3 weeks.

**Nucleic Acid and Protein Manipulations**

Genomic DNA was isolated with the DNAzol™ reagent. Total RNA of *L. infantum* promastigotes and axenic amastigotes was
isolated using the TRIzol™ reagent (Gibco BRL). Southern and Northern blot hybridizations were performed following standard procedures (33). Leishmania cells were lysed and protein lysates were sonicated (10 pulses). The proteins were quantified using Amido Black 10B (Bio-Rad), and 50 µg of total protein lysates were loaded onto 10% SDS-PAGE gels for Western blot analyses. The gels were transferred on a polyvinylidene difluoride membrane (Immobilon-P, Millipore) and incubated for 1 hour in blocking solution (PBS with 0.1% Tween 20 and 5% non-fat dry milk). Then the first antibody (a goat anti-luciferase pAB, Promega) was added for 90 min in 1:1000 dilution. Following a few washes with PBST (PBS supplemented with 0.1% Tween 20), a donkey anti-goat horseradish peroxidase conjugate antibody (Santa Cruz Biotechnology) diluted at1:5000 was added for 60 min. After additional washes, the blot was visualized by chemiluminescence using a Renaissance kit (New Life Science Products). LUC RNA and protein levels were estimated by densitometric analyses using a PhosphorImager with ImageQuant 3.1 software. An anti-α-tubulin antibody (Sigma) was also used to verify equal protein loading on SDS-PAGE gels for Western blot analysis.

Sucrose Gradient Analysis- Approximately 3x10^7 L. infantum axenic amastigotes grown up to late logarithmic phase were first incubated with 100 µg/ml cycloheximide (Sigma) for 10 min, washed with PBS and lysed with a Dounce homogenizer in lysis buffer [10 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl_2, 1 mM DTT, 0.5% IGEPAL, 100 µg/ml cycloheximide, 100 U/ml RNAGuard (Amersham), 1 mM PMSF, 15 µl/ml of protease inhibitor cocktail (Sigma)]. Leishmania lysates were pelleted by centrifugation at 12,000 rpm for 15 min at 4°C and the supernatant (40 OD_260 units) was layered on top of a 15% to 45% linear sucrose gradient (10 ml) in gradient buffer (50 mM Tris-HCl pH 7.4, 50 mM KCl, 10 mM MgCl_2, 1 mM DTT, 3 U/ml RNAGuard). The gradient was made with the Gradient Maker (#GM-100; CBS Scientific Co). Ribosomal subunits (40S and 60S), monosomes (80S) and polysomes were sedimented by centrifugation in a Beckman SW40 Ti rotor at 35,000 rpm for 2 hours and 15 min at 4°C as previously described (34,35). After centrifugation, approximately sixteen 0.6 ml fractions were collected at 4°C using an ISCO Density Gradient Fractionation System under constant monitoring of absorption at 254 nm. The position of the 40S, 60S, 80S and polysomal peaks was also corroborated by Northern blot analysis using a 18S rRNA specific probe. RNA was extracted from each fraction by phenol-chloroform and followed by ethanol precipitation and analyzed by Northern blot hybridization.

RESULTS

Two distinct 3’UTR elements contribute to stage-specific regulation of the amastin mRNA-

We recently characterized a large family of developmentally regulated genes in Leishmania encoding surface proteins, named amastins (24). In one of the L. infantum amastin gene homologs that we have studied in more detail, we identified a region of 770 nt in the 3’UTR (Fig. 1A) that regulates stage-specific gene expression most likely at the level of translation (15). Remarkably, the first ~450 nucleotides of this regulatory region (Fig. 1A) were found to be conserved among several known amastigote-specific mRNAs in Leishmania, including the majority of the amastin transcripts (15,24). With the nucleotide sequence of the L. major and L. infantum genomes now completed, in silico screening depicted several hundreds of intergenic sequences that share variable levels of homology with the 450 nt element (data not shown). We initially hypothesized that this 450 nt conserved element was responsible for the 770 nt-mediated translational regulation of the amastin mRNA. To test this hypothesis, we made a series of deletions within the 770 nt region that we cloned downstream of the firefly luciferase (LUC) reporter gene (Fig. 1B). These LUC-chimeric constructs were transfected into Leishmania as part of episomal expression vectors and LUC activities were measured in
recombinant parasites grown in both developmental life stages using axenic culture systems and macrophage infections in vitro (Fig. 1B). As previously shown (15), the 770 nt region in construct LUC-770 increases LUC activity by more than 16-fold, specifically in amastigotes. The complete 450 nt element in vector LUC-450, surprisingly, confers low levels of LUC induction (~2.5-fold). Recent BLAST analyses on the complete *Leishmania* genome (http://www.genedb.org/) and more careful sequence comparison studies between the 3'UTRs of the 45 amastin gene homologs (24) indicated that the region of homology extends by few nucleotides of each side of the 450 nt element (data not shown). Based on this updated information, we made a new construct with 550 nt that contains ~60 nt (5'-side) and ~40 nt (3'-side) of either side of the 450 nt element. The LUC-550 construct confers higher induction of LUC activity (~8-fold), that represents however, half the regulation conferred by the 770 nt region (Fig. 1B). Extending the 3'-side by 50 more nucleotides did not increase further LUC activity (data not shown). These results suggest that the full-length element is required for regulation and that the extremities of the element may be critical for stabilizing an RNA secondary structure. Moreover, our deletion data suggest that other sequences within the 770 nt region located downstream of the 450 nt element may be involved in translational regulation of the amastin transcript.

To examine this possibility, we fused the last ~300 nt of the 770 nt region to the *LUC* coding region (LUC-300) and tested whether this sequence was capable of promoting the induction of LUC activity specifically in amastigotes. The LUC-300 construct induces indeed LUC activity by 8-fold, similarly to the levels of regulation by the 450 nt element in construct LUC-550 (Fig. 1B). A deletion of 100 nucleotides of either side of the 300 nt region in constructs LUC-200L (L for left side) and LUC-200R (R for right side), respectively has no effect on regulation hence delimiting the second regulatory sequence of the amastin mRNA to an internal region of ~100 nt (see Fig. 1B). As indicated by *in silico* analyses, the 100 nt region was not as widespread as the 450 nt element in the *Leishmania* genome and it was found conserved only in seven other *Leishmania* amastin gene homologs ((24); data not shown). It is possible, however, that a short sequence motif within the 100 nt region may be responsible for regulation and that this motif could be indeed present in several other mRNAs. Further deletions within this region will be required to examine this possibility.

A novel putative regulatory element is implicated in stage-specific accumulation of the amastin mRNA

In light of our deletional analyses and luciferase reporter studies, we conclude that two distinct regions within the amastin 3'UTR region equally promote the induction of LUC activity specifically in the amastigote stage of the parasite. No regulation was seen in promastigotes with any of these regulatory elements (Fig. 1B). To further assess the individual contribution of the 450 nt and 100 nt 3'UTR elements to stage-specific posttranscriptional regulation, we first examined whether could promote mRNA accumulation. Northern blot analysis of RNA from recombinant parasites transfected with the LUC-chimeric constructs described in Figure 1B showed no differences in the accumulation between the *LUC* control mRNA and the LUC-770, LUC-550, LUC-300 and LUC-200R mRNAs (Fig. 2A). Moreover, no changes in RNA abundance between promastigotes and amastigotes were observed (Fig. 2A). However, Western blot analyses indicated that LUC-770, LUC-550, LUC-300 and LUC-200R mRNAs produce high levels of LUC protein compared to the LUC control RNA (Fig. 2B). The LUC-770 RNA harboring both 3'UTR elements produces twice as much protein, suggesting that the 450 nt and 100 nt elements contribute equally to translational regulation (Fig. 2B). Altogether these findings suggest that the induction of LUC activity by both 3'UTR elements identified within the 770 nt region of the amastin mRNA (Fig. 1B) is not due to an increase in mRNA...
accumulation but is probably the result of translational regulation.

It was previously shown that amastin mRNAs specifically accumulate in *Leishmania* amastigotes (24) due to an increase in mRNA stability (14). It is possible that mRNA stability is conferred by the first 1300 nucleotides of the amastin 3'UTR. However, we could not rule out the possibility that the 450 nt and 100 nt elements within the last 770 nucleotides of the 3'UTR played a role in this process as experiments were performed with episomal LUC-vectors (>30 copies per cell) hence complicating the interpretation of the data. In order to better mimic the endogenous situation of the amastin transcripts that are poorly expressed in promastigotes compared to amastigotes, we integrated a single copy of each of the regulatory 3'UTR elements by gene targeting into the constitutively expressed *BT1* (biopterin transporter 1) genomic locus of *Leishmania* (32,36). Linear cassettes with the LUC gene fused either to the 770 nt region containing the 450 nt and 100 nt elements (BT1-LUC-770) or to the 300 nt region containing only the 100 nt element (BT1-LUC-300) and to the control construct lacking both elements (BT1-LUC) (see also Fig. 1B) that were sedimented over a linear 15%-45% sucrose gradient and fractionated with continuous monitoring at A254 to separate the ribosomal subunits and monosomes from polysomal fractions according to their respective densities (Fig. 4A). RNAs isolated from these fractions (Fig. 4B) were hybridized to a LUC-specific probe in order to assess the effect of the amastin 3'UTR regulatory elements on mRNA association with polysomes (Fig. 4C-E). Polysome profile analyses indicate that the LUC-770 and LUC-200R transcripts are preferentially associated with heavy sedimenting polysomes known to be actively translating, similarly to the α-tubulin, which has a typical profile for a translationally active transcript (Fig. 4C-D and G). The combination of both elements increases polysomal association of the LUC message (Fig. 4C) in agreement with the LUC activity (Figs. 1B and 3D) and Western blot (Fig. 2B) data. Under the same experimental conditions, the LUC transcript lacking both elements is distributed uniformly throughout the sucrose gradient and at significantly lower levels (Fig. 4E). Polysome disruption by EDTA treatment results in a dramatic shift in the sedimentation pattern of LUC-770 mRNA from the heavy polysomes to the lighter and free fractions of the gradient.
(Fig. 4F), suggesting that LUC-770 is associated with the translation apparatus. Increased association of the 3'UTR regulatory elements-containing transcripts with polysomes is seen only in amastigotes (Fig. 4) and no differences in polysome distribution between the LUC-770, LUC-200R and LUC mRNAs were observed in promastigotes (data not shown).

Translational regulation of the amastin transcripts is not stimulated by 5'UTR sequences

Posttranscriptional regulation of stage-specific gene expression in *Leishmania* is mediated almost exclusively by sequences in the 3'UTRs (reviewed in (10,11)). It has been shown previously that 5'UTR and 3'UTR have a slight synergistic effect in the stage-regulated translation of *HSP83* mRNA in *Leishmania* (16). In higher eukaryotes, there is a growing body of evidence that the interaction of RNA binding factors and translation initiation factors with the 5'- and 3'-ends of mRNA could regulate translation initiation through transcript circularization (37,38). In order to investigate whether the 5'UTR of the amastin mRNA could alter in any way 3'UTR-mediated translational regulation, we compared the induction of LUC activity between recombinant *Leishmania* expressing the LUC gene fused to the amastin 5'UTR and the 3'UTR regulatory elements (5'UTR-LUC-770) (Fig. 5A) and *Leishmania* expressing the LUC-770 construct lacking the 5'UTR (see Fig. 1B). No differences in the induction of LUC activity were observed between these transfectants (Fig. 5B). Furthermore, the 5'UTR of amastin mRNA has no effect on mRNA accumulation as LUC-770 and 5'UTR-LUC-770 steady-state RNA levels are very similar (Fig. 5C). These results suggest that 5'UTR sequences are not required for stimulating translation initiation of the amastin mRNA, which seems to be mediated exclusively by sequences in the 3'UTR.

Distinct environmental signals stimulate 3'UTR-mediated amastin mRNA accumulation and translation

*Leishmania*'s differentiation from promastigotes in the sandfly to amastigotes in the phagolysosomes of macrophages is triggered by drastic environmental changes. Heat shock (from 25°C to 37°C) and acidic pH (~4.5-5.5) represent the major triggering factors for parasite’s differentiation (9,39,40). Therefore, we tested whether these two important signals could trigger 3'UTR-mediated posttranscriptional regulation of amastin mRNAs. Exposure of recombinant parasites expressing the LUC-770 transcript (both 3'UTR regulatory elements) to elevated temperature (37°C) for more than 24 hours resulted in ~28-fold induction of LUC activity (Fig. 6A). However, under the same conditions, the control LUC-expressing parasites showed no induction of LUC activity. Interestingly, acidic pH (~5.5) has no effect on LUC activity. Exposure of LUC-770-expressing parasites to combined high temperature and acidic pH conditions for ~24 hours induces LUC activity by more than 15-fold similarly to the fully differentiated amastigotes in axenic cultures or within macrophages (compare Figs. 1B and 6A). As expected, LUC activity in recombinant parasites expressing the LUC-200R transcript (only the 100 nt element) was induced at 50% the levels of the LUC-770 RNA (450 nt and 100 nt elements) (Fig 6A). Based on these results, we conclude that 3'UTR-mediated translational regulation is triggered by elevated temperature to which *Leishmania* promastigotes are exposed immediately following their entry into the mammalian host.

It was previously shown that the majority of amastin transcripts specifically accumulate in the amastigote stage (14,24). Here we show that both 3'UTR elements that regulate amastin mRNA translation specifically in amastigotes are not implicated in mRNA accumulation (see Fig. 3C). In agreement with these findings, exposure of *LUC*-770 recombinant parasites to either elevated temperature or acidic pH had no effect on mRNA accumulation (Fig. 6B). However, accumulation of the endogenous amastin mRNA that is controlled by a 2055 nt 3'UTR was significantly increased in response to acidic pH.
but not to heat shock (Fig. 6B). These important findings suggest that stage-specific amastin gene expression involves two levels of regulation, mRNA stability and mRNA translation, that are triggered by distinct environmental signals associated with promastigote to amastigote differentiation within macrophages.

DISCUSSION

Here we have presented data implicating a two-step posttranscriptional mechanism of regulation that relies on distinct 3’UTR elements to stimulate stability and translation of the developmentally regulated amastin transcripts in *Leishmania*. We have also investigated how these elements respond to key environmental signals inducing parasite’s differentiation within macrophages. This is an important question as developmental gene regulation in this organism relies primarily on environmental changes throughout its life cycle. Our results provide new insights into the complex 3’UTR-mediated mechanisms that control stage-specific regulation of gene expression in *Leishmania*.

We previously showed that amastigote-specific translational regulation of the *L. infantum* amastin mRNA is mediated by the last 770 nucleotides of the 3’UTR which contain a ~450 nt element that is highly conserved among several *Leishmania* mRNAs, including a number of known amastigote-specific mRNAs (15). We have hypothesized that this 3’UTR element may play an important role in stage-specific translational control. Here we report that the 450 nt element stimulates translation initiation of the amastin mRNA, specifically in the amastigote stage, as inferred by the increased mRNA association with heavy sedimenting polysomes that are part of the active translation apparatus. This increased polysomal association under conditions where the control mRNAs lacking this element do not associate is probably not facilitated by a direct interaction of the 450 nt RNA with the 40S or 60S ribosomal subunits, as suggested by sucrose gradient analyses of purified ribosomal subunits and radiolabeled RNA (data not shown). It is hypothesized that the 450 nt element may interact with cellular factors that stimulate translation initiation by facilitating either the recruitment of ribosomes and/or initiation factors to the mRNA. Ongoing and future experiments will aim to identify the responsible components and molecular interactions.

Deletional analyses in this study indicate that regulation by the 450 nt conserved element requires the entire region of homology and a short sequence of 40-60 nt of either side of the element, suggesting that an RNA secondary structure may be critical for regulation. It is possible that conformational changes of the 450 nt RNA due to elevated temperature or to the activation of cellular factors specifically in amastigotes could stimulate translation initiation through binding of these factors to the element. Preliminary experiments with computer-aided folding and covariation searches suggest the presence of structural subdomains that are conserved among the 450 nt elements from different mRNAs (data not shown). The widespread distribution of the 450 nt RNA element raises the interesting possibility that several *Leishmania* transcripts may be regulated by a common mechanism of translational control in response to an environmental stimuli. This unusual type of regulation could provide a great advantage to the parasite for a rapid adaptation within its mammalian host. Remarkably, it is shown here that the 450 nt-mediated translational regulation is triggered by a temperature shift from 25°C to 37°C to which the parasite is exposed upon its entry to the mammalian host.

Our findings provide also some new insights on the complexity of mechanisms that control stage-specific translational regulation in *Leishmania*. It is shown here that amastin translation is stimulated by two distinct 3’UTR regions. Indeed, in addition to the 450 nt element, a downstream region of ~100 nt that is present in ~20% of the amastin transcripts is shown to be involved in translational regulation. Similarly to the 450 nt element, this newly identified region enhances mRNA association with heavy sedimenting polysomes and stimulates translation in response to elevated temperature. The proximity of the 100 nt
regulatory element to the poly(A) tail might suggest a role of this sequence in polyadenylation. It is well documented that 3' poly(A) tails could stimulate translation initiation through the binding of factors (reviewed in (41)). Our data suggest that both 3'UTR-mediated mechanisms act in an additive manner to activate translation of the amastin transcripts. Amastin mRNAs are expressed at very low levels by phagocytosed promastigotes and regulation at the level of translation could provide a rapid and efficient way to ensure the production of the amastin surface proteins shortly following the entry of the parasite into macrophages.

Our polysome analyses were carried out in the presence of cycloheximide, a compound that inhibits elongation of translation. Under those conditions, the increased association of the message containing both 3'UTR elements with heavy mRNPs in contrast to the LUC mRNA lacking the elements suggests that regulation by these elements occurs probably at the level of translation initiation. Translation initiation is the rate-limiting step of translational regulation in most eukaryotes and is often the target of control (reviewed in (42)). 3'UTR elements of several cellular and viral mRNAs have been found to affect translation initiation under various conditions of developmental switches or stress (reviewed in (43,44)). Several 3'UTR-mediated mechanisms of translational control have been reported, including the recruitment of the 40S ribosomal subunit (45), 60S ribosomal subunit joining (46), binding to ribosomal protein L13a (47), recruitment of eIF4G (48), transcript circularization through 3'-5' base-pairing (49), a tRNA like structure (50), a tRNA-reach-back mechanism (51) and an internal ribosome entry site (IRES) like structure at the 3'-end of the message (52). In Leishmania, very few 3'UTR elements implicated in translational control have been identified thus far (15,17,53) and the molecular mechanisms are for the most part unknown.

Another important finding in this study is that developmental regulation of the amastin gene family is controlled not only at the level of translation but also at the level of transcript stability by distinct 3'UTR-mediated mechanisms that are triggered by different environmental signals during promastigote to amastigote differentiation within macrophages. It is shown here that elevated temperature triggers the mechanism(s) of translational regulation that rely on the 450 nt and 100 nt 3'UTR regulatory elements to selectively stimulate translation of amastin mRNAs. Using an elegant experimental system ensuring low steady-state levels of LUC-chimeric transcripts, we showed that neither of these 3'UTR regulatory elements plays a role in mRNA stability. Acidic pH that together with high temperature induce parasite's differentiation from promastigotes to amastigotes (9,39,40,54) has no effect on amastin translational regulation but remarkably stimulates mRNA stability. Our previous studies showed that the majority of amastin transcripts specifically accumulate in the amastigote stage of the parasite (14,24) and that this differential transcript accumulation is due to an increase in the half-life of the message (14). This increase in mRNA accumulation under conditions where amastin translation is still highly active via the 450 nt and 100 nt elements, could be explained by the threatening environment in the phagolysosomes that could either affect protein stability due to elevated proteolytic activity or slow down overall mRNA translation. It has been shown in other eukaryotes that diverse cellular stress conditions inhibit global protein synthesis (reviewed in (55-57)) but enhance translation of specific transcripts (58,59). Interestingly, our preliminary data show that a combination of acidic pH and elevated temperature impairs global mRNA translation in Leishmania (Laverdière M. et al., in preparation).

In conclusion, the studies in this report suggest a complex mechanism of regulation for the developmentally regulated amastin gene family in Leishmania that is achieved in two subsequent steps during parasite's differentiation within macrophages. These include a bipartite 3'UTR-mediated translational regulation that is triggered by elevated temperature followed by an increase in mRNA accumulation, via a distinct regulatory element
most likely within the 3′UTR, which is activated by acidic pH (summarized in Table I). This is the first time that distinct environmental signals associated with the process of parasite’s differentiation are reported to specifically trigger mechanisms of posttranscriptional regulation that control stage-specific gene expression in Leishmania. Future experiments will aim to identify components and the molecular interactions of these complex mechanisms.

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**FOOTNOTES**

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Fig. 1. Sequences involved in 3'UTR-mediated posttranscriptional regulation of the amastin mRNA. A, schematic representation of the Leishmania infantum amastin mRNA (LinJ34.0840) with the 596 nt coding region (ORF) flanked by the 211 nt 5'UTR and the 2055 nt 3'UTR. The IR corresponds to the 467 bp intercistronic region of the amastin gene. The conserved 450 nt 3'UTR element identified previously (15) is indicated by the left hatched box. The right hatched box represents a 100 nt region, which overlaps between the LUC-200L and LUC-200R constructs (see 1B). B, deleitional analysis within the 770 nt 3'UTR regulatory region of the L. infantum amastin mRNA. The different LUC-chimeric constructs tested are schematically represented with the corresponding name indicated on the left. In all these plasmids, LUC-chimeric transcripts are processed at the 5'-end by sequences from the α-tubulin intercistronic region (αIR) that provide signals for trans-splicing and at the 3'-end by the amastin IR region. Plasmids were introduced into L. infantum by electroporation and stable transfectants were analyzed by luciferase reporter assays. The effect of 3'UTR deletions on LUC activity was measured in L. infantum-LUC recombinant parasites grown as axenic promastigotes, axenic amastigotes and also as intramacrophage amastigotes. Similar results were obtained when using axenic and intramacrophage amastigotes. Results are presented as the relative luciferase fold increase compared to the control transfectant (LUC) for each growth condition. Values are mean ± standard error of five independent experiments.

Fig. 2. Effect of 3'UTR deletions on amastin mRNA accumulation and mRNA translation. A, Northern blot analysis of total RNA extracted from L. infantum promastigote (P) and axenic amastigote (A) cell lines expressing the different LUC-chimeric constructs described in Fig. 1B. Equal amounts (~15 µg) of total RNA were loaded on agarose gel prior to transfer onto a nylon membrane and to hybridization with a probe corresponding to the luciferase coding region. All chimeric transcripts have the expected size. Northern blot hybridization experiments were repeated three times and similar results were obtained. B, Western blot analysis of total protein lysates from L. infantum recombinant strains expressing the different LUC-chimeric constructs (see Fig. 1B). Total protein lysates were extracted from axenically grown amastigotes and subjected to Western blot analysis using the anti-LUC antibody as described under Experimental Procedures. Membranes were stripped and reacted with an anti-α-tubulin (α-tub) antibody to verify protein loading. Western blot analyses were carried out with three different cultures for each transfectant and similar results were obtained.

Fig. 3. Genomic integration of the amastin 3'UTR regulatory elements to evaluate their role in stage-specific posttranscriptional regulation. A, schematic representation of the targeting cassettes for the genomic integration of both 3'UTR regulatory elements of the amastin mRNA (450 nt and 100 nt) (BT1-LUC-770) or of the 100 nt region alone (BT1-LUC-300) fused to the LUC coding region into the L. infantum biotin transporter 1 (BT1) locus, which is constitutively expressed. BT1-LUC is the control construct lacking any 3'UTR regulatory sequence. For genomic integration by homologous recombination, the above targeting cassettes were excised with HpaI and HindIII digestion and introduced as linear fragments into L. infantum by electroporation as described in Experimental Procedures. Positions of key restriction enzymes for analyzing the genotypes of the different transfectants are indicated. B, Southern blot analysis to verify correct integration of LUC-chimeric constructs into the BT1 locus. Genomic DNA from the different transfectants (clones were used here) was extracted, digested with PstI or NcoI, transferred onto nylon membrane and hybridized to a probe specific for the BT1 gene. Homozygous BT1 mutants with the two BT1 alleles (the Leishmania genome is diploid) successfully disrupted by the LUC-chimeric constructs were obtained in all three cases. C, Northern blot hybridization with a probe corresponding to the LUC coding region in order to evaluate the effect of both 3'UTR regulatory elements (450 nt and 100 nt) on mRNA accumulation in both developmental stages of the parasite (Promastigotes and Amastigotes). The expression of the endogenous amastin transcript was verified by hybridization using a probe specific to the amastin 3'UTR (14). Equal amounts of total RNA were used as indicated by ethidium bromide staining. D, luciferase reporter assays with L. infantum recombinant parasites.
expressing the integrated LUC-chimeric constructs grown as axenic amastigotes. The results are presented as the relative luciferase fold increase compared to the control (BT1-LUC). Values are mean ± standard error of three independent experiments.

**Fig. 4.** Distribution of transcripts containing the amastin 3'UTR regulatory elements in polysome gradients. A, representative OD 254 nm profile of a 10 ml sucrose gradient with sedimentation from left to right. The 40S, 60S, 80S and polysome peaks are indicated. Cytoplasmic extracts from the control LUC, LUC-770, and LUC-200R recombinant parasites grown as axenic amastigotes were sedimented over a linear 15%-45% sucrose gradient and fractionated with continuous monitoring at A_{254nm} as indicated in Experimental Procedures. Fractions were numbered from 1 through 16 referring to the top and the bottom of the gradient, respectively. B, ethidium bromide staining to visualize the distribution of the ribosomes in the gradient. RNA was extracted from each fraction and analyzed by Northern blot hybridization. C-E, polysome profile distribution of LUC, LUC-770 (450 nt and 100 nt elements) and LUC-200R (100 nt element) transcripts. Northern blots were hybridized with a probe specific for the LUC gene. F, addition of EDTA to assess the specificity of LUC-770 association with polyribosomes. Treatment with 50 mM EDTA results in the dissociation of ribosomes from mRNAs and in the shift of LUC-770 mRNA to the top of the gradient. G, distribution of α-tubulin mRNA in polysomes. The α-tubulin mRNA has a typical profile for a translationally active transcript, with much of the mRNA in polysomal fractions and it was used here as a control. Polysome profile analyses were carried out three times for each transfectant, and similar results were obtained.

**Fig. 5.** Translational regulation of the amastin mRNA does not require sequences in the 5'UTR. A, schematic representation of the 5'UTR-LUC-770 vector in which the amastin 5'UTR and the 770 nt subregion of the amastin 3'UTR were fused to the LUC open reading frame (for more details see Fig. 1A and Experimental Procedures). B, the role of 5'UTR sequences on the translational regulation of the amastin mRNA was evaluated using a luciferase reporter system. LUC-chimeric constructs harboring (5'UTR-LUC-770) or lacking (LUC-770) the amastin 5'UTR sequence were transfected into L. infantum and the LUC activity was measured in axenically grown amastigotes. The results are presented as the relative luciferase fold increase compared to the control (LUC). Values are mean ± standard error of three independent experiments. C, Northern blot analysis of L. infantum axenic amastigotes expressing LUC-770 and 5'UTR-LUC-770 chimeric transcripts. Hybridization was carried out with a probe specific to the LUC coding region. RNA loading on the gel was monitored by hybridization to a 18S rRNA-specific probe. Northern blot analyses were repeated three times and similar results were obtained.

**Fig. 6.** Distinct environmental signals inducing Leishmania’s differentiation within macrophages independently trigger mechanisms that stimulate amastin mRNA stability and translation. A, elevated temperature specifically stimulates 3'UTR-mediated translational regulation of the amastin transcript. Late-log L. infantum promastigotes expressing the LUC-770 or the LUC-200R and/or the LUC (control) vectors were subjected for more than 24 hours to elevated temperature (a shift from 25°C to 37°C) or to acidic pH (5.8) or to a combination of both (37°C and pH 5.8), and the effect of these environmental changes on mRNA translation was evaluated by measuring the luciferase activity. Induction in LUC activity upon a temperature shift was detected at earlier time points but at lower levels (data not shown). B, acidic pH specifically activates the accumulation of amastin mRNA by a mechanism that is independent of the 450 nt and 100 nt elements. L. infantum recombinant parasites expressing the LUC-770 vector were subjected to the same growth conditions as in A and the steady-state levels of the LUC-770 and amastin transcripts were analyzed by Northern blot hybridization to gene specific probes (LUC and amastin). RNA loading was similar between the different conditions of growth as indicated by ethidium bromide staining of the gel (lower panel).
**Table I.** Amastin gene regulation in *Leishmania infantum*

| Developmental life stages of *Leishmania* | Environmental signals | mRNA accumulation | mRNA translation | 3'UTR elements |
|------------------------------------------|------------------------|-------------------|------------------|----------------|
| Extradcellular promastigotes in the sandfly midgut | T° 25°C, pH ~7.0 | _a_ | _ | no | no | yes b |
| Internalized promastigotes by macrophages | T° 37°C, pH ~7.0 | _a_ | + | yes | yes | yes b |
| Amastigotes in the phagolysosomes of macrophages | T° 37°C, pH 4.5 - 5.5 | + c | + | yes | yes | yes b |

*a* Decreased mRNA accumulation in promastigotes correlates to a decrease in mRNA stability compared to amastigotes (14).

*b* Other sequences within the 3'UTR unrelated to the 450 nt and 100 nt elements are likely to control either decreased mRNA stability in promastigotes or increased mRNA stability in amastigotes.

*c* Increased mRNA accumulation in amastigotes correlates to an increase in mRNA stability compared to promastigotes (14).
Fig. 1
**Fig. 2**

**A**

- LUC
- 2.7 kb →
- 2.0 kb →
- rRNA →

- LUC
- LUC-770
- LUC-550
- LUC-300
- LUC-200R

**B**

- LUC
- (61 kDa)
- α-tub
- (54 kDa)
Fig. 3
Fig. 4
Fig. 5

A. Diagram of Amastin 5' UTR with LUC and IR regions.

B. Graph showing relative LUC activity for LUC, LUC-770, and 5'UTR-LUC-770.

C. Gel image showing LUC bands at 2.9 kb and 2.7 kb, and 18S rRNA bands.
Fig. 6

Panel A: Graph showing relative LUC activity under different conditions. The x-axis represents different treatments: -, T', pH, T' + pH, and the y-axis represents relative LUC activity.

Panel B: Western blot analyses showing LUC-770 (2.7 kb), Amastin (2.8 kb), and rRNA bands under different conditions.
Distinct 3' untranslated region elements regulate stage-specific mRNA accumulation and translation in Leishmania
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