Deadenylation-independent stage-specific mRNA degradation in *Leishmania*

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ABSTRACT

The life cycle of *Leishmania* alternates between developmental forms residing within the insect vector (e.g. promastigotes) and the mammalian host (amastigotes). In *Leishmania* nearly all control of gene expression is post-transcriptional and involves sequences in the 3'-untranslated regions (3'UTRs) of mRNAs. Very little is known as to how these cis-elements regulate RNA turnover and translation rates in trypanosomatids and nothing is known about mRNA degradation mechanisms in *Leishmania* in particular. Here, we use the amastin mRNA of *Leishmania* to study the mechanisms of mRNA degradation. Overall, these results suggest that degradation of the amastin mRNA of *Leishmania* is likely to be bi-phasic, the first phase being stage-specific and dependent on an unusual URE-mediated pathway of mRNA degradation.

INTRODUCTION

Parasitic species belonging to the kinetoplastid group of protozoans are of high medical and veterinary importance mainly in tropical and subtropical countries. These include trypanosomatids such as *Leishmania* spp. (Leishmaniasis), *Trypanosoma brucei* (Sleeping sickness) and *Trypanosoma cruzi* (Chagas disease). Their life cycle involves a mammalian host and an arthropod vector. The parasites face disparate conditions during their life cycle, a drastic change in temperature between their insect (20–25°C) and mammalian (37°C) hosts being typically a shared challenge. Other changes can be unique to the life style of a given parasite (e.g. the acidic pH that *Leishmania* spp. face within the phagolysosomes of host macrophages). The parasites employ various adaptive strategies to be able to survive and grow in their distinct niches. These stage- or species-specific structural and metabolic adaptations are associated with significant changes in gene expression as evidenced by recent functional genomic analyses [Rochette et al., unpublished data; (1–4)].

Kinetoplastids branched very early from the eukaryotic lineage and perhaps because of these they display several unusual or unique biological features. Tens to hundreds of genes are collinearly transcribed into a polycistronic mRNAs (5–7). Individual mRNAs are resolved from nuclear pre-mRNAs via coupled co-transcriptional processes of trans-splicing and 3'-end cleavage/polyadenylation (8). The process of trans-splicing involves the addition of ~40-bases long capped mini-exon to the 5'-termini of all mRNAs from a separate RNA substrate (SL-RNA) [reviewed in (8)]. Transcription has been postulated to initiate at strand switch regions on each chromosome probably within the intercistronic regions (9,10). There is, however, no indication for specific RNA polymerase II promoters associated with protein-coding genes to-date and the genomes of the parasites are short of genes encoding major transcription factors and co-activators. Consequently, most of gene regulation in these parasites occurs at the post-transcriptional levels, mainly via mRNA stability and translation [reviewed in (11,12)].

Eukaryotic mRNAs are typically capped at their 5'-ends and possess poly(A) tail at their 3'-ends. Such modifications at the mRNA termini, plus the proteins they bind to, confer mRNA stability and translatability. The degradation of eukaryotic mRNA is typically triggered by poly(A) tail shortening [reviewed in (13)]. Deadenylation, in turn, invokes decapping and the cumulative outcome is unprotected ends that are readily attacked by 5'- and

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3′-exonucleases [reviewed in (13,14)]. Two 5′-exonucleases have been identified in yeast, the nuclear Rat1p/Xrn2p and the cytoplasmic Xrn1p (15,16). More than 10 conserved 3′-exonucleases have been characterized and some, together with other protein factors, are found in a complex called the exosome. Besides their role in determining mRNA half-lives in the cytoplasm, the 3′- and 5′-exonucleases are also involved in pre-mRNA quality control and the maturation of ribosomal, nuclear and small nucleolar RNAs [for examples see (17–19)]. In yeast, degradation from the 5′-end seems to be the major determinant of mRNA degradation and the 3′-pathway is only detectable in mutants of the 5′-pathway [reviewed in (13)]. In mammals, there is a growing consensus that both pathways are probably significant determinants of mRNA degradation (20–22).

The genomes of trypanosomes harbor genes that encode for deadenylases [reviewed in (11)]. A clear orthologue of a decapping enzyme has so far proven to be elusive even though decapping activity has been detected in the related organism Leptomonas seymouri (23). Degradation of mRNAs in T. brucei involves both 5′- and 3′-pathways (24) and homologues of all major 3′-exonucleases (25–27), and 5′-exonucleases (28) are well-characterized. Trypanosomes harbor four homologues of Xrn1p in T. brucei and Xrn2p/Rat1p (28). One of these, termed XRNA, is cytoplasmic and has been shown to be critical in the degradation of both stable and unstable mRNAs in T. brucei (28).

mRNA degradation cis determinants are predominantly found in the 3′-untranslated regions (UTRs) in all three species of trypanomatids. In Leishmania, in contrast to other trypanosomatids, several hundred of the 3′UTR cis-elements belong to two major classes of short interspersed degenerate retroposons, SIDER1 and SIDER2 (31), and several lines of evidence indicate that these elements modulate post-transcriptional gene expression in this parasite (31–33). These elements are thought to have evolved specifically in the Leishmania lineage perhaps under the selective pressure presented by the parasite’s unique niche. Whether or not the mechanisms through which these elements function are also unique remains to be determined. The degradation of several unstable mRNAs in T. brucei [reviewed in (11) and T. cruzi (34)] is dependent on U-rich elements (UREs). These elements seem to be structurally (35) and functionally (24,29,36–40) similar to the mammalian AU-rich elements (AREs) found in several proto-oncogene and inflammatory cytokine mRNAs (41). These elements confer instability in a number of mRNAs in procyclic trypanosomes, developmental forms of the parasite that reside in the mid-gut of the insect vector. In T. cruzi, protein factors (UBPs) that bind these elements with high affinity and avidity have been identified (34,42,43). Homologues of these RNA-binding proteins also exist in T. brucei even though these proteins seem to have broad RNA-binding specificity (44). No UREs have been reported in Leishmania so far and virtually nothing is known about mechanisms underlying mRNA degradation in these parasites. In this study, we demonstrate URE-mediated degradation of the stage-specific amastin mRNA in Leishmania, thereby embark on attempts to gain insight into the mechanistic basis of mRNA degradation in this organism.

MATERIALS AND METHODS

Plasmids

The parental plasmid used in this study, pSPBT1-YNEOzLUC, was previously described (33). This plasmid allows the integration of reporter constructs into the BT1 genomic locus. Sequence corresponding to the amastin (LinJ34_V3.1030) 3′UTR and downstream intergenic region was amplified by PCR from L. infantum LEM 1317 genomic DNA (gDNA) using the forward primer, amastin-3′UTR-F (5′-AAGCTACTCTGGATCTCGCGG-3′) and the reverse primer, amastin-IR-R (5′-GGATCCGGCTCGCCAGTTAGTAGGCGG-3′). The URE deletion construct was made by fusion PCR as described (45). Primers amastin-3′UTR-F and amastin-Up-URE-R (5′-CGTCCATGCGCTTCTCTTGCGC GTGCCTGTGTGGC-3′) were used to amplify the upstream region from the URE. Primers amastin-Down-URE-F (5′-CGACACACACGCACCGCGACAGAGA GCCGCATGGACG-3′) and amastin-IR-R, was used to amplify the downstream region of the URE. Primer amastin-Up-URE-R is the reverse complement of primer amastin-Down-URE-F. One hundred nanograms of the resulting PCR products, together with primers Amastin-3′UTR-F and amastin-IR-R, were used for a fusion PCR to generate amastin 3′UTR URE. The only difference between full-length 3′UTR and 3′UTRΔURE is, therefore, the deletion of the 100-nt long URE. The resulting PCR fragments were digested with BanHI (primers amastin-3′UTR-F and amastin-IR-R contain this site) and cloned into the BanHI site of the pSPBT1-YNEOzLUC plasmid, downstream of the firefly luciferase (LUC) open reading frame (Figure 2).

Parasite culture and transfections

Leishmania donovani infantum MHOM/MA/67/ITMAP-263, the parental strain for all the parasite lines employed in this study, was cultured in SDM-79 medium (pH 7), supplemented with 10% heat-inactivated fetal calf serum (Multicell, Wisent Inc.) and 5 mg/ml hemin at 25°C as promastigotes and were switched to MAA/20 medium (pH 5.8) at 37°C with 5% CO2 and passed ~2–3 times to generate axenic amastigotes (46). For genomic integration into the BT1 locus, ~2.5 μg of Hpal-HindIII digests (these enzymes only cut on either side of the BT1 targeting regions) were transfected into promastigote L. infantum by electroporation as described (47). Transfected cells were plated on SDM-79 medium with 1.5% agar and 0.01 mg/ml of G418 (Sigma), and individual clones were obtained after ~2–3 weeks.
RNA analysis

Parasites were treated with 0.5 μg/ml Actinomycin-D (Sigma) with/without 2.5 μM Sinefungin (Sigma) for mRNA decay rate determination. All time points include 5 min of centrifugation times. Total RNA was extracted from cultures with OD600nm = 0.3–0.5 after lysis with Trizol (Invitrogen) according to the manufacturer’s instructions. Ten to forty micrograms RNA was used for standard RNA blotting. Hybridization was done at 42°C with 50% formamide solution and RNA gels were transferred into Hybond-N membranes (Amersham Biosciences) by upward capillary movement. RNase-H (Invitrogen) digestions were carried out as described with 20–60 μg RNA as a starting material in the presence of a specific oligonucleotide 300R (5′-TTGTCTCCGTT CCTCC GGGATCCCG-3′) with or without oligo dT (Invitrogen) and the reaction contained RNase inhibitor (Invitrogen). RNase H samples were run typically on 25 cm × 20 cm, 2–2.4% agarose gels. Polyacrylamide gels were 5% and transfer was carried out using BioRad’s submarine system. Blots were probed with a fragment corresponding to the last 300 bases of the amastin mRNA. Ten to twenty micrograms RNA was used as a starting material for the Terminator (Epicentre Biotechnologies) treatments of RNA samples. The reactions were carried out according to manufacturer’s instructions at 30°C for 1–1.5 h and an RNase inhibitor (Invitrogen) was included in the reaction. Minus Terminator controls were treated equally except that the enzyme was not added. The reaction was stopped by adding 5 mM EDTA, and was directly analyzed by an RNA blot after denaturation in standard MOPS loading buffer. All quantifications of RNA blots were done using the ImageQuant 5.2 software.

Luciferase assay

10⁷ parasites were resuspended in 100 μl of lysis buffer (25 mM Tris–phosphate at pH 7.8, 2 mM dithiothreitol, 1% Triton X-100, 10% glycerol) and incubated at room temperature for 30 min, then transferred at −80°C for 2 h or overnight. After thawing, 20 μl of the parasite lysates was added to 96-well plates. One hundred microliters of luciferase assay buffer [20 mM tricine, 1.07 mM (MgCO₃)₄·Mg(OH)₂·H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 270 mM coenzyme A, 470 mM luciferin, 530 mM adenosine triphosphate, 33.3 mM DTT] was added to each well. Bioluminescence was measured using a Dynex MLX luminometer.

RESULTS

Quantitative analysis of the stage-specific mRNA regulation of the amastin mRNA

This study attempted to begin to elucidate mechanisms underlying mRNA degradation in Leishmania using the amastin mRNA as a model. The amastin gene encodes a putative surface antigen and is a member of a multi-gene family (49,50). What we refer to as amastin in this paper is the original gene that was first identified-LinJ34_v3.1030 (51). We previously demonstrated that most of the amastin mRNAs accumulate preferentially in amastigotes due to increased mRNA stability (49,51).

We first wished to obtain a quantitative data on the amastin mRNA regulation by using an additional probe that is specific to the mRNA’s 3′UTR, the last 300 bases (probe 300) to exclude possible cross-reactivity with other amastins. Overall the amastin mRNA is ~3.9 more abundant in axenic amastigotes than in promastigotes-the developmental forms of the parasite that reside in the mammalian host and the insect vector, respectively (Figure 1A). The values obtained using the amastin coding sequence as a probe and the probe 300 are comparable and Figure 1A is representative of both. The decay kinetics of mRNAs was followed upon inhibition of
Table 1. mRNA half-lives for the endogenous amastin and reporter mRNAs analysed in this study

| mRNA       | mRNA half-lives (min) | ACT-D     | ACT-D + SIN |
|------------|-----------------------|-----------|-------------|
|            |                       | Pro Ama   | Pro Ama     |
| Amastin    | 45 ± 20               | >120      | 45 ± 15     |
| LUC-3'UTR  | 31.3 ± 19.6           | 82.5 ± 10.6| 28 ± 15.9   |
| LUC-3'UTRΔURE | >120               | 76 ± 1.4  | 63 ± 21.3   |

Decay rates for the endogenous amastin, LUC-3'UTR and LUC-3'UTRΔURE mRNAs in promastigotes (Pro) and axenic amastigotes (Ama) were determined after transcriptional inhibition using Actinomycin D (ACT-D) with or without splicing arrest using Sinefungin (SIN).

Despite the variation of the absolute half-life values which are directly reflected in this Table, LUC-3'UTR mRNA was consistently more stable than LUC-3'UTR mRNA in promastigotes in each of the four independent experiments we carried out whereas no significant differences were observed between these two RNAs in amastigotes. Numerically, this parameter is reflected by the average of the relative differences (the half-life of LUC-3'UTRΔURE mRNA minus that of the LUC-3'UTR mRNA), which is 34 min ± SD of 8 min.

**de novo** transcription using Actinomycin D and in some cases together with the arrest of splicing using Sinefungin. Sinefungin is assumed to prevent the splicing of pre-made pre-mRNAs by inhibiting the de novo methylation of the cap structure of the Spliced-Leader RNA (SL-RNA) and/or mRNA maturation from incomplete transcriptional inhibition. The combination of the two drugs was previously suggested to be important in drawing a more accurate representation of decay rates (11,30). Based on an analysis using the probe 300, the amastin mRNA decays with an overall half-life of ~40 min in promastigotes versus >120 min in amastigotes (Table 1, Figure 1B).

**Stage-specific mRNA accumulation of the amastin mRNA is mediated through its 3'UTR**

We asked if the stage-specific accumulation of the amastin mRNA is mediated through sequences in the 3'UTR, as is the case for most trypanosomatid mRNAs studied to date. For this and subsequent analysis, we made a bicistronic construct into the Biopterin Transporter 1 (BT1) genomic locus by homologous recombination (data not shown).

The protein encoded by the BT1 gene is not required under culture conditions in biopterin-rich media but it is essential otherwise (52). The BT1 locus is, therefore, used routinely for the integration of transgenic constructs, particularly when a physiologically relevant expression level is desirable. All the other sequences except the 3'UTR have been used routinely in several published [e.g. (33)] and unpublished reporter gene analysis and they do not affect RNA levels between promastigotes and amastigotes. Any difference in the LUC-amastin 3'UTR mRNA (LUC-3'UTR) levels should therefore be due to the amastin 3'UTR. Figure 2B shows that LUC-3'UTR mRNA is ~3.2-fold higher in amastigotes than in promastigotes, comparable with the regulation of the endogenous amastin mRNA (Figure 1A). mRNA decay analysis of the LUC-3'UTR showed that its half-life is ~30 min in promastigotes and ~80 min in amastigotes (Table 1, Figure 3). In promastigotes, ~50–60% the LUC-3'UTR mRNA population are degraded with a half-life of ~10–15 min (Figure 3A and B). The rest of the population (40–50%) is degraded with a slower kinetics, the half-life being ~45 min (Figure 3A and B, data not shown). These results suggest that the stage specificity of the steady-state levels and degradation kinetics of the amastin mRNA are mediated mainly through sequences in its 3'UTR and that degradation is probably bi-phasic.

A U-rich ~100 bases region in the 3'UTR of the amastin mRNA contributes significantly to its stage-specific accumulation and its facilitated degradation in promastigotes

Next, we assessed whether the amastin mRNA regulation could be attributed to a distinct element in its 1.8 kb long 3'UTR. We have previously shown that the last 770 bases region of the amastin 3'UTR, in-and-by itself, does not account for stage-specific accumulation of the amastin mRNA (33). Sequence scanning of the rest of the 3'UTR revealed that it contains a ~100 bases long U-rich sequence (URE) (Figure 2A). Given the role of similar elements in the degradation of unstable mRNAs in other eukaryotes including T. brucei and T. cruzi [reviewed in (11,34)], we deleted this region in a construct which is otherwise identical to the LUC-3'UTR plasmid. The stable transfectant of this plasmid (LUC-3'UTRΔURE) was analyzed in parallel with that of the LUC-3'UTR. Copy number differences were ruled out by Southern blot analysis and comparison of NEO mRNA levels (data not shown). As shown in Figure 2B, the LUC-3'UTRΔURE mRNA steady-state levels are ~2.2-fold higher than that of LUC-3'UTR in promastigotes whereas no significant difference could be observed in amastigotes, suggesting that the URE accounts significantly for the stage-specific accumulation of the amastin mRNA. Decay analysis showed that the difference in the steady-state levels between LUC-3'UTR and LUC-3'UTRΔURE mRNA levels observed in promastigotes is accompanied by an overall increase in mRNA half-life from ~30 to ~60 min (Figure 3A and B, Table 1). No significant changes could be detected in amastigotes (Figure 3C, Table 1). A more detailed comparison revealed that differences in degradation kinetics are more apparent in the initial phase (the first 15–30 min) than the later phase (Figure 3A and B). Luciferase activity levels generally followed the mRNA trends (Figure 2C, data not shown) and the URE-mediated mRNA degradation is, therefore, unlikely to be due to differential translatability of the mRNA. Taken together, these results suggested that a significant part of the amastin mRNA degradation in promastigotes is...
URE-mediated and that this probably occurs during the initiation phase of the degradation.

Differential degradation of the amastin mRNA in promastigotes seems to be initiated via deadenylation-independent mechanisms

mRNA degradation in eukaryotes is typically initiated and modulated via differential deadenylation [reviewed in (13)]. Thus, we asked if the promastigote-specific degradation of the amastin mRNA is due to rapid deadenylation. The endogenous amastin or LUC-3′UTR mRNAs are relatively long (≈2.6 and ≈3.5 kb, respectively) and changes in mRNA sizes due to deadenylation in ordinary RNA blot analysis would be difficult to visualize. We, therefore, like in many other previous studies by others [e.g. (24)], exploited the DNA–RNA hybrid-dependent ribonuclease activity of RNase H to cleave these RNAs at specific sites and generate shorter 3′-end fragments. This was achieved by incubating RNA samples from several time points after ACT-D ± SIN treatments with RNase H in the presence of anti-sense oligonucleotide targeted to a region of ≈300 bases from the end of the amastin 3′UTR (Figure 4A). Oligo dT was added in some samples so that the poly(A) tail could be cleaved off and the resulting fragment served as a marker for a 100% deadenylated mRNA species. This analysis in promastigotes showed that the poly(A) tail of the LUC-3′UTR mRNA is still intact (relative to time 0) long after a significant part of the mRNA body has been degraded (Figure 4B, left panel). In contrast, a significant deadenylation clearly precedes the degradation of the mRNA in amastigotes (Figure 4B, right panel). Interestingly, the deadenylation pattern of the relatively more stable LUC-3′UTR DURE mRNA was not different between promastigotes (Figure 4B, middle panel) and amastigotes (data not shown) and it remarkably resembles that of LUC-3′UTR in amastigotes (Figure 4B, right panel). Noteworthy, a minor population of the LUC-3′UTR mRNA in promastigotes appears to be deadenylated at
later time points (the last lanes in Figure 4B, left panel and 4C) and is probably coincident with the second and slower phase of the mRNA degradation (Figure 3A and B).

We carried out a similar analysis for the endogenous amastin mRNA both in promastigotes and amastigotes. In order to detect the deadenylation pattern of the endogenous amastin mRNA in promastigotes (expression of the amastin mRNA is much higher in amastigotes compared to promastigotes), we had to increase the sensitivity of the assay and to expose the Northern blots much longer. This analysis shows that consistent with the reporter mRNA results described earlier (Figure 4B, left panel), the amastin mRNA in promastigotes—in contrast to the same mRNA in amastigotes—is degraded without detectable deadenylation (Figure 5A and C upper panel). We next wanted to assess the deadenylation profile of the amastin mRNA during differentiation. To induce differentiation in vitro, we subject amastigotes grown at 37°C and pH 5.8 to promastigote conditions (25°C and pH 7.0) of growth. Approximately 5 h following the switch from amastigote to promastigote conditions, a significant decrease in amastin mRNA levels could be observed (Figure 5B). Interestingly, under these conditions, the deadenylation pattern of the endogenous amastin mRNA (Figure 5C lower panel) was comparable to that seen using adapted promastigote cultures (Figure 5A). Overall, these results suggest that the initial phase of the degradation of the amastin mRNA in promastigotes per se is likely to be deadenylation-independent and that the stage-specific regulation of the amastin mRNA is unlikely to be a function of differential deadenylation rate.

Is decapping the rate-limiting step for the promastigote-specific degradation of the amastin mRNA?

Deadenylation-independent mechanisms of mRNA degradation are rare in eukaryotes and are mainly initiated via an endonuclease or deadenylation-independent decapping activities. A role for a decapping mechanism is usually demonstrated via genetic or RNA interference (RNAi) approaches. However, no clear homologue of a decapping
enzyme could so far be identified in the genomes of trypanosomatids. We, therefore, resorted in this study into an indirect assessment of the decapping rate of the LUC-30 UTR mRNA versus the LUC-30 DURE mRNA in promastigotes. To do this, we took advantage of the commercially available 5'-phosphate-dependent ribonuclease, Terminator, which has so far been used mainly to enrich mRNA preparation as the enzyme degrades all uncapped RNAs (e.g. decapped mRNAs, rRNAs, tRNAs) while capped mRNAs are protected.

A recent study has made use of the enzyme to qualitatively access the cap status of a deadenylated intermediate of the hsp70 mRNA (53). Here, we anticipated that relative mRNA decapping rates could quantitatively be inferred by comparison of Terminator treated versus untreated RNA samples derived from various time points after transcriptional arrest. For this assay, uncapped rRNAs that are cleaved by the enzyme served as positive controls (with 50–80% efficiency in our case). Histone 4A (Hist4A) mRNA is very stable within the time points used in the assay and therefore served as a negative control and indicated that the enzyme is highly specific (Figure 6A). Tube-to-tube variations in the efficiency of the Terminator activity were corrected with the levels of 18S RNA after loading normalization with the Hist4A mRNA (Figure 6A). Values from Terminator-treated samples were then divided by those from Terminator-untreated samples. The resulting numbers for each of the various time points after ACT-D treatment were expressed as a function of time 0. Such analysis indicated that there is no significant difference between the percentage of capped LUC-3'UTR and LUC-3'UTRAURE mRNAs at various times.

Figure 4. Determination of poly(A) tail status during reporter mRNA decay. (A) Schematic representation of the deadenylation assay. RNase-H digests DNA/RNA hybrid. In this case the DNA is an oligonucleotide (300R), which is reverse complementary to a region of ~300 bases upstream of the poly(A) tail of the reporter mRNA. The presence of oligo dT in the reaction allows the trimming of the poly(A) tails (see later). The vertical darker arrows indicate the sequence to which the oligonucleotide was targeted into. The last 300 bases of the 3'UTR is underlined and is where the probe for the RNA blots was targeted. (B) Deadenylation of reporter mRNAs in promastigotes (Pro) and amastigotes (Ama). RNA samples were collected from different time points after treatment with ACT-D and subjected to RNase-H treatment. 3' fragments were analysed by RNA blot. At time 0, RNA samples were also treated with RNase-H digestion in the presence of oligo dT (in addition to the specific oligonucleotide) to generate poly(A) minus 3-end markers (0 + dT' lanes). Hist 4A is used as a loading control. (C) As in (B) but RNA was derived from ACT-D and SIN treatment of LUC-30 UTR promastigotes. Data are representative of three experiments. (D) Quantitative representation of the decay rate of LUC-3'UTR polyadenylated 3'-end fragments in promastigotes (B, left panel) is compared to that of the full-length LUC-3'UTR mRNA in promastigotes (Figure 3A). The lateral bracket indicated in Figure 4B left panel shows the portion of the gel used for quantification of the largely poly(A)+ 3'-end fragments. Note that the full-length LUC-3'UTR mRNA used for this quantification was probed by the luciferase coding sequence, which is ~1.8 kb upstream of the polyadenylation site while the 3'-end products of the RNase H were probed by the last 300 terminal sequence of the 3'-end.
time points after transcriptional arrest (relative to time 0) (Figure 6B). These results, within the limits of the assay employed, suggested that no detectable difference in decapping rate can account for the URE-mediated facilitated degradation of the amastin mRNA in promastigotes.

DISCUSSION

In this study, we began a series of experiments aimed at dissecting the mechanisms of mRNA degradation responsible for the expression of stage-specific mRNAs in *Leishmania*. Specifically, we studied the degradation of the amastin mRNA, which was previously shown to be more abundant in amastigotes than in promastigotes (51). Further interest in undertaking this study stems from the observations that the amastin gene encodes a putative surface protein and is a member of multi-gene family with an expected role in the biology and/or pathobiology of the parasite (49,50).

In common with a number of other previously studied mRNAs, the stage-specific amastin mRNA is regulated via sequences in its 3'UTR at the level of mRNA stability. Sequence analysis of its 3'UTR revealed the presence of a U/C24 100 bases long U-rich sequence (URE) and our reporter mRNA analysis showed that this element contributes significantly to the stage-specific accumulation and degradation of the amastin mRNA. A similar role of UREs was previously shown for *T. brucei* and *T. cruzi* mRNAs [reviewed in (11,34)], suggesting that this mechanism is probably conserved in trypanosomatids. Noteworthy, the designation of this element as a URE, and its reference relative to elements in other eukaryotes, is solely based on the relatively high (50%) U-content of the region. Also, the specific regulatory region within the element, if distinct at all, might not necessarily be as U-rich.

The URE does not seem to account for all of the stage-specific regulation of the amastin mRNA. If any, the rest of the amastin mRNA regulation might be explained by other elements in its 1.8 kb 3'UTR. The 3'UTR of the amastin mRNA contains a sequence related to the
Leishmania specific SIDER1 retroelements (31). We have previously shown that the amastin SIDER1 alone does not alter reporter RNA levels (33). However, the possibility that it might have a context dependent function, perhaps in synergy with the URE, remains to be tested.

mRNA decay analysis of the amastin mRNA revealed a remarkable resemblance of degradation kinetics with that of unstable mRNAs in T. brucei. Unlike the degradation of the stable mRNAs, at least the initial phase of the degradation of unstable URE-containing mRNAs appears to be deadenylation-independent (24,29). At least at first glance, an alternative interpretation of the deadenylation analysis we employed in this study can be associated with the possibility that deadenylated species of the endogenous amastin or LUC-3'UTR mRNAs in promastigotes might be degraded too fast to be detectable. Our data do not rule out the singular possibility that deadenylated amastin mRNA species might be degraded faster in promastigotes. However, whether the differential degradation of the amastin mRNA is mediated through and/or preceded by undetectable deadenylation is altogether a different question. Several indications seem to go against this possibility. First, there are no appreciable levels of deadenylated species at time 0 and at early time points following actinomycin D treatment. Secondly, deadenylated species are actually detectable in the later time points (Figure 4B left panel and Figure 4C). Thirdly, as mRNAs get deadenylated, there must be a proportional decrease of the poly(A) plus mRNAs. This would imply that the remaining polyadenylated portion of the 3'-end fragments should disappear faster relative to the full-length mRNA. However, our results did not indicate that this is the case (Figures 3 and 4). In fact, the decay kinetics of the non-deadenylated 3'-end fragments was not distinguishable from those of the full-length, RNase-H-untreated mRNAs (Figure 4D). Fourthly, we did not observe detectable differences when we compared degradation from the 5' and 3'-ends in RNase H-RNA blots using an oligonucleotide that allowed cutting at ~800 bases upstream of the 3'-end and subsequent detection with region-specific probes (see Supplementary Data in Figure SI). Indeed, RNAi-mediated depletion of a homologue of a yeast deadenylase in T. brucei has minimal effects on the degradation kinetics of unstable mRNAs (including URE-mRNAs) while that of moderately and highly stable mRNAs is clearly altered (personal communication by Dr Clayton, ZMBH, Germany). These observations collectively compelled us to conclude that the URE-mediated promastigote-specific degradation of the amastin mRNA is deadenylation-independent. It is, therefore, tempting to suggest that the likely possibility that deadenylated species might be degraded faster in promastigotes can instead be viewed as further evidence to our conclusion; namely that URE amastin mRNAs are degraded faster in promastigotes regardless of whether they contain poly(A) or not (i.e. degradation is independent of deadenylation status).

Significant levels of deadenylated species are detectable at steady-state in both stages of the parasite. This is a surprising observation because deadenylated mRNAs are considered to be unstable. Interestingly, a recent global analysis of poly(A) length of mammalian mRNAs has revealed a surprisingly high proportion of the mRNAs (~25%) having oligoadenylated species with only <30 length of poly(A) (54). Even a more surprising finding of the study by Meijer et al. is that some stable mRNAs fall among these mRNAs. For example, 25% of the beta-actin mRNA, an mRNA known to be very stable, contained a short poly(A) tail. These observations collectively challenge the current notion that deadenylated mRNA species are unstable. A related observation in this study is that a higher proportion of deadenylated and oligoadenylated species are detected for stable mRNAs (the amastin or LUC-3'UTR mRNAs in amastigotes and the LUC-3'UTRAURE mRNA in both stages) than unstable mRNAs (the amastin and LUC-3'UTR mRNAs in promastigotes). This can be because the deadenylated species are degraded faster in promastigotes. Alternatively, this can be viewed as an indication for the possibility that the URE-mediated regulation of the amastin mRNA is not only deadenylation-independent but also polyadenylation-dependent. In other words, the poly(A) tail might have a destabilizing role rather than a stabilizing one. In bacteria, poly(A) tail serves as a ‘holding toe’ for 3'-exonucleases thereby facilitating degradation (55). However, this is not the case for almost all functional eukaryotic mRNAs studied so far. The few cases where poly(A) tail has a destabilizing function in eukaryotes fall within the realms of nuclear RNA degradation of certain pre-mRNAs and/or aberrant mRNAs as part of quality-control mechanisms (56–58).

Mechanisms of deadenylation-independent mRNA degradation, albeit rare, include deadenylation-independent decapping mechanisms such as the one associated with at least one pathway of the non-sense-mediated decay (59,60). Unfortunately, no clear homologue to any of the decapping enzymes from other eukaryotes has so far been identified in the genomes of trypanosomatids even though in vitro decapping activity was biochemically detected in a related species (23). In this study, we attempted to indirectly compare decapping rates by taking advantage of a 5'-phosphate-dependent enzyme, Terminator. This analysis did not show a significant difference between LUC-3'UTR and LUC-3'UTRAURE mRNAs in promastigotes suggesting the differential mRNA degradation is unlikely to be a function of an altered decapping rate. We, therefore, favor the scenario that an endonuclease-mediated cleavage might be the rate-limiting step, a well-studied example of which can be found in the degradation of the mammalian alpha-globin mRNA [reviewed in (61)]. Alternatively, a novel pathway might be awaiting a discovery.

Our current model of the amastin mRNA degradation based on this study, and in analogy with the situation in T. brucei, is that two mechanisms are likely to be involved: one that appears to be deadenylation- and decapping-independent, URE-mediated rapid degradation that is developmentally regulated, and the other is constitutive, which is likely to be slower and deadenylation-dependent (Figure 7). This study is the first not only in reporting a URE-mediated degradation in Leishmania but also in
attempting to draw the mechanistic basis of differential mRNA degradation in this parasite. The extent to which URE-mediated degradation differs from mammalian ARE-mediated mRNA instability and whether there are species-specific aspects of this mechanism among trypanosomatids remain to be determined.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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