Post-transcriptional Elements Regulating Expression of mRNAs from the Amastin/Tuzin Gene Cluster of Trypanosoma cruzi*

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The genome of Trypanosoma cruzi contains tandemly arrayed copies of the gene encoding amastin, an abundant protein on the surface of the amastigote stage of the parasite. The transcription rate of the amastin genes is the same in the different developmental stages, but the steady state level of the 1.4-kilobase amastin mRNA is 50-85 times higher in amastigotes than in epimastigotes or trypomastigotes (1). Here we show that the amastin genes alternate with genes encoding another protein, called tuzin, whose 1.7-kilobase mRNA is much less abundant in amastigotes. The 3'-untranslated region (UTR) of tuzin mRNA is only a few nucleotides in length or even nonexistent, in contrast with the 630-nucleotide 3'-UTR of amastin mRNA. No promoter elements were found upstream or within the amastin/tuzin gene cluster. However, in amastigotes, the protein synthesis inhibitor cycloheximide caused a 3-fold decrease in amastin mRNA and a 7-fold increase in tuzin mRNA. Furthermore, when the amastin 3'-UTR plus its downstream intergenic region were fused behind the luciferase coding region in a chimeric plasmid for transient transfections, luciferase activity increased 7-fold in amastigotes and decreased 5-fold in epimastigotes. Thus, developmental expression of these alternating genes is regulated by different mechanisms.

Trypanosoma cruzi, the protozoan parasite that causes Chagas’ disease, constitutes a major public health problem throughout much of Latin America. During its life cycle in the reduvid bug vector and a mammalian host, the parasite goes through two extracellular stages called the epimastigote and trypomastigote forms and one intracellular stage called the amastigote form. In mammalian hosts, the parasites exist mainly as intracellular amastigotes in a variety of cell types. Relatively little is known about the biochemical and immunological properties of this intracellular form compared with the epimastigote form, primarily for the practical reason that epimastigotes grow readily in cell-free culture, whereas amastigotes can only be obtained from infected animals or cultures of mammalian cell lines.

Recently, we showed that the amastigote form of T. cruzi has on its surface a family of closely related glycoproteins collectively called amastin whose mRNA level is at least 50-fold higher in amastigotes than in epimastigotes and trypomastigotes (1). The 174-amino acid sequence of nascent amastin has four distinct hydrophobic domains of 20-30 amino acids each, suggesting that the protein may span the outer membrane. The biological function of amastin is not known, but its abundance and surface location suggest that it plays an important role in the interaction between amastigotes and their cytoplasmic environment.

Amastin is encoded by eight or more tandem genes of 1.2 kb each that are located within 3-kb DNA repeats. These genes are constitutively transcribed at the same rate in all developmental stages, indicating that their elevated expression in amastigotes must be regulated post-transcriptionally (1). Many tandem genes in T. cruzi and other trypanosomatids, such as Leishmania species and African trypanosomes, are known to be transcribed into polycistronic precursor RNAs (2-7). These precursor transcripts are processed into individual mRNAs by intergenic cleavages followed by addition of a 39-nucleotide spliced leader (SL) to the 5' ends in a trans-splicing reaction and addition of a poly(A) to the 3' ends via polyanadenylation (8-10). Thus, if the amastin gene cluster is transcribed polycistronically, the post-transcriptional control of amastin mRNA abundance is likely to be regulated at these processing steps or by stability conferred on the mRNA by the 5'- or 3'-untranslated regions (UTRs).

Another unusual feature of gene expression in trypanosomatids is the apparent lack of promoters at the beginning of tandem gene clusters where transcription by RNA polymerase II could be initiated. To date, the only identified promoters for protein-encoding genes of trypanosomatids are in African trypanosomes. In these organisms, the promoters for genes encoding two different surface proteins, the variant surface glycoprotein (VSG) and the procyclic acidic repetitive protein (PARP), were detected by their ability to drive expression of a reporter gene in transfected cells (11, 12). However, these genes appear to represent a special case because their transcription is resistant to α-amanitin, suggesting that similar to rRNA genes, they are transcribed by RNA polymerase I or a modified form of RNA polymerase II (13, 14). In contrast, transcription of the amastin genes of T. cruzi and the other protein-encoding genes of trypanosomatids examined to date is inhibited by α-amanitin, indicating that they are transcribed by a conventional RNA polymerase II.

Two reports describing expression of foreign genes in T. cruzi indicated that the presence of properly oriented 5' sequences is
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essential for expression of a reporter gene. In one study a segment of the T. cruzi SL gene inserted in front of a chimeric neomycin phosphotransferase and chloramphenicol acetyltransferase (15), and in another report, expression of the neomycin phosphotransferase and chloramphenicol acetyltransferase genes was obtained following transfection with a plasmid bearing these genes flanked by regions of the glyceroldehyde-3-phosphate dehydrogenase gene (16). Since neither of these studies defined the sequences required for mRNA expression in T. cruzi, we undertook the present study to search for potential regulatory elements upstream of or within the amastin gene family. We discovered that the 3-kb repeats contain a previously unknown gene of 1.5 kb in addition to the 1.2-kb amastin gene, leaving intergenic regions (IRs) of only 111 and 145 bp between these two gene classes. The new gene potentially encodes a protein we have named tuzin for T. cruzi expressed protein. Since the steady-state levels of both amastin mRNA and tuzin mRNA vary dramatically among the three developmental stages, the signals for their respective post-transcriptional regulation likely occur within their UTRs or short IRs. We show here that indeed the 3’-UTRs and IRs contribute to the differential expression of amastin and tuzin mRNAs in the different developmental stages.

MATERIALS AND METHODS

Parasites—The Tulahuen strain of T. cruzi (17) was used for all experiments. Epimastigotes were maintained in logarithmic growth phase at 26°C in supplemented liver digest neutralized tryptose medium as described previously (18). Culture-derived trypomastigotes and amastigotes were obtained by infecting monolayers of the renal carcinoma cell line RA786 (19) grown in RPMI medium at 37°C and purified by centrifugation in metrizamide gradients as described previously (1). Hybridization to subcloned fragments 1–5 in Southern blots of Fig. 1 was carried out for 3 days at 65°C using 2–5× 10^6 cpm of labeled RNA that was purified using Sephadex G-50 QuickSpin columns (Boehringer Mannheim) in 5 mM of hybridization solution followed by filter washings as described previously (27).

DNA Transfections and Luciferase Assays—Plasmid DNAs were used for transfections of the same strain of T. cruzi. A 5′- and a 3′- probe, which is indicated in the legend between the amastin coding region (P1) and the 3′ end of the amastin coding region (P3). Primers P1 (see above) and P3 (5′-TGCTGTGCTGAATACAGGCTGCA-3′) were annealed to facilitate cloning. Finally, plasmid pHDT was constructed by deleting the 360-bp BamHI-PstI fragment containing the PARP 3′-UTR in the pHD1 plasmid (26) and inserting the 520-bp HindIII-Stul fragment containing the 3′-UTR of the TCR27 gene.

Transcription in Isolated Nuclei—Nuclei were isolated from epimastigotes, and nuclear run on transcripts were labeled with [α-32P]UTP as described previously (1). Hybridization to subcloned segments 1–5 in Southern blots of Fig. 1 was carried out for 3 days at 65°C using 2–5× 10^6 cpm of labeled RNA that was purified using Sephadex G-50 QuickSpin columns (Boehringer Mannheim) in 5 mM of hybridization solution followed by filter washings as described previously (27).

RESULTS

Identification of the 5′ End of the Amastin Gene Cluster—The amastin genes are located within tandem repeats of 3 kb in the genomes of several T. cruzi strains (1). To isolate the unique region immediately upstream of the amastin gene cluster, a genomic library of T. cruzi DNA was screened with a donned amastin cDNA. Several independent recombinant A.f. IX phage clones were identified and examined. All of them have 3-kb repeats, and one phage, α-A11, has an additional nonrepetitive region. Restriction digests, Southern blot analyses, and PCR amplifications were used to generate the restriction map shown in Fig. 1A. The 14-kb insert of phage α-A11 has two amastin genes and, as described below, 2.5 copies of the previously unknown tuzin gene. It also has 6.5 kb of unique sequence with T4 DNA polymerase and T4 DNA ligase resulted in a plasmid, named pLST, containing the 73-bp sequence immediately upstream of the amastin start codon. This 73-bp sequence includes the amastin 5′-UTR, a SL addition site and a polypyrimidine-rich region, and it precedes the luciferase gene in pLST.

To generate plasmid pLST containing the 5′-flanking region of the amastin cluster, a 2.9-kb HindIII-PstI fragment located upstream of the first amastin gene in recombinant phage α-A11 was used to replace a HindIII fragment containing the intergenic region in plasmid pLIT. Plasmid pL2T was prepared by replacing the same segment in pLIT with a 3-kb HindIII fragment in α-A11 containing both the amastin and tuzin genes. Plasmids containing the T. cruzi rRNA gene promoter (pLRT) were generated from plasmid pLS by inserting a 580-bp HindIII fragment into the unique HindIII site. This fragment was isolated from plasmid pTCB1S (24, 25). Plasmids pLRA and pLRC are derivatives of pLRT in which the TCR27 3′-UTR was replaced by fragments corresponding to the 3′-UTR and polyadenylation sites of an amastin gene and a tuzin gene, respectively. The amastin 3′-UTR fragment was obtained by digesting plasmid pAB3 with SphI and MscI, generating an 860-bp fragment containing the complete 3′-UTR plus a 200-bp segment spanning the polyadenylation site and SL addition site of the downstream tuzin gene. The tuzin 3′-UTR was obtained by PCR amplification of plasmid pAB3 using primers corresponding to the 5′ end of the amastin coding region (P1) and the 3′ end of the tuzin gene (P3). Primers P1 (see above) and P3 (5′-TGCTGTGCTGAATACAGGCTGCA-3′) were annealed to facilitate cloning. Finally, plasmid pHDT was constructed by deleting the 360-bp BamHI-PstI fragment containing the PARP 3′-UTR in the pHD1 plasmid (26) and inserting the 520-bp HindIII-Stul fragment containing the 3′-UTR of the TCR27 gene.
**A**

Diagram of the 5' region of the amastin/tuzin gene cluster and the sequence of an interior tuzin gene plus its flanking regions.

- Large and small shaded rectangles indicate the amastin coding regions and 3'9-UTRs, respectively.
- Open rectangles represent the tuzin genes, whose 3'9-UTRs of 0–20 bp are too small to show here.
- Restriction sites are for BamHI (B), BglII (Bl), EcoRI (E), PstI (P), and SphI (S).
- The open arrowhead indicates the position at which the sequence preceding the entire gene cluster diverges from the sequence preceding the interior tuzin genes and corresponds to the open arrowhead in panel B. The genomic DNA segment in bacteriophage clone λ-A11 is denoted by the hatched line.
- Three fragments derived from either the 5'9 end or interior regions of the gene cluster that were used in the transfection experiments shown in Fig. 4 are denoted by the patterned boxes.
- Lines labeled 1–5 indicate fragments that were probed in the nuclear run on experiment shown in Fig. 3. B, the sequence of a 2411-bp region between the coding regions of adjacent amastin genes with the amastin stop and start codons at the 5' and 3' ends, respectively. The start and stop codons of the intervening tuzin gene are denoted by the underlying black box and black oval, respectively.
- Boxed AG dinucleotides indicate SL addition sites. Polypyrimidine sites upstream of these SL sites are also boxed. Black arrowheads indicate post-transcriptional regulatory elements in T. cruzi.

**B**

- Sequence of a 2411-bp region between the coding regions of adjacent amastin genes with the amastin stop and start codons at the 5' and 3' ends, respectively.
- The start and stop codons of the intervening tuzin gene are denoted by the underlying black box and black oval, respectively.
- Boxed AG dinucleotides indicate SL addition sites. Polypyrimidine sites upstream of these SL sites are also boxed.

**C**

- Sequence of a 2411-bp region between the coding regions of adjacent amastin genes with the amastin stop and start codons at the 5' and 3' ends, respectively.
- The start and stop codons of the intervening tuzin gene are denoted by the underlying black box and black oval, respectively.
- Boxed AG dinucleotides indicate SL addition sites. Polypyrimidine sites upstream of these SL sites are also boxed.

**Fig. 1.** Diagram of the 5' region of the amastin/tuzin gene cluster and the sequence of an interior tuzin gene plus its flanking regions. A, large and small shaded rectangles indicate the amastin coding regions and 3'9-UTRs, respectively. Open rectangles represent the tuzin genes, whose 3'9-UTRs of 0–20 bp are too small to show here. Restriction sites are for BamHI (B), BglII (Bl), EcoRI (E), PstI (P), and SphI (S). The open arrowhead indicates the position at which the sequence preceding the entire gene cluster diverges from the sequence preceding the interior tuzin genes and corresponds to the open arrowhead in panel B. The genomic DNA segment in bacteriophage clone λ-A11 is denoted by the hatched line. Three fragments derived from either the 5' end or interior regions of the gene cluster that were used in the transfection experiments shown in Fig. 4 are denoted by the patterned boxes. Lines labeled 1–5 indicate fragments that were probed in the nuclear run on experiment shown in Fig. 3. B, the sequence of a 2411-bp region between the coding regions of adjacent amastin genes with the amastin stop and start codons at the 5' and 3' ends, respectively. The start and stop codons of the intervening tuzin gene are denoted by the underlying black box and black oval, respectively. Boxed AG dinucleotides indicate SL addition sites. Polypyrimidine sites upstream of these SL sites are also boxed. Black arrowheads indicate post-transcriptional regulatory elements in T. cruzi.
upstream of the first gene in the cluster. The sequence extending from the first EcoRI site indicated in Fig. 1A through most of the first amastin gene was determined. This sequence has a point mutation in the first amastin gene that abolishes an EcoRI site located 172 bp downstream of the start codon in all but one of the amastin cDNAs previously sequenced (1). Moreover, the presence of a 5.5-kb EcoRI fragment at the 5’ end of the cluster is consistent with the results of Southern blots of T. cruzi genomic DNA probed with amastin cDNA, in which weakly hybridizing EcoRI fragments of 5.5 and 1.0 kb were observed (not shown).

In addition to the above characterization, 3-kb fragments from BgIII- or EcoRI-digested T. cruzi DNAs were eluted from an agarose gel and ligated into pBluescript. After colony hybridization with an amastin cDNA, two plasmids with 3-kb inserts, named pAB3 and pAE3, were chosen for study. In both inserts, the sequence between the stop codon of the upstream amastin gene and the start codon of the downstream amastin gene was determined, and this segment of the pAE3 insert is shown in Fig. 1B. This intergenic sequence was compared with the sequence upstream of the first amastin gene in the cluster. The two sequences are completely different upstream of the open arrowhead shown in Fig. 1, A and B, and nearly identical downstream of this position. In the intergenic sequence shown in Fig. 1B, distinctive poly pyrimidine tracts occur just upstream of the point of divergence. Poly pyrimidine tracts have been found to be essential for trans-splicing of the SL in Trypanosoma brucei and Leishmania enrietti (29, 30), and this motif provided the first hint that the 3-kb repeats might contain another gene besides the amastin gene.

Another Gene Is Located Between Adjacent Amastin Genes—The distance between the 3’ poly(A) addition site of one amastin gene, and the 5’ SL addition site of the adjacent downstream amastin gene is about 1,743 bp (these addition sites vary slightly in different cDNAs). Analysis of this sequence in pAE3 revealed the presence of a 447-codon open reading frame potentially encoding a 45-kDa protein that we have named tuzin. The deduced amino acid sequence, shown in Fig. 1C, had no substantive similarity to protein sequences in the data bases in April, 1995. Southern blots of partially restricted genomic DNAs probed with this coding region confirmed that the tuzin and amastin genes alternate head-to-tail in the same 3-kb repeats (not shown). The corresponding sequence of the 3-kb repeat in pAB3 as well as partial sequences of several tuzin cDNAs (see below) indicate that, similar to amastin genes, the tuzin genes are heterogeneous in sequence with frequent base substitutions.

To determine if the tuzin region is transcribed, we prepared Northern blots of RNAs isolated from the epimastigote, amastigote, and trypomastigote stages. The blot shown in Fig. 2 was probed in succession with the coding regions for tuzin (panel B), amastin (panel A), and 24 S, rRNA (31) (panel C). The amastin and tuzin RNAs are 1.4 and 1.7 kb, respectively, indicating from a comparison with their respective gene lengths that each has a 3’ poly(A) tail of about 200 nucleotides. A densitometer tracing of a short exposure of the autoradiogram shown in panel A indicated that the ratio of amastin RNA in epimastigotes, amastigotes, and trypomastigotes is 1:85:4.8, respectively. The corresponding ratio for the tuzin RNA was 1:3.5:0.1, whereas that for rRNA was 1:1:1, demonstrating that equal amounts of total RNA were added to each lane. In addition, the tuzin autoradiogram shown in Fig. 2 was exposed about 10 times longer than the amastin autoradiogram, indicating that in the amastigote and trypomastigote stages amastin RNA is much more abundant than tuzin RNA. Our earlier measurements suggested that amastin RNA is about 50-fold more abundant in amastigotes than in epimastigotes (1), but the difference between that value and the 85-fold found here could reflect differences in the growth phases of the different developmental stages when the RNA was collected. Thus, it is likely that the two alternating gene classes are transcribed into large polycistronic precursor RNAs, which are processed into amastin and tuzin mRNAs whose steady state levels are quite different in the amastigote and trypomastigote stages but much more similar in the epimastigote stage. Since the specific activities of the radioactive amastin and tuzin gene probes used in panels A and B may be different, we cannot use the Northern blots to quantitate the relative abundances of amastin and tuzin mRNAs in epimastigotes. However, about the same number of amastin and tuzin cDNA clones were detected in a T. cruzi epimastigote cDNA library, suggesting that the levels of their corresponding mRNAs are similar at this developmental stage.

To map the 5’ and 3’ ends of the tuzin mRNA, five tuzin cDNAs were isolated from about 10,000 clones of an amastigote cDNA library. The same number of library clones yielded more than 100 amastin cDNA clones, consistent with the Northern blot that suggested amastin mRNAs are about 20 times more abundant in amastigotes than tuzin mRNAs. Partial sequencing of the five tuzin cDNA clones demonstrated that in four cases the polyadenylation sites were located either seven or 20 nucleotides beyond the stop codon, and in one case polyadenylation occurred at the A residue within the UAG stop codon itself, as indicated in Fig. 1B. The RNA molecule that gave rise to this latter cDNA apparently did not have any 3’-UTR nucleotides other than the poly(A), but its translation termination was preserved because the UAG was converted to a UAA stop codon.

Since none of the five tuzin cDNAs contained a 5’ SL, PCR amplification was performed on an sample of the cDNA library
using a SL primer and a primer complementary to nucleotides 886–906 in the tuzin coding region. The sequence of the resulting 140-bp amplification product demonstrated that the AG dinucleotide at position 777 serves as the main splice acceptor site and the start codon, so some tuzin mRNAs might have alternative SL addition sites as is the case with amastin mRNAs (1).

Search for a Transcription Initiation Site at the 5' End of the Amastin/Tuzin Gene Cluster—Since the amastin and tuzin genes are transcribed to an equal extent in all developmental stages (1), we examined the region at the 5' end of the amastin/tuzin gene cluster for a site where this transcription might begin. Nuclear run on assays were conducted using nuclei prepared from epimastigotes, and 32P-labeled nascent RNAs were used to probe Southern blots containing DNA fragments 1–5 indicated in Fig. 1A. Fig. 3 shows that no detectable transcription occurs from fragments 1 and 2, which contain approximately 3 kb of unique sequence located upstream of the first tuzin gene in the cluster. As expected, strong hybridization to fragments 3–5, which contain the tuzin and amastin genes, occurred. Strong hybridization also occurred to control DNA fragments containing a rRNA gene and the TCR27 gene, which we have previously shown to be a single copy gene encoding a T. cruzi cytoskeletal protein (22, 23, 32). Thus, if a unique transcription start site exists for this gene cluster, it is located within the approximately 400-bp sequence between the upstream EcoRI site indicated in Fig. 1A and the start of the first tuzin gene. Further efforts to demonstrate transcription within this region were not successful. Therefore, it is not possible to distinguish from these experiments whether transcription begins a short distance upstream of the first gene and extends through the cluster or whether transcription initiation occurs at one or more sites within the cluster. Hybridization of nuclear run on RNA to the individual strands of the amastin gene cloned into bacteriophage M13 demonstrated that only the coding strand of these genes is transcribed (1), so at the minimum there must be a signal that imparts a directionality to the transcription of this gene cluster.

Identification of 5' Sequences Important for Expression Using Transient Transfection Assays—Since the approximate location of a promoter for the amastin/tuzin gene cluster was not identified in the nuclear run on experiments, we conducted transient transfections to look for 5' sequences important for amastin and tuzin expression using a series of constructs derived from pGEM-luc (Promega). This plasmid contains a luciferase reporter gene flanked by restriction sites into which different fragments were inserted. In the plasmids tested, a 520-bp fragment that includes the entire 3'-UTR and 135 bp beyond its polyadenylation site was cloned downstream of the luciferase gene. T. cruzi epimastigotes were transfected with 100 μg of each of the depicted plasmids and assayed for luciferase activity 48 h later.

No luciferase activity above background was detected when unmodified pGEM-luc or pLT, a derivative containing the TCR27 3'-UTR, were introduced. However, when a 73-bp seg-
ment containing the polyprimidinetracts and SL addition site immediately preceding the amastin coding region was placed in front of the luciferase gene of pLT, a 400-fold increase in luciferase activity over the background level was detected (pLST). This result provides strong evidence that a SL addition site is necessary for the production of luciferase mRNA, and this 73-bp segment was included in all subsequent plasmid constructs.

The three plasmids shown in the middle of Fig. 4 (pL3T, pL2T, and pLIT) each contain a different region of the amastin/ tuzin gene cluster cloned in front of the luciferase gene and the 73-bp segment. Thus, if any of these three regions contains promoter activity, the amount of luciferase detected when its plasmid is introduced into T. cruzi epimastigotes should increase relative to that of pLST. As indicated in Fig. 1, the cloned region in pL3T includes the first tuzin gene in the gene cluster plus about 1.5 kb of unique sequence preceding this gene. The region in pL2T is a complete internal 3-kb repeat including an amastin gene, a tuzin gene, and their downstream IRs. The third region, cloned in pLIT, contains only a tuzin gene and its 5′- and 3′-flanking intergenic regions. For each of these constructs, the amount of luciferase activity is about 700-fold above background (range = 692–747-fold) compared with 400-fold above background for pLST. This 1.75-fold increase in luciferase activity might be due to a small amount of promoter activity or due to the presence of additional sequences upstream of the 73-bp segment that also influence splicing. In either event, no region that clearly contains substantial promoter activity was detected (see Fig. 5 for an example of a T. cruzi DNA sequence that does have promoter activity). In particular, note that in pL3T the 1.5-kb region immediately upstream of the gene cluster does not have promoter activity. Regions still further upstream were not investigated because the nuclear run on experiments (Fig. 3) indicated that these regions, encompassing fragments 1 and 2 shown Fig. 1, were not transcribed. When the orientation of these three fragments was reversed, the luciferase activity was about the same as that for pLST (not shown).

The only trypanosomatid in which promoters for protein-encoding genes have been identified is the African trypanosome, T. brucei, and in these cases the promoters have been shown to be resistant to α-amanitin, which is a characteristic of RNA polymerase I promoters (11–14). Since our results suggest that the signals for SL addition alone are sufficient for luciferase activity in transfected epimastigote cells, we tested whether the promoter for one such α-amanitin resistant gene of African trypanosomes, the PARP gene (12, 26), would stimulate luciferase expression in T. cruzi. As shown at the bottom of Fig. 4, plasmid pHDT has a 290-bp fragment containing the PARP promoter and SL addition site (26) inserted upstream of the luciferase gene, and the 3′-UTR of the TCR27 gene inserted downstream. This plasmid directs about 900-fold more luciferase activity than background, or only 1.3-fold (900– versus 700-fold) more than pLIT, pL2T, or pL3T, which contain regions derived from the amastin/tuzin gene cluster. Although it is not possible to interpret unambiguously the result of this heterologous PARP transfaction, it does suggest that the 290-bp fragment of T. brucei contributes functional SL addition signals for the luciferase gene but little, if any, promoter activity in T. cruzi. Thus, a promoter for a protein-encoding gene of African trypanosomes is not recognized as a promoter by epimastigotes of T. cruzi.

The Role of the Amastin and Tuzin 3′-UTRs Plus IRs in the Developmental Expression of Their RNAs—The 3′-UTR of amastin mRNA (630 nucleotides) is larger than its coding region (525 nucleotides). The presence of this large 3′-UTR in the abundant, amastigote-specific amastin mRNA and a small or nonexistent 3′-UTR in the less abundant tuzin mRNA, suggests that the amastin 3′-UTR might be important in the control of the stage-specific expression of amastin. This prediction is consistent with the fact that all developmentally regulated genes studied so far in trypanosomatids appear to be controlled at least in part by post-transcriptional events (26, 33, 34). Thus, we examined whether the amastin and tuzin 3′-UTRs plus their downstream IRs affect expression of the luciferase gene when transfected into T. cruzi amastigotes and epimastigotes. Transfections into trypanosomatids were not attempted because of difficulties in obtaining sufficient numbers of trypanosomatids for this type of experiment.

Unfortunately, repeated attempts to transiently transfec amastigote cells under a variety of electroporation conditions with plasmids pLIT and pLST shown in Fig. 4, or with corresponding plasmids containing the amastin 3′-UTR + IR, did not detect any luciferase activity above background (not shown). These negative results suggest that electroporation is much less efficient for transfection of amastigotes than for epimastigotes. Therefore, we had to construct an improved vector that directed much higher levels of luciferase activity. The new construct was based on the observation that in African trypanosomes high expression levels of a reporter gene are only achieved using promoters for genes encoding rRNA, PARP, and the VSG, each of which is a promoter for RNA polymerase I or a RNA polymerase-II-like enzyme. Since the promoter for a T. cruzi rRNA gene has recently been reported (24, 25), we inserted a 580-bp fragment containing this promoter in front of the amastin SL addition site of pLST and obtained the results shown in Fig. 5. The presence of this T. cruzi rRNA promoter increased the amount of luciferase activity in transfected epimastigotes so dramatically that we reduced the amount of plasmid in a typical transfection from 100 to 20 μg and conducted the luciferase assays with a 1:50 dilution of the cell extracts. Thus, the results with pLST shown in Fig. 5 are not directly comparable with the results shown in Fig. 4 because of the differing transfection and assay conditions.

When transfected into epimastigotes, pLRT containing the T. cruzi rRNA promoter and the 3′-UTR of the TCR27 gene stimulated about 2,050 times more luciferase activity (271,098 versus 132-fold) than did the corresponding plasmid without the promoter (pLST) or with the promoter in the opposite orientation. When transfected into amastigotes, pLRT containing the correctly oriented promoter resulted in only a 94-fold increase in luciferase activity rather than the 2,050-fold increase seen in epimastigotes, consistent with the prediction that transfection efficiency is much lower in amastigotes than in epimastigotes. However, this value in amastigotes still represents a substantial number of luciferase units above background (15,000 versus about 170), so plasmids in which luciferase expression is driven by the rRNA gene promoter were used to compare the effects of different 3′-UTRs + IRs. As shown in Fig. 5, in epimastigotes the 3′-UTR of the constitutively expressed TCR27 gene (pLRT) conferred the highest level of luciferase expression, whereas the presence of the amastin 3′-UTR + IR (pLRA) resulted in a 5-fold drop in luciferase expression. The opposite effect was seen in amastigote cells. Luciferase activity was 7.2-fold higher with the amastin 3′-UTR + IR than with the TCR27 3′-UTR. As indicated in the right-hand column of Fig. 5, the resulting normalized difference between amastigotes and epimastigotes is 36-fold (5 × 7.2), which is not much less than the 50–85-fold difference in the steady-state level of amastin mRNA observed on Northern blots (see Fig. 2). Likewise, for the tuzin 3′-UTR + IR, the normalized ratio in luciferase activity between amastigotes and
epimastigotes is 3, consistent with the Northern blot in Fig. 2, indicating that there is 3.5 times more tuzin mRNA in amastigotes than epimastigotes. Furthermore, in amastigotes, the presence of the tuzin 3'-UTR + IR (pLRC) had the opposite effect of the amastin 3'-UTR IR on luciferase activity. When transfected into amastigotes, the tuzin region caused the luciferase activity to drop to 0.3 of that expressed with the TCR273-UTR, whereas the amastin 3'-UTR + IR increased it by 7.2-fold. In contrast, in epimastigotes the tuzin and amastin 3'-UTRs + IRs had similar affects. Compared with the TCR27 3'-UTR, the tuzin 3'-UTR + IR caused a 10-fold drop, and the corresponding amastin region caused a 5-fold drop. Thus, the tuzin 3'-UTR + IR resulted in a lower level of the chimeric luciferase mRNA in both epimastigotes and amastigotes, whereas the amastin 3'-UTR + IR caused an increase in amastigotes and a decrease in epimastigotes.

The Effect of Inhibition of Protein Synthesis on the Steady State Levels of Amastin and Tuzin mRNAs—Inhibitors of protein synthesis have been shown to affect the steady-state levels of mRNAs encoding several mammalian proteins (35), gp63 in Leishmania (36), and PARP in African trypanosomes (37). For the gp63 and PARP genes, the 10-fold or more increase in their mRNAs in the presence of cycloheximide suggests that a labile negative regulator might target these transcripts for degradation (36, 37). Since gp63 and PARP are both abundant stage-specific surface proteins, as is amastin (1), we decided to investigate the effect of cycloheximide on the accumulation of amastin and tuzin transcripts in T. cruzi. A cycloheximide concentration of 250 ng/ml has been shown previously to inhibit protein synthesis in T. cruzi by 97% (38).

T. cruzi epimastigotes and amastigotes were incubated in the presence of either 200 or 500 ng/ml cycloheximide for 4 h and total RNA isolated for Northern blots that were probed in succession with the coding regions of tuzin, amastin, and rRNA (Fig. 6). Again, trypomastigotes were not used because of the difficulty in obtaining a sufficient number for this experiment. After this normalization, the relative signal intensities in panels A and B were determined and several conclusions were drawn. First, the two different cycloheximide concentrations had very similar effects in all cases, so the RNA levels in the two concentrations could be averaged. Second, the tuzin autoradiogram shown in Fig. 6 was exposed about 10 times longer than the amastin autoradiogram, again reflecting the lower abundance of tuzin mRNA. Third, in amastigotes the effect of cycloheximide on amastin RNA and tuzin RNA was quite different. At this developmental stage, the drug caused a 3-fold drop in amastin RNA and a 7-fold increase in tuzin RNA. The magnitude of this 7-fold increase is not readily apparent in panel B because of the adjustment that must be made for more RNA in the 0 drug lane of amastigotes as shown in panel C. Fourth, in epimastigotes the presence of cycloheximide did not affect substantively the steady-state level of amastin mRNA (i.e. 1.3-fold more with cycloheximide), whereas the tuzin mRNA level increased by 4.3. Finally, no differences were observed between 4 h of incubation in cycloheximide (Fig. 6) and 2 or 8 h of incubation (not shown).

The interpretation of these cycloheximide results is tempered by several factors (see “Discussion”), but one clear observation is that in amastigotes the inhibition of protein synthesis...
be important. This region contributes to the differential expression of the three gene classes encoding the surface protease gp63 of Leishmania chagasi, whereas the 3′-UTRs by themselves have little effect (34). Likewise, within the IR following the dihydrofolate reductase-thymidylate synthase gene of Leishmania major, and between the β- and α-tubulin genes of T. brucei, the SL addition site of the downstream gene directs the location of the upstream gene's poly(A) addition site (8, 48).

Amastin mRNA has a short 5′-UTR (17 nt) and a long 3′-UTR (630 nt), whereas tuzin mRNA has a long 5′-UTR (137-nucleotide) and a short (~20-nucleotide) or nonexistent 3′-UTR. None of these UTRs nor the intervening IRs of 145 and 111 bp display any obvious sequence similarities, consistent with the possibility that these sequences participate in the differential expression of amastin and tuzin mRNAs. In the case of tuzin mRNA, its short or nonexistent 3′-UTR suggests that this sequence does not contribute much to the differential regulation of tuzin mRNA. The complete absence of a 3′-UTR in one tuzin cDNA and the creation of its UAA stop codon by 3′-polyadenylation is reminiscent of a similar phenomenon in mammalian mitochondrial DNA in which the termination codons in the mRNAs of 9 of the 13 protein-encoding genes are created during poly(A) addition (49).

Two general models have been proposed to account for those cases in other biological systems where cycloheximide affects the level of an mRNA in a post-transcriptional manner (42, 50). One model invokes the existence of a labile regulatory protein that either stabilizes a specific mRNA or targets it for degradation (50). The other model, shown for mammalian β-tubulin mRNA, involves the co-translational degradation of mRNAs by a ribosome-associated RNase (51, 52). In T. cruzi amastigotes, cycloheximide caused a decrease in the abundant amastin mRNA and an increase in the less abundant tuzin mRNA, consistent with the presence of both the positive and negative regulatory aspects of the first model. In epimastigotes, the cycloheximide had no effect on amastin mRNA and caused an increase in tuzin mRNA similar to that observed in amastigotes. This result suggests that the cycloheximide-affected mechanism regulating tuzin mRNA levels may be the same in amastigotes and epimastigotes, whereas the cycloheximide-affected mechanism contributing to the elevated amastin mRNA level in amastigotes does not operate in epimastigotes. More experiments will be required to elucidate the details of these cycloheximide-affected mechanisms, including measurements of the mRNA decay rates and the use of other translation inhibitors such as pactamycin or puromycin that act on different steps in protein synthesis than does cycloheximide (53).

Transient transfections of the luciferase reporter gene containing the different 3′-UTRs + 1Rs also indicated that different regulatory mechanisms control the amastin and tuzin mRNA levels (Fig. 5). From these experiments, we cannot determine whether it is the 3′-UTRs or the 1Rs, or both, that are responsible for this difference. However, when similar reporter gene transfections were conducted in Leishmania using the different 3′-UTRs and the downstream 1Rs of the three gp63 gene classes of L. chagasi, it was found that the 3′-UTRs and the 1Rs each contribute to the differential expression of the three gene classes (34). By analogy, it seems likely that a similar scenario will hold for the differential expression of the amastin/tuzin gene cluster.

Finally, the deduced amino acid sequence of tuzin (Fig. 1C) does not have any substantive similarities with the protein sequences currently in the data bases. In contrast to amastin, which is an extremely hydrophobic protein, tuzin is a highly charged molecule lacking a signal peptide or other sequence motifs that might provide insight into its cellular location and
biological function. The relatively low abundance of its mRNA suggests that tuzin is a rare protein that will be difficult to detect in Western blots of subcellular fractions or in cellular immunolocalization assays. It also is difficult to say whether the alternating arrangement of the amastin and tuzin genes means that their gene products are associated physiologically or structurally. Other known co-transcribed, multi-gene clusters of trypanosomatids encode proteins such as ubiquitin and calmodulin (6), a phosphatase, and an RNA polymerase subunit (7), and the VSG, a transferrin receptor and adenylate cyclase (54). It is possible that these gene products are associated, i.e. the phosophatase could act on the RNA polymerase, or the transferrin and adenylate cyclase might be attached to the same membrane as the VSG, but in no case has such a relationship been demonstrated. Thus, the reason for the alternating nature of the amastin and tuzin genes remains unclear.

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REFERENCES

1. Teixeira, S. M. R., Russell, D. G., Kirkhoff, L. V., and Donelson, J. E. (1994) J. Biol. Chem. 269, 20509–20516
2. Mühich, M. L., and Boothroyd, J. C. (1988) Mol. Microbiol. 2, 569–571
3. Zomerdijk, J. C. M. B., Ouellette, M., ten Asbroek, L. M. A., Kieft, R., Bommer, M. A. M., Clayton, C. (1993) Mol. Biochem. Parasitol. 57, 317–330
4. Dietrich, P., Soares, M. B., Affonso, M. H. T., and Floeter-Winter, L. M. (1993) Mol. Biochem. Parasitol. 62, 237–245
5. Dietrich, P., Soares, M. B., Affonso, M. H. T., and Floeter-Winter, L. M. (1993) Mol. Biochem. Parasitol. 62, 237–245