Expression of trans-Sialidase and 85-kDa Glycoprotein Genes in *Trypanosoma cruzi* Is Differentially Regulated at the Post-transcriptional Level by Labile Protein Factors*

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To adapt to different environments, *Trypanosoma cruzi*, the protozoan parasite that causes Chagas’ disease, expresses a different set of proteins during development. To begin to understand the mechanism that controls this differential gene expression, we have analyzed the levels of amastin and trans-sialidase mRNAs and the mRNAs encoding members of the 85-kDa glycoprotein gene family, which are differentially expressed in the *T. cruzi* stages found in the mammalian host. *Amastin* mRNA is expressed predominantly in intracellular and proliferative amastigotes. *Trans-Sialidase* mRNAs are found mostly in forms undergoing transformation from amastigotes to trypomastigotes inside infected cells, whereas mRNAs encoding the 85-kDa glycoproteins appear only in the infective trypomastigotes released from the cells. The genes coding for these mRNA species are constitutively transcribed in all stages of *T. cruzi* cells, suggesting that expression is controlled post-transcriptionally during differentiation. Inhibition of transcription by actinomycin D revealed that each mRNA species has a relatively long half-life in stages where it accumulates. In the case of the trans-sialidase and 85-kDa glycoprotein genes, mRNA accumulation was induced by treatment with the protein synthesis inhibitor cycloheximide at the stages that preceded the normal accumulation. Therefore, mRNA stabilization may account for mRNA accumulation. mRNA degradation could be promoted by proteins with high turnover, or stabilization could be promoted by forming a complex with the translational machinery at defined times in development. Identification of the factors that induce mRNA degradation or stabilization is essential to the understanding of control of gene expression in these organisms.

Gene expression in *Trypanosoma cruzi* as well as in other trypanosomes is largely controlled at the post-transcriptional level (1–6), although there are a few exceptions where promoters and transcriptional activation have been described (7). Well defined RNA polymerase II initiation sites have not been found, and most genes are transcribed as part of polycistrionic units and are processed by trans-splicing (8, 9). During processing, capped spliced leader sequence is added to the 5′-end, and a polyadenosine tail is added to the 3′-end of mRNA (10), probably ensuring mRNA stability and transport to the cytoplasm (11). In particular, correct splicing and polyadenylation, as well as the existence of specific 3′-untranslated portions of the transcribed genes, have been shown to promote either mRNA stability (12) or an increase in the translational efficiency (13). However, the nature of controlling factors and how environmental modifications induce differential expression remain obscure.

*T. cruzi* provides an attractive model to study how modifications in the environment induce differential gene expression. In the insect host, the epimastigote form of the parasite proliferates in the gut. In the posterior gut, where the nutrients become scarce, the parasite transforms into metacyclic trypomastigote forms, which are nonproliferative and highly infective to the mammalian host. Within the mammalian host, the parasite proliferates only inside the cell cytoplasm, as amastigote forms. Once the infected cell is full of parasites, and probably deprived of nutrients, the amastigote form transforms into nonproliferative and infective trypomastigote forms, which rupture the cell, escaping to the bloodstream. The differentiation between noninfective and infective forms is related to the shutdown of the proliferative machinery and the expression of a set of genes involved in cell invasion and induction of resistance to host defenses (14, 15). These changes are correlated to the activation of adenylate cyclase (16–21) via transducing pathways involving G-proteins (22, 23), phospholipase C (20), and calcium internalization (24). The molecules expressed during this phase of differentiation are members of a large family of 85-kDa glycoproteins (14) that are localized on the surface of infective forms and are thought to participate in adhesion to the host cell surface during invasion (25–27). The large family of 85-kDa glycoproteins, collectively baptized as gp85 (14), encompasses a subfamily of several members, earlier defined as Tc85, that are capable of binding to wheat germ agglutinin (28) and are recognized by a specific monoclonal antibody (29). The most acidic component of the Tc85 subfamily was characterized as a laminin ligand (30), which may be directly involved in the interaction of trypomastigotes with the mammalian cell (25, 29).

Another component expressed in large amounts in differ-
Entiatated trypanomastigotes is the enzyme trans-sialidase (TS). This enzyme transfers host sialic acid to abundant surface molecules characterized as mucin-like glycoproteins (31-34). which form a protective coat on the trypanomastigote surface. Several pieces of evidence suggest that TS is involved in cell invasion (35) and escape from the phagosome (36) and is an important virulence factor mediating the immune response to the parasite in the mammalian host (37, 38). TS is encoded by a family of ~80 genes that have a common amino-terminal domain, which includes the catalytic portion of the enzyme, and a variable number of 12-amino acid repeats in the carboxy-terminal region (39).

To investigate how expression of the Tc85 and TS genes is regulated during T. cruzi differentiation, the steady-state levels of mRNA coding for these proteins have been analyzed. This study revealed that the corresponding mRNAs accumulate at different time periods during the differentiation of intracellular amastigotes to extracellular trypanomastigotes. The time of accumulation depends on the number of infected parasites per cell, suggesting that interaction with the host cell environment is involved in the control of TS and Tc85 gene expression. To initiate a systematic characterization of the controlling mechanism, the rate of transcription in lysosome-permeabilized parasites and the mRNA stability in the presence of transcription and translation inhibitors have been studied.

**EXPERIMENTAL PROCEDURES**

**Parasites**—The Y strain (40) of T. cruzi was used in this study. Epimastigote forms were maintained in liver infusion tryptose medium containing 10% fetal bovine serum at 28 °C (41). Intracellular amastigotes, intracellular intermediate forms, and trypanomastigotes were obtained from infected L6E6 cells (American Type Culture Collection, Rockville, MD) grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 28 °C (41). Intracellular amastigotes (shown in Fig. 1) a probe for the amastin gene, which was a 1.8-kilobase fragment derived from plasmid pDS154-0 (46); 3) a probe corresponding to the catalytic domain of TS, which consisted of an NcoI-PstI fragment derived from plasmid pTc85 (45); 4) a probe for a- and b-tubulin, which was a 2.3-kilobase BamHI fragment derived from plasmid pDC1 (provided by Yara Traub-Cseko, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil (48); and 5) a probe for the amastin gene, which was a 1.8-kilobase XbaI-XhoI fragment derived from plasmid pTc433 (provided by Dr. Santos Teixeira (49)). The DNA fragments were purified by elution after agarose gel electrophoresis and radiolabeled with random hexanucleotide priming with DNA polymerase I (Klenow fragment) and [y-32P]dCTP (3000 Ci/mmol) for 3 h at room temperature. At the end of this period, the labeled probes were purified from free nucleotides by Sephade G-50 spin columns.

**Transcription in Permeable Cells**—T. cruzi parasites in different stages were collected and washed as described (50). Briefly, parasites (2 × 10^8) were washed twice at room temperature with 20 mM potassium glutamate, 3 mM MgCl₂, 150 mM sucrose, 10 μg/ml leupeptin, and 1 μM dithiothreitol (transcription buffer) and resuspended in 800 μl of the same buffer. The parasite suspension was chilled on ice for 5 min, and 100 μl of sialic acid phenol/guanidine isothiocyanate; and the labeled RNA was recovered as described above. The labeled RNA was then hybridized in 5 × saline/sodium phosphate/EDTA, 5 × Denhardt's solution, 0.1 mg/ml yeast tRNA, and 0.1% SDS to dot blots containing 5 μg of the indicated DNA. For preparation of the dot blots, plasmid DNA was denatured in 0.3 M NaOH for 30 min at 55 °C, neutralized by adding ammonium acetate to 2 ×, and loaded onto cellulose membranes using a minifilter fractionation apparatus. The following plasmids were linearized and adsorbed to the filters: 1) pTc185 (18 S ribosomal gene (51), 2) pTS87 (TS catalytic domain), 3) pTCTR (TS repeat), 4) pTc85 (amastin), 5) pDC1 (tubulin), 6) pTc85 (Tc85), 7) bluscript SK- containing the region between the open reading frame of two TS genes (TS intergenic); this was generated by polymerase chain reaction amplification of a cosmid containing TS genes (COS-7) (39) using 5'-GGCCGCCT-GACCCCTGTGTTCCCTCGGTT-3' and 5'-GGCCGCTCAGTGA GTAGTGGAGCTCCTC-3' (as primers), and 8) a plasmid generated by the removal of the internal KpnI fragments of plasmid 154 (46) (this contains the 89 base pairs upstream of the splice site of TS (TS 5'-end)). The filters were washed with 6 × SSC, incubated 2 h at 80 °C, and prehybridized with the hybridization solution described above. Hybridization was carried out for 4 h at 65 °C, then the filters were washed three times at 65 °C with 2 × SSC and 0.1% SDS and once with 2 × SSC and treated with 10 μg/ml RNase A to reduce nonspecific hybridization. The membranes were then exposed to x-ray films.

**RESULTS**

The morphology of T. cruzi stages found during differentiation of intracellular amastigotes to extracellular trypanomastigotes is shown in Fig. 1. As the infected cell becomes full of amastigotes (shown in Fig. 1a), intracellular parasites start to elongate, and a clear flagellum can be recognized (Fig. 1b). However, these intermediate forms are morphologically dis-
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Fig. 1. Morphological differences of T. cruzi developmental stages. Intracellular amastigotes (a), intermediate forms (b), released trypomastigotes (c), and cultured epimastigotes (d) were fixed with formaldehyde, stained with 4,6-diamidino-2-phenylindole, and observed under fluorescent light (left) and phase contrast (right). The arrows indicate the nuclei, and the arrowheads indicate the kinetoplast in selected images. Bar = 2 μm.

Distinct from the released trypomastigotes. They show a migration of the flagellum insertion point (flagellar pocket) to the anterior end of the parasite, as is typical of trypomastigote forms (Fig. 1c). The kinetoplast (mitochondrial DNA) follows the apparent migration of the flagellar pocket and is found at several orientations with respect to the nucleus. Mechanically released intermediate forms do not swim and are clearly different from the other stages. Intermediate forms are also distinct from epimastigotes, which have the kinetoplast in a position similar to intracellular amastigotes (Fig. 1d). To follow the molecular events occurring during this differentiation process, expression of the amastin, TS, and Tc85 genes was studied in typical amastigotes, in intermediate forms, and in trypomastigotes released from infected cells. Trypomastigotes released from over-infected cells were discarded, as they contained round forms, morphologically similar to amastigotes.

By measuring the steady-state levels of the transcripts corresponding to amastin, TS, and Tc85 mRNAs in these different stages, we found that amastin mRNA is mainly expressed in intracellular amastigotes, as shown previously (5). TS mRNA is mainly expressed in the intermediate forms, and Tc85 mRNA is detected only in extracellular trypomastigotes (Fig. 2). All these mRNAs are weakly expressed in epimastigotes. Tubulin mRNA levels are approximately constant in epimastigotes and intermediate forms and slightly reduced in amastigotes and trypomastigotes. The reduced levels of tubulin mRNA in amastigotes may be related to this being a spherical stage; thus, amastigotes contain less tubulin. In released trypomastigotes, the reduced levels of tubulin mRNA may be related to the decrease in the overall amount of mRNA in this differentiated nondividing stage. In fact, large numbers of trypomastigotes have been used to obtain equivalent amounts of total RNA compared with the other stages. When the same number of parasites, instead of the same amount of total RNA or poly(A)+ RNA of each stage, were used in the blot, similar qualitative results were obtained (data not shown). In this case, TS mRNA was found also mainly in intracellular trypomastigotes, and Tc85 mRNA only in extracellular trypomastigotes.

To investigate at which level the expression of the TS and Tc85 genes is regulated, we have analyzed transcription of these genes in lysolecithin-permeable parasites. Under these conditions, nascent RNAs are elongated and processed by trans-splicing and polyadenylation (50). As shown in Fig. 3a, transcription occurred intensely in all stages for the TS, amastin, and Tc85 genes. There was no decrease in transcription of the genes that showed diminished steady-state levels, suggesting that these genes are constitutively transcribed to similar extents in all developmental stages. As TS genes are organized in long tandem repeats, with each open reading frame separated by ~2 kilobases (Fig. 3c), the possibility of transcription along the intergenic region was also examined. As shown in Fig. 3b, comparable levels of transcription were found in coding and noncoding regions of TS genes. Moreover, no mature mRNA was detected at any stage using the intergenic region to probe Northern blots (data not shown), suggesting that this region probably does not produce stable mRNA.

Our results indicate that TS genes are highly expressed in the transition forms arising from differentiation between amastigotes to trypomastigotes, just before parasites are released from the infected cell. This finding suggests that the TS gene expression mechanism is associated with the differentiation process. Two possibilities could explain this association: 1) TS gene expression is a programmed event and occurs after a given number of amastigote divisions; and 2) TS gene expression is activated by a mechanism that depends on factors produced by the infected cell. Such factors would become available when a certain degree of infection is reached, and this would induce the accumulation of TS mRNA. To distinguish between these two possibilities, L6E6 cells were infected with increasing numbers of parasites, and after different periods of time, the percentage of intermediate forms and the level of TS mRNA were measured in the same amount of intracellular parasites. The time of appearance and the prevalence of intermediate forms were dependent on the size of the initial inoculum. The larger the infective load, the sooner intermediate forms were dependent on the size of the initial inoculum. The larger the infective load, the sooner intermediate forms were detected, at least 48 h after infection (Fig. 4). Furthermore, the accumulation of TS mRNA paralleled the accumulation of intermediate forms. This suggests that the switch from amastigote to trypomastigote depends on the parasite burden within the infected cells and does not seem to be programmed by the number of cell divisions of the amastigote forms.

Treatment with actinomycin D, which blocks transcription in T. cruzi (52), was used to study the stability of amastin, TS, and Tc85 mRNAs during intracellular development of the parasite. Amastigote, intermediate, and trypomastigote forms were treated for different incubation periods with actinomycin D, and the amount of each mRNA species was studied by Northern blot analysis. The concentration of actinomycin D used in the experiments is known to block transcription in all stages of T. cruzi (5). The RNA loaded in each lane corre-

FIG. 2. Steady-state levels of amastin, TS, and Tc85 mRNAs during T. cruzi development. 5 μg of total RNA from epimastigotes (E; equivalent to 1 × 10⁶ parasites), intracellular amastigotes (A; equivalent to 2 × 10⁶ parasites), intracellular intermediate forms (IT; equivalent to 1 × 10⁷ parasites), and trypomastigotes (T; equivalent to 4 × 10⁸ parasites) was fractionated on formaldehyde-containing 1% agarose gels; transferred to nylon membranes; and probed successively with an α/β-tubulin coding sequence, a DNA probe coding for amastin, and a probe coding for the Tc85 glycoprotein subfamily. The numbers on left represent the sizes of RNA markers (in kilobases (Kb)).
responded to total RNA isolated from $1 \times 10^8$ extracellular trypomastigotes, $3 \times 10^7$ amastigotes, or $3 \times 10^7$ intermediate forms. As shown in Fig. 5, amastin mRNA was quite stable in amastigotes with a half-life of 2–3 h, whereas tubulin mRNA had a shorter half-life (data not shown). Very little TS and Tc85 mRNAs were detected in amastigotes, and therefore, their half-lives could not have been estimated accurately. In contrast, TS mRNA was stable in intermediate forms (half-life longer than 4 h) (Fig. 5b). In this case, tubulin mRNA decayed much faster (half-life of ~1 h; data not shown).

The mRNA stability of the amastin, TS, and Tc85 genes was examined in trypomastigotes released from infected cells. In the absence of actinomycin D, the level of mRNAs coding for the TS gene decreased, whereas the Tc85 mRNA levels were constant in recently released trypomastigotes (Fig. 6). These trypomastigotes were collected no more than 3 h after they were released from the infected cell. When the parasites were incubated in the presence of actinomycin D, Tc85 mRNA was the most stable (half-life of ~3 h), followed by TS mRNA (half-life of 1.5 h) and tubulin mRNA (half-life of 0.7 h) (Fig. 6).
In trypanomastigotes collected after 12 h, the levels of TS, Tc85, and tubulin mRNAs rapidly decayed (half-life of ~0.7 h; data not shown).

In higher eukaryotes, the abundance of some unstable mRNAs (e.g., encoding histones, oncogene products, and cytokines) can be increased dramatically by incubating cells in the presence of protein synthesis inhibitors (53), and blocking protein synthesis can reveal at what stage the control is exerted in the gene expression pathway. Thus, we studied the effect of cycloheximide, an inhibitor of polypeptide chain elongation, on the levels of TS and Tc85 mRNAs during intracellular development of T. cruzi. Different parasite stages were incubated in the presence of 250 ng/ml cycloheximide, which blocks protein synthesis in the T. cruzi stages studied (5, 43); after different periods of treatment, the total RNA was extracted, and the amounts of TS and Tc85 mRNAs were analyzed by Northern blotting. In the amastigote forms, TS (but not Tc85) transcripts increased with time in the presence of the drug (Fig. 7, a and b). Tc85 RNA was not detected in amastigotes even after long exposures. In contrast, cycloheximide did not affect the level of TS mRNA, but increased the amount of Tc85 transcripts by a factor of 3 in intermediate forms after 2 h of treatment (Fig. 7, c and d). In intermediate forms, TS RNA was stable. The addition of cycloheximide to recently released trypomastigotes promoted an increase in TS mRNA levels and only a small increase in Tc85 mRNA levels (Fig. 7, e and f). Thus, cycloheximide induces strong accumulation of mRNA species mainly in the developmental stages preceding those in which expression of a specific mRNA is maximal.

**DISCUSSION**

We have shown that during the intracellular development of T. cruzi, mRNAs coding for amastin, TS, and Tc85 are sequentially expressed during intracellular differentiation of amastigotes to trypomastigotes. The amastin mRNA level is high in proliferating amastigotes; TS mRNA is found mainly in the intermediate forms undergoing transformation from amastigotes to trypomastigotes; and Tc85 mRNA is observed only in extracellular trypomastigote forms. Fig. 8 schematically represents a model for the timing of the expression of these three genes. It is noteworthy that TS gene expression anticipates the expression of Tc85, suggesting that these two proteins act at different times, despite the fact that both have been implicated in cell invasion. It is important to note that the amount of mRNA may not reflect directly the level of protein or enzymatic activity. Nevertheless, TS might be mostly required at the moment the parasite is released from the infected cell, whereas Tc85 may be important for the adhesion to new host cells, as suggested before (25, 54). This hypothesis agrees with findings that recently released parasites are poorly invasive, and invasion competence is acquired after a certain time (55). Expression of some members of the 85-kDa glycoprotein gene family might be related to the acquisition of this invasive capacity (26). In addition, the presence of TS in recently released parasites could be important to promote parasite exit from the infected cell, spreading from the local site of infection, and access to the bloodstream.

The data we present indicate that the morphological transformations observed during parasite development occur together with the differential expression of the amastin, TS, and Tc85 genes, and we propose that this process is influenced by parasite-host cell interactions. The results shown in Fig. 4 suggest that the number of T. cruzi divisions in the host cell does not control expression of these genes since intermediate forms and TS mRNA appear earlier in infection when the cells are infected with a higher number of parasites. We found that the larger the number of infecting parasites, the earlier we could detect intermediate forms and the accumulation of TS mRNA. The nature of the signals that induce the transformation and accumulation of TS mRNA is unknown. They might be related to the limitation of nutrients, as shown for the differentiation of epimastigotes to metacyclic forms (56). Alternatively, products derived from the host cell might act directly or indirectly as signals for differentiation. For example, peptides generated by the catabolism of hemoglobin are able to induce
activation of the epimastigote adenylate kinase via a G protein pathway (23). Furthermore, an increase in cAMP seems to promote differentiation into infective metacyclic forms, whereas activation of protein kinase C seems to be important for T. cruzi proliferation (18, 20).

We have provided evidence supporting the notion that differential expression of the TS and Tc85 genes is mostly controlled at the post-transcriptional level, as shown for several other genes differentially expressed in trypanosomes. The transcription assays using permeable cells suggest that transcription is constitutive for all these genes, and that the TS gene is transcribed as a polycistronic RNA. In addition, inhibition of transcription by actinomycin D revealed that amastin, TS, and Tc85 mRNAs are stable in the developmental stages where they are known to accumulate. Specifically, amastin mRNA is stable in amastigotes; TS mRNA is stable in intermediate forms; and Tc85 mRNA is stable in recently eclosed trypomastigotes. On the other hand, we found that at least in trypomastigotes, TS mRNA decays faster than tubulin mRNA, indicating that at this stage, TS mRNA is unstable. The data presented derive from trypomastigotes recently released from infected cells. However, when trypomastigotes age, the amount of RNA decreases due to a general decrease in transcription. Thus, decay of TS and Tc85 mRNAs is much faster in trypomastigotes that are collected 12 h after eclosion (data not shown), even in absence of actinomycin D, in agreement with the fact that the level of these mRNAs is substantially decreased in aged trypomastigotes. The highly invasive forms that arise after this aging period are known to have much lower levels of cellular RNA than at other parasite stages.

The mechanism that promotes TS and Tc85 mRNA stabilization could occur by the removal of labile factors. Protein synthesis inhibition by cycloheximide treatment shows that the level of TS mRNA increases in amastigotes and that Tc85 mRNA increases in intermediate stages. In both cases, the increase is observed immediately before the stage at which stabilization occurs. The addition of cycloheximide at the stages where mRNA levels normally start to decrease also results in some accumulation. Just after the period in which Tc85 mRNA stabilization occurs, a small increase in mRNA accumulation was observed (see Fig. 7f), but after longer incubation, the levels of mRNA rapidly decreased, suggesting that other factors may be present to promote degradation and removal of the stable mRNAs. At the stages that show maximal RNA accumulation, the stabilization could be additionally supported by formation of stable polysomes. The effect of cycloheximide in this case could be a consequence of inhibition of translation, without release of the mRNA from the polysomes, as found in other trypanosomes. Procyclin-encoding mRNA accumulates in bloodstream forms of Trypanosoma brucei after treatment with cycloheximide (57). In Leishmania, protein synthesis inhibition induces accumulation of the mRNA encoding the major surface glycoprotein (gp63), which has been shown to be negatively regulated by a labile, sequence-specific protein that targets this RNA for rapid degradation (58). Recently, it has been shown that inhibition of proteasome function prevents transformation in T. cruzi (59), arguing that stabilization of labile proteins can also influence differentiation.

RNA stability seems to be mediated by the interaction of molecules with regulatory elements present in the 3′-untranslated region (3′-UTR) of mRNAs. These elements have been identified in T. brucei procyclin mRNA as 16-mer stem-loop and 26-mer polypyrimidine tract sequences in the 3′-UTR, which, upon interaction with certain proteins, promote stabilization and translation of the target RNA, respectively (12, 13). In T. cruzi, the stability of mRNA coding for reporter genes is also modulated by the 3′-UTR of some members of the 85-kDa glycoprotein family (6). 3′-UTRs from members of the gp85 gene family (Tri4c-1, SA85.1, and Tsa-1), which accumulate in trypomastigotes, exhibit an inhibitory effect on the expression of reporter genes in epimastigotes, metacyclic trypomastigotes, and amastigotes, but not in trypomastigotes. This probably represents a common mechanism to down-regulate gp85. The 3′-UTR of amastin mRNA, which is constitutively transcribed, also decreases the steady-state levels of reporter mRNAs 6–14-fold in epimastigotes as compared with epimastigote-specific 3′-UTR sequences. This inhibitory effect is not observed in amastigotes in which amastin mRNA accumulates (5).

We cannot exclude the possibility that a mechanism besides mRNA stability controls the expression of TS and Tc85. In fact, control at the translational level has been found for Tc85 (6). The rates of transcription initiation, elongation, trans-splicing, polyadenylation, and RNA transport could also participate in the control. For example, binding of splicing factors to unprocessed RNAs could inhibit RNA export from the nucleus, leading to rapid degradation, as shown in other eukaryotes. An increase in transcription initiation and elongation rate, although not detected using the lysolecithin-permeable cell assay, could also modulate expression.

In summary, these results suggest that stage-specific proteins expressed in the intracellular forms of T. cruzi are post-transcriptionally regulated by factors involving stabilization of mRNAs. The differential expression of the TS and Tc85 genes also provides a convenient system to study the signaling mechanism occurring in T. cruzi differentiation. Expression of these genes can be measured after addition of molecules that interfere with signaling mechanisms known to induce differentiation through an increase in cAMP levels (17, 18, 20, 21, 60–63), induction of calcium transients (62, 63), or inhibition of protein kinases.

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