Developmental stages of the trypanosome life cycle differ in their morphology, biology, and biochemical properties. Consequently, several proteins have to be tightly regulated in their expression to allow trypanosomes to adapt rapidly to sudden environmental changes, a process that might be of central importance for parasite survival. However, in contrast to higher eukaryotic cells, trypanosomes do not seem to regulate gene expression through regulation of transcription initiation. These parasites make use of post-transcriptional regulatory mechanisms and modification of mRNA half-life is a relevant one. Trans-acting factors binding to cis-elements that affect mRNA stability of mature transcripts have not been identified in these cells. In this work, a novel U-rich RNA-binding protein (TcUBP-1) from *Trypanosoma cruzi*, the agent of Chagas disease, was identified. Its structure includes an RNA recognition motif, a nuclear export signal, and auxiliary domains with glycosyl- and glutamine-rich regions. TcUBP-1 recognizes the 44-nucleotide AU-rich RNA instability element located in the 3′-untranslated region of mucin SMUG mRNAs (Di Noia, J. M., D’Orso, L., Sanchez, D. O., and Frasch, A. C. (2000) J. Biol. Chem. 275, 10218–10227) as well as GU-rich sequences. Over-expression of TcUBP-1 in trypanosomes decreases the half-life of SMUG mucin mRNAs in vivo but does not affect the stability of other parasite mRNAs. Because TcUBP-1 is developmentally regulated, it might have a relevant role in regulating protein expression during trypanosome differentiation, allowing a correct expression pattern of U-rich-containing mRNAs.

Messenger RNA degradation/stabilization is one of the mechanisms that control gene expression in higher eukaryotic cells (1). One well characterized cis-element that regulates mRNA stability is an AU-rich element (ARE) found in the 3′UTR of short-lived mRNAs (2, 3) such as those of proto-oncogenes and cytokines (interleukin-1, -2, -3, and -10) (4–6). Each ARE represents a combination of functionally and structurally distinct sequence motifs such as the AUUA pentamer, the UUUUAUAUA(U/A) nonamer, and stretches of uridines and/or U-rich domains that range in size from 50 to 150 bp. It was shown that ARE-directed mRNA decay is linked to cell transformation, cell growth and differentiation, cell adhesion, and immune response regulation (7). An effect of the ARE on translation efficiency was also described both positively (8) and negatively (9). RNA-binding proteins that exhibit affinity for, and interact with, ARE sequences have been identified in higher eukaryotes. Many of these factors contain highly conserved RNA binding domains that place them within RNA recognition motif (RRM) superfamily (10). Two kinds of trans-acting factors were described that have opposite functions. The ELAV protein HuR increases stability of ARE-containing mRNAs when over-expressed in transfected fibroblasts (11). Conversely, AUF-1/hnRNP D (12) mediates a destabilizing activity of the mRNAs containing ARE sequences within their 3′UTR (13). It was proposed that ELAV proteins may bind the ARE-containing mRNAs in the nucleus and transport them to the cytoplasm, where they either find access to the translational apparatus or are released for rapid degradation (14). Other hnRNPs, such as hnRNP A1 and C (15), hnRNP A0 (16), and some RNA-binding proteins displaying catalytic activities such as glyceraldehyde-3-phosphate-dehydrogenase (17), AUH, an enoyl-CoA hydratase (18), were also shown to be AU-rich RNA-binding proteins.

Trypanosomes, protozoan parasites from the order Kinetoplastida, cause widespread diseases in humans, domestic animals, and wildlife that include infections of medical and veterinary importance. Studies of trypanosomes have revealed a number of unusual mechanisms occurring during transcription and RNA maturation. Among them are the RNA editing in mitochondrially encoded transcripts (19), polycistronic transcription, and trans-splicing. Polycistronic transcripts are processed to mature mRNAs through trans-splicing on the 5′-end and poly(A) addition on the 3′-end (20). This scenario makes it difficult to consider that trypanosomes regulate protein expression through regulation of transcription initiation, because unrelated mRNAs might be present in the same polycistron (21). In addition, RNA polymerase II promoters have been difficult to detect in trypanosomes. Two putative promoter regions were identified in the SMUG and the SMUG mucin (Di Noia et al., 2000) gene families.
described as transcriptionally void regions upstream from the highly transcribed actin and Hsp70 genes (22, 23). However, one example of RNA polymerase II promoter was recently identified in the spliced leader genes of the parasite Leptomonas seymouri (24). Thus, trypanosomes are interesting organisms in the analysis of post-transcriptional regulatory mechanisms affecting mRNA expression patterns (25). Cis-regulatory elements altering the half-life of mature mRNAs have been identified, most of them located on the 3′-untranslated regions (3′UTR) (26, 27). One example is the 16-mer loop localized in the 3′UTR of procyclic mRNAs of Trypanosoma brucei that confers mRNA stability and improves translation efficiency (28). Also, different 3′UTRs and intergenic regions from trypanosome mRNAs were shown to influence luciferase expression by changing the steady-state level and/or the translation efficiency of a luc mRNA reporter (29). Trypanosoma cruzi transcribers, such as the surface proteins PL-160 (30), amastin (27), gp72, and gp85 (29), also contain regulatory sequences that modulate mRNA stability. Although these results suggest the possible importance of cis-acting elements in mRNA stability or instability, the trans-acting factors involved in these processes have not yet been identified in trypanosomes.

*T. cruzi* is the protozoan parasite agent of Chagas disease. The parasite has two hosts, an insect vector and mammals (including man). The parasite is covered by highly O-glycosylated mucins that are regulated developmentally. The stage of the parasite present in the insect vector (epimastigote) expresses members of a small mucin family named TcSMUG whose core proteins are encoded in about 70 different genes (31). The forms of the parasite present in the bloodstream of the mammalian host have larger mucins (60–200 kDa), compared with 35–50 kDa in epimastigotes) encoded by about 500 different genes (32, 33). Developmentally regulated expression of these mucins in the different parasite stages is relevant because they might accomplish different functions in relation to parasite survival (34). In previous work, we have identified an ARE on the 3′UTR of the mRNAs encoding the core protein of *T. cruzi* small mucins (TcSMUG), which affects mRNA stability. Transfection experiments, using a cat reporter gene flanked by mucin intergenic regions bearing or not ARE sequences within its 3′UTR, showed that this cis-element destabilizes mucin mRNAs in a stage-specific manner (31). These results suggest that different trans-acting factors might bind mucin transcripts in vivo and selectively regulate the stability in a different manner throughout parasite development (35).

In this work, we have identified a gene encoding a developmentally regulated U-rich RNA-binding protein named TcUBP-1. It specifically recognizes the 44-nucleotide SMUG mucin AU-rich mRNA instability element previously described by us (31), as well as GU-rich sequences. Transfection experiments allowed us to demonstrate that TcUBP-1 is able to selectively destabilize SMUG mucin transcripts in vivo but not mRNAs lacking AU-rich elements within their 3′UTR. These results suggest that TcUBP-1 is involved in the regulation of mRNA expression mediated by U-rich elements.

**EXPERIMENTAL PROCEDURES**

**Parasite Cultures and Drug Treatments**—*T. cruzi* CL-Brener cloned stock (36) was used. Different forms of the parasites were obtained as described previously (37). Epimastigote cultures were taken in logarithmic growth phase at a cell density of 3 × 10†/ml and treated with actinomycin D (Sigma) at a final concentration of 10 μg/ml, which is known to inhibit transcription in trypanosomatids (28, 38).

**DNA Constructions and Parasite Transfections**—TcUBP-1 open reading frame (GenBank accession number AF372519) was cloned in the BamHI and EcoRI restriction sites of pRIBOTEX vector (39), kindly provided by Dr. R. Hernandez (Universidad Nacional de Mexico). Transfections were carried out as described previously (31).

**Network Blot Analysis**—RNA was purified using TRIzol reagent following the manufacturer’s instructions (Life Technologies, Inc.). Northern blots were carried out as described (40). The SMUG and 24SorRNA DNA probes were obtained as described previously (31). Ductin DNA probe was obtained from a clone with high homology to mammalian ductin. Hsp70 probe was obtained from the E. coli clone number TENG-0042 (GenBank accession number A1034982).

**Southern Blot Analysis**—Genomic DNA of *T. cruzi* CL-Brener clone was digested with the indicated restriction enzymes (New England Biolabs). The DNA fragments were separated by electrophoresis in 0.8% agarose gel, transferred by capillarity (41) to Zeta Probe Nylon-N membranes (Bio-Rad), and UV cross-linked using a UVP CL-1000 cross-linker (Stratagene). These products were hybridized with the probes described below (UTR and 5′UTR) using hybridization solution containing 0.5 M NaHPO₄, 7% SDS, 1 mM EDTA, and 1% bovine serum albumin, at 58 °C for low stringency conditions and 65 °C for high stringency conditions. The RR probe was made from clone TcUBP-1 by polymerase chain reaction using oligonucleotides NH₂-L (5′-CGGGATCCATGAGCCAAATT- GGTGGTTC-3′) and RNP-II (5′-CGGAAATGCTACCCCTGAGG-3′). Probe AUX was made using oligonucleotides NES-sec (5′-AACACGCGTTAGGATCCG-3′) and COO-HL (5′-CGGAATTTCACTTCGACAAGGGCGCC-3′) and was labeled with [32P]dCTP (PerkinElmer Life Sciences).

**Reverse Transcription-Polymerase Chain Reaction**—Reverse transcription was performed on total RNA from the epimastigote stage of the parasite using the Superscript II kit following the manufacturer’s instructions (Life Technologies, Inc.). Primer oligo(dT)₅ (5′-GGCAGTCTGCGGCGCGCCG(T)₃′) was used for CDNA synthesis. The first polymerase chain reaction was performed using the oligonucleotides ME (5′-TACAGTTCTTCTGACTATATTG-3′) and RNP-I (5′-AAATTCTCAAATCATGACG-3′). These products were cloned in pGEMT-Easy vector (Promega) and sequenced.

**In Vitro Transcription**—All plasmids for in vitro transcription were constructed as follows. Complementary DNA oligonucleotides, corresponding to the sense and antisense strands of each RNA, were annealed and cloned into the EcoRI and HindIII sites of the vector pBS(–) (Stratagene). All of the RNA probes had the same flanking region, GGATCC, in the 5′-end and one A at the 3′-end. Transcription of sense and antisense RNAs was performed on total RNA from the epimastigote stage of *T. cruzi* using the T7 RNA-polymerase (Promega) in the presence of [α-32P]UTP (800 Ci/mmol, PerkinElmer Life Sciences) and 500 μM ATP, CTP, and GTP. All transcripts were purified on an 8% urea-12% polyacrylamide gel.

**Protein Extract Preparation**—For total extract preparation, parasites were resuspended in lysis buffer (0.75% CHAPS detergent, 1 mM MgCl₂, 1 mM EDTA, 5 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 7.6, and 10% glycerol) and supplemented with protease inhibitors phenylmethylsulfonyl fluoride, 1 mM, and *trans*-epoxysoycycnil-t-leucyl-aminomethyl-4guanidino), 50 μM (Sigma). After 30 min on ice, the extract was centrifuged at 10,000 × g and the supernatant was stored at −70 °C.

**Recombinant Protein Expression and Purification**—TcUBP-1 cDNA was cloned into the BamHI and EcoRI restriction endonuclease sites of the pGEX-2T vector (Amersham Pharmacia Biotech) generating a glutathione S-transferase (GST) fusion and transformed in Escherichia coli strain DH5αF′. Cultures were induced with 0.2 mM isopropyl β-d-thiogalactopyranoside for 3 h at 37 °C. Recombinant proteins were purified using GST-agarose columns (Sigma).

**Analysis of RNA-Protein Interactions**—Binding reactions were performed with different amounts of TcUBP-1 protein, 10,000 cpm of RNA probe, 10 mM Tris-HCl (pH 7.6), 5% glycerol, 100 mM KCl, 5 mM MgCl₂, 1 μg/ml bovine serum albumin, and 50 ng/μl tRNA (Sigma) in a 20-μl final volume reaction mixture and incubated for 10 min at 37 °C. When indicated, heparin was added at a concentration of 1 μg/ml. Each reaction was loaded directly onto a 7% polyacrylamide gel (acrylamide/bisacrylamide, 38:2) 0.5× TBE (Tris borate-EDTA), to perform an electrophoretic mobility shift assay (EMSA). For competition assays, TcUBP-1 recombinant protein was incubated simultaneously with the indicated amounts of unlabeled and labeled RNAs. All homoribopolymers were from Sigma. For the determination of the *K*₅₀ between TcUBP-1 and different RNAs, increasing amounts of protein were incubated with the labeled RNA and resolved as described above.

**Antibody Production and Western Blot Analysis**—Purified GST-TcUBP-1 was injected into mice with Freund’s complete adjuvant at 3-week intervals. For Western blot analysis, protein samples fractionated by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech), probed with anti GST-TcUBP-1 antibodies, and developed using horseradish peroxi-
idase-conjugated anti-rabbit antibodies and the Supersignal® West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer’s instructions. Signals were quantitated using the 1D Image Analysis Software (Kodak Digital Science).

RESULTS
Identification of a Putative U-rich RNA-binding Protein in T. cruzi—The presence of functional AU-rich elements in mucin mRNAs from trypanosomes (31, 35) suggests the existence of trans-acting factors binding to these elements. During a search for new T. cruzi genes (42), we identified ESTs and Genome Sequence Surveys having homologies with eukaryotic RNA-binding proteins. One of these cDNA clones has a deduced amino acid sequence with identities with Sex lethal (SXL) (43), Rbp9 (44), and members of the ELAV family (45) (Figs. 1 and 2). The putative protein encoded by this T. cruzi cDNA clone, for which the complete sequence was obtained by reverse transcription-polymerase chain reaction, was named TcUBP-1 for U-rich RNA-binding protein. This 1017-base pair clone had a 638-base pair coding region. TcUBP-1 presents a short Gln-rich amino-terminal composed of 34 amino acids followed by one RNA recognition motif having the canonical RNP-2 and RNP-1 motifs (46). The RRM motif from the T. cruzi protein (Fig. 1, A and B) highly resembles those present in higher eukaryote RNA-binding proteins, such as Rbp9, HuC, and SXL among others (Fig. 2A). The similarity between them ranges between 40–55%, and the RNP-1 and -2 motifs are the most conserved regions. This degree of conservation might be because of the tertiary structure of the RRM motif required for RNA recognition (47). After the RRM motif, a putative NES, or LEU-rich nuclear export signal (48), is present (Fig. 1). This region has three of the four LEU expected to be conserved (49) as compared with the functional NES sequences present in MAPK, mPKIα, HIV1-Rev, and cyclin B1 proteins (50) (Fig. 2B). Toward the carboxyl terminus, two auxiliary domains are present. First is a short Gly-rich region that resembles the repeats in hnRNP A1; an ARE-binding protein in which the intracellular localization determinant for targeting it to the nucleus has been identified in the Gly-rich domain of the protein (51). Second, a glutamine-rich carboxyl-terminal region is present. Both Gly- and Gln-rich auxiliary domains, like the ones present in TcUBP-1, were described as being involved in protein-protein interactions in other eukaryotic cells (52).

TcUBP-1 Belongs to a Gene Family Having Conserved RNP Motifs—A Southern blot of T. cruzi genomic DNA using the RRM region as a probe (Fig. 3A) revealed a number of bands under low stringency, whereas only a few bands were observed under high stringency conditions (Fig. 3B, panels I and II, respectively). A probe for the auxiliary region gave similar results, although fewer bands were observed under low stringency conditions (data not shown). These results indicated that TcUBP-1 might be part of a gene family whose members have greater homologies in the RRM motif than in the auxiliary domains. Two clones related in sequence and structure to TcUBP-1, containing the same RRM motif and different auxiliary domains, were recently identified in our laboratory.3 Searches in the Genome Sequence Survey and EST T. cruzi and T. brucei data bases were conducted using the RRM sequence. Four new sequences were identified (GenBankTM accession numbers AZ050633, AQ638822, AQ637856, and AQ654405). These clones share about 35–50% identity in the RRM motif, and the RNP-1 and RNP-2 motifs are the most conserved region of the proteins. Additionally, a search in the Sanger Center Leishmania major and T. brucei (www.sanger.ac.uk/Projects/) data bases was performed. Two clones highly similar to TcUBP-1 were identified (GenBankTM accession numbers AQS45665 and 0.15543) (see Fig. 3C). The similarity index between them is about 70%, the RRM motif and the putative NES sequence being the most conserved regions of the proteins. These results suggest that highly similar sequences of

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3 I. D’Orso and A. C. C. Frasch, unpublished data.
TcUBP-1 are conserved in different protozoan parasite species.

The 44-nt Mucin AU-rich RNA Instability Element Is Recognized in Vitro by TcUBP-1—We have shown that a 44-nt AU-rich cis-element localized on the 3′/H11032 UTR of the TcSMUG mucin family was important in conferring mRNA instability in a stage-specific manner (31). To determine whether TcUBP-1 recognizes this RNA sequence (named SMUG-L-AU), an in vitro binding reaction was performed (Fig. 4A). The in vitro transcribed SMUG-L-AU RNA was incubated with GST, GST-TcUBP-1, or without protein, and the reactions were resolved in a native EMSA. TcUBP-1 bound to the SMUG-L-AU RNA and the binding was not affected by the addition of heparin, an unspecific competitor. Control experiments using GST protein were negative (Fig. 4A). The ribonucleoprotein complex formed by TcUBP-1 and the SMUG-L-AU RNA template was efficiently competed with poly(U) but not with poly(A) or poly(C) RNA homopolymers. Poly(G) somewhat reduced the binding of TcUBP-1 to the RNA probe but much less efficiently than poly(U) (Fig. 4B). The binding of TcUBP-1 to the SMUG-L-AU probe seemed to be to a single RNA site, because only one shifted band was obtained within a large range of TcUBP-1 protein amount (Fig. 4C). We also searched for the minimal sequence requirement for TcUBP-1 to bind the SMUG-L-AU RNA. Three RNAs from SMUG-L-AU were used to perform in vitro binding and EMSA (Fig. 4D). The binding to the 31-nt SMUG-L-AU-1 RNA was reduced as compared with the complete SMUG-L-AU RNA, showing that the two AUUUUA motifs in the boundaries of this RNA are important for an efficient binding of TcUBP-1. The binding to the UUAUUUAU nonamer (SMUG-L-AU-2 RNA) was detectable only after over-exposing the film. This interaction was slightly improved when the nonamer was present within a short U-rich context, as happens with the SMUG-L-AU-3 RNA, which has two additional uridines outside the nonamer RNA oligonucleotide (Fig. 4D). Thus, the entire 44-nt SMUG-L-AU RNA seems to be required for an efficient binding, since deletion of some U-rich stretches within this sequence greatly affects TcUBP-1 recognition.

Because a number of T. cruzi transcripts have AU-rich sequences within their 3′/H11032 UTR, we tested whether TcUBP-1 specifically recognized the SMUG-L-AU RNA or was also able to recognize AU-rich sequences present in other mRNAs. We have previously identified mRNAs bearing AU-rich sequences in their 3′UTRs, like phospolipase-1 (PLC-AU), hexose transporter (HEX-T-AU), and the mucin SMUG-S group (SMUG-S-AU) among others (31). RNA probes from these AU-rich sequences were prepared and analyzed by EMSA. TcUBP-1 clearly recognized AU-rich regions within the 3′UTR of SMUG-L and -S mucin transcripts, having AUUUA, AUUUUA, and AUUUUUA motifs (Fig. 4D). On the other hand, TcUBP-1 failed to recognize either the hexose transporter AU-rich region (HEX-T-AU RNA) or a poly-(AU)11 sequence having two AUUUA pentamers in the 3′ end of the PLC-1 3′UTR (PLC-AU RNA) (Fig. 4D). Thus, although many similar AU-rich sequences might be present in different parasite transcripts, TcUBP-1 seems to recognize some, but not all, of these motifs.

TcUBP-1 Selectively Binds to Specific AU- and GU-rich Sequences—To further analyze the specificity of TcUBP-1 for the small 18-mer SMUG-S-AU RNA that is present in the mucin
SMUG-S 3' UTR, four modified oligonucleotides based on SMUG-S-AU were tested for in vitro recognition (Fig. 4D). In the RNA oligonucleotide P1, all the adenosines within the U-rich region were changed to guanosines. P2 and P3 RNA oligonucleotides have one or two additional guanosines instead of the second or the second and third uridines, respectively, in the GUUUUG motif. Finally, in the P4 RNA, the uridine of the middle of AUUUUUUA heptamer was replaced by an adenosine, rendering a typical (AUU)₅A sequence found in the 3′ UTR of T. cruzi transcripts such as Hsp70 (53). TcUBP-1 recognized the P1 RNA oligonucleotide as well as the SMUG-S-AU RNA template itself. In contrast, TeU-BP-1 did not recognize the (GUU)₅G RNA template (P2), P3, and P4 probes. These results confirmed the importance of the U-rich nature of the RNA oligonucleotides for the in vitro binding of TeU-BP-1 and showed that GU-rich sequences are also recognized.

**Discussion**

**Expression of TeU-BP-1 Is Developmentally Regulated**—To analyze the TeU-BP-1 expression pattern on the different stages of the *T. cruzi* life cycle, a rabbit anti TeU-BP-1 serum was obtained and reacted with a Western blot made with total protein parasite extracts (Fig. 6A). A band with an apparent molecular mass of about 45 kDa (named TeU-BP-1) was detected in all parasite stages. Densitometric analysis showed that TeU-BP-1 was expressed 10-fold more in the mammalian host stages (amastigotes and cell-derived trypomastigotes) than in the epimastigote form and 5-fold more than in the metacyclic trypomastigote stage of the parasite. A band of an apparent molecular mass of 80 kDa was also detected. This band was uninformative because of anti-GST antibody cross-reactivity, as shown by antiserum depletion with GST (data not shown). When the gel was over-exposed a specific band of about 27 kDa was detected (Fig. 6A).

The estimated size of the protein encoded by *TeU-BP-1* gene is 25 kDa and thus is much smaller than the apparent molecular mass of the protein detected in parasite extracts. To interpret this discrepancy, we analyzed the effect of transfecting *T. cruzi* epimastigotes with the *TeU-BP-1* gene and compared the protein profiles with mock-transfected parasites. Detection was performed with an anti-*TeU-BP-1* antibody as shown in Fig. 6A. The proteins detected in pH (parasite population transfected with plasmid alone) and pR-*TeU-BP-1* (parasite population transfected with *TeU-BP-1* gene) were of the same apparent molecular mass, about 45 kDa (Fig. 6B). However, the intensity of the 45-kDa product was about 4.5-fold higher in pH-*TeU-BP-1* than in pH-transfected parasites. Because cruzipain (used as an internal control) was expressed at similar levels in both transfected parasite populations (Fig. 6B), we assumed that over-expression of *TeU-BP-1* resulted in the over-expression of a 45-kDa protein, the same molecular mass as the endogenous *TeU-BP-1*. Thus, the difference in the protein molecular mass might be due to *in vivo* post-translational modifications, covalent oligomerization (54, 55) within the *TeU-BP-1* product itself or with another protein (see Discussion). The protein detected in *E. coli* transformed with *TeU-BP-1* had an apparent molecular mass of 25 kDa (data not shown). Thus, the difference in mass might be due to post-translational modifications occurring in trypanosomes.

**Over-expression of TeU-BP-1 Selectively Destabilizes SMUG mRNAs Containing AU-rich Sequences within Their 3' UTR**—We next analyzed the stability of ARE-containing mRNAs in parasites over-expressing *TeU-BP-1*. After stable selection, parasites were treated with actinomycin D; half-lives of two ARE-
containing mRNAs (SMUG-L and -S), as well as transcripts lacking ARE sequences (ductin and Hsp70), were analyzed by Northern blot hybridization (Fig. 7A). The data normalized to the rRNA internal control was plotted in Fig. 7B. Two independent experiments were performed using independent cell lines that were transfected and processed separately. All pR-TcUBP-1 transfected parasite populations expressed similar levels of TcUBP-1 as detected by Western blot analysis (data not shown), being 4.2- and 4.5-fold more abundant than in the pR population. The plots shown in Fig. 7B were done using the mean value from all experiments, which differed by less than 5% in each of the indicated points. The initial time, displaying the stronger signal, was taken as 100% to allow a comparison of the mRNA half-lives.

The negative control, the pR-transfected populations, showed that the SMUG mRNA half-lives of S and L group members were 5.5 and 5.75 h, respectively, which was similar to the values obtained in a previous work (31). Conversely, the mRNA decay of mucin SMUG mRNAs in cells over-expressing TcUBP-1 (pR-TcUBP-1) was faster, exhibiting a half-life of 3 h for the S group (54% of pR) and 3.75 h for the L group (65% of pR). The half-lives of transcripts from ductin and Hsp70, which do not have AU- or GU-rich motifs in their 3′UTR, were analyzed. None of these transcripts were affected by TcUBP-1 over-expression (Fig. 7, A and B). The half-life of ductin mRNA was 1.5 and 1.65 h in parasites transfected with control pR and pR-TcUBP-1, respectively. Hsp70 mRNA turnover was also shown not to be affected by TcUBP-1 over-expression, with similar half-lives of 5.2 and 4.8 h for pR and pR-TcUBP-1 parasite populations, respectively.

**DISCUSSION**

In this work we have identified a protein that is involved in the selective control of mRNA stability in *Trypanosoma cruzi*. Although a number of cis-regulatory elements were detected in transcripts from kinetoplastid parasites, to our knowledge this is the first identification of a functionally active trans-acting factor that modulates mRNA levels in these parasites. Indeed, the presence of highly similar sequences coding for putative U-rich RNA-binding proteins in *L. major* and *T. brucei* suggests their conservation among different protozoan parasite species (see Fig. 3C). The number of protein factors interacting with mRNA in *T. cruzi* is difficult to estimate at present. However, 6 genes are likely to code for proteins having RRM motifs, and at least one of them, TcUBP-1, is an RNA-binding protein, as shown in this work. But this might be a minimal number because other protein families having this biological activity but lacking RRM motifs might be present in the parasite as is the case in higher eukaryotic cells (46). Few other RNA-binding proteins were identified in kinetoplastid parasites, but none of them was shown to be involved in modulation of mRNA stability. A serine/arginine (SR)-rich protein having RNA recognition motifs was suggested to be involved in trans-splicing (56). Recently, a protein binding to amastin 3′UTR mRNA was detected in *T. cruzi* (27), but it has not been further characterized in terms of its biological activity in vivo.

TcUBP-1 is composed of two regions, an amino-terminal sequence having the RRM motif and a carboxyl-terminal ex-
within their 3'UTR destablizes mucin mRNAs containing U-rich sequences within their 3'UTR. A, Northern blot analysis from epimastigotes transfected with vector alone (pR) or over-expressing TcUBP-1 protein (pR-TcUBP-1). Parasites were treated with 10 μg/ml actinomycin D (ActD), and total RNA was prepared at the indicated time points. The same filter was hybridized sequentially with the different probes indicated at the left. *, cDNA clone having high homologies with mammalian duetin (R. Verdün, unpublished data). B, quantitation of mRNA levels from the different Northern blot hybridizations shown in A, normalized to the rRNA signal. Closed circles, pR; open circles, pR-TcUBP-1. The data were expressed as the mean relative amount of mRNA ± S.E. (n = 2) at each time point after correction for the level of rRNA. Differences between pR and pR-TcUBP-1 were significant when comparing the means by Student's t test (p < 0.05; **, p < 0.01). The same transfected parasite populations analyzed by Western blot in Fig. 6B were used for the in vivo analysis of mRNAs half-life.

fig 7. TcUBP-1 over-expression in the epimastigote stage of the parasite selectively destabilizes mucin mRNAs containing U-rich sequences within their 3'UTR. A, Northern blot analysis from epimastigotes transfected with vector alone (pR) or over-expressing TcUBP-1 protein (pR-TcUBP-1). Parasites were treated with 10 μg/ml actinomycin D (ActD), and total RNA was prepared at the indicated time points. The same filter was hybridized sequentially with the different probes indicated at the left. *, cDNA clone having high homologies with mammalian duetin (R. Verdün, unpublished data). B, quantitation of mRNA levels from the different Northern blot hybridizations shown in A, normalized to the rRNA signal. Closed circles, pR; open circles, pR-TcUBP-1. The data were expressed as the mean relative amount of mRNA ± S.E. (n = 2) at each time point after correction for the level of rRNA. Differences between pR and pR-TcUBP-1 were significant when comparing the means by Student's t test (p < 0.05; **, p < 0.01). The same transfected parasite populations analyzed by Western blot in Fig. 6B were used for the in vivo analysis of mRNAs half-life.

U-rich RNA-binding Proteins in Trypanosomes

Tension having Gly- and Gln-rich auxiliary domains, both separated by a hinge region bearing a putative NES sequence (Fig. 1). RRMs are found in three types of RNA-binding proteins: hnRNPs, splicing regulators, and development stage-specific regulatory factors (10). These proteins have been demonstrated to be involved in many different post-transcriptional events including RNA processing, transport, and translation (57). An example is the splicing regulator SXL, in which the consensus binding site is UUUUUGUU(U/G)U(U/G)UUU(U/G)UU (58) and which resembles some of the RNA oligonucleotides recognized by TcUBP-1 (Figs. 4 and 5). Auxiliary domains might serve as sites for protein-protein interactions and also for post-translational modifications, which can modulate TcUBP-1 function (52). In fact, our preliminary analysis of few members of the TcUBP family suggests that they differ in the auxiliary domains. Further work will be required to define whether these differences have a functional correlate, which is the minimal domain required for an efficient RNA binding, and which is the function for the auxiliary domains.

TcUBP-1 bound to U-rich RNA oligonucleotides, in particular to those having either G or A intercalated at defined positions (Fig. 4). GU-rich sequences are bound with higher affinity than AU-rich sequences (Fig. 5). The specificity and affinity of this recognition was shown through the use of a number of different oligoribonucleotides and competition experiments (Figs. 4 and 5). More important, the specificity for binding of the U-rich elements is also observed in experiments performed in vivo. TcUBP-1 decrease the stability of two transcripts from the SMUG mucin family bearing U-rich elements within their 3'UTR but do not affect the stability of mRNAs lacking this target motif (Fig. 7, A and B). Thus, TcUBP-1 might not only recognize in vitro specific sequences in the 3'UTR but also result in the modulation of the biological activity. This scenario might be, however, an over-simplification of what is actually occurring in vivo. We have recently found that the combination of protein factors binding to different cis-elements present within the same UTR result in either stabilization or destabilization of mRNAs (35). Thus, a coordinated interaction between different negative and positive cis-elements might regulate the final cellular destination of a mRNA in order for it to be stabilized, translated, or rapidly degraded.

We have found evidence showing the linkage between the modulation of mRNA stability and the regulation of the RNA-binding protein expression pattern in different stages of the parasite. Antibodies raised to TcUBP-1 detect a protein in parasite extracts having an apparent molecular mass of 45 kDa. This product is present in larger amounts in the stages present in the mammalian host than in the insect epimastigote stage. Although the deduced size of the product from the TcUBP-1 gene is about 25 kDa, we confirmed by Western blot analysis that overexpression of the transfected gene in T. cruzi epimastigote stage generates a product of 45 kDa, indistinguishable from that of the endogenous gene (Fig. 6B). Alternative explanations are that the product of TcUBP-1 is post-translationally modified through phosphorylation, methylation, or covalent homo- or heterooligomerization (54, 59). Post-translational modifications can enhance, reduce, or eliminate the binding specificity or affinity for U-rich elements by altering the nucleocytoplasmic distribution or by changing their interactions with auxiliary cellular factors that are necessary for substrate recognition and binding (13). Dityrosine or isodityrosine formation by covalent bond was identified in several proteins of the human immune system (54) and in a large variety of organisms including insects, plants, and animals (55). Additionally, the anomalous electrophoretic migration
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might be due to the estimated high pl or to the particular amino acid composition of TcUBP-1. Glutamine comprises about 23% of the protein. In any case, it is possible to think that the 45-kDa protein is the endogenous homologue of TcUBP-1, which is involved in the selective destabilization of mucin SMUG-L and -S ARE-containing mRNAs (Fig. 7). An inverse correlation between TcUBP-1 levels and SMUG mucin mRNA steady-state levels is shown during parasite development. Thus, there is more SMUG mRNA in the epimastigote stage, when TcUBP-1 is expressed at lower levels.

As stated above, TcUBP-1 RRM presents a close similarity with members of the ELAV-like protein family (45), which stabilizes ARE-containing mRNAs. However, as shown in this work, TcUBP-1 is involved in the down-regulation of SMUG mRNAs when over-expressed in epimastigotes, the stage in which there is a lower level of TcUBP-1 protein. Therefore, it is tempting to speculate that TcUBP-1 might antagonize the in vivo effect produced on ARE-containing mRNAs by an ELAV homologue like HuR or HuC (45). Some of the U-rich transcripts, such as (AUUUU)₄₄A, are recognized by both TcUBP-1 and HuR. However, TcUBP-1 did not bind some of the U-rich elements that HuR did, such as (AUUUU)₄₄A and (AUGU)₄₄A (60).

Thus, TcUBP-1 might be more related to hnRNP D in terms of function on mRNA stability, since it is a destabilizing effect produced on ARE-containing mRNAs by an ELAV protein of the zygote and ookinete stages of the chicken malaria, P. falciparum (11). Orso and A. C. C. Frasch, unpublished data.

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Acknowledgments—We are indebted to Berta Franke de Cazzulo and Liliana Sforo for parasite cultures, Antonio Gonzalez and Alberto Delgado (Instituto de Parasitología y Biomedicina López-Neyra, Granada, Spain) for providing the ESTs TENG-0035 and TENG-0042, Fabio Fragà for animal care, Minoru Yoshida (University of Tokyo, Japan) for providing leptomycin-B, and J. De Gaudenzi for his help in part of the electrophoresis work. pHIBOTEX vector was kindly provided by Dr. R. Hernández. We also thank Drs. J. J. Cazzulo, S. Silverstein, and J. Di Noia for critical reading of the manuscript.

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