Post-transcriptional regulatory mechanisms have been suggested to be the main point of control of gene expression in kinetoplastid parasites. We have previously shown that Trypanosoma cruzi SMUG mucin mRNA steady-state level is developmentally regulated by post-transcriptional mechanisms, being stable in the epimastigote insect vector stage, but unstable in the trypomastigote infective stage of the parasite. Its turnover is controlled by an AU-rich element (ARE) localized in the 3'-untranslated region, since a reporter gene lacking this sequence was stable in the trypomastigote stage (Di Noia, J. M., D'Orso, L., Sanchez, D. O., and Frasch, A. C. (2000) J. Biol. Chem. 275, 10218–10227). Here, we show by gel mobility shift assay that the 44-nt ARE sequence interacts with a set of stage-specific AU-rich element RNA-binding proteins (ARE-BPs). The epimastigote stage AU-rich element RNA-binding protein, named E-ARE-BP, and the trypomastigote stage ARE-BPs, named T-ARE-BPs, are efficiently competed by poly(U). UV cross-linking analysis showed that E-ARE-BP has an apparent molecular mass of 100 kDa and is different from the 45–50-kDa ARE-BP's present in other stages of the parasite. Transfection experiments allowed the identification of a novel cis-element that might be responsible for a positive effect on mRNA stability. It is a G-rich element, named GRE, composed by two contiguous CGGGG pentamers. The factors that recognize GRE were different from the ones that bind to ARE, in both molecular masses and subcellular localization. Thus, ARE and GRE are functionally different cis-elements, which might regulate mucin expression throughout the parasite life cycle.

Kinetoplastid parasites control gene expression at the level of mRNA maturation by post-transcriptional mechanisms (reviewed in Refs. 2 and 3). α-Amanitin-sensitive RNA polymerase II from trypanosomes transcribes large polycistronic units containing a number of coding sequences (4). Transcriptional start sites have been extremely difficult to detect; only two putative promoter regions were described as transcriptionally void regions upstream from the actin and Hsp70 genes (5, 6). The maturation of polycistronic RNA precursors to render individual mRNA molecules is achieved by cleavage in the intergenic region by a coupled processing of 5' end trans-splicing and 3' end polyadenylation (7). Both processes seem to depend on the recognition of polyuridylicate tracts present in the intergenic regions (8), which acts as a bifunctional element affecting RNA processing both upstream and downstream from itself (7).

In vivo treatment of parasites with protein synthesis inhibitors induces an accumulation (9) or a decrease in the mRNA levels of some transcripts (1), and this effect is not due to an increase or a reduction in transcriptional levels, respectively. Therefore, these results point to the presence of labile factors, affected by protein synthesis inhibitors, that might be negative or positive regulators of mRNA maturation. However, the mechanisms of interference in pre-mRNA processing, unbalanced nucleo-cytoplasmic transport, or unusual mRNA stability control processes remain to be identified. It is known that both 5'- and 3'-untranslated regions (UTRs) are responsible for stabilization/destabilization mechanisms, up- or down-regulating mRNA levels in a developmentally regulated manner (10, 11). In transient and stable parasite transfection experiments, the 3'-UTRs of some mRNAs were found to influence the expression of a reporter gene in a stage-specific manner (1, 10, 11). The way in which the 3'-UTR differentially influences the mRNA steady-state levels is still unknown. Furthermore, few cis-elements responsible for these post-transcriptional regulatory mechanisms have been defined (12–14).

Several cis-elements and trans-acting factors controlling mRNA stability have been characterized in higher eukaryotes (15, 16). A well known example is the case of AU-rich elements or AREs, cis-sequences localized in the 3'-UTR of short-lived mRNAs, such as proto-oncogenes and cytokines (17). These elements are recognized by different positive or negative RNA-binding proteins, like HuR and AUF-1/heterogeneous nuclear ribonucleoprotein D, respectively (18–20), causing rapid
chances in mRNA stability. Another example is the ribonucleo-
protein complex associated with human α-globin mRNA (21). A
cytidine-rich (C-rich) segment within the 3′-UTR of α-globin is
critical for mRNA stability through the interaction with different
trans-acting factors that mediate this effect (22). However,
it has been shown that neither αCP1 nor αCP2 complex-
forming proteins can bind the C-rich element unless they are
complexed with the remaining non-poly(C)-binding proteins,
such as AUFl/heterogeneous nuclear ribonucleoprotein D (23).
Thus, a protein implicated in ARE-mediated mRNA decay is
also an integral component of the mRNA stabilizing α-complex.

Trypanosoma cruzi, the protozoan parasite agent of Chagas
cdisease, is covered by a dense mucin coat (24), at least in two of
its developmental stages. Mucins are highly O-glycosylated
proteins having relevant roles in cell protection and in cell-cell
interactions, especially in immune cell migration in vertebrate
cells (25). Mucins from T. cruzi were classified into two differ-
ent protein families that differ between parasite stages. The
form of the parasite present in the insect vector, epimastigote,
expresses a small mucin family named TeSMUG (35–50 kDa)
whose core proteins are encoded in about 70 different genes (1),
while the forms of the parasite present in the mammalian host,
 bloodstream trypanomastigotes, have larger mucins encoded by
500 different genes (26). Developmentally regulated expression
of these mucins in the different parasite stages is relevant
because they might accomplish different functions related with
cyst stage (27).

We have previously demonstrated that a 44-nucleotide ARE
sequence within the 3′-UTR of SMUG mucin family was a desta-
abilizing cis-element acting in a stage specific manner (1). These
results suggest that different trans-acting factors might bind
mucin transcripts in vivo, and selectively regulate its mRNA
stability throughout parasite development. We have now iden-
tified a novel G-rich element, named GRE, which might be
responsible for a stage-specific stabilization of SMUG mRNA
family in the epimastigote form of the parasite. Transfection
experiments show that GRE and ARE sequences have opposite
functions in terms of mRNA stabilization in the different stages
of parasite development. We have now identified a novel G-rich
element, named GRE, which might be

Experimental Procedures

Parasite Cultures and Drug Treatments—Trypanosoma cruzi CL-
Brener cloned stock (28) was used. Different forms of the parasites
were obtained as described previously (29). Purity of the different parasite
forms was determined by conventional microscopy and was at least
95%. Epimastigote cultures were taken in logarithmic growth phase at
a cell density of 3 × 10^7/ml and treated with actinomycin D (ActD)
(Sigma), at a final concentration of 10 μg/ml, which is known to inhibit
transcription in trypanosomatids (12, 30). Aliquots were taken at dif-
ferent times after addition of the inhibitor. Cycloheximide (Sigma)
was used at a final concentration of 50 μg/ml (31). Parasite viability was
confirmed by microscopy at every time point of the experiments.
Culture aliquots were harvested by centrifugation, washed with phos-
tate-buffered saline, and frozen at −70 °C until RNA extraction.

Chloramphenicol Acetyltransferase (CAT) Assay—An equal number of
parasites from each transfected population was harvested, washed
once with 0.25 M Tris-HCl (pH 8), and cellular extracts were prepared
by four freeze-thaw cycles and heat inactivation. Cell lysates were
assayed for CAT activity as described previously (32). Reactions were
conducted for 1 h at 37 °C with cellular extracts prepared from 10^7
parasites. This time was previously adjusted to fit within the linear
range of the assay. Conversion of [14C]chloramphenicol to acetylated
forms was analyzed by thin layer chromatography and quantified by
densitometry.

DNA Constructions and Parasite Transfections—The chlorampheni-
col acetyltransferase (cat) gene, the complete TeSMUG intergenic
region, and the SMUG-L and SMUG-L/AU constructs were amplified by
PCR as described previously (1). All 3′-UTR deletions were created by
PCR and fused downstream from cat into the HindIII and XhoI sites.

The PCR primers contained restriction enzymes sites to facilitate the
subsequent cloning steps as indicated. All the primers used to generate
internal deletion mutants of the 3′-UTR are listed below. For clone
SMUG-L/AU: SMUG-L/AU: 5′-ggatctGAGGAGGGGCGGGCGCTTGTTG-3′
and 3′-ggatctGTCGAGGAATTAATATGCCGCGTCGCGCGTCGCGTGTTG-3′.
For clone SMUG-L: 5′-ggatctGTCGAGGAATTAATATGCCGCGTCGCGCGTCGCGTGTTG-3′.
Two oligonucleotides containing the first 27 base pairs with a HindIII
site: 5′-ggatctGTCGAGGAATTAATATGCCGCGTCGCGTGTTG-3′ and sensA, 3′-ggatctGTCGAGGAATTAATATGCCGCGTCGCGTGTTG-3′.

Each DNA fragment was cloned in the pTEK vector (33), kindly
provided by Dr. J. M. Kelly (London School of Hygiene and Tropical
Medicine, London, United Kingdom). Transfections were carried out as
described previously (1). The neo resistance gene was used for selection
and as an internal control of transfection levels since it is transcribed
polyestrionically from the same promoter (33). The polyadenylation
site of the cat mRNA was determined by reverse transcription-PCR using
the oligonucleotide anchor d(T) (5′-GGAGGGGCGGCGCTTGTTG-3′)
using the Superscript II enzyme (Life Technologies, Inc.). PCR was
formed with 10 μl of trypanosome total extract (prepared as
above) 10,000 cpm of RNA probe, 10 μl Tris-HCl (pH 7.6), 5% glycerol,
100 mM KCl, 5 μg MgcL, 1 μg/ml bovine serum albumin, 500 ng/ml tRNA
(Sigma) in a 20 μl final volume. The incubation time was 10 min
at 25 °C. Heparin was added at a concentration of 1 μg/ml. Each
reaction was loaded directly onto a 7% acrylamide-bisacrylamide (38:2),
0.5× TBE nondenaturing gel to perform an electrophoresis mobility

In Vivo Transcription—All plasmids for in vivo transcription were
constructed as follows. Complementary oligonucleotides, corresponding
to the sense and antisense strands of the RNAs transcribed, were
annealed and cloned into the EcoRI and HindIII sites of the vector
pBS (Stratagene, La Jolla, CA). Transcription of sense sequences was
performed with 1 μg of HindIII-digested plasmids using T7 RNA-po-
ymerase (Promega) in the presence of [α-32P]UTP (800 Ci/mmol,
PerkinElmer Life Sciences), 500 μM ATP, CTP, and GTP. Antisense
transcripts were synthesized with T3 RNA polymerase. All transcripts
were purified on a 8 M urea, 12% polyacrylamide gel and eluted over-
night in RNA elution buffer (0.3 M NaOAc, 10 μg MgCl₂, and 1 μl
EDTA). After elution, RNAs were ethanol-precipitated and resus-
pended in 50 μl of water. Preparative in vitro transcription was done as
described previously (34) and detected by UV shadowing.

Protein Extract Preparation and Subcellular Fractionation—For to-
tal parasite extracts, parasites were prepared in lysis buffer (0.75% CHAPS detergent, 1 mM MgcL, 1 mM EGTA, 5 mM β-
mercaptoethanol, 10 mM Tris-HCl (pH 7.6), and 10% glycerol)
supplemented with protease inhibitors: 1 mM phenylmethylsulfonyl flu-
oride and 50 μM E-64 (Sigma). After 30 min on ice, the extract was
centrifuged at 19,000 rpm (SS-34 rotor) and the supernatant stored at
70 °C. For subcellular fractionation, nuclear and cytoplasmic frac-
tions were prepared as described previously for another kinetoplastid
parasite, Crithidia fasciculata (35). Briefly, parasites were twice
washed in Buffer A (10 mM Tris-HCl (pH 7.6), 1.5 mM MgcL, 10 mM KCl)
and resuspended in Buffer B (Buffer A plus 1 mM dithiothreitol, 1 mM
EDTA, and 0.5% Nonidet P-40) in the presence of protease inhibitors.
After 20 min on ice and vortexing each 3 min, the preparation was
centrifuged for 15 min at 5000 rpm. The supernatant containing the
cytosolic fraction was mixed with an equal volume of Buffer D (10 mM
Tris-HCl (pH 7.6), 10 mM KCl, 1 mM MgcL, 1 mM EDTA, 10% glycerol).
The pellet was resuspended in an equal volume of Buffer C (Buffer D
plus 20% glycerol) and passed through a 21-gauge needle and frozen
several times on liquid N₂ to lyse nuclei. After centrifugation to remove
debris, the supernatant was mixed with an equal volume of Buffer D
(nuclear fraction). Polysomes were prepared as previously described
(36). Polysome extract was pre-treated at 25 °C for 15 min with ribo-
nuclease A (37) when indicated, and the RNAse was inactivated with
the ribonuclease inhibitor RNAasin (Promega), prior incubation of the
extract with the labeled RNA. The amount of RNAse A used was
determined by titration.

Analysis of RNA-Protein Interactions—Binding reactions were per-
fomed with 10 μl (3 μg/ml) of trypanosome total extract (prepared as
above), 10,000 cpm of RNA probe. 10 mM Tris-HCl (pH 7.6), 5% glycerol,
100 mM KCl, 5 mM MgcL, 1 μg/ml bovine serum albumin, 500 ng/ml tRNA
(Sigma) in a 20 μl final volume. The incubation time was 10 min
at 25 °C. Heparin was added at a concentration of 1 μg/ml. Each
reaction was loaded directly onto a 7% acrylamide-bisacrylamide (38:2),
0.5× TBE nondenaturing gel to perform an electrophoresis mobility
shift assay (EMSA). The gels were dried and exposed to film at -70 °C.
For competition experiments, the extract was incubated simultaneously
with the indicated amounts of unlabeled and labeled RNAs. All homori-
bopolymers (poly(A), poly(C), poly(G), and poly(U)) were from Sigma.

**UV Cross-linking Analysis**—

$^{32}$P-Labeled RNA was incubated with a trypanosome total extract as described above. The *in vitro* binding

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**Fig. 1.** Half-life determinations of cat mRNA fused to complete mucin SMUG-L 3'-UTR and deletion mutants. A, schematic representations of complete SMUG-L and 3'-UTR deletion mutants are shown. All constructs were done by PCR as described under “Experimental Procedures” using PCR primers with restriction endonuclease sites (BamHI; SmaI; H, HindIII; E, EcoRI; X, XhoI). The 5' and 3' intergenic regions (IR) contain the original trans-splicing site (ag) and polypyrimidine tract (pPy) for efficient mRNA processing. Epimastigote forms of the parasite were transfected with the indicated DNA constructs cloned in pTEX vector (33). B, epimastigotes transfected with the recombinant DNAs described in A were treated with 10 μg/ml ActD and total RNA was prepared at the indicated times (0, 60, 120, and 180 min). Equal amounts of RNA were analyzed by Northern blot. The same filter was sequentially hybridized with cat, neo, and rRNA probes. The hybridization performed with the neo probe serves as an internal control of the experiment since this gene is expressed from the same vector. C, quantitation of the bands from the Northern blots shown in B. The half-life of each transcript is indicated below the graphic. D, epimastigotes transfected with SMUG-L and SMUG-LΔGRE constructions were treated with 10 μg/ml ActD and total RNA was prepared at the indicated times (0, 15, 30, 45, and 60 min). E, quantitation of the bands from the Northern blots shown in D. In panels C and E, the data were expressed as the mean relative amount of mRNA ± the standard error of the media (n = 3) at each time point after correction for the level of rRNA. Differences between SMUG-L and each deletion mutant were significant when comparing the means by Student’s t test (*, p < 0.05; **, p < 0.01).
Comparing the means by Student’s t test were significant (*, between SMUG-L, SMUG-L1, and SMUG-L2 expressed as the mean value normalized to rRNA signal) is shown. Results are expressed as the mean value ± the standard error media (n = 3). Differences between SMUG-L, SMUG-L1, and SMUG-L2 were significant (*, p < 0.05, when comparing the means by Student’s t test).

**Northern Blot**—RNA was purified using TRIzol reagent following the manufacturer’s instructions (Life Technologies, Inc.). Northern blots were carried out as described previously (38). Zeta-Probe nylon membranes (Bio-Rad) were used for all blottings. Probes were radioactively labeled with [α-32P]dCTP (PerkinElmer Life Sciences) by PCR as in Ref. 39. Densitometry was done using 1D Image Analysis Software (Kodak Digital Science).

**RESULTS**

*Both Positive and Negative cis-Elements within the 3′-UTR of SMUG-Mucin mRNA Modulate Translation Efficiency*—To SMUG mucin family was previously shown to be post-transcriptionally regulated and an ARE within its intergenic regions of SMUG-L group, which contains sequences that ensure correct splicing and polyadenylation, cloned in the pTEX vector. The transcript from the complete construct SMUG-L had a half-life of about 70 min. Conversely, SMUG-L Δ GRE transcript had a shorter half-life (t1/2 = 30 min), that is about 42% of that from SMUG-L clone. GRE sequence is a G-rich element that contains the first 27 nt of the 3′-UTR downstream the stop codon and is composed by two contiguous CCGGGG pentamers (see below). Transcripts from two other constructs, SMUG-L2 and SMUG-L3, had similar half-lives to those of SMUG-L (t1/2 = 75 min and t1/2 = 65 min, respectively). Finally, SMUG-L Δ A1 and SMUG-L Δ Sire deletion mutants were transcribed into RNAs having increased half-lives (t1/2 = 140 min) (Fig. 1, B and C). Since the short interspersed repeat element (SIRE) retrotransposon (40) is a large element (450 base pairs), some partial deletion would be required to better define the region causing this effect. Since in the half-life determination of clone SMUG-L Δ GRE (Fig. 1B), less than 50% of the mRNA levels remained at the first sampling time (60 min), the experiment was repeated taking samples between 0 and 60 min. Thus, the half-life of SMUG-L Δ GRE was better calculated and shown to be 30 min, identical to that indicated in Fig. 1C (Fig. 1, D and E). These results suggest that the sequences in the 3′-UTR could be divided into several functional regions: 1) a positive G-rich element named GRE; 2) one negative element between nucleotides 28 and 62 downstream stop codon, named E1 for element 1; and 3) an AU-rich element between nucleotides 272 and 318 involved in selective mRNA destabilization in a stage-specific manner (see next section). The 3′-UTRs of SMUG-L and SMUG-L Δ GRE were modeled using the Genequest program (Lasergene Package, DNAstar Inc.) to predict if the deletion of GRE sequence would have an effect on the three-dimensional structure of the RNA. Both transcripts were
found to share the same modeled structure, including all loops
of the 3′-UTR (data not shown). Thus, it is likely that the
sequence of the G-rich element is the one that confers the effect
reflected on mRNA stability, and not any modification of the
whole 3′-UTR of the RNA molecule.

In order to determine if the different domains of the 3′-UTR

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**Fig. 3.** A novel GRE localized in the 3′-UTR of SMUG-L confers mRNA stability in a stage-specific manner and is functionally different to the AU-rich element. A, schematic representation of SMUG-L (complete construct) and SMUG-L△GRE and SMUG-L△AU deletion mutants used to transfect epimastigote stage of the parasite. The sequence that was deleted in clone SMUG-L△GRE and SMUG-L△AU is indicated in the SMUG-L scheme. B, Northern blot of total RNA from epimastigotes transfected with the constructs shown in A. Epimastigotes were treated with 10 μg/ml ActD, and total RNA was prepared at the indicated times (0, 45, 60, 90, and 120 min). The same filter was sequentially hybridized with cat, neo, and rRNA probes. C, quantitation of cat mRNA levels from the Northern blot shown in B. The data were expressed as the mean relative amount of mRNA ± the standard error of the media (n = 3) at each time point after correction for the level of rRNA. Differences between SMUG-L△GRE and SMUG-L and between SMUG-L△GRE and SMUG-L△AU were significant (*, p < 0.05; **, p < 0.01 when comparing the means by Student’s t test). D, epimastigote-derived metacyclic trypomastigotes were treated as indicated in B. E, quantitation of cat mRNA levels from the Northern blot shown in D. The data were expressed as the mean relative amount of mRNA ± the standard error of the media (n = 2) at each time point after correction for the level of rRNA. Differences between SMUG-L△AU and SMUG-L and between SMUG-L△AU and SMUG-L△GRE were significant (**, p < 0.01, when comparing the means by Student’s t test). In panels C and E, the half-life of each transcript is indicated below the graphic.
also influence expression at the translational level, the CAT activity from control and deletion mutants was measured and the values obtained were normalized to cat mRNA steady-state levels from each construct (Fig. 2). Enzymatic activity was expressed as the percentage of that obtained with the complete construct SMUG-L. The value obtained with the parasite population transfected with SMUG-L \( \Delta \) GRE was similar (117% of SMUG-L) than the one obtained from parasites transfected with the complete construct SMUG-L, suggesting that this G-rich element does not modulate translation efficiency. Conversely, SMUG-L \( \Delta 1 \) deletion mutant, whose transcript has a larger half-life, also presented an increase in translation (185% of SMUG-L). This suggests that SMUG-L \( \Delta 1 \) regulates both mRNA stability and translation efficiency in a negative manner. Moreover, SMUG-L \( \Delta 2 \) and SMUG-L \( \Delta 3 \) did not show a considerable effect on translational activity (88% and 112% of SMUG-L, respectively) (Fig. 2). Finally, the retrotransposon SIRE seems to produce a positive effect on translation, since its deletion causes a decrease in the ratio CAT activity/cat mRNA (15% of SMUG-L construct). This result is interesting, because it was suggested that SIRE exhibits another function in the process of mRNA maturation (see “Discussion”). Sites for 5’ end trans-splicing and 3’ end polyadenylation were the same in all the mRNAs derived from the constructs made, as indicated under “Experimental Procedures.”

**A Novel GRE Confers mRNA Stability in a Stage-specific Manner and Is Functionally Different from the AU-rich Instability Element**—The effect of the GRE deletion (SMUG-L \( \Delta \) GRE construct) on mRNA stability was analyzed in different parasite stages and the results compared with those obtained with the constructs SMUG-L (complete 3’-UTR) and SMUG-L \( \Delta \) AU (lacking the 44-nt AU-rich instability element) (Fig. 3A). Epimastigote forms were differentiated into the infective form of the parasite, metacyclic trypomastigotes, and incubated with ActD to determine half-lives of the transcripts (Fig. 3). The probe used in the Northern blot analysis corresponds to the cat open reading frame. In the epimastigote stage, both SMUG-L and SMUG-L \( \Delta \) AU transcripts bearing the GRE sequence within its 3’-UTR have similar half-lives (\( t_{1/2} = 70 \) and \( t_{1/2} = 68 \) min, respectively). On the other hand, transcripts from SMUG-
LD GRE are less stable ($t_{1/2} = 30$ min) (Fig. 3C). It might be concluded that: 1) the GRE sequence in the epimastigote stage is involved in a selective mRNA stabilization process, and 2) the ARE sequence seems not to be involved in mRNA stabilization in this parasite form, since transcripts from both SMUG-L and SMUG-LΔGRE constructs have similar half-lives (Fig. 3B and C) (see “Discussion”).

Analysis of the infective metacyclic trypomastigotes stage, derived from differentiation of epimastigotes, also revealed differences in the mRNA steady-state levels. Both SMUG-L and SMUG-LΔGRE RNAs were extremely short-lived ($t_{1/2} < 10$ min) as compared with those from SMUG-LΔAU, which have a $t_{1/2} > 30$ min (Fig. 3E). Thus, the instability of SMUG-L and SMUG-LΔGRE transcripts in the metacyclic trypomastigotes stage could be due to the presence of the ARE sequence within its 3′-UTR. Additionally, the same filter used to detect cat transcripts was hybridized with a neo probe. Since the neomycin gene is flanked by glyceraldehyde-3-phosphate dehydrogenase intergenic regions (33) in the same plasmid bearing the cat reporter, it serves as an internal control of half-life determinations. As seen in Fig. 3 (B and D), neomycin half-lives are similar in each parasite stage independently of the construct tested.

The 27-nt GRE That Confers mRNA Stability Specifically Interacts with Different Nuclear and Cytoplasmic Complex-forming RNA-binding Proteins—The identification of this novel cis-element involved in a mRNA stabilization process allowed the searching for trans-acting factors able to recognize G-rich sequences. The 27-nt GRE sequence was transcribed in vitro as described under “Experimental Procedures” and used to perform RNA-protein binding reactions and EMSA. The
SMUG-L-GRE RNA oligonucleotide revealed the same three ribonucleoprotein complexes in all four parasite forms tested (Fig. 4A). As controls, no bands corresponding to the G-complexes 1, 2, and 3 were observed after incubation of SMUG-L-GRE RNA with RNase A or the protein extract with proteinase K (data not shown). To determine the apparent molecular masses of the proteins that compose the GRE-ribonucleoprotein complexes, a total protein extract from the epimastigote form of the parasite was incubated with an excess of 32P-labeled SMUG-L-GRE RNA oligonucleotide. The in vitro binding reactions were run in a native polyacrylamide gel and after UV cross-linking, the complexes were treated as under “Experimental Procedures” and further electrophoresed in a 10% SDS-PAGE (Fig. 4B). G-complex 1 gave rise to a single band having an apparent molecular mass of 80 kDa, while G-complexes 2 and 3 are composed by several proteins with apparent molecular masses of 35, 39, and 66 kDa. The RNA-binding proteins that compose G-complex 2 are present in different abundance in the epimastigote total lysate. One low abundant protein of about 66 kDa is detected together with two highly abundant factors of 35 and 55 kDa (Fig. 4B).

Competition experiments were conducted to further characterize the sequence specificity of all complexes formed. Each of the four homoribopolymers was used to compete with the SMUG-L-GRE RNA oligonucleotide in an in vitro binding reaction. Poly(G) selectively blocks the assembly of two ribonucleoprotein complexes, G-complex 1 (smaller band) and G-complex 2 (Fig. 4C). This result is in agreement with the G-rich nature of the cis-element, used in the in vitro binding reaction. G-complex 1 is effectively competed out with a molar excess of 10-fold, whereas the formation of G-complex 2 partially disappeared at a molar excess of 1000-fold. This result could be due to differences in the concentration of protein-forming complexes in the epimastigote lysate and is also suggested by the UV cross-linking analysis (Fig. 4B), where G-complex 1 is barely detectable comparing with the amount of proteins forming G-complex 2. Conversely, complex 3 was not efficiently competed by any homoribopolymer and, thus, might be unspecific.

The minimal size of the SMUG-L-GRE RNA element recognized by the proteins forming G-complexes 1 and 2 was analyzed. The RNA sequence was divided into two separate sequences: (a) SMUG-L-GRE-1, with the sequence GGACGGGG-CGGGG; and (b) SMUG-L-GRE-2, which presents a CG-rich content, GCGCGUGCGCCG (Fig. 5A). The SMUG-L-GRE-1 RNA is sufficient to interact with both trans-acting factors (Fig. 5B). This result suggest that the minimal sequence for G-complex 1 and 2 formation is the first half of the element, which is composed of two contiguous CGGGG pentamers. G-complex 1 is localized in the cytoplasm, whereas G-complex 2 is equally
The 44-nt AU-rich Instability Element Interacts with Stage-specific, Developmentally Regulated, RNA-binding Proteins—
The 44-nt AU-rich cis-element was important in conferring mRNA instability in a stage-specific manner (1) (Fig. 3). Therefore, to know if the RNA-binding proteins that recognized in vitro this element, named here SMUG-L-AU, are developmentally regulated, protein extracts from the four different parasite stages were incubated with the RNA template in an in vitro binding reaction. The complexes formed were identified in a native polyacrylamide gel (Fig. 6A). A stage-specific pattern of RNA binding to this motif was observed. In the epimastigote stage, an RNA-binding protein named E-ARE-BP, for epimastigote AU-rich element binding protein, migrated much more slowly in the polyacrylamide native gel than the ribonucleoprotein complex detected in the other three parasite stages. To determine the apparent molecular masses of these RNA-binding proteins, the total protein lysate of each parasite stage was incubated with an excess of SMUG-L-AU RNA probe and the ribonucleoprotein-complexes identified in the EMSA were UV-cross-linked and further electrophoresed in SDS-PAGE. The E-ARE-BP had an apparent molecular mass of ~100 kDa. In contrast, the ARE-BPs range between 45 and 50 kDa (Fig. 6B). The addition of increasing amounts of unlabeled antisense RNAs were also tested in competition experiments (Fig. 7B). The addition of increasing amounts of unlabeled sense SMUG-L-AU RNA to the reaction mixture resulted in a concentration-dependent reduction in the formation of the ribonucleoprotein complex containing E-ARE-BP, whereas the addition of unlabeled antisense SMUG-L-AU RNA had little effect on the formation of this complex. Trypomastigote ARE-BPs (T-ARE-BPs) are also efficiently competed by poly(U) RNA, and not by any other homoribopolymer (Fig. 7C). Additionally, we tested the competition with unlabeled in vitro transcribed SMUG-l-AU sense and antisense RNAs. The SMUG-l-AU sense RNA, as was shown for the E-ARE-BP, abolished the binding of the ARE-BPs in a concentration-dependent manner. This result confirmed that the T-ARE-BPs selectively and specifically recognized the AU-rich sequence of SMUG mRNAs (Fig. 7D) and that the U-rich nature of the oligoribonucleotide is important for the binding.

Different Subcellular Localization of ARE RNA-binding Proteins—
The presence of both AU and G-rich binding activities was analyzed in a nuclear and cytoplasmic preparation of T. cruzi epimastigotes and trypomastigotes. Subcellular fractionation was done as described under “Experimental Procedures.” These experiments showed that the E-ARE-BP is mainly cytosolic or that the E-ARE-BP might recognize the SMUG-l-AU RNA only in the cytoplasm and not in the nucleus (see “Discussion”) (Fig. 8A). In contrast, the 45–50-kDa T-ARE-BPs are localized in similar amounts in both compartments, nucleus and cytoplasm (Fig. 8C).

ARE-binding proteins of higher eukaryotes were shown to be associated with polysomes, and this particular localization was due to a translational regulatory mechanism conferred by those trans-acting factors (41–43). In a previous work, we reported that the ARE motif positively regulates translation efficiency in the epimastigote stage of the parasite (1), as is the case with the ARE sequences in TNF-α and some cytokine and proto-oncogene mRNAs (44). A polysome fraction (P) of T. cruzi epimastigotes was prepared as described previously (36) in the presence of cycloheximide to freeze ribosomes. After extract preparation and centrifugation through a sucrose cushion, the supernatant was saved as a postribosomal supernatant (PS) and the pellet was saved as polysomes (P). All the extracts were analyzed in an in vitro binding reaction with the SMUG-l-AU RNA template. The polysome extract was shown to have some AU-rich sequence binding activity, but minimal in comparison to the one observed in the postribosomal fraction (Fig. 8A). To further determine if the lack of a strong shifted band in the polysome fraction was due to the presence of some endogenous U-rich RNA competitor that might be sequestering part of E-ARE-BP, the extract was pre-treated with ribonuclease A (RNase A) as described previously (37), and the nuclease was inactivated prior to perform the in vitro binding reaction with...
the SMUG-L-AU RNA probe. The result shown in Fig. 8B demonstrate that, in the presence of RNase, the binding of E-ARE-BP is increased 4.5-fold, suggesting that there might be some RNA competing with the labeled AU-rich RNA in the polysome fraction. Moreover, the RNA probe remains intact after incubation with the polysome extract. Thus, the absence of such a strong band in these fraction was not due to the presence of a polysome-associated nuclease that could recognize the ARE sequence (Fig. 8B).

We conclude that E-ARE-BP is mainly cytoplasmic and may be partially associated to polysomes, whereas T-ARE-BPs are localized in both compartments and may be nuclear-cytoplasm shuttling RNA-binding proteins.

DISCUSSION

In this work we have obtained evidence for the existence of novel cis-elements localized in the 3’-UTR of SMUG mucins from *T. cruzi* that control both mRNA stability and translation efficiency. In the presence of nucleotides 28 and 62 of the 3’-UTR, increases the half-life of a cat reporter mRNA (Fig. 1), suggesting that this sequence acts as a negative element. Finally, deletion of the 450-base pair retrotransposon SIRE also produces the same effect as the element E1, but, given the large size of SIRE sequence, further work is required to confirm this effect. It was shown previously that SIRE is responsible for the down-regulation of gene expression of the TCP2β ribosomal protein by altering its trans-splicing efficiency (45). Thus, different functions might be assigned to sequences within this retrotransposon. Indeed, it was reported that U-rich regions and also the length of the 3’-UTR positively regulate mRNA polyadenylation and the translation efficiency of a reporter gene (11). Although GRE sequence is sufficient to up-regulate SMUG mRNA abundance, E1 has a dual effect on mRNA stability and translation, regulating both processes in a negative manner. It is not unprecedented for a single element to have two functions, since AU-rich sequences within the 3’-UTR of TNF-α affect both mRNA abundance and translation efficiency (46–48).

Two functionally different cis-elements, ARE and GRE, were identified. The ARE was involved in mRNA destabilization in the infective stage of the parasite, but not in the replicative epimastigote stage, because mRNAs from SMUG-L and SMUG-LΔAU constructs have similar half-lives in the latter stage (Fig. 3). These results further support the idea that the RNA-binding protein(s) that recognize the ARE in the epimastigote stage of the parasite, might provide resistance to endo- or exonucleolytic cleavage rather than providing actively mRNA protection. Conversely, GRE sequences have a different effect on mRNA stability throughout parasite development. It up-regulates SMUG mRNA abundance in the epimastigote stage, since deletion of the GRE motif makes the mRNA more labile (Fig. 3, B and C). The presence of the ARE sequence within the 3’-UTR of mucin SMUG mRNA have also been shown to modulate translation efficiency in a positive manner (1). In contrast, GRE had no considerable effect on translational levels, suggesting that both elements might coordinate cooperate in the *in vivo* regulation of SMUG mRNA abundance in the epimastigote stage of the parasite, but not in translation. Coordinated interaction between different negative and positive cis-elements was observed in the 3’-UTR of procyclic mRNAs of African trypanosomes, affecting both mRNA abundance and translation efficiency (12).

Cellular factors interacting with RNA motifs that regulate mRNA stability have not been identified yet in trypanosomes. Evidence showing that GRE and ARE sequences interact with different cellular trans-acting factors has now been obtained (Figs. 4 and 6, and summarized in the model of Fig. 9). Three GRE-forming ribonucleoprotein complexes were detected. Two of them, named G-complex 1 and G-complex 2, were specifically and efficiently competed by poly(G) homoribopolymer (Fig. 4C). G-complex 1 is formed by a single protein band whose apparent molecular mass is 80 kDa, and G-complex 2 is composed of several factors whose molecular masses were about 35, 39, and 66 kDa. This suggests that the 80-kDa protein of G-complex 1 directly recognized GRE sequence. In the case of G-complex 2, the three proteins might also be involved in protein-protein interactions. The presence of large complexes might regulate mRNA expression in a coordinated way, depending on the proteins that compose it or the protein-protein interactions that are produced during the different stages of the parasite. Since the presence of the ARE within SMUG-L...
3′-UTR led to a rapid mRNA decay, it is possible that a coordinated interaction between GRE-binding proteins with ARE-BPs and/or other protein factors not identified yet might determine the final mucin SMUG mRNA stability (Fig. 9).

A model for the post-transcriptional regulatory mechanism acting on mucin SMUG mRNA and mediated by ARE and GRE RNA-binding proteins is shown in Fig. 9. E-ARE-BP, only expressed in the epimastigote stage, might be a positive trans-acting factor interacting with the ARE and protecting SMUG mRNA from degradation. E-ARE-BP binding could also prevent the association of the destabilizing factor(s) to those mRNAs, possibly through competition for binding to similar cis-elements. Indeed, E-ARE-BP might be one of the proteins involved in the modulation of the translation activity mediated by the ARE motif (1), probably through the interaction with other cellular factors of the translational apparatus. On the other hand, GRE RNA-binding proteins are always present during the life cycle of T. cruzi (Fig. 9). The possibility that an ARE-GRE-complex exists in vivo (Fig. 9), and that this whole complex or some complex-forming proteins interact with a poly(A)-binding protein or other cellular factor(s) to prevent the attack of a deadenylase activity, remains to be investigated. It is well known that, in mammalian cells, a large complex is formed by several proteins having different affinities for poly(C) homoribopolymer, such as the assembly of the α-globin mRNA stability complex in the pyrimidine-rich region of the globin 3′-UTR (22).

The results obtained by subcellular fractionation suggest that E-ARE-BP is localized in the cytoplasm or only recognized the RNA in this cellular compartment, where mRNA decay or translational processes take place. Future experiments on Western blot analysis would permit us to determine if E-ARE-BP is also present in the nucleus and, thus, is recruited by some complex-forming proteins. Conversely, the ARE-BPs, at least in the trypomastigote stage, are present in similar amounts in both nucleus and cytoplasm and might be shuttling RNA-binding proteins (Fig. 8). G-complexes forming proteins, at least G-complex 2, might be formed by RNA-binding factors that showed a shuttling behavior between nucleus and cytoplasm. Consequently, it is possible that those GRE RNA-binding proteins might protect the messenger during transport between both compartments. Several proteins in higher eukaryotes were shown to present a shuttling behavior between nucleus and cytoplasm (18, 20, 49). In trypanosomes a classical nuclear localization signal was identified and shown to be functional in the La and histone H2B proteins (50). A regulated nuclear-cytoplasm export pathway mediated by CRM1 also might be present in kinetoplastid parasites, since leptomyctin B affects the axenic growth of the epimastigote form of the parasite.2 Leptomyctin B inhibits the formation of the complex formed by nuclear export signal-containing proteins, RanGTP, and the receptor CRM1 (51).

Post-transcriptional regulatory mechanisms, such as the ones mediated by ARE or GRE sequences, may be required for a quick response to change mucin core molecules expression pattern, triggering parasite adaptation to sudden changes on the environment. In this regard, expression of the correct surface mucin coat may be of central importance for parasite survival. Identification of an in vivo role for these ARE and GRE RNA-binding proteins in the mRNA stability of T. cruzi transcripts may allow proposal of a model of RNA metabolism and maturation in parasites that are deficient in the regulation by RNA polymerase II transcription.

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