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Evidence for a negative feedback control mediated by the 3′ untranslated region assuring the low expression level of the RNA binding protein TcRBP19 in *T. cruzi* epimastigotes

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ABSTRACT

Because of their relevant role in the post-transcriptional regulation of the expression of a multitude of genes, RNA-binding proteins (RBPs) need to be accurately regulated in response to environmental signals in terms of quantity, functionality and localization. Transcriptional, post-transcriptional and post-translational steps have all been involved in this tight control. We have previously identified a *Trypanosoma cruzi* RBP, named TcRBP19, which can barely be detected at the replicative intracellular amastigote stage of the mammalian host. Even though protein coding genes are typically transcribed constitutively in trypanosomes, TcRBP19 protein is undetectable at the epimastigote stage. Here, we show that this protein expression pattern follows the steady-state of its mRNA. Using a *T. cruzi* reporter gene approach, we could establish a role for the 3′ UTR of the *tcrbp19* mRNA in transcript down-regulation at the epimastigote stage. In addition, the binding of the TcRBP19 protein to its encoding mRNA was revealed by *in vitro* pull down followed by qRT-PCR and confirmed by CLIP assays. Furthermore, we found that forced overexpression of TcRBP19 in *T. cruzi* epimastigotes decreased the stability of the endogenous *tcrbp19* mRNA. These results support a negative feedback control of TcRBP19 to help maintain its very low concentration of TcRBP19 in the epimastigote stage. To our knowledge, this is the first RBP reported in trypanosomatids capable of negatively regulating its own mRNA. The mechanism revealed here adds to our limited but growing number of examples of negative mRNA autoregulation in the control of gene expression.

1. Introduction

Differential expression is a result of multiple layers of complex molecular interactions mediated by gene regulatory signals. In particular, the relevance of post-transcriptional regulation of mRNA levels has been largely recognized and the number and nature of the factors, as well as the mechanisms involved, are constantly growing. Kinetoplastids have proved to be interesting models for the study of gene expression regulation, leading to the early discovery of general molecular mechanisms. While post-transcriptional regulation is envisioned as the main step in the control of gene expression in trypanosomatids, the *cis* elements, *trans* acting factors, and molecular mechanism involved are not yet understood in depth [1,2].

The protozoan *Trypanosoma cruzi* is the causative agent of Chagas' disease. This is a major health problem in Latin America, now spreading to areas such as Canada, the US, and Spain, due to migration of infected people into these non-endemic countries [3]. *T. cruzi* has a complex life cycle alternating through at least 4 stages between 2 hosts: a mammal and an insect. In the mammalian host, the non-replicative metacyclic trypomastigotes, which are highly motile, can invade damaged skin tissue, or mucosa, and can be ingested by macrophages. Inside the cell, metacyclic trypomastigotes differentiate into amastigote forms that divide actively and are released in the mammalian bloodstream as the non-replicative trypomastigote form. Triatomine insects ingest host blood which contains circulating infective bloodstream trypomastigotes. Once in the midgut of the insect, the trypomastigote transforms into the dividing epimastigote form. At the posterior end of the digestive tract, epimastigotes differentiate to the infective and non-dividing metacyclic trypomastigotes, which are eliminated in the insect feces. To allow for a fast adaptation to the different environments constantly faced by the parasite, a rapid regulation of gene expression is needed [1].

Trypanosomatids display a number of distinctive features regarding mechanisms controlling gene expression. Coding genes are organized in polycistronic transcription units, from where individual mRNAs are processed by *trans*-splicing and polyadenylation. There are no canonical promoters for RNA pol II protein coding

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genes identified so far ([1] and references therein). Primary transcripts from non-related genes adjacent in one polycistronic unit are produced at similar levels. Although, transcription initiation has been associated to polycistronic strand switching and chromatin histones modifications ([4] and references therein), regulation of trypanosomatid gene expression is highly dependent on post-transcriptional mechanisms. Thus, quantity, localization and functionality of *trans* acting factors need to be accurately regulated. RNA-binding proteins (RBPs) are prominent *trans* acting factors that assist post-transcriptional processes such as mRNA maturation, stability, transport, and translatability [5,6].

Previously, we have identified a *T. cruzi* RBP (≈17 kDa), named TcRBP19 (Acc Number:XP_814431), which is only detected in the amastigote stage showing a predominant cytoplasmic location [7]. It contains an RNA-binding domain called RNA-recognition motif (RRM). Though some RRM RBPs have been characterized in *T. cruzi*, their RNA targets as well as their role in gene expression regulation have been reported only for a few cases [8]. TcRBP19 function could not be directly attributed *in silico*, since orthologues are restricted to kinetoplastids and not characterized so far [7].

As a first approach to understand the differential expression pattern of TcRBP19, we investigated the mechanisms underlying its mRNA abundance. We found that *tcrbp19* mRNA is three-fold down-regulated in the epimastigote stage compared to the cellular amastigote stage. In addition, we observed that the *tcrbp19* 3′UTR has a down-regulatory effect on the expression of a *cat* reporter gene in epimastigotes, but not in amastigotes. Using *in vitro* approaches we demonstrated that the *tcrbp19* transcript constitutes a binding target for the TcRBP19 protein itself. We also found that *tcrbp19* mRNA stability was decreased when TcRBP19 was ectopically over-expressed in epimastigotes. These results suggest the existence of a negative feedback mechanism affecting *tcrbp19* mRNA levels that may contribute to the control of TcRBP19 abundance in *T. cruzi* epimastigotes.

2. Materials and methods

2.1. Parasites

The *T. cruzi* Dm28 clone [9] was used. Epimastigotes were maintained at 28 °C in liver infusion tryptose (LIT) medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Metacyclic trypomastigotes and *in vitro* extracellular amastigotes were prepared according to established protocols [10].

2.2. RNA preparation and quantification

Total RNA was extracted from 1×10^9 *in vitro* differentiated amastigotes or 1×10^8 epimastigotes, using the RNeasy mini kit (Qiagen) followed by DNase I treatment with DNA-free (Ambion). cDNA was synthesized from 1 μg of total RNA using Superscript III kit first strand synthesis (Invitrogen) and oligodT primer. In order to define the tcrbp19 5′ UTR, spliced leader and tcrbp19 specific primers (SL: 5′-CGCTATTATTGATACAGTTTCTG-3′ and TcRBP19rev: 5′-CGATTTGCCAAGAAGTTTGTG-3') were used. In the same way, to define the tcrbp19 3′UTR, oligodT and tcrbp19 specific primers (Tc19lp1Fw: 5′-TGAGGATTATCGCAGCGCCATAC-3′) were used (Fig. S1).

For the quantification of retrotranscribed products (qRT-PCR), cDNAs were amplified using *tcrbp19* CDS-specific primers (TcRBP19Fw: 5′-AGGCATTCAGCGTTACAAGAAC-3′ and TcR BP19rev: 5′-CGATTTGCCAAGAAGTTTGTG-3′), and *tcrbp19* 5′UTR specific primers (5′UTRfw: 5′-GGTGACGGTTGGTGCT TCTGGC-3′ and 5′UTRrev: 5′-GCACCCGATTTGCCGTTCTTG-3′) (Fig. S1) in a real time rotary analyzer RotorGene 6000 (Corbett). Relative amounts of the target gene were normalized to the *gapdh* housekeeping gene using CDS specific primers (GapdhFw: 5′-CGACAAC GAGTGGGGATACT-3′ and GapdhRev: 5′-CTACAACCTT GCCGAAC-GAT-3′). The PCR reaction mixture containing 0.9 μM of each primer, 1× QuantiTect SYBR Green PCR Master Mix (Qiagen), and 2 μL of cDNA template was performed in a final volume of 10 μL. GST-pull down captured mRNAs and total RNA, as well as RNA from wild type and TcRBP19 over-expressing parasites, were compared using the $2^{-\Delta\Delta CT}$ method [11].

2.3. Reporter analysis

The pTEX-CAT-3′UTR construct was generated from the pTEX vector [12] with the *cat* reporter gene (pTEX-CAT), by substitution of the vector reporter 3′UTRs (*Hind*III and *Xho*I sites), with *tcrbp19* 3′UTRs obtained by RT-PCR using specific primers (tc19-1FW_hind: 5′-CAAAGCTTAAAGGAAATGCGTTGCCTGC-3′; tc19-1REV_Xho: 5′-CCTCGAGCTGCCGCACCCAACATTTCACCG-3′) (Fig. S1). Both pTEX-CAT-3′UTR and pTEX-CAT were used to transfect epimastigotes in replica experiments as indicated above and described previously [7]. In order to analyze *cat* mRNA levels in amastigotes, HeLa cells were infected with transfected parasites as described. Total RNA from the transfected parasites was purified and analyzed by qRT-PCR. Relative amounts of *cat* mRNA (amplified using primers cat_fw: 5′-GCGTGTTACGGTGAAAACCT-3′ and cat_rev: 5′-GGATTGGCTGAGACGAAAAA-3′) normalized to *gapdh* mRNA, were compared between pTEX-CAT-3′UTR and pTEX-CAT transfected parasites using the $2^{-\Delta\Delta CT}$ method [11]. Plasmid copy number was assessed by qPCR measuring the relative amounts of *cat* DNA over *gapdh* DNA in each transfectant.

2.4. RNA pull-down assay

Fusion protein GST-TcRBP19 was produced and purified from *Escherichia coli* cultures as described [7]. Recombinant protein GST-TcRBP19 (or GST alone used as a negative control) was attached to glutathione Sepharose beads (GE Healthcare) for 1 h at 4 °C. 90 μg of total amastigote RNA in binding buffer (10 mM Tris–HCl, 10 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA) were incubated for 1 h at 4 °C with agitation in the presence of 100 mM heparin and 100 mM spermidine as non-specific competitors. After gently washing with PBS, bound RNA was eluted by a 5 min boiling step. Eluted RNAs were purified using the RNeasy mini kit (Qiagen) and treated with DNase I (Ambion). TcRBP19 interacting RNA and non-bound RNA were analyzed by qRT-PCR as indicated above. As a negative control we set reactions using *tcpuf6* primers (PufF 5′-CCTGTATTTTCAGGGCATGTCGGAGTGGGACAACATTGCC-3′ and PufR 5′-GTACAAGAAAGCTGGGTAAATGCAGATGCAGCATTTATACC-3′).

2.5. Cross-linking and RNA immunoprecipitation (CLIP)

Total protein extract from 1×10^8 over-expressing epimastigotes was prepared as described [13] in the presence of 1.000 U of RNAse OUT (Invitrogen) and UV cross-linked for 30 min at 254 nm. Cross-linked RNA–protein complexes were immunoprecipitated with anti-TcRBP19 polyclonal antibody overnight and then, 150 μL Protein A Sepharose (GE Healthcare) was added. A control reaction without antibody addition was also performed. Ribonucleoprotein complexes were washed three times with 200 μL of binding buffer (10 mMTris–HCl, 10 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA). DNase treatment was performed using DNA free kit (Ambion).

cDNA was prepared from immunoprecipitated RNA and RT-PCR was done using Tc19lp1Fw: 5′- TGAGGATTATCGCAGCGCCATAC -3′ and oligodT primers.

2.6. TcRBP19 ectopical over-expression

The plasmid pTEX-TcRBP19 was constructed using the pTEX plasmid [12], in which the *tcrbp19* open reading frame was inserted using the *Bam*HI and *Xho*I sites. Two biological replicas of electroporated epimastigotes with each plasmid (pTEX-TcRBP19 and pTEX alone) were cultured in the presence of Genetecin 500 μg/mL, and checked by optical microscopy and Western blot using the specific antibody anti-TcRBP19, as previously described [7].

2.7. TcRBP19 target mRNA stability assays

Actinomycin D (Sigma) was added to TcRBP19 over-expressing and control pTEX transfected epimastigote cultures (2×10^7 cells/mL) at a 10 μg/mL concentration. Two millilitre aliquots were removed at different time points (0, 10, 20, 40 and 80 min), and RNA was extracted as described above. The primers for qRT-PCR were designed to anneal with the *tcrbp19* 5′UTR region to ensure the exclusive amplification of the endogenous *tcrbp19* mRNA. The fold change of the endogenous *tcrbp19* normalized to *gapdh* and relative to the expression at time zero was calculated using the $2^{-\Delta\Delta CT}$ method as described previously [11].

3. Results and discussion

3.1. The tcrbp19 mRNA is down-regulated in T. cruzi epimastigotes

Trying to get an insight into the molecular mechanisms involved in the differential expression of the TcRBP19 protein in *T. cruzi,* we studied the *tcrpb19* transcript abundance. Despite failure to detect TcRBP19 protein, we found *tcrpb19* transcripts in the epimastigote stage. This is not surprising due to the constitutive transcription of protein genes in kinetoplastids. Sequencing analysis of the amplified products using primers directed to the gene and the spliced leader or polyA tail enabled us to experimentally define the 5′ and 3′ UTR regions (Fig. S1).

Mirroring the protein profile, quantification of the *tcrbp19* steady-state mRNA level revealed a down-regulation in epimastigotes compared to amastigotes (Fig. 1). In accordance with this observation, transcriptome analysis has revealed a diminished content of *tcrbp19* mRNA in epimastigotes compared to the cumulative expression in the four developmental parasite life cycle stages [14]. Even though the involvement of translational and post-translational processes may also contribute to the actual protein content, this finding points out to the existence of a mRNA stability mechanism acting on the regulation of the differential expression and provide a possible explanation for the TcRBP19 protein pattern previously observed [7].

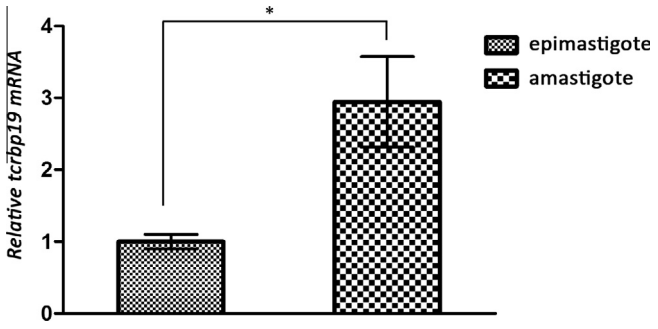

Fig. 1. Quantification of endogenous *tcrbp19* mRNA levels in *T. cruzi* amastigotes and epimastigotes. mRNA quantification relative to *gapdh* mRNA was assessed by qRT-PCR in total RNA obtained from *T. cruzi* epimastigote and amastigote. Results are expressed as means of triplicates and s.d. values are indicated. Normality and homogeneity of the data were evaluated by analysis of variance (ANOVA). *t*-test was used to determine the statistical significance. **Statistical significance difference* (*$p < 0.05$).

3.2. The tcrbp19 3′UTR down-regulates reporter gene transcripts in T. cruzi epimastigotes

It is widely recognized that the *cis* signals that participate in the control of mRNA stability are mainly located at the 3′UTRs. Such is also the case for *T. cruzi*, where the regulatory role of the 3′UTRs of some protein coding genes, differentially expressed during the parasite life cycle has been already reported [2,15–18]. Therefore, we studied the putative involvement of the *tcrbp19* 3′UTR in its post-transcriptional regulation. To address this issue, we analyzed the expression of a reporter gene (*cat*) from an episomal vector (pTEX) both in the epimastigote and the amastigote stage. Reporter mRNA levels were compared between two transfected populations: pTEX-CAT, which bears the *gapdh* 3′UTR and pTEX-CAT-3′UTR, carrying the *tcrpb19* 3′UTR downstream the reporter coding region. Epimastigote transfectant parasites were cultured at low antibiotic pressure to maintain a low copy number of plasmid and, hence, minimize the effect of over-expression. Nonetheless, plasmid copy number per parasite was calculated from total DNA by qPCR using *cat* and *gapdh* specific primers, yielding in average, one copy of the vector per transfectant genome.

The effect of *tcrbp19* 3′UTR on the abundance of *cat* mRNA, was quantified by qRT-PCR using *gapdh* as a control (Fig. 2). We found that in epimastigotes, the substitution of the reporter gene 3′UTR by the *tcrbp19* 3′UTR significantly reduces reporter mRNA levels. On the contrary, in the amastigote stage, a significant increase of *cat* mRNA levels comparing data from pTEX-CAT-3′UTR with the pTEX-CAT parasites is found. These results supports the existence of a regulatory element in the *tcrbp19* 3′UTR that remains to be delimitated.

Though the *tcrbp19* 3′UTR contains U-rich tracts, a sole AU rich core element AUUUA (ARE) was found. This 3′UTR element, widespread in eukaryotes, has been involved in the regulation of mRNA stability [19,20]. In *T. cruzi*, the role of AREs in the 3′UTR of TcSMUG mRNA mediating developmental regulation has been suggested [21,22]. Besides, a 3′UTR 43-nt U rich element, which also includes an ARE core pentamer, has been involved in the modulation of mRNA abundance in the intracellular amastigote stage [23]. However, no significant similarity of the *tcrbp19* 3′UTR to this 43-nt U element was found. This is not surprising since different subset of genes responding to different signals may have similar patterns of expression during development.

3.3. The tcrbp19 transcript constitutes a TcRBP19 target

Previous analysis of the *in vitro* amastigote RNA targets of TcRBP19 protein by GST pull-down assays followed by competitive hybridization of the eluted and bound RNAs to a *T. cruzi* DNA microarray (to be published elsewhere), revealed *tcrbp19* as the most represented mRNA. Since these data suggest that the TcRBP19 protein may assist its own mRNA metabolism, we here focused on this interaction.

Firstly, the amounts of *tcrbp19* relative to *gapdh* obtained from amastigote total or captured RNA in the TcRBP19 GST pull-down were quantified (Fig. 3A) to verify those previous results. As a control, the relative amount of the unrelated *tcpuf6* transcript [24] was also determined. The specific detection of *tcrbp19* mRNA in the pull-down supports that TcRBP19 protein associates with its own endogenous mRNA *in vitro*.

Secondly, to confirm the direct interaction of TcRBP19 with its encoding mRNA, we performed CLIP assays with total extract from TcRBP19 over-expressing parasites. The ectopic over-expression

was achieved by cloning the TcRBP19 coding region in the pTEX vector (pTEX-TcRBP19) (Fig. S2). Cross-linking followed by immunoprecipitation, using a previously characterized specific TcRBP19 antibody [7], enabled the detection of *tcrbp19* mRNA in the immunoprecipitated fraction (Fig. 3B).

3.4. The endogenous tcrbp19 mRNA is down-regulated when TcRBP19 expression is forced in epimastigotes

Since no functional RNA interference mechanism has been demonstrated in *T. cruzi* [25] and only a few cases of gene knock down have been reported in this parasite, over-expression constitutes a major approach to study protein function. Indeed, ectopic over-expression is helping to understand RBP functions/mechanisms in trypanosomatids (for a recent example see [26]). In order to understand the biological significance of TcRBP19 binding to its own mRNA, we studied the effect of its over-production. In the TcRBP19 over-expressing parasites the level and functionality of the ectopic *tcrbp19* transcript relies under *gapdh* UTR and intergenic region control (Fig. S2). As previously reported, the forced over-expression of TcRBP19 did not produce any evident morphological changes in *T. cruzi* epimastigotes [7]. However, under these conditions, a significant fall in the endogenous steady-state of *tcrbp19* transcript relative to *gapdh* mRNA levels was observed (≈190-fold, $p < 0.05$). For the unequivocal quantification of the endogenous *tcrbp19* transcripts, primers driving the amplification of part of the *tcrbp19* 5′UTR, absent in the TcRBP19 over-expressing vector, were designed. In addition, the amount of endogenous *tcrbp19* transcript relative to *gapdh* gene transcript was measured by qRT-PCR at different time points after actinomycin D treatment, and normalized to time 0 (Fig. 4). A decrease of the endogenous *tcrbp19* mRNA stability can be deduced for TcRBP19 over-expressing parasites when compared to the pTEX control ones. Therefore, the involvement of TcRBP19 in the destabilization of its cognate transcript is strongly suggested.

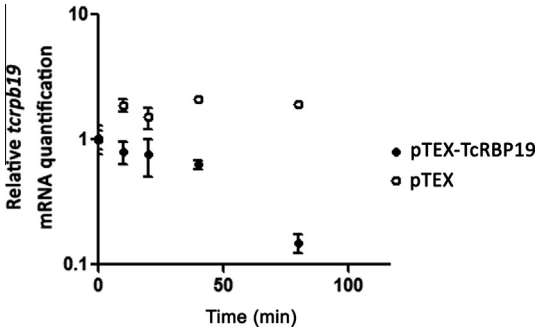

Fig. 4. Effect of TcRBP19 over-expression on *tcrbp19* mRNA stability. The amount of endogenous *tcrbp19* transcript was determined in triplicates at different time points after actinomycin D treatment by qRT-PCR in TcRBP19 over-expressing epimastigotes (pTEX-TcRBP19), using *tcrbp19* 5′UTR primers (filled circles). Control parasites transfected with pTEX only were processed in the same way (empty circles). mRNA quantification was calculated relative to *gapdh* mRNA and normalized to time 0 as described [11].

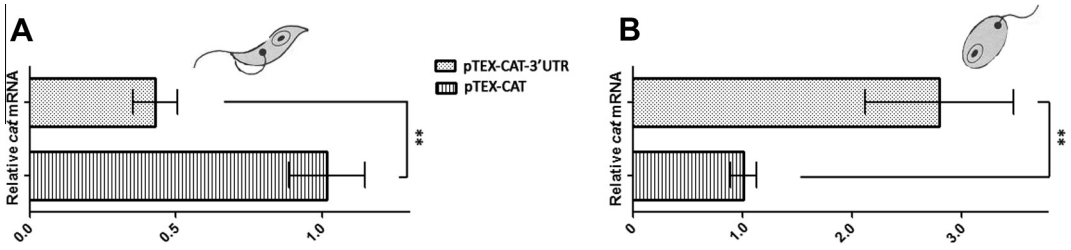

Fig. 2. Effect of *tcrbp19* 3′UTR on reporter gene mRNA steady state levels. mRNA quantification relative to *gapdh* mRNA was assessed by qRT-PCR in total RNA obtained from parasites transfected with pTEX-CAT-3′UTR and control pTEX-CAT, normalized to the latter both in *T. cruzi* epimastigotes (A) and amastigotes (B). Each point corresponds to the average obtained from triplicates of parasite populations derived from two independent pTEX-CAT-3′UTR plasmid transfection and sd. values are indicated. Normality and homogeneity of the data were evaluated by analysis of variance (ANOVA). *t*-test was used to determine the statistical significance. **Statistical significance difference* (**$p < 0.005$).

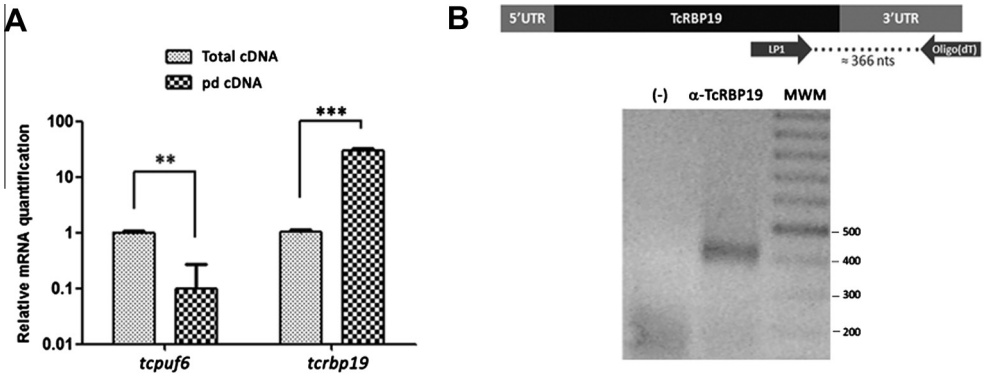

Fig. 3. Binding of *tcrbp19* mRNA to TcRBP19 protein. (A) Quantification of *tcrpb19* mRNA bound *in vitro* to the recombinant GST-TcRBP19. qRT-PCR analysis of *tcrpb19* transcript relative to *gapdh* mRNA was assayed in total cDNA and in the *pull-down* (pd) fraction. The amount of the unrelated *tcpuf6* transcript was determined as a negative control. Average values of triplicates are shown and the corresponding sd. are indicated. Normality and homogeneity of the data were evaluated by analysis of variance (ANOVA). *t*-test was used to determine the statistical significance. **Statistical significance difference* (**$p < 0.005$; ***$p < 0.0001$). (B) Protein–RNA crosslinking and immunoprecipitation with (α-TcRBP19) or without (−) anti-TcRBP19 affinity purified polyclonal antibody was performed on TcRBP19 over-expressing epimastigotes, followed by RT-PCR using the primers shown in the upper scheme. MWM: Molecular Weight Marker GeneRuler™ 100 bp DNA Ladder (Fermentas®).

Negative feedback of mRNA by their encoded products provides a fine-tuning mechanism for the precise control of their expression. In higher eukaryotes, some RBP coding genes have been involved in these regulatory mechanisms modulating transcription, alternative splicing, 3′ end processing and translation efficiency (see for example [27–31]) establishing auto- or hierarchical regulation. Since trypanosomes do not regulate transcription initiation [5,6], control of mRNA abundance is entirely dependent on post-transcriptional events among which, exploiting mRNA down-autoregulation constitutes a plausible recourse.

We have very recently shown that over-expression of TcRBP19 in *T. cruzi* impairs differentiation into the metacyclic infective form and subsequent host cell infectivity [32]. Thus, to avoid impairment of progression through the parasite's life cycle, TcRBP19 levels in the epimastigote stage must remain tightly controlled. In this context, the specific down-autoregulatory mechanism here revealed could serve to monitor the *tcrbp19* mRNA levels in the epimastigote stage. This mechanism might enable a close tracking of TcRBP19 dose granting its under representation in epimastigotes. The delimitation of the 3′UTR elements required for mRNA modulation, as well as the study of stage-specific TcRBP19 mRNA targets and protein partners would contribute to further understand TcRBP19 function.

In conclusion, our work not only shows that the abundance of *tcrbp*19 mRNA in *T. cruzi* epimastigotes is controlled *via* its 3′UTR, but also identifies its protein product, TcRBP19, as a *trans* acting factor exerting a negative control on *tcrbp19* mRNA stability in *T. cruzi* epimastigotes. This finding underscores the importance of RBPs and their participation in multiple layers of control of gene expression, being TcRBP19 the first reported protein that is involved in mRNA negative autoregulation in trypanosomatids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.096.

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