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ORIGINAL ARTICLE

Insights into the Regulation of mRNA Processing of Polycistronic Transcripts Mediated by DRBD4/PTB2, a Trypanosome Homolog of the Polypyrimidine Tract-Binding Protein

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ABSTRACT

Trypanosomes regulate gene expression mostly by posttranscriptional mechanisms, including control of mRNA turnover and translation efficiency. This regulation is carried out via certain elements located at the 3′-untranslated regions of mRNAs, which are recognized by RNA-binding proteins. In trypanosomes, trans-splicing is of central importance to control mRNA maturation. We have previously shown that *Tc*DRBD4/PTB2, a trypanosome homolog of the human polypyrimidine tract-binding protein splicing regulator, interacts with the intergenic region of one specific dicistronic transcript, referred to as *TcUBP* (and encoding for *Tc*UBP1 and *Tc*UBP2, two closely kinetoplastid-specific proteins). In this work, a survey of *TcUBP* RNA processing revealed certain *Tc*DRBD4/PTB2-regulatory elements within its intercistronic region, which are likely to influence the trans-splicing rate of monocistronic-derived transcripts. Furthermore, *Tc*DRBD4/PTB2 overexpression in epimastigote cells notably decreased both UBP1 and UBP2 protein expression. This type of posttranscriptional gene regulatory mechanism could be extended to other transcripts as well, as we identified several other RNA precursor molecules that specifically bind to *Tc*DRBD4/PTB2. Altogether, these findings support a model in which *Tc*DRBD4/PTB2-containing ribonucleoprotein complexes can prevent trans-splicing. This could represent another stage of gene expression regulation mediated by the masking of trans-splicing/polyadenylation signals.

TRYPANOSOMA cruzi is a digenetic protozoan parasite that suffers continuous morphological changes throughout its complex life cycle. Developmental stages include the proliferative forms epimastigotes (in the gut of the arthropod vector) and amastigotes (within mammalian cells), and the infective forms, called metacyclic trypomastigotes in the vector and blood trypomastigotes in the mammalian host. Current evidence stresses that, to control gene expression, these microorganisms make use essentially of posttranscriptional processing (Clayton 2014; Freire et al. 2014; Kramer and Carrington 2011), which includes a basic mechanism of mRNA localization (Alves et al. 2013). Translation initiation has been reported to be important for the growth control and differentiation in trypanosomes through regulation of a membrane-bound eIF2 alpha kinase (da Silva Augusto et al. 2015). Recently, ribosome profiling has revealed that translation regulation, in addition to control of the steady-state level of mRNA, is a major mechanism affecting parasite differentiation in *T. cruzi* (Smircich et al. 2015). Within this context of genetic programs, trypanosomes are quickly emerging as one of the best studied biological systems in which RNA regulons (Keene 2014; Mansfield and Keene 2009) coordinate posttranscriptional gene expression (Archer et al. 2009; Blackinton and Keene 2014; De Gaudenzi et al. 2013; Fernandez-Moya and Estevez 2010; Li et al. 2012).

In these parasites, most protein-coding genes are arrayed in long multigene transcription units. This structural peculiarity has been particularly studied for large gene families such as amastin surface glycoproteins (Jack-

son 2015; Kangussu-Marcolino et al. 2013). Trypanosome genetic information is transcribed into large polycistrons and individual mRNAs are generated by 5′-trans-splicing and 3′-polyadenylation of precursor RNAs (Clayton 2013). Co-transcriptional RNA processing is governed by a common polypyrimidine tract located between two neighboring open reading frames, which is the signal sequence recognized by both trans-splicing and polyadenylation machineries (Matthews et al. 1994). During this processing, a spliced-leader (*SL*) sequence is added to all RNA molecules only a few nucleotides upstream the start codon, thus generating short 5′-untranslated regions (UTRs). These 5′-UTRs and the coding sequence are usually under structural constraints to accommodate the translational machinery (Conne et al. 2000). Therefore, the main elements involved in transcript stability and translation efficiency are located within the 3′-UTRs. Linear or structured RNA elements located in the 3′-UTR of certain mRNAs have been shown to function as *cis*-acting motifs that bind different *trans*-acting factors (Araujo and Teixeira 2011; Bayer-Santos et al. 2012; Pastro et al. 2013; Perez-Diaz et al. 2013), including components of the kinetoplastid superfamily of RNA-recognition motif (RRM)-containing RNA-binding proteins (RBPs) (De Gaudenzi et al. 2005; Guerra-Slompo et al. 2012). Two small RRM-type protein family members, termed *T. cruzi* uridine-rich RBP 1 (*Tc*UBP1) and *Tc*UBP2, have been extensively studied by our group, providing evidence on gene structures (D'Orso and Frasch 2002; De Gaudenzi et al. 2003, 2005; Jäger et al. 2007) and biological functions (Cassola et al. 2015; De Gaudenzi et al. 2011, 2013; Li et al. 2012; Noe et al. 2008). Both *UBP1* and *UBP2* are encoded in a single stable dicistronic unit, referred to as *T. cruzi* uridine-rich RNA-binding protein (*TcUBP*), separated by an intercistronic region (ICR) of ~3.4-kb. This transcript has the canonical 39-nt *SL* sequence at its 5′-end but appears either to lack or to have a very short poly(A) tail at its 3′-end (Jäger et al. 2007). The dicistronic RNA is highly expressed whereas monocistrons are barely detectable in Northern blot assays. Moreover, *TcUBP* pre-mRNA is present in all life cycle stages of *T. cruzi* but only UBP2 expression is developmentally regulated (De Gaudenzi et al. 2003). This fact prompted us to evaluate whether the *TcUBP* dicistron can be further processed into mature monocistronic transcripts and whether protein translation can initiate directly from this dicistronic molecule.

Although it is clear that the polypyrimidine tract contributes to the selection of the splicing site (Campos et al. 2008), there is not much information about the key elements determining trans-splicing efficiency (Siegel et al. 2005). To modulate trans-splicing, there should be factors binding to specific signals to either activate or repress this process. In agreement with this, the presence of the SR and hnRNP protein families in trypanosomatids (reviewed in He and Smith 2009; Shepard and Hertel 2009), previously described as modulators of splicing, suggests the presence of the machinery necessary for a functional alternative splicing in these parasites. Another important factor to analyze is the presence of the polypyrimidine tract-binding protein (PTB), a key modulator of alternative splicing in higher eukaryotes (Kafasla et al. 2012). It has been shown that mammalian PTB can repress not only splicing but also polyadenylation (Le Sommer et al. 2005). Although its localization is mainly nuclear, this protein can shuttle into the cytoplasm to stabilize different mRNAs (Estevez 2008; Stern et al. 2009) and regulate the localization and translation of transcripts through internal ribosome entry sites (Bushell et al. 2006).

Two variants of this protein have been described in *T. brucei* and *T. cruzi*: double RNA-binding domain 3 (DBDR3) and DBDR4, the trypanosome homologs of the mammalian PTB1 and PTB2, respectively (De Gaudenzi et al. 2005). Both proteins have been shown to be essential but differentially involved in regulation of splicing, as DRDB3/PTB1 but not DRDB4/PTB2 affects trans-splicing as well as *cis*-splicing (Stern et al. 2009). The functions of *T. cruzi* DRBD4/PTB2 (TcCLB.511727.160) have not been well studied yet. Changes in the localization of this protein (from nuclear speckles to the nucleolus) have been observed upon induction of different stresses (Nazer and Sanchez 2011; Nazer et al. 2011, 2012) and a previous work of our group reported a possible role for this protein in trans-splicing (Jäger et al. 2007). Here, we identified pre-mRNA targets binding specifically to *Tc*DRBD4/PTB2. In addition, we particularly describe the structure of one of these targets, the dicistronic unit *TcUBP*, and the role of *Tc*DRBD4/PTB2 in the trans-splicing processing of this type of RNA precursor molecules.

MATERIALS AND METHODS

Parasite cultures and drug treatments

The *T. cruzi* CL-Brener cloned stock was used (Zingales et al. 1997). Culture conditions and protein extract preparations were according to Di Noia et al. (Di Noia et al. 2000). Actinomycin D (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 10 μg/ml for 3 or 6 h. For polysome analysis, cycloheximide (Research Organics Inc., Cleveland, OH) was used at a final concentration of 50 μg/ml. All parasite aliquots were harvested by centrifugation and washed with phosphate-buffered saline.

*Tc*DRBD4/PTB2-associated RNAs

Cell lysis, immunoprecipitation assay, RNA purification, and identification of *Tc*DRBD4/PTB2 targets were performed as described before (Noe et al. 2008). Briefly, anti-*Tc*DRBD4/PTB2 polyclonal rabbit serum was used at 1:500 dilution and RNA was extracted from immunoprecipitation complexes with TriZol reagent (Invitrogen Argentina S.A., CABA, Buenos Aires) and used to perform RT-PCR with random primers and the BD Smart™ PCR cDNA synthesis Kit (BD Biosciences, San Jose, CA). PCR products were cloned and sequenced. As a control, RNA isolated by immunoprecipitation with antibodies toward the non-RBP protein, *T. cruzi* Ribose 5-phosphate isomerase Type B (Stern et al. 2007) was used. RT-PCRs of RNA extracted

from ribonucleoprotein (RNP) complexes were performed to confirm the presence of target pre-RNA candidates using gene specific oligonucleotides (see Table 1). All PCR products had the expected size and were fully sequenced for their validation.

Northern blots

Hybridizations were performed as described in Di Noia et al. (2000) with DNA probes radioactively labeled by PCR with [α-^{32}P] dCTP (Perkin-Elmer Life Sciences, Akron, OH) using the specific primers: NH2-UBP1-*Bam*HI and NH2-TcUBP1/AS for the probe *Tcubp1*, NH2-UBP2-*Bam*HI and NH2-TcUBP2/AS for the probe *Tcubp2*, 28S-Fwd and 28S-Rev for the probe *28S rRNA*, and Neo-Fwd and Neo-Rev for the Neo probe (see Table 1 for details). The *neo* resistance gene was used for selection and as an internal control of transfection levels and 28S rRNA probe was used as loading control.

Bioinformatic analysis

Sequence similarity searches were performed using the BLAST algorithm at the Internet site of the National Center for Biotechnology Information. Sequence comparisons were performed using the TriTryp database (http://tritrypdb.org/tritrypdb/) (Aslett et al. 2010).

DNA constructs and parasite transfections

The DNA construct named pR-ICR was obtained by cloning a cDNA fragment corresponding to the complete *TcUBP* ICR (GenBank accession no. AY210411) into the *Bam*HI site of the pRIBOTEX vector, fusing the reporter gene *Red Fluorescence Protein (RFP)* to *UBP2* and *UBP1* to *Green Fluorescence Protein (GFP)*. Deletion of the first ~200-nt at the 5′-end of the ICR was obtained by PCR using specific primers (see Table 1), checked by sequencing and termed ICRΔ5. Similarly, deletion of 156-nt at the 3′-end of the ICR was also obtained by PCR using specific primers (see Table 1) and termed ICRΔ3. Parasite transfections were carried out as previously described (Di Noia et al. 2000).

Sucrose gradients and polysome purification

Epimastigote cultures were treated with cycloheximide for 20 min to arrest protein synthesis, as described above. Parasite lysates corresponding to 500 × 10^6 cells were loaded onto a sucrose step gradient (15–45% sucrose) or

Table 1. Constructions made for *Tc*DRBD4/PTB2 cloning, ICR deletions, and DNA/RNA probe synthesis

Clone or construct	Primer name	Sequence (5′ to 3′)[a]
*Tc*DRBD4/PTB2	Fwd-*Eco*RI	cgaattcATGATGTCCGTGGTCTTGCG
	Rev-*Bam*HI	cggatccTCACTCCTCTTCAGTTGGTTTCTCC
ICR	UBP2-STOP/SE	cgcggatccGTTCTCTCTGCTCTTTGGTGTGTGT
	P4_*Bam*HI	cgggatccTTCCCACAATCAAGCAAAC
ICRΔ5	UBP2_3UTR-SE	AGAAGGGGCGAAGGCAG
	P4_*Bam*HI	cgggatccTTCCCACAATCAAGCAAAC
ICRΔ3	UBP2-STOP/SE	cgcggatccGTTCTCTCTGCTCTTTGGTGTGTGT
	C2/AS	CAGATGTGAGGCAAGTACACG
Tcubp1 probe	NH2-UBP1-*Bam*HI	cgggatccATGAGCCAAATTCCGTTGGTTTC
	NH2-UBP1/AS	TTGTTGTTGCTGCTGCTGCAGC
Tcubp2 probe	NH2-UBP2-*Bam*HI	cgggatccATGTCTCAACAGATGCAATAC
	NH2-UBP2/AS	ATCGGGCTCGGGGTTCATCTG
28S rRNA probe	Fwd	GTAGTATAGGTGGAAGCGCAAG
	Rev	CCAGCTCACGTTCCCTGTCA
Neo probe	Fwd	ATGGGATCGGCCATTGAACAAG
	Rev	GGCTAGCATACTCTAGCGGCATATACAC
Neg. ctrl. (100 nt)	T7 primer	GTAATACGACTCACTATAGGG
	SP6 primer	TATTTAGGTGACACTATAG
TcCLB.509795.20/30	ICR_Fwd	AGACACGGAAGATGATTTGGGCGA
	ICR_Rev	TGCACCAACGCCATTCGTTTCACG
TcCLB.504431.64/50	ICR_Fwd	TCCAAAGAGGTCCATGTCTGCCTA
	ICR_Rev	CGTGAAAGTTGTCGCACACAAGCA
TcCLB.506625.70/80	ICR_Fwd	AAAGACGGCGTGATCGAAGTTACGGT
	ICR_Rev	TTTGACACCTTCGTCGGAATGCCC
TcCLB.509549.110/120	ICR_Fwd	GACGAACGGAACTATTGGCT
	ICR_Rev	CAAGACGTTCTTCTTCGGCA
TcCLB.511391.180/170	ICR_Fwd	TGCTGCTGCTGCTGGTGGT
	ICR_Rev	AAACCGACTCTTCACCCGCATCT
TcCLB.509551.40/30	ICR_Fwd	CGGTGGCACTGGTAAGAAGT
	ICR_Rev	CTCATCCTCCAGCACCTCTC

[a]The restriction sites used for molecular cloning are lowercase/underlined.

a 30% sucrose cushion. For EDTA treatment, samples were resuspended in 20 mM EDTA (final concentration) prior to sucrose gradient fractionation. To isolate purified polysomes, the lysates were clarified by centrifugation at 4 °C for 10 min at 10,000 *g*. The supernatant was layered onto a 30% sucrose cushion and centrifuged for 2.5 h at 4 °C at 100,000 *g* in a SW41 rotor. The supernatant (S100) was harvested and the pellet (P100, polysomes) was resuspended in protease inhibitor. Gradients were centrifuged at 150,000 *g* in a Beckman SW40Ti rotor for 3 h at 4 °C. Fractions (1 ml) were collected from the top of the vial. Aliquots of each fraction were analyzed for protein composition. Protein samples were precipitated with 20% (w/v) trichloroacetic acid and cold acetone and used in Western blotting experiments. All fractions were stored at −80 °C.

Western blots

Protein immunoblot experiments were performed as previously (De Gaudenzi et al. 2003). The antibodies used were: anti-alpha Tubulin antibody (Sigma-Aldrich), rabbit polyclonal anti-*Tc*DRBD4/PTB2 antibody (kindly provided by Lena Åslund, Uppsala University, Sweden), rabbit polyclonal antibody directed toward the complete RBD of *Tc*UBP1 (anti-RRM) (D'Orso and Frasch 2002), rabbit polyclonal antibody against *Tc*RBP3 (anti-*Tc*RBP3) (De Gaudenzi et al. 2003), and mouse polyclonal antibody against the 40S ribosomal protein S12 (anti-*Tc*S12) (Cassola et al. 2007).

Microscopic analysis

Parasites were harvested, washed in phosphate-buffered saline, adhered to polylysine-coated microscope slides, fixed with 4% paraformaldehyde in phosphate-buffered saline followed by 10-min incubation with 25 mM NH_4Cl. DNA was stained with 1 mg/ml DAPI and slides were mounted with 10 μl of Fluor Save reagent (Calbiochem, San Diego, CA). Microscopic analysis was performed in a Nikon Eclipse E600 microscope coupled to a SPOT RT color camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Immunofluorescence staining was performed as previously described (Jäger et al. 2007). Antibodies used were: mouse polyclonal anti-*Tc*DRBD4/PTB2 antibody, rabbit polyclonal anti-acetylated Histone H4 (Abcam, Cambridge, MA), rabbit polyclonal anti-Ribose 5-phosphate isomerase type B (anti-*Tc*RPIB, Stern et al. 2007; kindly provided by Juan J. Cazzulo, IIB-UNSAM, Buenos Aires), and rabbit polyclonal anti-heat shock protein 70 (anti-*Tc*HSP70, Jäger et al. 2007). Secondary antibodies used were: goat anti-mouse IgG Alexa Fluor 488 conjugate (Invitrogen), and goat anti-rabbit IgG Alexa Fluor 568 conjugate (Invitrogen).

DRBD4/PTB2 cloning, expression, and purification

A 1,473-nt fragment corresponding to *tcdrbd4/ptb2* coding sequence (TcCLB.511727.160) was amplified by PCR with oligonucleotides Fwd-*Eco*RI and Rev-*Bam*HI (see Table 1) and cloned into the pGEX system (GE Healthcare Bio-Sciences, Pittsburgh, PA), generating a glutathione S-transferase (GST) fusion and transformed in *Escherichia coli* strain DH5αF'Iq. Cultures were induced with 0.15 mM isopropyl β-D-thiogalactopyranoside for 16 h at 18 °C. Recombinant proteins were purified using GST-agarose columns (Sigma). The PCR product was also cloned into the pRIBOTEX vector (Martinez-Calvillo et al. 1997).

Dihydrazide-agarose RNA cross-linking

Poly(U) homoribopolymer (Sigma-Aldrich) and in vitro transcribed RNAs were oxidized with $NaIO_4$ and cross-linked to adipic acid dihydrazide-agarose beads (Sigma-Aldrich) as indicated previously (Caputi et al. 1999). Purified proteins or parasite protein extracts were incubated for 1 h with RNA cross-linked beads and washed. Elution was done with 2X Laemmli buffer and samples were resolved by electrophoresis in SDS-PAGE gels and finally detected by Coomassie Blue staining or Western blotting assays. A cDNA fragment containing the complete ICR was cloned into the pGEM-T Easy vector system (Promega Corporation, Madison, WI) downstream the T7 promoter and in vitro transcribed using the MEGAscript kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. Negative control was PCR amplified using T7/SP6 primers and pGEM-T Easy (Promega Corporation) as template, digested with *Nco*I and in vitro transcribed with SP6 MEGAscript kit (Ambion Inc.).

RESULTS

*Tc*DRBD4/PTB2 recognizes RNA fragments within the *TcUBP* ICR

Genome organization in trypanosomes is characterized by the presence of gene clusters that are co-directionality transcribed into polycistronic RNAs (De Gaudenzi et al. 2011; Jackson 2015). Here, we focused on the study of a dicistronic precursor molecule that encodes *Tc*UBP1 and *Tc*UBP2 (Fig. 1A). A previous in silico study identified several putative motifs within the ICR that might be involved in *TcUBP* dicistronic RNA processing, among them: AU- or GA-rich elements and pyrimidine-rich tracts (Jäger et al. 2007). AU-rich elements have been previously reported to be required for epimastigote stage-specific expression of mRNAs (Suganuma et al. 2013) and 14 of these above-mentioned polypyrimidine tracts were of particular interest because they contain putative recognition sites for PTB (Fig. 1A).

According to this, we sought to determine if the splicing factor double RNA-binding domain 4 (DRBD4)/PTB2 binds specifically to the *TcUBP* ICR. Thus, both the recombinant DRBD4/PTB2 and the trypanosome protein extract were independently incubated with the full ICR transcript immobilized to dihydrazide-agarose beads. Eluted proteins were separated by SDS-PAGE and stained by Coomassie Blue or transferred to nitrocellulose for immunodetection using

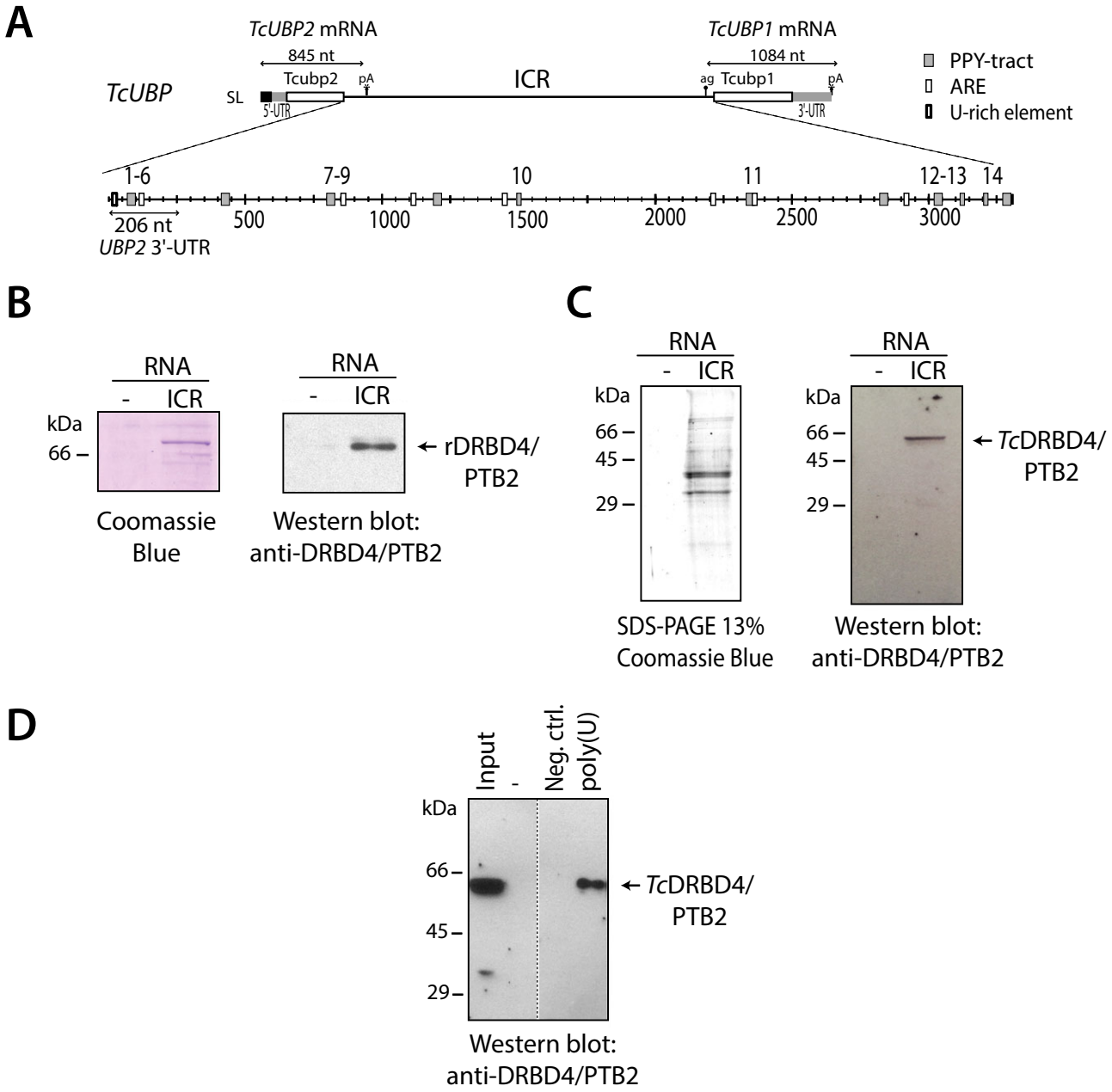

Figure 1 Cis-acting elements within *TcUBP* intercistronic region interact in vitro with *Tc*DRBD4/PTB2. **A.** Schematic representation of the primary structures of dicistronic and monocistronic RNA species. Detail of the *TcUBP* ICR showing the AU-rich (ARE), U-rich elements, polypyrimidine (PPY) tracts, and putative PTB-binding sites (numbers 1–14). The relative position of each element is indicated by the numbers on the bottom of the scheme. The 206-nt sequence corresponding to UBP2 3′-UTR that contains the first six PPY-tracts is also shown. **B** and **C.** *TcUBP* ICR was in vitro transcribed, cross-linked to adipic acid dihydrazide-agarose beads, and incubated with GST-*Tc*DRBD4/PTB2 (B) or total parasite protein extracts (C). One fraction of the remaining bound proteins was separated by SDS-PAGE and stained with Coomassie Blue, and an equivalent fraction was transferred to nitrocellulose filters and probed with anti-*Tc*DRBD4/PTB2 antibodies (1:500 dilution). **D**. Negative control was in vitro transcribed and processed as in C. As positive control poly(U) homoribopolymer was included. Input = total protein extract; – = beads alone; ICR = complete ICR transcript; Neg. ctrl. = negative control; poly(U) = poly(U) homoribopolymer. The arrows indicate the position of the GST-tagged (rDRBD4/PTB2) and native (*Tc*DRBD4/PTB2) proteins. Protein molecular weight markers are indicated on the left margins.

specific anti-*Tc*DRBD4/PTB2 antibodies. The recombinant GST-*Tc*DRBD4/PTB2 interacted in vitro with the complete ICR fragment of *TcUBP* (Fig. 1B). As expected, the native endogenous *Tc*DRBD4/PTB2 bound to the complete ICR dicistronic RNA (Fig. 1C) but failed to bind to a nonspecific RNA fragment transcribed from a pGEM-T Easy polylinker

(negative control, Fig. 1D). Also, the interaction to pyrimidine poly(U) nucleic acid used as a positive experimental control was detected (Fig. 1D).

DRBD4/PTB2 regulatory elements found within *TcUBP* ICR affect RNA processing

To further molecularly characterize the *TcUBP* RNA, the original ICR region (pR-ICR) was now replaced with a mutated construct (pR-ICRΔ5) that lacks a short 5′-intergenic pyrimidine-rich fragment of about 200-nt corresponding to the 3′-UTR of UBP2, thus deleting 6 of the 14 PTB-binding sites detected within the *TcUBP* ICR (Fig. 2A). In this regard, we found that the majority of the predicted PTB-binding motifs identified in the ICR have a position bias. If we split the ~3.4-kb sequence into two segments, more than 70% of the PTB putative elements were preferentially located proximal to the *TcUBP2* coding sequence. Stably transfected parasites were treated for 6 h with actinomycin D to block transcription. Northern blot analysis of total RNA from these parasites showed that although recombinant *RFP-UBP2* and *UBP1-GFP* monocistronic mRNA levels are barely detectable in the ICR transfection (Fig. 2B), the ICRΔ5 construct yielded higher monocistronic expression levels (Fig. 2B). As the DNA constructs were designed as fusion proteins with the reporter genes *RFP* and *GFP* (fused to *UBP2* and *UBP1*, respectively), we were able to analyze protein expression in transfected parasites by fluorescence microscopy. Parasites transfected with the ICRΔ5 construct showed a higher fluorescence signal than the corresponding control with the natural ICR, which correlates with an increase in the levels of monocistronic RNA production (Fig. 2C). As a control, these samples were also used for immunofluorescence staining with alpha Tubulin, where no visible differences were detected (Fig. 2C, S1). To rule out the influence of the length of this region, we performed another transfection experiment with a new construct, named ICRΔ3, containing a mutated ICR with a 3′ deletion of 156-nt length that removes the 5′-UTR of *Tc*UBP1. Northern blot analysis showed that this deletion does not alter the dicistron RNA processing (Fig. S2). Altogether, these results show that the ~200-nt ICR 5′ deletion from the construction ICRΔ5 can prevent trans-splicing processing and suggest that *Tc*UBP1 and *Tc*UBP2 protein levels are directly related to *TcUBP1* and *TcUBP2* monocistrons. Given that six PTB-binding sites were deleted with this fragment, it is tempting to speculate that DRBD4/PTB2 might be involved in ICR processing, thus influencing monocistronic RNA maturation.

Overexpression of *Tc*DRBD4/PTB2 reduces *Tc*UBP1 and *Tc*UBP2 protein expression

In agreement with the presence of several putative PTB-binding sites found along *TcUBP* ICR, we have previously demonstrated by co-immunoprecipitation experiments that *Tc*DRBD4/PTB2 is associated in vivo with *TcUBP* RNA (Jäger et al. 2007). Thus, we next analyzed whether this RBP is able to specifically affect the expression of *Tc*UBPs proteins by overexpressing *Tc*DRBD4/PTB2 in epimastigote cells. It should be noted that *Tc*DRBD4/PTB2 overexpression has a deleterious effect on the viability of the cells, precluding the purification of the transfected population that is required for qPCR analysis of dicistronic and monocistronic levels. Alternatively, we analyzed the transfected population by indirect immunofluorescence using anti-*Tc*DRBD4/PTB2 and anti-*Tc*UBP antibodies (Fig. 3A and B). The parasites overexpressing *Tc*DRBD4/PTB2 protein (pR-DRBD4/PTB2, parasites showing a high level of nuclear green fluorescence) showed a significant reduction of red fluorescence corresponding to the signal of UBP1 and UBP2 RRM-type proteins comparing to the empty vector-transfected control parasites (pR, which showed a low nuclear green fluorescence corresponding to the expression of the endogenous protein) (*t*-test, $p < 0.0001$; Fig. 3C). We conclude that DRBD4/PTB2-overexpressing parasites show a marked reduction in the number of monocistronic-derived *Tc*UBP1 and *Tc*UBP2 proteins, which could be due to a decrease in the trans-splicing rate (see Discussion). We also evaluated the expression of other nonrelated proteins, such as the nuclear Histone H4 acetylated and also two cytoplasmic proteins: heat shock protein 70 and ribose 5-phosphate isomerase type B. These results, showed in the Fig. S3, indicated that only the fluorescence signal for RRM is visibly diminished on parasites overexpressing *Tc*DRBD4, not affecting the expression of the other proteins.

*Tc*DRBD4/PTB2-containing RNP complexes are not involved in mRNA translation

To further analyze the association of *Tc*DRBD4/PTB2 with its pre-RNA targets, we examined whether these ribonucleoparticle assemblies can also occur in polysomes. For this purpose, we prepared a polysome fractionation in solutions containing either magnesium or EDTA (Fig. 4A) and analyzed both the S100 and P100 fractions by immunoblot with several anti-RBP antibodies in the presence of magnesium (required for ribosome stability) or EDTA (required for the dissociation of ribosomal subunits) (Ji et al. 2003). As shown in the figure, the *Tc*S12 ribosome protein, a component of the 40S small subunit, is associated to polysomes, whereas *Tc*DRBD4/PTB2 shows a polysome-free localization. As additional controls, other two RRM-containing RBPs were analyzed: UBP1, which is associated with polysomes as the signal was detected in the P100 fraction, and RBP3, which is excluded from polysomes, as shown by the signal detected in the S100 fraction (Fig. 4A). Next, full polysome profiles were obtained once more in sucrose solutions containing either magnesium or EDTA (Fig. 4B). In the presence of EDTA, the polysome profile is shifted to the first two peaks corresponding to the 40S and 60S ribosomal subunits (Ji et al. 2003). The distribution of S12 protein is observed in 40S, 60S, 80S, and polysomes as expected (Fig. 4B). A different localization pattern was seen when using *Tc*DRBD4/PTB2 anti-serum. The *Tc*DRBD4/PTB2 signal was mainly

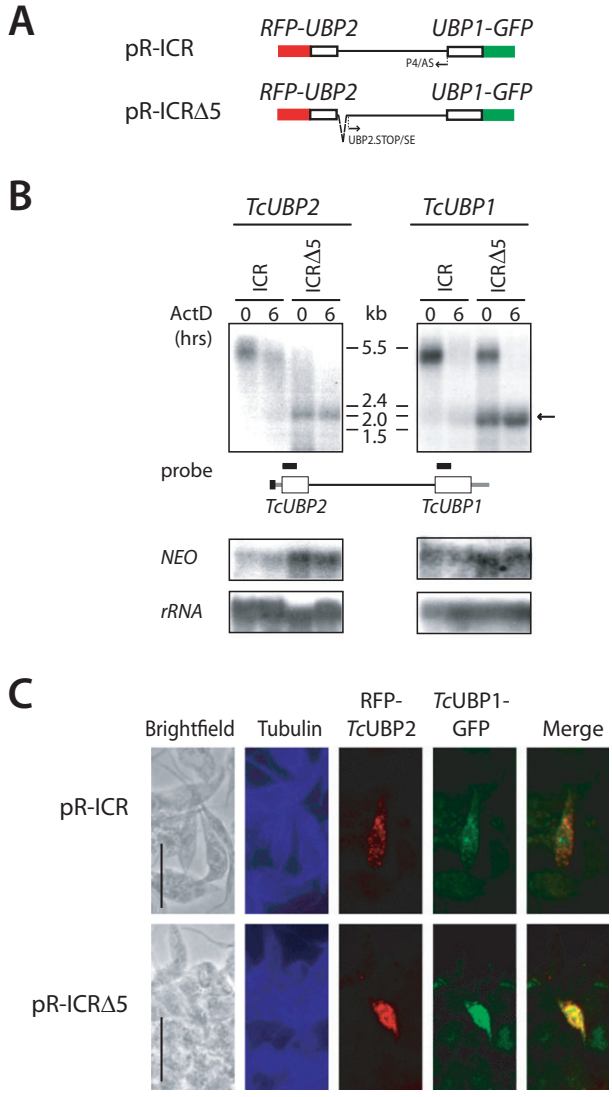

Figure 2 *Tc*DRBD4/PTB2 regulatory elements present in the 5′-end of *TcUBP* ICR affect mRNA processing/stability and translation. **A**. Schematic representation of the constructs used for epimastigote transfections. The pR-ICR construct includes the complete *TcUBP* dicistron with the coding sequences for *TcUBP2* and *TcUBP1* fused to the reporter genes Red Fluorescence Protein (RFP) and Green Fluorescence Protein (GFP), respectively. The pR-ICRΔ5 lacks about 200-nt from the 5′-end of the dicistronic ICR. The primers used for DNA construct synthesis are indicated. **B.** Northern blots of pR-ICR- and pR-ICRΔ5-transfected parasites are shown. Epimastigotes were incubated with actinomycin D and total RNA was extracted from aliquots at 0 and 6 h of incubation. Samples were separated onto agarose gels, transferred and hybridized with probes indicated below the panels (the same filter was sequentially hybridized with Neo and rRNA probes). The molecular weight markers including the three ribosomal RNA bands (18S, 24Sα, and 24Sβ) are shown. The arrow indicates the position of *UBP1* and *UBP2* monocistrons. Neo = neomycin probe; rRNA = 28S ribosomal rRNA probe. **C**. Fluorescence microscopy analysis of the same parasite populations used in the experiment shown in B (pR-ICR and pR-ICRΔ5 constructs). Brightfield photographs, RFP-*Tc*UBP2 (red), *Tc*UBP1-GFP (green) and merge of red and green signals are shown. Red and green fluorescence correspond to residual RFP and GFP signal postfixation, respectively. The expression of the nonrelated protein alpha Tubulin was also tested by indirect immunofluorescence using specific anti-alpha Tubulin antibody (signal shown in blue). ActD = Actinomycin D. Bars = 10 μm.

distributed among the 40S and 60S fractions from top to bottom aliquots (Fig. 4B). In the high-density polysomal fractions, the *Tc*DRBD4/PTB2 signal was barely detected in the presence of magnesium and only when the filter was extensively overexposed (not shown). Taken together, these findings indicate that *Tc*DRBD4/PTB2 is excluded from polysomes, as previously reported for *T. brucei* DRBD3 (Fernandez-Moya et al. 2012), and suggest that *Tc*DRBD4/PTB2-containing RNP complexes are not involved in translation events.

DISCUSSION

In this work, we studied the role of *Tc*DRBD4/PTB2, the trypanosome homolog of PTB, in the regulation of trans-splicing, an important mechanism involved in trypanosomatid gene expression regulation (Michaeli 2011). PTB has been well described in mammals as a key factor controlling alternative splicing of specific transcripts (Sawicka et al. 2008). Many eukaryotic proteins with well-defined roles in splicing also recognize conserved motifs in 3′-UTRs, suggesting diverse posttranscriptional regulatory functions for these RBPs (Ray et al. 2013). Strikingly, the *Tb*DRBD4/PTB2 ortholog has been extensively studied as an essential factor affecting the stability of distinct sets of mRNAs (Stern et al. 2009) and also found in an RNP complex with the nuclear RBP *Tb*RRM1 (Naguleswaran et al. 2015). A recent high-throughput sequencing analysis showed that *Tb*DRBD3/PTB1 binds to transcripts encoding ribosomal proteins and enzymes required to obtain energy through the oxidation of proline to succinate (Das et al. 2015). Also, in response to oxidative or starvation-induced stresses, this RRM-type RBP forms a complex that changes its composition and subcellular localization (Fernandez-Moya et al. 2012).

Our previous study based on a computer-assisted approach predicted the presence of several putative binding sites for *Tc*DRBD4/PTB2 in the pre-mRNA *TcUBP*, a dicistronic unit containing two gene members of the RRM family in *T. cruzi* (Jäger et al. 2007). Although *TcUBP* RNA is detected in all life cycle stages of the parasite (De Gaudenzi et al. 2003), the protein expression of both cistrons (*TcUBP1* and *TcUBP2*) is developmentally regulated. *Tc*UBP2 is expressed in epimastigote cells, whereas *Tc*UBP1 is expressed in all parasite forms. The levels of the mature monocistrons are also different among the three developmental stages of the parasite: the steady-state level of the *TcUBP1* transcript is nearly 10-fold higher than *TcUBP2* in epimastigotes and about fourfold higher in trypomastigotes and amastigotes (Fig. S5).

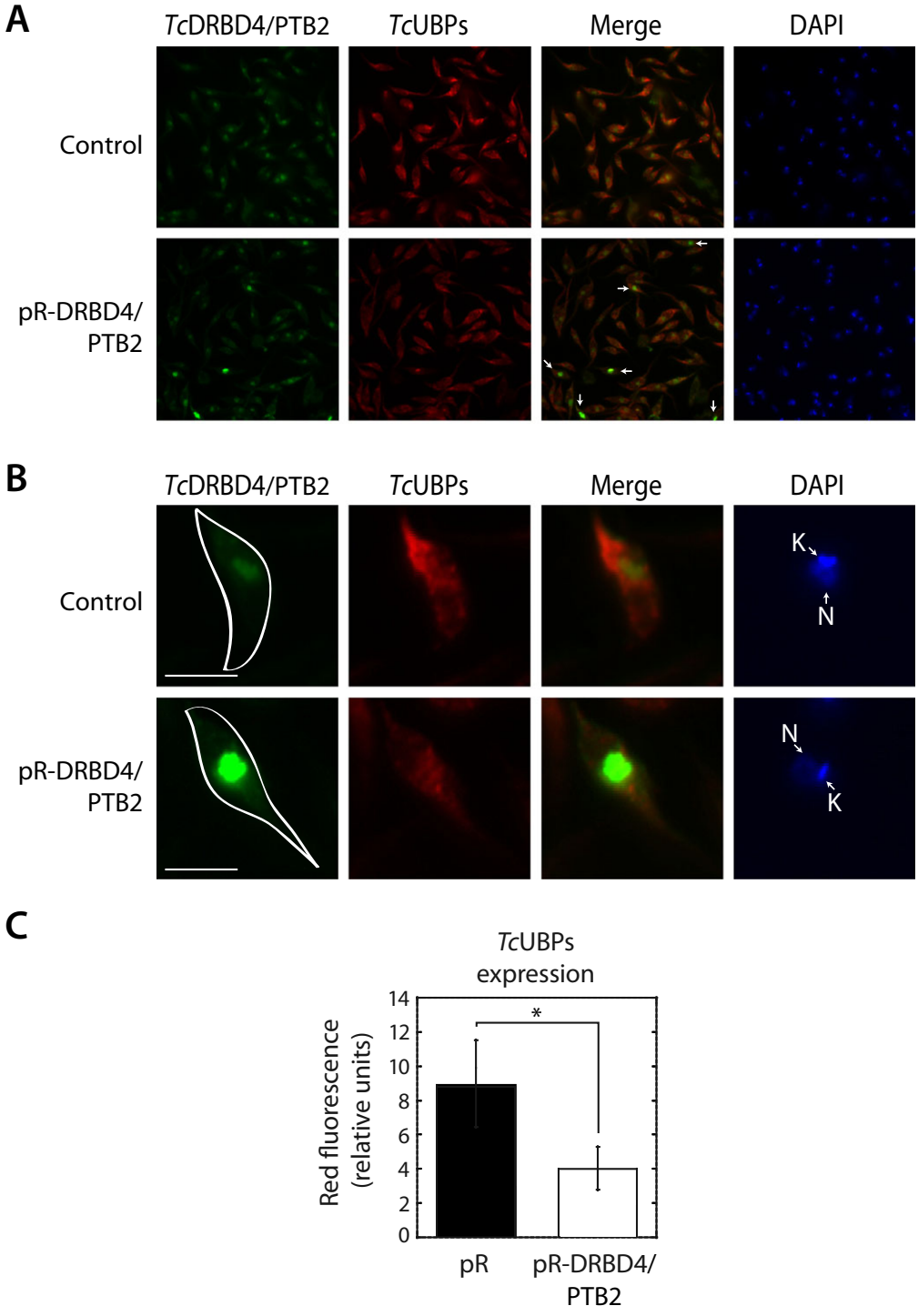

Figure 3 *Tc*DRBD4/PTB2 overexpression in epimastigote cells affects UBP1 and UBP2 protein synthesis. **A.** Representative images of *Tc*UBPs/*Tc*DRBD4/PTB2 immunofluorescence detection using anti-RRM and anti-*Tc*DRBD4/PTB2 antibodies on pR-DRBD4/PTB2 and empty vector-transfected parasites (Control). *Tc*DRBD4/PTB2 signal (Alexa-488) is shown in green and *Tc*UBPs signal (Alexa-568) in red. Merge of both panels (red and green) and DNA stained with DAPI are also shown. DRBD4/PTB2 overexpressing parasites are indicated by white arrows in the merge image panel. N = nuclear DNA; K = kinetoplast DNA. **B**. Detail of a single control epimastigote and DRBD4/PTB2-overexpressing parasite. The key for the image panel is as for (A). **C.** Red fluorescence levels corresponding to RRM-type *Tc*UBPs of transfected cells with the pR-DRBD4/PTB2 construct or the control vector pR were significantly different (*t*-test, *p < 0.001). Quantification was performed by densitometry using ImageJ 1.41 software and error bars represent the mean ± standard deviation of fluorescence signals of 30 cells from two independent experiments. Control, empty vector-transfected parasites; pR-DRBD4/PTB2-, pRIBOTEX-*Tc*DRBD4/PTB2-transfected parasites. Bars = 10 μm.

Although both *TcUBP1* and *TcUBP2* transcripts are generated from a single dicistronic unit, strong differences were observed between their steady-state levels, indicating that *cis*-elements within the ICR might be involved in trans-splicing events controlling the monocistronic production. In particular, we found that narrowing down the ICR by deleting a small segment containing almost half of the PTB putative binding elements (ICRΔ5 construct, Fig. 2) increased the levels of both mature *TcUBP* transcripts with the concomitant increase in the expression of the proteins. In addition, overexpression of *Tc*DRBD4/PTB2 in epimastigote cells down-regulated *Tc*UBP1 and *Tc*UBP2 protein levels (see Fig. 3). We propose that this is not a unique case. In searching for RNA precursor molecules, we extended our analysis to other possible ICRs bound to DRBD4/PTB2 in *T. cruzi* (see Materials and Methods). As a result, we obtained six hits that matched to ICRs that should not be present in mature transcripts, thus we proposed them as other possible dicistronic candidates (Fig. S4). The presence of these intergenic regions, which should not be detected if these molecules were fully processed by polyadenylation/trans-splicing into the corresponding monocistrons, constitutes indirect evidence that they are indeed stable cytosolic molecules. These findings suggest that *Tc*DRBD4/PTB2 is a multifunctional RBP that forms stable RNP complexes with cytosolic pre-RNAs.

As previously reported, *TcUBP* dicistronic RNA half-life is about 5 h (Jäger et al. 2007), a long cytoplasmic half-life when comparing with other trypanosome mRNAs (Di Noia et al. 2000). By protein-RNA co-immunoprecipitation analysis, we also showed that the *Tc*DRBD4/PTB2-RNA interaction takes place in vivo, thus suggesting a role for this RBP in trans-splicing regulation. In conclusion, the parasite homolog of PTB, which is involved in exon skipping in

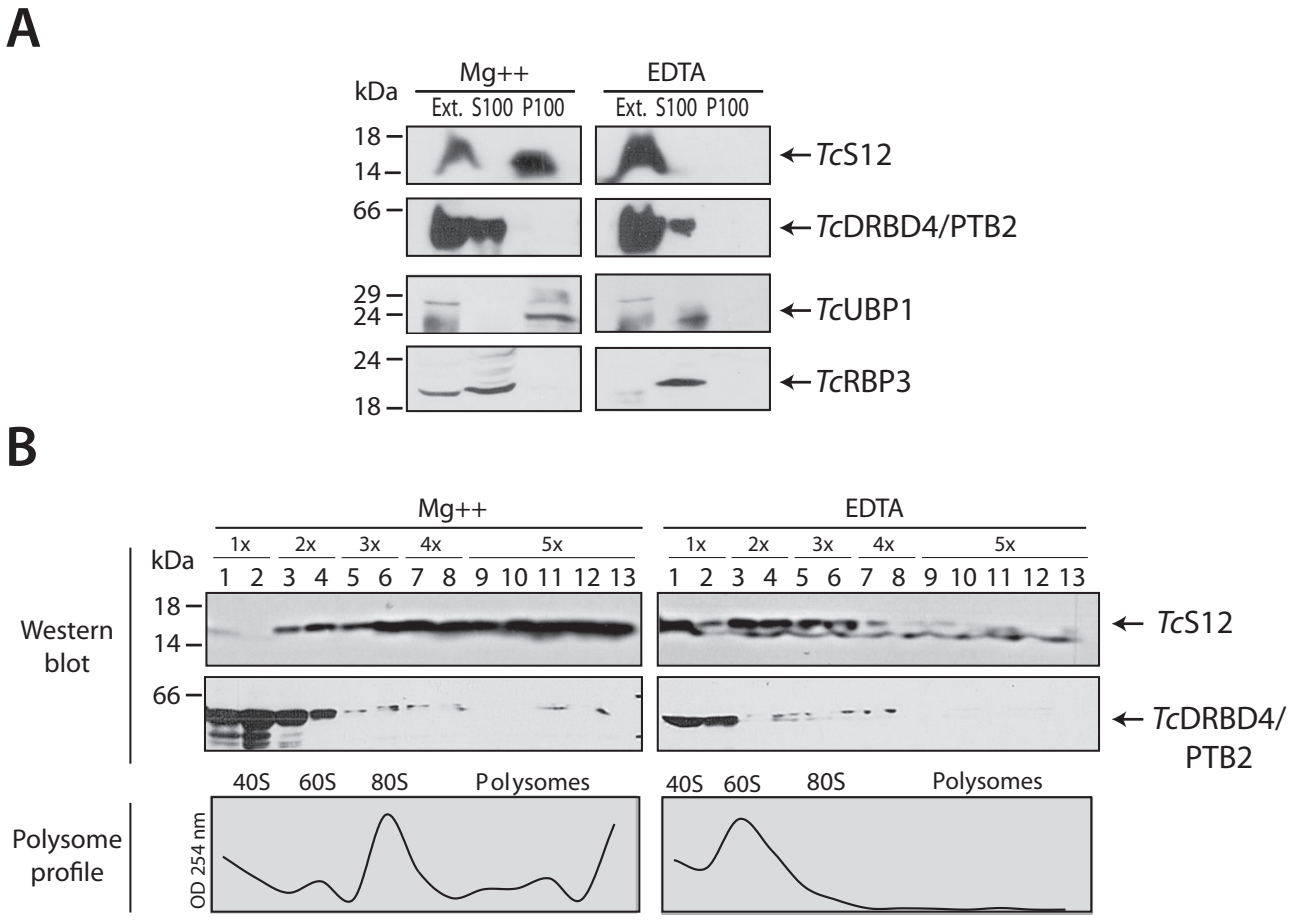

Figure 4 *Tc*DRBD4/PTB2 does not associate with polysomes. **A.** Western blots of clarified trypanosome cell cytoplasmic extract (Ext), prepolysomal (S100) and polysomal (P100) fractions obtained by sedimentation through a 30% sucrose cushion in the presence of magnesium or EDTA. Equal aliquots of Ext and S100 fractions and fivefold-concentrated aliquots of the P100 fraction were probed with an anti-DRBD4/PTB2 serum. The distribution of *Tc*DRBD4/PTB2 protein was compared to that of proteins known to be polysome-associated (*Tc*S12 and *Tc*UBP1) or polysome-free (*Tc*RBP3). For *Tc*S12, an overexposed film showed its presence in the S100 fraction in the presence of EDTA. **B.** Trypanosome full polysome profiles (1X to 5X) in the presence of magnesium or EDTA were analyzed by Western blot using anti-*Tc*DRBD4/PTB2 serum and anti-*Tc*S12 serum for comparison. The proteins of interest are indicated by arrows and molecular mass markers (kDa) are also indicated on the left margin. Absorption at 254 nm was measured to show the polysome profile plot. Mg^{++} = magnesium.

other cells (Wagner and Garcia-Blanco 2001), might preclude the trans-splicing reaction in trypanosomes and thus avoid the production of mature *TcUBPs* monocistronic RNAs able to be translated. The results of this study reinforce the concept that RNA processing is of crucial importance in the regulation of protein synthesis in trypanosomes. Other authors have demonstrated that under severe endoplasmic reticulum stress, *T. brucei* induces a novel SL silencing pathway mediated by phosphorylation in the nucleus of TATA-binding protein/TRF4, which blocks SL RNA transcription and thus inhibits trans-splicing and protein synthesis (Michaeli 2015). In *Leishmania*, tRNA-derived small RNAs and a number of novel transcripts that are processed alongside other RNAs during trans-splicing appear to be specifically present in exosomes (Lambertz et al. 2015). In *T. cruzi*, a variety of small RNAs contained in extracellular vesicles are also originated from multiple sources, including tRNAs (Bayer-Santos et al. 2014).

Several reports describing trans-RNA splicing events in precursor RNAs suggest that alternative trans-splicing exerts a key role in the control of gene expression in trypanosomatids (Benabdellah et al. 2007; Helm et al. 2008; Jäger et al. 2007). Widespread stage-regulated alternative splicing patterns have been recently discovered in the transcriptome of *T. brucei* by using parallel sequencing (Nilsson et al. 2010). Although most eukaryotic mRNAs are monocistronic molecules, stable dicistronic units have been described before, first in the nervous system (Lee 1991) and later in *Caenorhabditis*, *Drosophila*, and plants (Blumenthal 2004). In trypanosomatids, these molecules have been found in *T. brucei* (LeBlanc et al. 1999; Radwanska et al. 2000; Vanhamme et al. 1998) and in *T. cruzi* (Jäger et al. 2007). Alternative trans-splicing can generate dicistronic units, when an acceptor site is skipped during the initial polycistronic processing. In *T. cruzi*, alternative splicing of the LYT1 gene results in the expression of two proteins: mLYT1, which is expressed in the mammalian stages and localizes on the plasma membrane, and kLYT1, which is most abundant in epimastigotes and localizes between the kinetoplast and the flagellum (Benabdellah et al. 2007). Another example, also in *T. cruzi*, is the proline racemase gene *TcPRACA*, which is processed in a secreted form expressed only in metacyclic trypomastigotes and a cytoplasmic form expressed in epimastigotes (Chamond et al. 2003). Given the lack of promoters, alternative trans-splicing might be an important mechanism in these organisms for producing differentially expressed and localized proteins from the same gene or for regulating mRNA abundance and stability. Further experimental approaches are necessary to explore whether exon skipping is related to the generation of alternative trans-splicing forms in trypanosomatids.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative microscopic images of pR-ICR- and pR-ICRΔ5-transfected parasites. Brightfield photographs, RFP-*Tc*UBP2 (red), *Tc*UBP1-GFP (green), and merge of red and green signals are shown. Red and green fluorescence correspond to residual RFP and GFP signal postfixation, respectively. The expression of the nonrelated protein alpha Tubulin was also tested by indirect immunofluorescence using specific anti-alpha Tubulin antibody (signal shown in blue). Bars = 10 μm.

Figure S2. *Tc*DRBD4/PTB2 regulatory elements present in the 3′-end of *TcUBP* ICR do not affect mRNA processing/stability. **A**. Schematic representation of the construct ICRΔ3 used for epimastigote transfections. The pR-ICRΔ3 lacks 156-nt from the 3′-end of the dicistronic ICR. Primer C2/AS used for DNA construct synthesis is indicated. **B.** Northern blots of pR-ICRΔ3-transfected parasites are shown. Epimastigotes were incubated with actinomycin D and total RNA was extracted from aliquots at 0 and 6 h of incubation. Samples were separated onto agarose gels, transferred, and hybridized with the *TcUBP1* probe (the same filter was sequentially hybridized with Neo and rRNA probes). The molecular weight markers including the three ribosomal RNA bands (18S, 24Sα and 24Sβ) are shown. Neo = neomycin probe; rRNA = 28S ribosomal rRNA probe.

Figure S3. *Tc*DRBD4/PTB2 overexpression in epimastigote cells does not affect Histone H4, HSP70 nor RPIB protein expression. **A.** Representative microscopic images of *Tc*DRBD4/PTB2 immunofluorescence detection using anti-*Tc*DRBD4/PTB2 and anti-RRM (*Tc*UBPs), anti-AcH4, HSP70, and anti-RPIB. *Tc*DRBD4/PTB2 signal (Alexa-488) is shown in green and *Tc*UBPs, Histone H4, HSP70, and RPIB signal (Alexa-568) in red. Merge of all panels (red, green, and DNA stained with DAPI) are also shown. DRBD4/PTB2 overexpressing parasites are indicated by white arrows in the green image panel. N = nuclear DNA; K = kinetoplast DNA. Bars = 10 μm.

Figure S4. Identification of *Tc*DRBD4/PTB2-associated pre-RNA molecules. Schematic representation of pre-RNA candidates. The length corresponding to the ICR and total pre-RNA is indicated for each candidate (nucleotides).

Figure S5. Expression of monocistronic *TcUBP1* and *TcUBP2* along the parasite life cycle. Mature *UBP1* and *UBP2* transcript levels were quantified by qRT-PCR and normalized to β-tubulin. The *UBP1/UBP2* transcript ratio is shown among the different stages of *Trypanosoma cruzi* life cycle. E = epimastigote; T = trypomastigote; A = amastigote.