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The RNA-binding protein *Tb*DRBD3 regulates the stability of a specific subset of mRNAs in trypanosomes

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ABSTRACT

In trypanosomes, the apparent lack of regulation of RNA polymerase II-dependent transcription initiation poses a challenge to understand how these eukaryotes adjust gene expression to adapt to the contrasting environments they find during their life cycles. Evidence so far indicates that mRNA turnover and translation are the major control points in which regulation is exerted in trypanosomes. However, very little is known about which proteins are involved, and how do they regulate the abundance and translation of different mRNAs in different life stages. In this work, an RNA-binding protein, *Tb*DRBD3, has been identified by affinity chromatography, and its function addressed using RNA interference, microarray analysis and immunoprecipitation of mRNA–protein complexes. The results obtained indicate that *Tb*DRBD3 binds to a subset of developmentally regulated mRNAs encoding membrane proteins, and that this association promotes the stabilization of the target transcripts. These observations raise the possibility that *Tb*DRBD3-mRNA complexes act as a post-transcriptional operon, and provide a framework to interpret how trypanosomes regulate gene expression in the absence of transcriptional control.

INTRODUCTION

Messenger RNA stability and translation are emerging as important mechanisms to control gene expression (1). These processes depend largely on RNA-binding proteins (RBPs). The association of RBPs with mRNAs is determined by sequence elements located usually within the 3′-untranslated regions (3′-UTRs) of regulated transcripts, and leads to intense changes in stability and/or translation of target mRNAs (2). Many reports have shown that RBPs do not bind to individual mRNAs randomly, but they associate with specific subsets of mRNAs coding for functionally related proteins. These observations led to the hypothesis of the existence in eukaryotes of 'post-transcriptional operons', by which single RBPs would be able to regulate the fate of multiple mRNAs in a coordinate manner (3,4). Indeed, an increasing number of instances exist where mRNA clusters that encode components of the same macromolecular complex or cellular process have been found associated with RBPs (4). This is best exemplified by the Puf family of RBPs in yeast, where each of the five members preferentially associates to mRNAs coding for membrane-associated proteins (Puf1p and Puf2p), mitochondrial proteins (Puf3p), ribosomal RNA-processing factors (Puf4p) or components of the spindle pole body (Puf5p) (5).

Post-transcriptional regulation of gene expression is of exceptional importance in trypanosomatid protozoa. These flagellated eukaryotes branched-out very early in the eukaryotic lineage (6), and some species, like *Trypanosoma brucei*, *Trypanosoma cruzi* or *Leishmania* spp., pose an important problem for the economy and public health in underdeveloped countries (http://www.who.int/tdr/diseases/). These parasites exhibit complex life cycles alternating between an invertebrate and a vertebrate, and are able to undergo intense morphological and biochemical changes in response to changes in temperature, pH, nutrients and defenses they encounter within one or the other host (7). All these adaptations require major changes in the gene expression program of the parasite. However, trypanosomatids seem to have completely lost the ability to regulate transcription initiation by RNA polymerase II (8). This is due to the unusual arrangement of chromosomes, in which most protein-coding genes are found in arrays of tens to hundreds units all orientated on the same strand (9,10). A single region seems to drive the expression of the whole chromosome (11,12), producing long polycistronic pre-mRNAs that are resolved into individual, mature mRNAs by coupled *trans*-splicing and polyadenylation reactions (13). Thus, regulation of gene expression in these parasites needs to

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be exerted posttranscriptionally. Messenger RNA processing and transport seem to be unregulated in trypanosomatids; instead, evidence so far indicates that the main control points of gene expression in these organisms are at the level of mRNA degradation and translation (8,14).

Trypanosoma brucei, the causative agent of sleeping sickness in man and nagana in cattle, has several life stages within the Tse-tse fly or the mammal. The two major replicative stages are the procyclic and the bloodstream slender forms. Transcriptome analysis of these two life forms has revealed that at least 200 transcripts show developmental regulation (15), and it is assumed that the differential transcript abundance during the parasite's life cycle is due to the interaction of RBPs to specific mRNA populations (14). Indeed, various regulatory sequences have been identified in trypanosomatids that confer stage-specific mRNA destabilization, translation or cell-cycle regulation (14), and some mRNA–protein complexes with a possible role in mRNA turnover have been partially characterized (16,17). However, virtually nothing is known about the RBPs involved in the control of mRNA abundance and translation in trypanosomatids and, to date, no RBP has been found associated with a specific subset of mRNAs coding for functionally related proteins.

The present work describes the identification of several proteins in *T. brucei* that bind to the 3′-UTR of a developmentally regulated mRNA *in vitro*, and the functional characterization of one of them, *Tb*DRBD3. The study of *Tb*DRBD3-depleted cells by means of RNA interference in combination with microarray analysis and immunoprecipitation of mRNA–protein complexes, indicates that this protein controls the abundance of a specific subset of developmentally regulated mRNAs encoding membrane proteins.

MATERIALS AND METHODS

Trypanosomes and cell culture

Procyclic-form 427 *T. brucei* cells were grown at 27°C in SDM-79 medium (18) containing 10% fetal bovine serum. Procyclic-form 449 trypanosomes stably expressing the Tn10 *tet* repressor (19) were grown in the presence of 0.5 μg/ml phleomycin. Bloodstream-form 427 cells were cultured at 37°C and 5% CO_2 in HMI-9 medium (20) containing 10% fetal bovine serum.

Purification of RBPs and mass spectrometry

To obtain cytosolic extracts, procyclic 427 cells were harvested at a cell density of 5–8 × 10^6/ml, washed in phosphate-buffered saline, resuspended in 1 mM HEPES, pH 7.4, 1 mM DTT and EDTA-free protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain), and disrupted for 10 min in a Potter-Elvehjem homogenizer equipped with a Teflon pestle. After adding HEPES buffer (pH 7.4) and NaCl to 20 and 100 mM, respectively, the lysate was centrifuged at 3000*g* for 10 min. The supernatant was ultracentrifuged for 1 h at 100 000*g*. NP-40, EDTA and glycerol were added to the final supernatant to 0.1% (w/v), 1 mM and 10% (w/v), respectively, and the mixture was frozen at −80°C until use. The RNA baits used were the phosphoglycerate kinase B (*PGK-B*) 3′-UTR and a modified version thereof in which the U-rich regulatory element was deleted (21). DNA fragments corresponding to both 3′-UTRs were PCR-amplified from plasmids pHD869 or pHD1044 (21) and cloned as EcoRI–BamHI fragments into plasmid pGR24 (a pBluescript SK + derivative containing the sequence corresponding to the *T. brucei* spliced-leader cloned between the KpnI and the EcoRI sites). The resulting plasmids, named pGR25 (intact 3′-UTR) and pGR26 (deleted version) were linearized at the BamHI site and *in vitro* transcribed by T7 RNA polymerase. Transcribed RNAs were bridged to streptavidin–agarose columns using a biotinylated deoxyoligonucleotide complementary to the spliced-leader sequence. Cytosolic extracts (30–40 mg of protein) were thawed, supplemented with 50 μg/ml tRNA and 100 U/ml of RNasin (Promega, Madrid, Spain) and split into two identical fractions; one was passed through a column containing the intact version of *PGK-B* 3′-UTR, and the other through a column containing the deleted version. Proteins were allowed to bind for 20 min at room temperature, followed by an incubation of 30 min at 4°C. After extensive washing in buffer K (20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% NP-40, 10% glycerol), RBPs were eluted in buffer K containing 500 mM NaCl, loaded in a 12% SDS–PAGE gel and visualized by Sypro Ruby staining. Those proteins appearing only in the intact 3′-UTR chromatography eluate were excised, subjected to MALDI-TOF/TOF and identified using MASCOT software (http://www.matrixsicence.com). Mass-spectrometry analysis was performed in the Proteomic Unit of the Scientific Park/Madrid Complutense University (Madrid, Spain).

Antibody production

A fragment of the *Tb*DRBD3 open reading frame (ORF) corresponding to the first 83 amino acids, or a fragment of *Tb*RBP33 corresponding to the last 160 amino acids, were cloned into pHD1306 (22), expressed as His_6-GST fusions, purified using glutathione- and nickel-affinity resins, and buffer exchanged into phosphate-buffered saline. Immunizations in rabbits were performed according to standard procedures (23). Polyclonal antibodies were affinity-purified using full-length versions of the proteins coupled to AffiGel-15 (Bio-Rad Laboratories, Madrid, Spain). To express full-length *Tb*DRBD3 and *Tb*RBP33 in *Escherichia coli* for antibody purification, ORFs were cloned into pGR36 (a pET24a derivative containing a DNA fragment coding for the calmodulin-binding peptide and the His_6 tag cloned between the HindIII and XhoI sites). Bacteria were grown at room temperature to an OD_{600} of 0.2, induced with 1 mM isopropyl-β-D-thiogalactopyranoside and incubated at the same temperature to an OD_{600} of 1.5. Recombinant proteins were purified using both calmodulin-affinity (Stratagene, La Jolla, CA, USA) and nickel-affinity (GE Healthcare, Barcelona, Spain) resins, according to the manufacturers' instructions. Western blots of total cell extracts were done as described (24).

Immunofluorescence assays

Immunolocalization studies in bloodstream and procyclic 427 trypanosomes were carried out in cells fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 20 min (22), using affinity-purified anti-*Tb*DRBD3 serum and goat anti-rabbit Alexa 488 secondary antibody (Molecular Probes, Invitrogen, Barcelona, Spain). Permeabilization with Igepal CA-630 (25), or fixation/permeabilization with methanol [using the protocol described in ref. (26), but substituting methanol for ethanol] gave similar results.

RNA interference

A stem–loop strategy was used to generate dsRNA (27). A fragment of the *Tb*DRBD3 ORF corresponding to nucleotide positions 2–502 was PCR-amplified and cloned into pGR19 (27) as two inverted repeats flanked by an 'stuffer' fragment to yield pGR69. There are no other sequences in the *T. brucei* genome that showed significant similarity to this dsRNA. Procyclic 449 cells were transfected with pGR69 linearized with NotI and selected in the presence of 50 μg/ml hygromycin. RNAi was induced by adding tetracycline to the culture medium at a concentration of 1 μg/ml.

Microarray analysis

RNAi was induced for 48 h, and total RNA was obtained from either uninduced or *Tb*DRBD3-depleted cells using Trizol (Invitrogen, Barcelona, Spain) and treated with DNase I (Promega). A total of 15 μg of RNA were reverse transcribed in the presence of 100 ng of oligo$(dT)_{12–18}$, 0.25 mM dATP, 0.25 mM dTTP, 0.25 mM dGTP, 0.05 mM dCTP, 0.05 mM Cy3- or Cy5-dCTP, 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 40 U of RNaseOUT (Invitrogen) and 400 U of Superscript III reverse transcriptase (Invitrogen). Reactions were incubated for 3 h at 50°C, stopped by heating at 70°C for 15 min and treated with 5 U of RNase H for 20 min at 37°C. cDNA was purified using the MinElute kit (Qiagen, Madrid, Spain), ethanol precipitated and resuspended in hybridization buffer. Genomic *T. brucei* microarray glass slides containing ca. 24 000 independent random genomic clones (15) were pre-hybridized and hybridized as described (28). Four hybridizations (two biological replicates with a dye swap each) were analyzed. Slides were scanned using a ScanArray 5000 laser-scanner (Packard BioScience, Dreieich, Germany). The resulting images were analyzed using GenePix (Axon Instruments, Sunnyvale, CA, USA) and MCHIPS software (29,30) essentially as described (15,28). Only those clones satisfying the following parameters—fitted intensities: at least 150 000; ratio: ±2; min/max-separation: 0.1—were selected. Regulated clones were sequenced from one end and identified in the *T. brucei* genome database (http://www.genedb.org/genedb/tryp/). Microarray data have been submitted to GEO under accession number GSE8734.

Northern analysis

A 10–15 μg of total RNA obtained from three independent RNAi experiments were electrophoresed in agarose–formaldehyde gels, transferred to Nytran membranes (Millipore, Madrid, Spain), hybridized following standard procedures (31) and analyzed using PhosphorImager screens and ImageQuant software. Transcript signals were normalized relative to a signal recognition particle (SRP) probe (32). To rule out unspecific effects on mRNA abundance due to RNAi, total RNA was also obtained from a procyclic cell line depleted of the peroxisomal protein PEX2 by means of RNAi (33) and analyzed in parallel. Table 1 shows a list of the probes used in this work.

mRNA degradation experiments

Total RNA was obtained from uninduced or RNAi-induced samples (48 h in the presence of tetracycline) of 5×10^7 cells taken at different times after the addition of 10 μg/ml actinomycin D (from a stock of 5 mg/ml in DMSO). RNA extractions and analysis were performed as described above from three independent RNAi inductions. mRNA half-lives were estimated after measuring PhosphorImager signals on northern blots, normalizing to SRP signal, plotting on semilogarithmic scales and calculating the amount of time required for a transcript to decrease to 50% of the initial amount. Half-life values were compared using Student's *t*-tests. A P-value < 0.05 was considered statistically significant.

Luciferase constructs and assays

A modified version of plasmid pHD1437 (34), named pGR86, was constructed by replacing the CAT gene for a luciferase ORF. The 3′-UTRs of amino acid transporter 11 (*AATP11*), glutamine synthetase (*GS*) and actin (*ACT*) genes were cloned downstream the luciferase gene to generate pGR88, pGR98 and pGR108, respectively. These plasmids were digested with NotI and stably integrated in the tubulin locus of procyclic trypanosomes by electroporation. After selecting in the presence of 1 μg/ml puromycin, three independent clones from each transfection were selected for northern and luciferase assays. *AATP11* 3′-UTR was obtained from plasmid pHD1470 (35), and *ACT* 3′-UTR, from plasmid pHD1034 (36). *GS* 3′-UTR was obtained by RT–PCR using the anchored oligo$(dT)_{18}$ primer CZ1584 (36) and *GS* gene-specific primers. Total protein extracts from 2×10^6 cells were assayed for luciferase activity in a Berthold FB 12 luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) using the Luciferase Assay System (Promega) according to the manufacturer's instructions.

Immunoprecipitation of mRNA–protein complexes

For each immunoprecipitation, 5×10^7 procyclic 427 cells were washed in serum-free SDM-79 culture medium and lyzed in 10 mM Tris–HCl, pH 7.4, 2 mM DTT, 0.1% (w/v) NP-40, 100 U/ml RNasin (Promega), 2 mM vanadyl ribonucleoside complexes (Sigma-Aldrich, Madrid, Spain) and EDTA-free protease inhibitor

Table 1. Probes used in northern hybridizations

Gene ID	Protein	Probe	Poly(A) site[a]
Tb09.211.0560	DRBD3	ORF + 3′-UTR [510–1034]	
Tb10.406.0110	GCS1-like	ORF [1561–1842]	
Tb927.1.2850[b]	Pteridin transporters	3′-UTR [1950–2578]	622
Tb927.4.4730	Amino acid transporter 11	3′-UTR [1423–2255]	
Tb927.3.4500	Cytosolic fumarase	3′-UTR [1695–3236]	
Tb927.7.4970	Glutamine synthetase	3′-UTR [1256–1614]	351
Tb09.244.2510[c]	BARP	ORF [370–557]	
Tb10.6k15.1350	Pteridin transporter	3′-UTR [1919–2240]	976
Tb927.7.5960[d]	MFS	ORF + 3′-UTR [1716–2025]	
Tb10.6k15.0070	PAG4	ORF [8–932]	
Tb927.8.3620[e]	Folate transporters	3′-UTR [1902–2177]	793
Tb11.02.2700	Mitochondrial fumarase	ORF + 3′-UTR [1629–2380]	
Tb09.211.0630	Actin	ORF [1–1128]	
Tb09.160.5480	NT10	ORF [59–689]	

Fragments of open-reading frames (ORF) and/or 3′-UTRs were obtained by PCR or restriction endonuclease digestion, and radiolabelled using the random primer PrimeIt kit (Stratagene) and [α-^{32}P]dCTP. The start and end positions of the probes relative to the translation start codon are indicated in brackets.

[a]Polyadenylation sites were estimated for some mRNAs by nested RT–PCR using two sense transcript-specific primers and the anchored oligo(dT) primer CZ1584 (see Materials and methods section). Nucleotide positions are relative to the stop codon (included).

[b]There are three genes in tandem coding for pteridine transporters in chrosomose I (http://www.genedb.org/genedb/tryp/), and have nearly identical 3′-UTRs. The probe was designed to detect all three transcripts.

[c]There are 14 BARP genes in tandem in chromosome IX (http://www.genedb.org/genedb/tryp/). The probe used shares 60–70% identity with other BARP genes.

[d]There are at least eight MFS genes in tandem in chromosome VII (http://www.genedb.org/genedb/tryp/). The probe used is predicted to hybridize also to MFS genes 927.7.5960, 927.7.5980 and 927.7.6000.

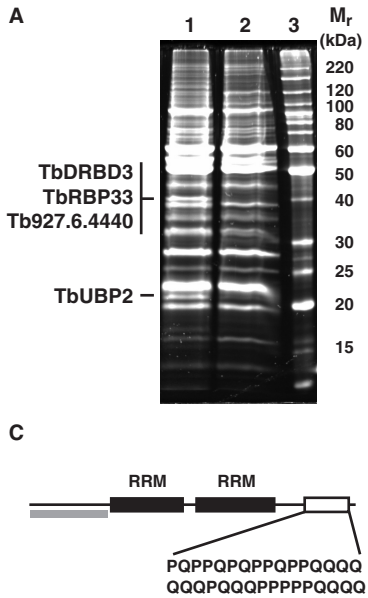
[e]There are three folate transporter genes in chromosome VIII with 3′-UTRs nearly identical up to 185-nt downstream to the stop codon (http://www.genedb.org/genedb/tryp/). The probe used was designed to detect all three transcripts. Mapped polyadenylation site is that of Tb927.8.3620 transcript.

cocktail (Roche Diagnostics) using a Potter-Elvehjem homogenizer equipped with a Teflon pestle. After adding NaCl to 150 mM, the lysate was centrifuged at 4°C for 15 min at 14 000*g*. The supernatant was subjected immediately to immunoprecipitation using protein G beads and affinity-purified antibodies for 3 h at 4°C. After extensive washing in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% (w/v) NP-40, the RNA was extracted from the immunoprecipitated material using TRI-Reagent (Sigma-Aldrich) and treated with DNase I. One half of the final RNA sample was reverse transcribed using the anchored oligo(dT)$_{18}$ primer CZ1584 (36) and Superscript III reverse transcriptase (Invitrogen); the other half was treated identically, except that the reverse transcriptase was omitted (minus RT control). cDNA was amplified using mRNA-specific primers pairs and 95°C (30 s), 55°C (30 s) and 72°C (30 s) for 30 cycles, then 5 min at 72°C. Total RNA was also obtained from an equivalent amount of extract to serve as a positive control (input RNA). Control immunoprecipitations and RT–PCR experiments were performed in parallel using either anti-*Tb*RBP33 antibodies or purified rabbit IgGs (Sigma-Aldrich). To test the effect that deletions in the 3′-UTR of *AATP11* mRNA have on *Tb*DRBD3 binding, mRNA–protein complexes were also immunoprecipitated from extracts obtained from permanent cell lines expressing CAT reporter mRNAs fused to different deletions of *AATP11* 3′-UTR (35). In this case, cells were disrupted by vortexing in the presence of 0.5% Igepal CA-630. PCR reactions were carried out using the sense oligonucleotide 5′-GC AAGGCGACAAGGTGCTGATGC-3′ that hybridizes close to the 3′-end of CAT ORF, and the antisense primer 5′-GCAGCTCAGTGTCTCTATAAATCAAT GC-3′, which is complementary to a region present in all deletions (nucleotides 261–289, see Figure 7B).

RESULTS

Identification of proteins that bind to the 3′-UTR of *PGK-B* mRNA

There are very few RNA *cis*-acting elements identified in *T. brucei* that determine regulation of gene expression (8,14) and that could be used as baits for RNA-affinity purification. One of them is a sequence located in the 3′-UTR of the *PGK-B* mRNA, which resembles mammalian regulatory AU-rich elements (21). Versions of this 3′-UTR containing or lacking the regulatory element were bound to streptavidin–agarose beads (see Materials and methods section). RBPs able to bind the intact 3′-UTR but not the version lacking the regulatory sequence were affinity-purified from procyclic cytosolic extracts, resolved in SDS–PAGE gels, stained with Sypro Ruby (Figure 1A) and identified by MALDI-TOF/TOF (Figure 1B). The protein band migrating at approximately 40 kDa consisted of a mixture of three polypeptide species, *Tb*DRBD3, *Tb*RBP33 and *Tb*927.6.4440, whereas the protein band migrating at ~22 kDa was identified as *Tb*UBP2. The latter is a putative homolog of *Tc*UBP2, an RBP involved in the regulation of transcript abundance in trypanosomes (17,37). With the exception of *Tb*927.6.4440, all proteins contain canonical RNA recognition motifs (RRM) within their sequences (Figure 1B). This work concentrates in the characterization of one of these proteins, *Tb*DRBD3.



B

Protein	kDa	coverage MALDI-TOF	No. of assigned peptides	Motifs
TbDRBD3	37	43%	3	RRM
TbRBP33	34	58%	4	RRM
Tb927.6.4440	38	36%	1	none
TbUBP2	20	41%	3	RRM

Figure 1. Identification of proteins that bind to *PGK-B* 3′-UTR. (**A**) Procyclic cytosolic extracts were chromatographed on streptavidin–agarose columns containing the *PGK-B* 3′-UTR (lane 1) or a version lacking the regulatory sequence (lane 2), electrophoresed in SDS–PAGE gels and stained with Sypro Ruby. Proteins appearing only in the intact 3′-UTR chromatography eluate were excised and subjected to mass spectrometry analysis. (**B**) Identification of RBPs by MALDI-TOF/TOF. The predicted protein molecular masses, the coverage of tryptic peptides obtained by MALDI-TOF analysis, the number of peptides identified by peptide fragmentation and the conserved PFAM motifs found in the protein sequences are indicated. (**C**) Schematic diagram of *Tb*DRBD3; RRM motifs are shown as black boxes, the C-terminal proline/glutamine-rich region as an empty box and the protein fragment used to raise antibodies as a thin gray box.

This RBP has two RRM motifs and a proline/glutamine-rich region near the C-terminus (Figure 1C). Work is in progress to characterize the other identified proteins following the experimental approach described in this article.

Subcellular localization of *Tb*DRBD3 and effects of RNA interference on trypanosome growth

Polyclonal antibodies were raised in rabbits against *Tb*DRBD3. The affinity-purified antiserum recognized a single band of ~40 kDa in total cell extracts, which was similarly expressed in bloodstream and procyclic trypanosomes (Figure 2A). This protein band decreased clearly in intensity after induction of RNAi targeting *TbDRBD3* mRNA (Figure 3B, see below). *Tb*DRBD3 was found predominantly in the cytosol of wild-type bloodstream and procyclic cells, as observed by immunofluorescence microscopy, although some signal could also be observed within the nucleus (Figure 2B). *Tb*DRBD3 fluorescence signal decreased to background levels after induction of RNAi (Supplementary Figure 1A). Different permeabilization/fixation protocols gave similar staining patterns (Supplementary Figure 1B). The localization of *Tb*DRBD3 was also analyzed by subcellular fractionation (Figure 2C). In this case, the protein was found in both the nuclear and the cytosolic fractions. Taken together, these results indicate that *Tb*DRBD3 can be found in both compartments, with a predominant cytosolic localization.

To study the function of *Tb*DRBD3 in procyclic trypanosomes, the protein was depleted *in vivo* using RNA interference in a tetracycline-inducible manner. A marked reduction of both mRNA and protein levels was readily observed after 48 h of tetracycline induction (Figure 3A and B). As seen in Figure 3C, depletion of *Tb*DRBD3 resulted in a growth arrest phase followed by cell death.

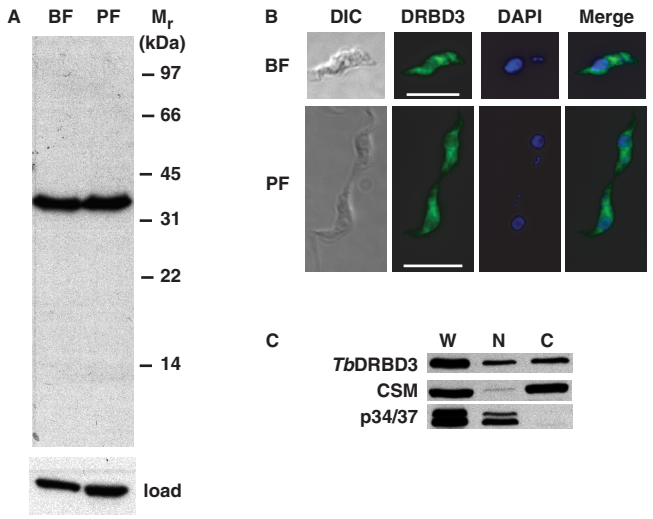


Figure 2. *Tb*DRBD3 is expressed in bloodstream and procyclic trypanosomes and localizes in both the cytosol and the nucleus. An affinity-purified anti-*Tb*DRBD3 antiserum was used to detect the protein by (**A**) western blot analysis of total cell extracts or (**B**) immunofluorescence assays. (**C**) Western blot analysis of samples obtained by cell fractionation. Cytosolic fractions (C) were obtained as described in Materials and methods section. Nuclear fractions (N) were obtained by resuspending the 3000*g* pellet in the original volume. W, whole-cell lysates. To check for proper fractionation, blots were also decorated with the cytosolic marker CSM (33) and the nuclear markers p34/37 (67).

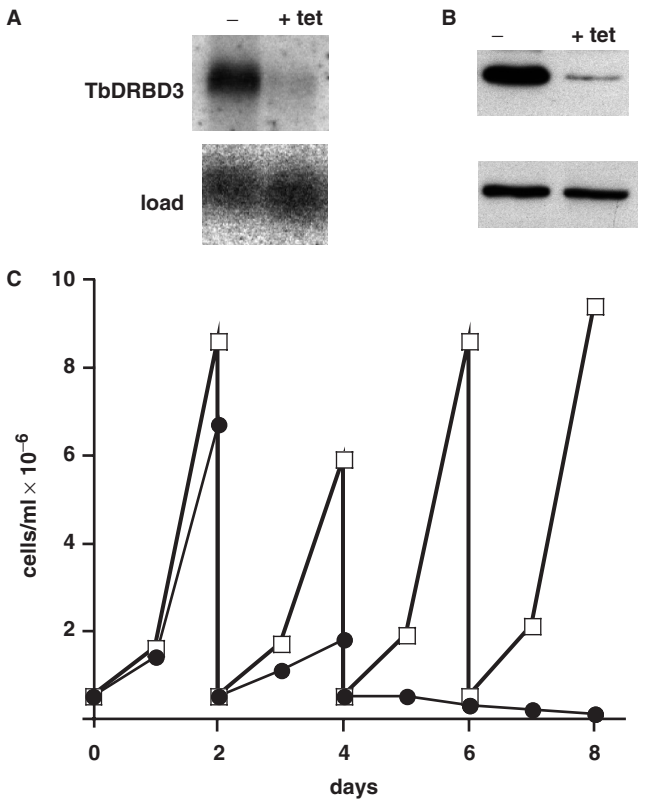


Figure 3. Depletion of *Tb*DRBD3 by RNA interference and effects on growth. A cell line was generated that expressed *TbDRBD3*-specific dsRNA in a tetracycline-inducible manner. The levels of *TbDRBD3* mRNA (**A**) or protein (**B**) were analyzed in cells incubated with tetracycline for 48 h. Loading controls were the signal-recognition particle RNA or the CSM protein. (**C**) The *Tb*DRBD3 RNAi cell line was grown in the absence (open squares and thick lines) or presence (filled circles and thin lines) of 1 μg/ml tetracycline to induce RNAi. Cultures were followed up to 8 days and diluted to 0.4×10^6 cells/ml every 2 days as required.

*Tb*DRBD3 regulates the abundance of a specific subset of mRNAs coding for membrane transporters and intermediate metabolism enzymes

In order to analyze whether *Tb*DRBD3 has a role in the regulation of mRNA turnover, the transcriptome of procyclic trypanosomes depleted of *Tb*DRBD3 was compared to that of uninduced cells using genomic microarrays. RNA samples were isolated from cells harvested after 48 h of induction with tetracycline. At this point, cell growth was barely affected (Figure 3C) and trypanosomes remained highly motile (data not shown). Thirty-four regulated spots showed reproducible regulation in *Tb*DRBD3-depleted cells (see Supplementary Figure 2 and Materials and methods section), which already suggested that *Tb*DRBD3 was involved in the regulation of the abundance of a small group of mRNAs. All clones were sequenced and found to encode 22 proteins, including *Tb*DRBD3 itself (Figure 4A). Regulation was confirmed by northern analysis for all tested mRNAs (Figure 4B). With the exception of a transcript encoded by the procyclin-associated gene 4 (*PAG4*, see below), all regulated mRNAs decreased 2- to 5-fold in abundance upon depletion of *Tb*DRBD3. To rule out unspecific effects on mRNA abundance due to RNAi, RNA was obtained from a control RNAi cell line depleted of the peroxisomal protein PEX2 (33) and analyzed in parallel. There was no apparent effect on the abundance of any mRNA in these cells (Figure 4B).

Interestingly, the majority (64%) of the regulated transcripts were found to encode membrane proteins (indicated in bold face in Figure 4A). These included the amino acid transporters AATP11 and Tb927.8.7740, transporters belonging to the major facilitator superfamily (MFS), surface protein BARP (38), a putative membrane protein with high similarity to plant generative cell-specific protein 1 (*GCS1*) (39), *trans*-sialidase Tb*TS* (40), three receptor-type adenlyate cyclases, thimet oligopeptidase A and two different pteridine transporters. There are four genes encoding pteridine transporters in the genome of *T. brucei*. Three are found as nearly identical tandem repeats in chromosome I, whereas a single gene is present in chromosome X that is 57% identical to its chromosome I counterparts. The mRNAs transcribed from both loci were downregulated upon depletion of *Tb*DRBD3 (Figure 4B). Other regulated mRNAs encoded the intermediate metabolism enzymes *GS* and cytosolic fumarase (*cFUM*).

Notably, some of these mRNAs are upregulated in procyclic forms, such as the transcripts coding for pteridin transporter *Tb*10.6k15.1350, *AATP11*, *trans*-sialidase *Tb*TS, thimet oligopeptidase A, *GS* and *cFUM* [(15,40,41), see also http://www.zmbh.uni-heidelberg.de/Clayton/pc_bs_OligoArray.xls, and data not shown], which suggests that *Tb*DRBD3 is involved, at least in part, in the developmental regulation of these transcripts during the parasite's life cycle. Although not detected in the microarray analysis, the abundance of *Tb*NT10, a purine nucleoside transporter also known to be developmentally regulated (15,42), was checked for regulation by northern analysis and found to be decreased by 2.4-fold after depletion of *Tb*DRBD3 in procyclic forms (Figure 4B). It is also worth mentioning that mRNAs for folate transporters, which encode proteins similar to pteridine transporters (41% identical), were barely affected by *Tb*DRBD3 depletion (Figure 4B). Likewise, the abundance of the mRNA encoding mitochondrial fumarase (*mFUM*), which is also upregulated in procyclic forms (41), was not affected by *Tb*DRBD3 RNAi (Figure 4B). The differential regulation of both fumarases was also confirmed by western blot analysis using monoclonal antibodies (41) (Figure 4C). *Tb*DRBD3 depletion did not affect the levels of either *PGK-B* mRNA or protein (Figure 4B and D). Moreover, *PGK-B* transcript was not detected in *Tb*DRBD3 mRNP complexes (see below). These results were unexpected, since *Tb*DRBD3 was identified based on its ability to bind *PGK-B* mRNA *in vitro* (Figure 1A), and suggest that *Tb*DRBD3 is not likely to be involved in the regulation of *PGK-B* mRNA abundance. The different behavior of *Tb*DRBD3 *in vitro* and *in vivo* could be due to posttranslational modifications, RNA secondary structure or association to other protein factors. Differential binding abilities *in vitro* and *in vivo* have been described for other RBPs (16,43).

A

Systematic name	Positive spots	Description of gene product	Regulation factor
Tb927.1.2820, 2850, 2880	4	**Pteridin transporters**	–2.2, –1.6, –2.9, –2.3
Tb927.3.1860, 1870	1	**Function unknown**	–1.5
Tb927.3.4500	1	Cytosolic fumarase	–1.8
Tb927.4.4730	2	**Amino acid transporter 11**	–2.9, –2.2
Tb927.7.1460	1	Function unknown	–2.0
Tb927.7.190	1	**Thimet oligopeptidase A**	–1.5
Tb927.7.2180	1	Function unknown	–2.1
Tb927.7.4970	1	Glutamine synthetase	–1.5
Tb927.7.5980	2	**MFS**	–2.1, –1.6
Tb927.7.6040	1	**Receptor-type adenylate cyclase**	–1.7
Tb927.7.6850	1	***Tb*TS *trans*-sialidase**	–1.5
Tb927.8.7740	1	**Amino acid transporter**	–2.2
Tb927.8.7940	1	**Receptor-type adenylate cyclase**	–1.5
Tb09.211.0560	3	DRBD3	–6.2, –6.1, –4.9
Tb09.244.2510	1	**BARP**	–2.6
Tb10.406.0110	1	**GCS1-like**	–2.6
Tb10.6k15.0070	1	**PAG4**	+2.6
Tb10.6k15.1290	1	Putative splicing factor	–2.0
Tb10.6k15.1350	1	**Pteridin transporter**	–1.9
Tb11.01.1540	1	Function unknown	–1.6
Tb11.01.7490	1	Putative rRNA/tRNA methyltransferase	–1.6
Tb11.02.3740	3	**Receptor-type adenylate cyclase**	–2.7, –2.2, –2.6

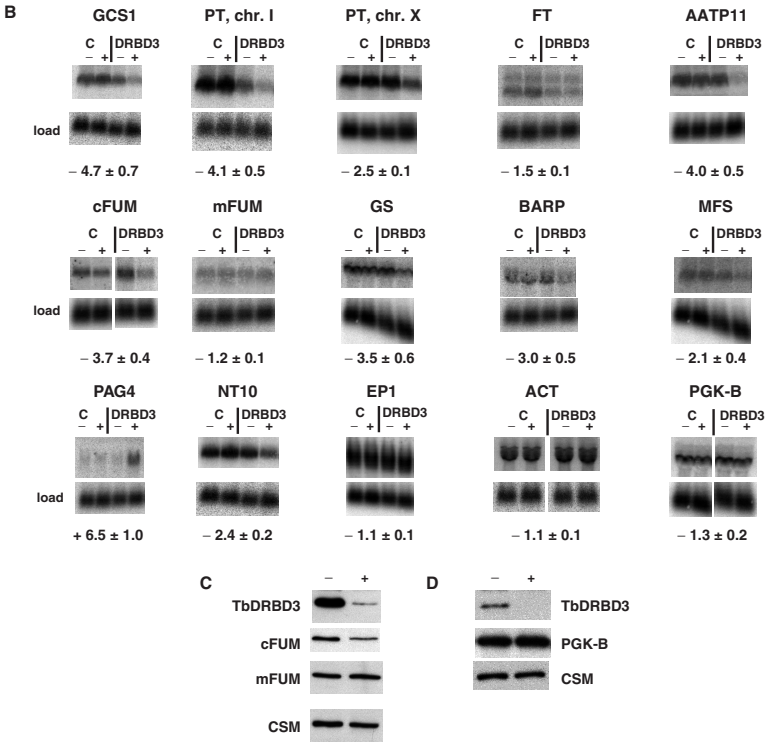


Figure 4. Effect of *Tb*DRBD3 depletion on the transcriptome of *T. brucei*. (**A**) Genomic clones showing the highest regulation in microarray hybridizations after depletion of *Tb*DRBD3. Systematic names and predicted protein products are shown according to GeneDB *T. brucei* genome database annotations (http://www.genedb.org/genedb/tryp). The number of independent regulated microarray spots is shown in the second column. Regulation factors obtained for each spot after analyzing microarray data with the MCHIPS software (29,30) (see Materials and methods section) are indicated in the last column. Predicted membrane proteins are indicated in boldface. In the case of Tb927.3.1860/1870, the genomic clone sequence spanned two ORFs; Tb927.3.1870 gene encodes a putative membrane protein. (**B**) Northern analysis was performed to confirm microarray results, and to analyze the effect of *Tb*DRBD3 depletion on the levels of other mRNAs. Total RNA was obtained from procyclic parasites grown in the absence (–) or the presence (+) of tetracycline for 48 h, transferred to nylon membranes and hybridized with specific probes. Representative blots are shown; the average fold regulation ± SEM is indicated below individual panels, and was calculated after analyzing samples from three independent RNAi experiments and normalizing to signal-recognition particle RNA levels (load). Total RNA was also obtained from a control cell line expressing a dsRNA targeting an mRNA encoding the peroxisomal protein PEX2, which was used as a control in all hybridizations (labeled as 'C' above each blot). The transcripts analyzed were *GCS1*; pteridine transporters (*PT*); folate transporters (*FT*); *AATP11*; cytosolic and mitochondrial fumarases (*cFUM* and *mFUM*); *GS*; *brucei* alanine-rich protein (*BARP*); *MFS*; *PAG4*; purine nucleoside transporter 10 (*NT10*); *ACT* and *PGK-B*. (**C** and **D**) The effect of *Tb*DRBD3 depletion on the levels of cFUM, mFUM and PGK proteins were also assessed by western blot analysis. Blots were decorated with anti-CSM antibodies to confirm equal loading.

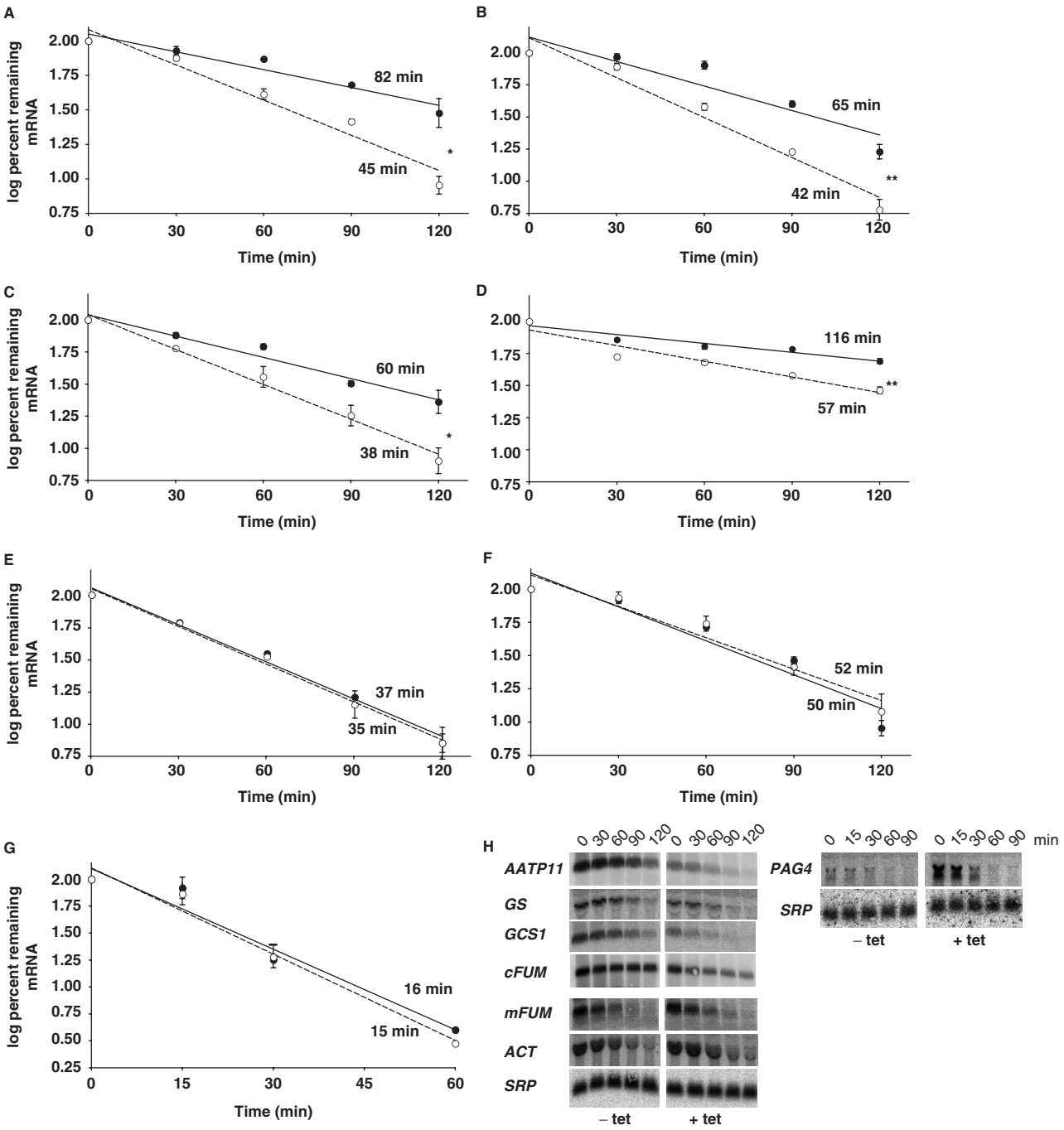


Figure 5. Effect of *Tb*DRBD3 depletion on the stability of transcripts encoding (**A**) *AATP11*, (**B**) *GS*, (**C**) *GCS1*, (**D**) *cFUM*, (**E**) *mFUM*, (**F**) *ACT* and (**G**) *PAG4*. Actinomycin D was added to *Tb*DRBD3-depleted (empty circles) or uninduced trypanosomes (filled circles). RNA samples were obtained at various time points and analyzed by northern blotting. *Tb*DRBD3 depletion significantly decreased the half-lives of *AATP11*, *GS*, *GCS-1* and *cFUM* transcripts (Student's *t*-test, $^{*}P < 0.02$, $^{**}P < 0.002$). *SRP* transcript was used to normalize northern signals. Representative blots are shown in (**H**).

*Tb*DRBD3 acts by stabilizing regulated mRNAs

A possible role of *Tb*DRBD3 could be the stabilization of target mRNAs, given the predominant cytosolic localization of the protein and the fact that most regulated transcripts decreased in abundance in *Tb*DRBD3-depleted cells. To test this hypothesis, the decay of four regulated transcripts coding for *AATP11*, *GS*, *cFUM* and *GCS1*, was monitored in RNAi-induced cells using actinomycin D to block transcription. Degradation kinetics of the transcript coding for *mFUM* and *ACT* mRNAs, whose abundance is not altered by *Tb*DRBD3 ablation (Figure 4B), were also analyzed as a control. As shown in Figure 5, the decreased *AATP11*, *GS*, *cFUM* and *GCS1* mRNA steady-state levels were due, at least in part, to the lower stability of these mRNAs in cells with reduced levels of *Tb*DRBD3. The half-lives of *mFUM, ACT* mRNAs were unaffected by *Tb*DRBD3 RNAi, as expected (Figure 5E and F).

The decay of *PAG4*, the only transcript whose abundance increased upon *Tb*DRBD3 ablation (Figure 4A and B), was also analyzed. As seen in Figure 5G, no apparent changes in the stability of *PAG4* transcript could be detected in *Tb*DRBD3-depleted cells. *PAG4* gene is located at the end of a polycistronic unit transcribed by RNA polymerase I (in trypanosomes there are a few protein-coding genes that are transcribed by this polymerase). This unit also contains three other *PAG* genes, and begins with two genes coding for main surface proteins in the procyclic stage, the procyclins EP1 and EP2 (44). The steady-state levels of *PAG* transcripts are over two orders of magnitude lower than those of *EP*s (45). Depletion of *Tb*DRBD3 in procyclic forms also resulted in an increase in the abundance of the other *PAG* transcripts, but *EP1* and *EP2* mRNA levels remained unchanged (Figure 4B and data not shown). Since *EP*s and *PAG*s genes are transcribed in the same polycistronic unit, these results suggest that *Tb*DRBD3 depletion affects pre-mRNA processing or transcription elongation. However, *PAG4* mRNA could not be detected in *Tb*DRBD3–mRNA complexes (see below), and therefore the increase in the abundance of this transcript could be an indirect effect of *Tb*DRBD3 depletion, for example, due to a drop in the intracellular levels of amino acids or other metabolites transported by the membrane proteins whose mRNAs are regulated by *Tb*DRBD3 (see Discussion section).

*Tb*DRBD3-dependent regulation is mediated through sequences localized in the 3′-UTR

Regulation of mRNA abundance is usually determined by sequences found in the 3′-UTR of target transcripts (46). To investigate whether the same is true for *Tb*DRBD3, the 3′-UTRs of two regulated transcripts, *AATP11* and *GS*, were placed downstream of a luciferase reporter gene and stably integrated in the *Tb*DRBD3 RNAi cell line. As shown in Figure 6, a ca. 3-fold reduction in the steady-state abundance of luciferase mRNA could be observed upon depletion of *Tb*DRBD3. As expected, the levels of a reporter mRNA bearing *ACT* 3′-UTR were unaffected by *Tb*DRBD3 ablation. Reduction in luciferase activity, although evident, was not as marked as for mRNA levels, which could be due to the high stability of luciferase within *T. brucei* cells (47) or to a more efficient translation of decreased mRNA levels to sustain the overall levels of the protein. Decay kinetics of *LUC-AATP11* and *LUC-ACT* reporter mRNAs were also analyzed (Figure 6C and D). The half-life values of both mRNAs measured in uninduced cells were lower than those observed for the respective endogenous mRNAs (i.e. compare Figure 5A with Figure 6C and Figure 5F with Figure 6D), which could be due to intrinsic instability of luciferase transcript in trypanosome cells. Importantly, the half-life of *LUC-AATP11* mRNA was significantly decreased in cells with reduced levels of *Tb*DRBD3 (Figure 6C). Decay of *LUC-GS* reporter mRNA could not be analyzed due to the very

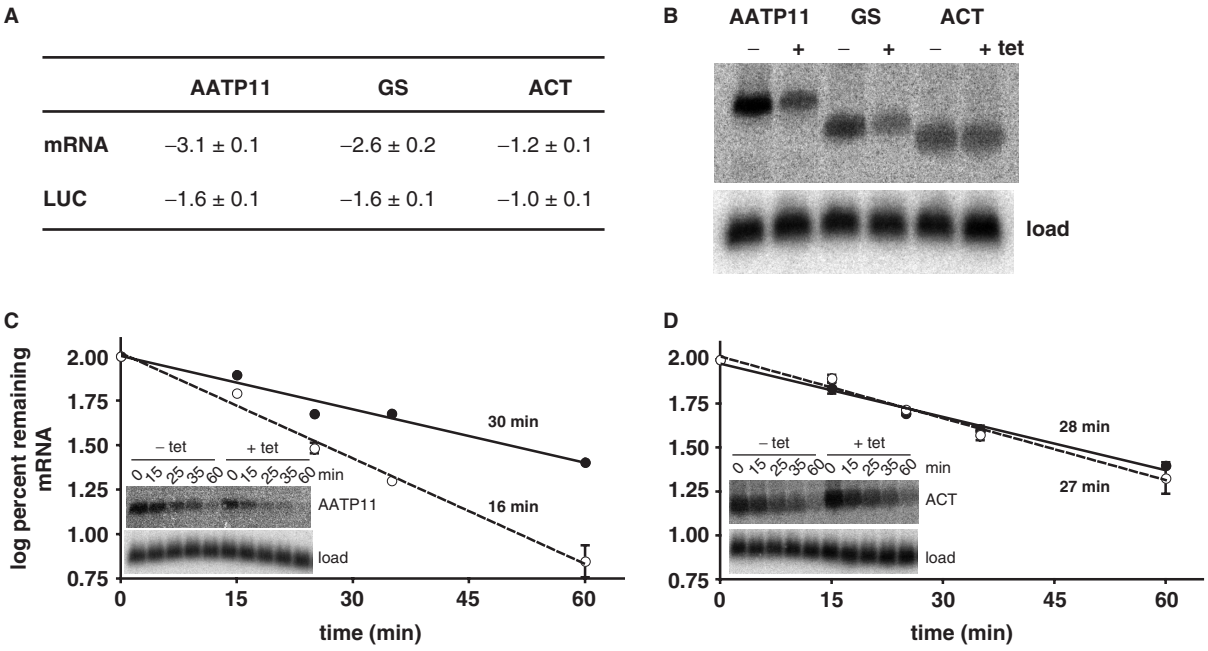


Figure 6. *Tb*DRBD3 knockdown affects the abundance of reporter mRNAs bearing *AATP11* and *GS* 3′-UTRs. Expression vectors containing a luciferase gene (*LUC*) fused to 3′-UTRs of *AATP11*, *GS* or *ACT* mRNAs were stably integrated into *Tb*DRBD3 RNAi procyclic cells. Cultures were treated with tetracycline for 48 h to deplete *Tb*DRBD3 protein (which was confirmed by western analysis, data not shown). (**A**) The levels of the different reporter mRNAs were analyzed by northern blotting using a probe complementary to the luciferase gene. Luciferase activity was assayed enzymatically. Both the average fold decrease in mRNA abundance and luciferase activity were calculated after analyzing three independent clones from each stable transfection. (**B**) Representative northern blot. Hybridization with an SRP probe (load) was included for normalization. The effect of *Tb*DRBD3 knockdown on the half-lives of (**C**) *LUC-AATP11* and (**D**) *LUC-ACT* reporter mRNAs was analyzed as described in Figure 5 legend. *Tb*DRBD3 depletion significantly decreased the half-life of *LUC-AATP11* reporter mRNA (Student's *t*-test, $P = 0.01$). Insets, representative blots; SRP transcript (load) was used to normalize northern signals.

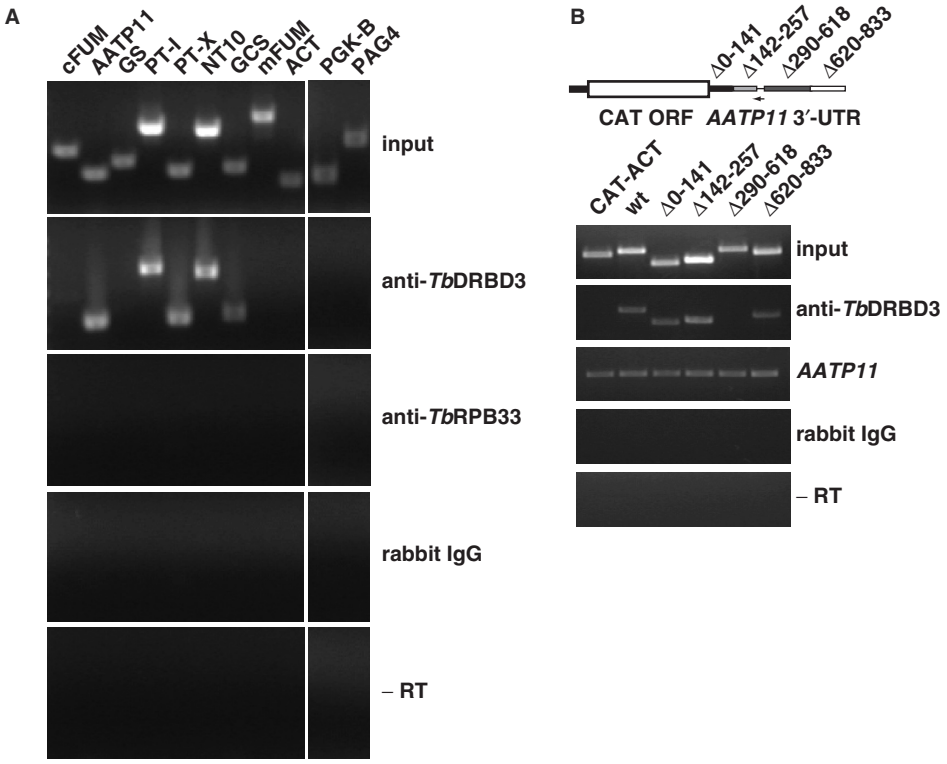


Figure 7. *Tb*DRBD3 associates with target transcripts *in vivo*. (**A**) mRNA–protein complexes were immunoprecipitated from *T. brucei* extracts using anti-*Tb*DRBD3 antibodies. The presence of different transcripts in the complexes was assessed by RT–PCR, and the reaction products were loaded in agarose gels. Immunoprecipitation analyses using antibodies against *Tb*RBP33 (another RBP bearing an RRM motif, Figure 1) or purified rabbit IgGs (Sigma-Aldrich) were performed in parallel to monitor for nonspecific binding. Total RNA was also isolated from an equivalent amount of extract before immunoprecipitation (input). Immunoprecipitated RNA was converted to cDNA in the presence of oligo(dT) and reverse transcriptase, and PCR reactions were performed using mRNA-specific primer pairs. RT–PCR reactions were also performed with immunoprecipitated *Tb*DRBD3 RNA in the absence of reverse transcriptase (−RT). Transcripts names are as in Figure 4. (**B**) Effect of deletions in the 3′-UTR of *AATP11* mRNA on *Tb*DRBD3 binding. mRNA–protein complexes were immunoprecipitated from extracts obtained from cell lines stably expressing *CAT* reporter mRNAs fused to different deletions of *AATP11* 3′-UTR (35), which are schematically shown in the figure. PCR reactions were carried out using a sense oligonucleotide that hybridizes in the CAT ORF and an antisense primer (indicated by an arrow) complementary to a region present in all deletions (see Materials and methods section). The 3′-UTR with deletion Δ620–833 also has its first 39 nucleotides removed. Immunoprecipitation + RT–PCR reactions were also carried out using extracts from a cell line expressing the CAT ORF fused to the *ACT* 3′-UTR (CAT–ACT), which served as a negative control. In this case, the antisense primer was complementary to the *ACT* 3′-UTR. The presence of endogenous *AATP11* mRNA was assessed in all samples to check for immunoprecipitation efficiency.

short half-life of this transcript (<5 min, data not shown). The half-life of *LUC-ACT* mRNA was unaffected by *Tb*DRBD3 RNAi, as expected (Figure 6D).

*Tb*DRBD3 is found in a ribonucleoprotein complex containing target mRNAs

Proteins that regulate the degradation and translation of mRNAs are found associated with their target mRNAs to form ribonucleoprotein complexes (mRNPs) (4), and indeed it was also the case for *Tb*DRBD3, as judged by immunoprecipitation assays and RT–PCR analysis. As shown in Figure 7A, most of the downregulated mRNAs were effectively amplified from *Tb*DRBD3 immunoprecipitated material. These include transcripts coding for *AATP11*, both pteridine transporters, the adenosine transporter NT10 and the *GCS1* protein. *cFUM* mRNA was barely detectable in the *Tb*DRBD3 sample, whereas *GS* mRNA) could not be amplified; therefore, the observed changes in the abundance of *cFUM* and *GS* mRNAs upon *Tb*DRBD3 depletion could be explained by loose association to the mRNP complex or by indirect effects due to changes in cell metabolism (see Discussion section). *PGK-B* and *PAG4* mRNAs were not detected in this assay either, indicating that *Tb*DRBD3 does not interact with these mRNAs *in vivo*. Immunoprecipitation analyses using either an antiserum raised against a different RBP, *Tb*RBP33, or purified rabbit IgGs, yielded undetectable or low-level amplification signals (Figure 7A).

No signal could be detected for transcripts encoding *mFUM* and *ACT*, two transcripts that were not regulated by *Tb*DRBD3 (see above), underscoring the specificity of *Tb*DRBD3–mRNA interactions. In addition, a chloramphenicol acetyl transferase (CAT) reporter mRNA could be immunoprecipitated if fused to the 3′-UTR of *AATP11*, but not when *ACT* 3′-UTR was used (Figure 7B). Most importantly, when reporter *CAT* mRNAs bearing different deletions of *AATP11* 3′-UTR were tested (35), it was observed that a region encompassing nucleotides 290–618 was required to immunoprecipitate the reporter mRNA (Figure 7B), indicating that *Tb*DRBD3 binds specifically to sequences and/or structural elements within this particular

region. Deletion of nucleotides 260–618 did not result in changes in the abundance of the mRNA [Figure 7C, (35) and data not shown]. Since the analyzed *CAT* reporter transcripts seem to have altered polyadenylation patterns (35), the transcription of a reporter mRNA with deletion 290–618 could result in the production of an mRNA with an aberrant 3′-UTR that makes it less prone to degradation.

DISCUSSION

There have been major advances in elucidating the mechanism of mRNA degradation in trypanosomes *in vivo* (36,48–51). However, information about RBPs responsible for the differential abundance of mRNAs during the parasite's life cycle is still very scarce. In this work, an RBP, *Tb*DRBD3, was identified based on its ability to bind the 3′-UTR of *PGK-B* mRNA *in vitro*. Depletion of *Tb*DRBD3 by means of RNAi resulted in a decrease in the steady-state abundance of a specific group of mRNAs encoding membrane proteins and intermediate metabolism enzymes, as seen by microarray and northern analysis. The small number of regulated transcripts, and the fact that the abundance of other mRNAs encoding similar proteins was not apparently affected, highlights the specificity of *Tb*DRBD3-dependent regulation. The results obtained from reporter mRNAs bearing different 3′-UTRs, from the decay rates of several regulated transcripts, and from the immunoprecipitation analysis all suggest that *Tb*DRBD3 binds target mRNAs and protects them from degradation. The fact that a reporter mRNA fused to the 3′-UTR of *AATP11* could be efficiently immunoprecipitated using anti-*Tb*DRBD3 antibodies (Figure 7B) suggests that *Tb*DRBD3-binding site/s localize within the 3′-UTR of regulated transcripts. In the case of *AATP11* 3′-UTR, the binding region seems to be located within nucleotides 290 and 618 (Figure 7B). Interestingly, this region was shown to be important for developmental regulation of *AATP11* transcript, but shorter sequence motifs could not be identified (35). All regulated mRNAs found to be associated with *Tb*DRBD3 contain the UAUUUUUU element shown to be overrepresented among mRNAs that are more abundant in the procyclic stage (52). Since the motif is also present in the *PGK-B* regulatory sequence used to identify *Tb*DRBD3 (Figure 1) (21), it is possible that this protein has affinity towards U-rich elements. Indeed, there is an UAUUUUUU motif within nucleotides 290 and 618 in *AATP11* 3′-UTR (35). However, this motif is also found in many other transcripts that were not detected in the microarray analysis. Moreover, two of these mRNAs, encoding *GS* and *mFUM*, contain UAUUUUUU sequences within their 3′-UTRs, and they do not bind to *Tb*DRBD3, as shown in Figure 7A. Taken together, these observations suggest that there are additional sequence and/or secondary structure determinants that are required for *Tb*DRBD3 to bind to its target mRNAs. This is a likely possibility, since *Tb*DRBD3 contains two RNA-binding domains (Figure 1), and some of such RBPs are able to recognize a long binding platform or to bind simultaneously to distantly positioned sequence motifs within the same RNA molecule (53).

Due to its cytosolic localization, *Tb*DRBD3 could inhibit deadenylation (the first step in the mRNA degradation pathway) by modulating the affinity of the poly(A)-binding protein to the poly(A) tail of regulated transcripts (1). Since *Tb*DRBD3 was also detected in the nucleus of trypanosomes, this protein could additionally facilitate the transport of target transcripts from the nucleus to the cytoplasm, as it has been proposed for ELAV/Hu stabilizing proteins (54).

AATP11, *PTX*, *NT10, TbTS* and *cFUM* mRNAs are abundant in the procyclic (insect) form of the parasite and downregulated in bloodstream forms. Therefore, *Tb*DRBD3-mediated stabilization could be responsible, at least in part, for the developmental regulation of the abundance of these transcripts during *T. brucei* life cycle. Interestingly, *Tb*DRBD3 is similarly expressed in both life forms. Thus, downregulation of the aforementioned transcripts in bloodstream trypanosomes cannot be due to a drop in the levels of *Tb*DRBD3 in the mammalian stage. Instead, *Tb*DRBD3 binding ability could be modulated in different life forms as the result of stage-specific posttranslational modifications, differential RNA secondary structure or association with distinct protein partners.

Importantly, *Tb*DRBD3 was found to be associated with regulated transcripts in mRNA–protein complexes *in vivo*, which indicates that the stability of all bound transcripts is coordinately regulated, and that *Tb*DRBD3–mRNA particles act as an posttranscriptional operon, as it has been proposed for other RBPs in yeast and mammals (4). The presence of RNA operons in such divergent eukaryotes suggests that this kind of complexes were beneficial and ancient modules that conferred additional and advantageous flexibility in the regulation of gene expression early in the eukaryotic lineage.

Yeast Puf proteins are probably the best example of a posttranscriptional operon (4,5). Interestingly, 52–57% of the mRNAs identified in yeast Puf1p and Puf2p mRNPs complexes were found to encode membrane proteins, and over 60% of the mRNAs regulated by *Tb*DRBD3 depletion also code for membrane proteins. Some of them are known to be involved in the transport of small molecules such as amino acids, biopterin or adenosine nucleosides. *Tb*927.7.5960 protein is probably involved in metabolite transport as well, since it contains the MFS motif present in a great variety of small molecule transporters (55). Protein *Tb*10.406.0110 is similar to plant *GCS1*, a membrane protein involved in pollen tube guidance and fertilization (39,56). The role of this protein in trypanosomes is unknown, but it might transduce environmental cues, as it has been proposed for plants (56). There are about sixty genes that encode putative receptor-type adenylate cyclases in the *T. brucei* genome. Three of them, sharing identities of 44–48%, seem to be regulated by *Tb*DRBD3 depletion, according to microarray data. Receptor-type adenylate cyclases in trypanosomes appear to function as enzyme-linked cell surface receptors involved in cAMP signalling (57). All these observations give reason to think that *Tb*DRBD3 regulates at the mRNA turnover level a group of membrane proteins involved in signal

transduction and transport in an orchestrated and stage-specific manner. Whether or not the transported molecules converge in a common metabolic pathway is a matter of future research. The amino acid specificity of AATP11 transporter is not known. Since *GS* mRNA is also down-regulated in procyclic *Tb*DRBD3-depleted cells, and this enzyme catalyzes the interconversion of glutamate and glutamine, it is possible that AATP11 transports glutamine, glutamate or both. Glutamate and glutamine are the sources of all cellular nitrogen (58), and in trypanosomes they could act as precursors of pyrimidines, polyamines, folylpolyglutamates and trypanothione (a thiol equivalent to glutathione in trypanosomes). Interestingly, a proposed role for *cFUM* in trypanosomes is to provide fumarate for the *de novo* synthesis of pyrimidines (41,59), so the possibility exists that *Tb*DRBD3 not only regulates nutrient transport but also the biosynthesis of nitrogen compounds. However, the mRNA coding for *GS* could not be detected in *Tb*DRBD3–mRNA complexes, and the transcript encoding *cFUM* was barely detectable. Although these results can be explained by a lower affinity of *Tb*DRBD3 towards these particular mRNAs, it is also plausible that (as in the case of *PAG*s transcripts described in the Results section) the reduction in the levels of both mRNAs in *Tb*DRBD3-depleted cells is the indirect consequence of nutrient starvation due to reduced transport activities. Interestingly, the expression of *GS* is controlled by the intracellular concentration of glutamine in yeast (58). Alternatively, *Tb*DRBD3 could regulate the abundance of an mRNA (not necessarily any of those shown in this work), which in turn would control *GS*, *cFUM and PAG4* transcripts. Be what it may, regulation of the expression of *GS* and *cFUM* seems to be exerted, at least in part, at the mRNA degradation level, since the half-lives of both transcripts decreased upon depletion of *Tb*DRBD3 (Figure 5).

The apparent absence of RNA polymerase II-dependent transcriptional regulation, the very unusual chromosomal organization and the pre-mRNA processing mechanism involving coupled *trans*-splicing and polyadenylation reactions all pose a challenge to understand how trypanosomes regulate gene expression. The functional characterization of *Tb*DRBD3 described in this work illustrates how it can be achieved, and provides a framework to study posttranscriptional regulation in these parasites. Thus, trypanosome genes could be simultaneously controlled at the mRNA level by association of transcripts encoding stage-specific, functionally linked or cytotopically related proteins to regulatory RBPs, as per the post-transcriptional operon hypothesis (3,4).

The finding that *Tb*DRBD3 is an essential protein, which associates with a specific group of mRNAs underscores the importance of this RBP in posttranscriptional regulation of gene expression in trypanosomes, and opens up the realistic possibility of discovering additional mRNA–protein complexes with a role in the control of transcript abundance and/or translation. Due to the high degree of conservation of RBPs among different organisms, the potential of these proteins as antitrypanosomal drug targets is dubious, but that might not be the case for the proteins encoded by the regulated mRNAs. In fact, pteridine transporters, *trans*-sialidase and *cFUM*, whose transcripts show *Tb*DRBD3-dependent regulation, are known to be important in cell viability, virulence and infectivity in trypanosomatids (41,60–62). Thus, the study of RNA operons in trypanosomes should be of great help in the identification of essential proteins amenable to drug inhibition.

With over 100 predicted RBPs of unknown function in the trypanosomes genomes (14,63), new *cis*-acting motifs discovered (52,64–66) and the experimental approaches described in this work, the identification of RBPs acting as 'posttranscription factors' in trypanosomes should be a feasible task.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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