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Trypanosoma brucei RBP16 Is a Mitochondrial Y-box Family Protein with Guide RNA Binding Activity*

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***Trypanosoma brucei* mitochondria possess a unique mechanism of mRNA maturation called RNA editing. In this process, uridylate residues are inserted and deleted posttranscriptionally into pre-mRNA to create translatable messages. The genetic information for RNA editing resides in small RNA molecules called guide RNAs (gRNAs). Thus, proteins in direct contact with gRNA are likely to catalyze or influence RNA editing. Herein we characterize an abundant gRNA-binding protein from *T. brucei* mitochondria. This protein, which we term RBP16 (for RNA-binding protein of 16 kDa), binds to different gRNA molecules. The major determinant of this interaction is the oligo(U) tail, present on the 3′-ends of gRNAs. RBP16 forms multiple, stable complexes with gRNA *in vitro*, and immunoprecipitation experiments provide evidence for an association between RBP16 and gRNA within *T. brucei* mitochondria. Mature RBP16 contains a cold shock domain at the N terminus and a C-terminal region rich in arginine and glycine. The presence of the cold shock domain places RBP16 as the first organellar member of the highly conserved Y-box protein family. The arginine and glycine rich C terminus in combination with the cold shock domain predicts that RBP16 will be involved in the regulation of gene expression at the posttranscriptional level.**

Kinetoplastid organisms possess a unique RNA processing mechanism called kinetoplastid RNA (kRNA)[1] editing. In this system, certain mitochondrial pre-mRNAs are posttranscriptionally modified by the insertion and deletion of exclusively uridylate residues to otherwise cryptic transcripts (reviewed in Refs. 1–3). Uridylate insertion and deletion is presumably a requirement for translation of these kinetoplast mRNAs, as it often creates start codons, stop codons, and in many cases entire open reading frames. The genetic information for kRNA editing is contained in short, mitochondrially encoded transcripts called guide RNAs (gRNAs) (4, 5). Each gRNA transfers the genetic information for specific uridylate insertion and deletion at multiple, adjacent sites in the pre-mRNA through base pairing interactions. In addition, multiple gRNAs are required for the complete editing of most pre-mRNAs.

A complex of seven proteins that contains editing activity as well as many of the enzyme activities previously postulated to be involved in kRNA editing has been purified from *Trypanosoma brucei* mitochondria (6). However, given the extensive interactions between biological macromolecules that must take place during kRNA editing, it is likely that proteins exist other than those found within the purified editing complex that affect the efficiency and/or accuracy of this process. A likely target for such protein factors is gRNA, given the central role of gRNA in this process. Evidence for the existence of gRNA-binding proteins in kinetoplastid mitochondria has been presented by several laboratories (7–10). Initial experiments using gel retardation methods revealed four ribonucleoprotein complexes that associate specifically with gRNAs (7, 8). Subsequent UV cross-linking experiments identified a set of mitochondrial proteins ranging from 9 to 124 kDa in *T. brucei* (8, 9) and from 30 to 88 kDa in *Crithidia fasciculata* (10) that interact with different gRNAs. A cDNA encoding a 21-kDa, high affinity gRNA-binding protein, gBP21, was recently cloned from *T. brucei* (11). Immunoprecipitation experiments have since revealed an association between gBP21 and the editing machinery (12). A 90-kDa protein with oligo(U) binding activity, TBRGG1, was found to co-sediment with editing activity (13). Finally, from *L. tarentolae,* gRNA-binding proteins of 18 and 110 kDa were identified as the ATPase subunit b and glutamate dehydrogenase, respectively (14, 15). These enzymes could represent a regulatory link between metabolism and RNA editing in kinetoplastids. Because gRNAs exhibit little primary sequence similarity, the basis of gRNA recognition by these proteins is unclear. Common determinants on gRNA molecules that may have a role in recognition by these proteins include a posttranscriptionally added 3′ oligo(U) tail (16) and a common secondary structure (17, 18).

To further understand the process of kRNA editing, we focused our efforts on identifying and characterizing other protein factors that interact specifically with gRNAs. We report here the identification of a gene encoding a protein with an apparent molecular mass of 16 kDa that can be UV cross-linked specifically to gRNA. We designated this protein RBP16 (RNA-binding protein of 16 kDa). RBP16 was purified based on its affinity for poly(U). Subsequent experiments confirmed that RBP16 is capable of interacting with different gRNAs through the oligo(U) tail. Furthermore, immunoprecipitation experiments provide strong evidence that RBP16 interacts with gRNA *in vivo*. The cDNA sequence of RBP16 predicts a protein with three domains: a cleaved mitochondrial import sequence, an N-terminal cold shock domain (CSD), and a C-terminal arginine- and glycine-rich region. The CSD of RBP16 shows extensive homology to bacterial cold shock proteins and to

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF042492.

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[1] The abbreviations used are: kRNA, kinetoplastid RNA; gRNA, guide RNA; CSD, cold shock domain; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; MBP, maltose-binding protein; PCR, polymerase chain reaction.

eukaryotic Y-box proteins. Bacterial cold shock proteins are proposed to function as RNA chaperones by preventing disadvantageous RNA secondary structures (19, 20). The eukaryotic Y-box proteins function in both transcriptional and posttranscriptional regulation of gene expression (21). Possible roles of RBP16 in kRNA editing and other aspects of trypanosome mitochondrial gene expression are discussed.

EXPERIMENTAL PROCEDURES

Cell Culture, Mitochondrial Vesicle Isolation, and Nucleic Acid Preparation—Procyclic form *T. brucei brucei* clone IsTaR1 stock EATRO 164 was grown as described (22). Mitochondrial vesicles were isolated and stored using a previously described method (23). Constructs encoding the gRNAs gA6[14] and gCYb[558], with 17 and 15 nucleotides (nt) oligo(U) tails respectively, were previously described (8). A gA6[14] construct lacking the 3′ oligo(U) tail was generated by PCR (8). Synthetic gRNA molecules possess 10 nt of vector sequence on their 5′-ends. For competition assays, a control transcript, approximately the size of a synthetic gRNA, was produced by run-off transcription from pBluescript (Stratagene) linearized with *Bam*HI. RNAs were synthesized *in vitro* with T7 polymerase and gel-purified on 6% acrylamide/7 M urea.

Isolation of RBP16 Protein—Purified mitochondrial vesicles from approximately 4×10^{11} cells were resuspended in Buffer A (25 mM Tris-Cl (pH 8.0), 15 mM MgOAc, 50 mM KCl) at a concentration of 1×10^{11} cells/ml. All buffers contained 1 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml leupeptin, and 5 mM iodoacetamide to minimize proteolysis. Vesicles were lysed by addition of 0.2% Nonidet P-40, and insoluble material was cleared from mitochondrial extracts by centrifugation. After addition of 1 mM $CaCl_2$, the cleared extract was incubated with micrococcal nuclease (100 units/1×10^{11} cells) for 15 min at 27 °C. Micrococcal nuclease was thereafter inhibited by the addition of EGTA to 5 mM. A poly(U)-Sepharose (Amersham Pharmacia Biotech) column with a 1-ml bed volume was equilibrated in Buffer A containing 50 mM KCl and 10% glycerol. The cleared, micrococcal nuclease treated extract was loaded onto the column, and the column was washed with 10 column volumes of Buffer A containing 300 mM KCl. Bound proteins were eluted with 10 column volumes of Buffer A containing a linear salt gradient ranging from 300 mM-800 mM KCl. Fraction volumes of 0.5 ml were collected. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by UV cross-linking to synthetic gA6(14) internally labeled with [α-^{32}P]UTP. Protein was incubated for 20 min at room temperature with 10 fmol of radiolabeled gA6[14], and UV cross-linking was performed as described previously (8). For determination of internal peptide sequence, poly(U)-Sepharose fractions containing RBP16 were separated by SDS-PAGE, stained with Coomassie Brilliant Blue, and excised from the gel. Internal peptide sequence was obtained from trypsin digestion products of RBP16 by the Harvard Microchemistry facility. RBP16 N-terminal sequence was obtained with a ProSeq Protein Microsequencing after transfer to a nylon membrane. RBP16 was further purified on poly(A)-Sepharose (Amersham Pharmacia Biotech) as follows: poly(U)-Sepharose fractions containing RBP16 were combined and dialyzed twice against 1 liter of Buffer A containing 150 mM KCl. RBP16 was collected in the flow through fraction of a 0.5-ml poly(A)-Sepharose column equilibrated in Buffer A containing 150 mM KCl. The purification procedure was analyzed by SDS-PAGE on a 15% polyacrylamide gel and stained with silver (24). Protein quantification was performed with the Bio-Rad protein assay using bovine immunoglobulin as a standard.

Cloning of RBP16 cDNA—Internal peptide sequences were used for the construction of degenerate oligonucleotides based on previously reported *T. brucei* codon usage (25). Restriction sites at the 5′-ends of oligonucleotides are indicated by underlining. Total procyclic form cDNA was generated by reverse transcription primed with (dT)-RXS (5′-GAGAATTCTCGAGTCGACTTTTTTTTTTTTTTT-3′). Internal cDNA sequence was obtained by PCR amplification of procyclic cDNA with the degenerate primers TB16-D (5′-GCGGATCCNACYTCRAAYTCVACYTCYTGRCCNAC-3′) and TB16-E (5′-GCGGATCCGGNTTYATYGARGAYGAYGCNGAY-3′). Nested PCR was then employed to selectively amplify 5′- and 3′-ends of RBP16 as follows. The 5′-end was first amplified using a primer corresponding to a portion of the splice leader sequence, E-SL22 (5′-GCGAATTCGCTATTATTAGAACAGTTTCTG-3′), and the primer TB16K-I (5′-GCGGATCCGTTTGAAGAGCTGAGAAATGC-3′). The 5′-end was further amplified with the primers E-SL22 (above) and TB16F-OE (5′-GCGGATCCRTCNGCRTCRTCYTCRATRAANCC-3′) using 1 μl of the previous reaction as template. The 3′-end of RBP16 cDNA was amplified using primer (dT)-RXS (above) and the primer TB16K-G (5′-GCGGATCCGAAGCAACACTTTGTGCATTTCTC-3′). Further amplification of the 3′-end of RBP16 cDNA was accomplished with the primers (dT)-RXS (above) and TB16K-J (5′-GCGGATCCGCTCTTCAAACGGAAACGGGGGG-3′) using 1 μl of the previous reaction as template. All reaction products were digested and ligated into the *Eco*RI/*Bam*HI site of pBluescriptII SK− (Stratagene) with the exception of the internal cDNA segment, which was cloned into the *Eco*RI site. Ligated products were transformed into *Escherichia coli* strain DH5α (Life Technologies, Inc.), and transformants were selected on MacConkey agar (Difco Laboratories) containing 100 μg/ml ampicillin. Plasmid DNA was isolated from amp^r colonies, and two clones for each cDNA segment were sequenced in both directions by automated DNA sequencing at the State University of New York at Buffalo Center for Advanced Molecular Biology and Immunology Nucleic Acid Sequencing facility. Sequences were analyzed using the GCG software package (26), and sequence comparisons were done with CLUSTAL W (27).

Bacterial Expression of RBP16—The full-length RBP16 open reading frame (beginning with the nucleotide sequence corresponding to the N-terminal peptide sequence) was amplified by PCR from total procyclic cDNA using primers Tb16K-O (5′-GCGGATCCATGGGTAACAAGGGTAAGGTGATATCG-3′) and Tb16K-P (5′-GCGTCGACCTAAAAGTCATCGCTGAAGCTC-3′), which were constructed based on the cDNA sequence. The reaction products were inserted into the *Bam*HI/*Sal*I site of pMal-C2 (New England BioLabs) and transformed into *E. coli* strain BL21 pLysS (Novagen). Expression and purification of the resulting maltose-binding protein (MBP) fusion was done by standard protocol (28). The expressed fusion protein (MBP-RBP16) was further purified by poly(U)-Sepharose chromatography.

Characterization of Nucleic Acid Binding Properties—Partially purified RBP16 (100 ng/reaction) and purified MBP-RBP16 (680 ng/reaction) were analyzed by UV cross-linking to a synthetic gRNA in the presence of various nucleic acid competitors. Ribohomopolymers, riboheteropolymers, and the double-stranded RNA polymer (poly(A)·U)) were purchased from Sigma. Oligo-$(dT)_{20}$ was purchased from Integrated DNA Technologies, Inc. A Bio-Rad model GS-700 imaging densitometer was used for the quantification of UV cross-linking signals in combination with Molecular Analyst software (version 1.5). For gel retardation assays, reaction conditions were identical to UV cross-linking experiments. After incubation at room temperature for 20 min, RBP16-gRNA complexes were separated by electrophoresis on a native 4% acrylamide gel (acrylamide/bisacrylamide ratio 19:1) in 50 mM Tris-glycine (pH 8.8). Shifted bands were detected by autoradiography.

Immunoprecipitation Experiments—The MBP-RBP16 fusion protein was used for the production of a polyclonal rabbit serum in coordination with the State University of New York at Buffalo Monoclonal Antibody center. Protein A-Sepharose (Amersham Pharmacia Biotech) was used for the purification of IgG molecules from both immune and preimmune sera. For immunoprecipitation experiments, mitochondrial vesicles from 1×10^{10} cells were lysed in 1 ml of buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 2.5 mM EDTA, 0.5 mM dithiothreitol, 0.2% Nonidet P-40), and insoluble material was cleared by centrifugation. One hundred micrograms of purified IgG from immune or preimmune sera was added to 300 μl of extract along with 100 units of RNAguard RNase inhibitor (Amersham Pharmacia Biotech) and incubated for 1 h at 4 °C with gentle rocking. Reactions were added to 50 μl of protein A-Sepharose equilibrated in wash buffer (as above with 0.1% Nonidet P-40), and incubated at 4 °C for 1 h with gentle rocking. Immune complexes were separated from supernatants by centrifugation and washed five times with 500 μl of wash buffer. RNA was isolated from protein A-Sepharose pellets and supernatants by incubation for 15 min at 37 °C in wash buffer containing 0.5% SDS and 50 μg/ml proteinase K. The reactions were extracted twice with phenol-chloroform, and RNA was precipitated with ethanol using glycogen as a carrier. RNA was detected by labeling the 5′-ends with [γ-^{32}P]ATP (NEN Life Science Products; 3000 Ci/mmol) and T4 polynucleotide kinase (Life Technologies, Inc.) following dephosphorylation with calf intestinal alkaline phosphatase (Life Technologies, Inc.). gRNA was identified by labeling 5′-ends with guanylyl transferase (a generous gift from Dr. Ed Niles). Guanylyl transferase reactions were carried out in a 15-μl volume in the presence of 50 μCi of [α-^{32}P]GTP (NEN Life Science Products; 800 Ci/mmol), 50 mM Tris-Cl (pH 8.0), 1.5 mM $MgCl_2$, 6.0 mM KCl, 2.5 mM dithiothreitol, and 25 units of RNAguard RNase inhibitor. Labeled RNA was separated on a 6% acrylamide/7 M urea gel and visualized by autoradiography.

RESULTS

Identification of RBP16—All gRNA molecules possess a 3′ oligo(U) extension that could potentially act as a protein recognition site (16). In addition, experiments from other laboratories demonstrated that poly(U) is an efficient competitor for

the UV cross-linking of mitochondrial proteins to gRNA (9–10). Based on these observations, we fractionated mitochondrial proteins from procyclic form trypanosomes according to poly(U) affinity to obtain a chromatographic fraction enriched for gRNA-binding proteins. The protein profile of the poly(U)-Sepharose fractionation procedure is shown in Fig. 1*A*. An abundant protein with an apparent molecular mass of 16 kDa eluted in fractions 9–16. Fractions were assayed for gRNA-binding proteins by UV cross-linking to synthetic gA6[14] internally labeled with [α-^{32}P]UTP. gA6[14] is the gRNA molecule that specifies the editing of the 3′-most editing block of the ATPase subunit 6 pre-mRNA (29). UV cross-linked proteins were resolved by SDS-PAGE and detected by autoradiography (Fig. 1*B*). An intense UV cross-linking signal was present in *lanes 9–16,* corresponding in size and elution pattern to the 16 kDa band observed in Fig. 1*A*. We named this protein RBP16. To further purify RBP16 for UV cross-linking studies, relevant poly(U) fractions were combined and loaded onto a poly(A)-Sepharose column, and RBP16 was collected in the flow-through (Fig. 1*C, lane A*). By silver stain, we estimated the flow-through fraction to consist of 75% RBP16. Using this procedure, mitochondrial vesicles from 4×10^{11} cell equivalents yield approximately 40 μg of RBP16.

To examine the gRNA binding capabilities of RBP16, partially purified RBP16 was UV cross-linked to radiolabeled gA6[14] in the presence of increasing amounts of unlabeled competitors (Fig. 2*A*). The quantification of UV cross-linking signals from Fig. 2*A* is shown in Fig. 2*B*. The gA6(14) UV cross-linking signal was competed >50% with 1000-fold molar excess unlabeled gA6[14] or gCYb[558], indicating that RBP16 has the capacity to bind different gRNAs. To examine the role of the oligo(U) tail in gRNA binding, the UV cross-linking signal was competed with increasing levels of unlabeled gA6[14]NT, a gRNA lacking the oligo(U) tail. A 4000-fold molar excess of this RNA was required to achieve the level of competition observed with unlabeled gA6[14]. To a slightly lesser extent, competition was observed at a 4000-fold molar excess of unlabeled transcript from a Bluescript plasmid. The difference in the ability of gA6[14]NT and the pBluescript transcript to compete the gA6[14] UV cross-linking signal was small but reproducible. This experiment demonstrated that RBP16 preferentially binds to gRNA over a random RNA molecule of the same size and that the major determinant of the interaction is the oligo(U) tail.

Cloning of RBP16 cDNA—Internal as well as N-terminal peptide sequences were obtained from the purified protein. Using degenerate oligonucleotide primers constructed from internal peptide sequences, a portion of the RBP16 cDNA was amplified by PCR from total procyclic cDNA followed by 5′ and 3′ amplification of cDNA ends. The RBP16 cDNA sequence predicts an RNA molecule with an open reading frame of 423 nt, as well as 5′ and 3′ untranslated regions of 123 and 578 nt, respectively (Fig. 3*A*). Northern analysis of total procyclic RNA detected a single transcript of ~1200 nt (data not shown), which is in good agreement with our cDNA sequence. Southern blot analysis of *T. brucei* genomic DNA demonstrated that RBP16 was encoded as a single copy (data not shown). Fig. 3*A* shows the RBP16 cDNA sequence aligned with the deduced amino acid sequence. The comparison of the N-terminal peptide sequence, which begins with asparagine 18, to the expected amino acid sequence suggests that the first 17 amino acids encode a cleaved mitochondrial import sequence. In light of this observation, the cDNA sequence predicts a mature protein of 13.2 kDa with a pI of 9.29. An overall positive charge of RBP16 at pH 8.8 may explain the slightly slower electrophoretic mobility than is predicted by its size.

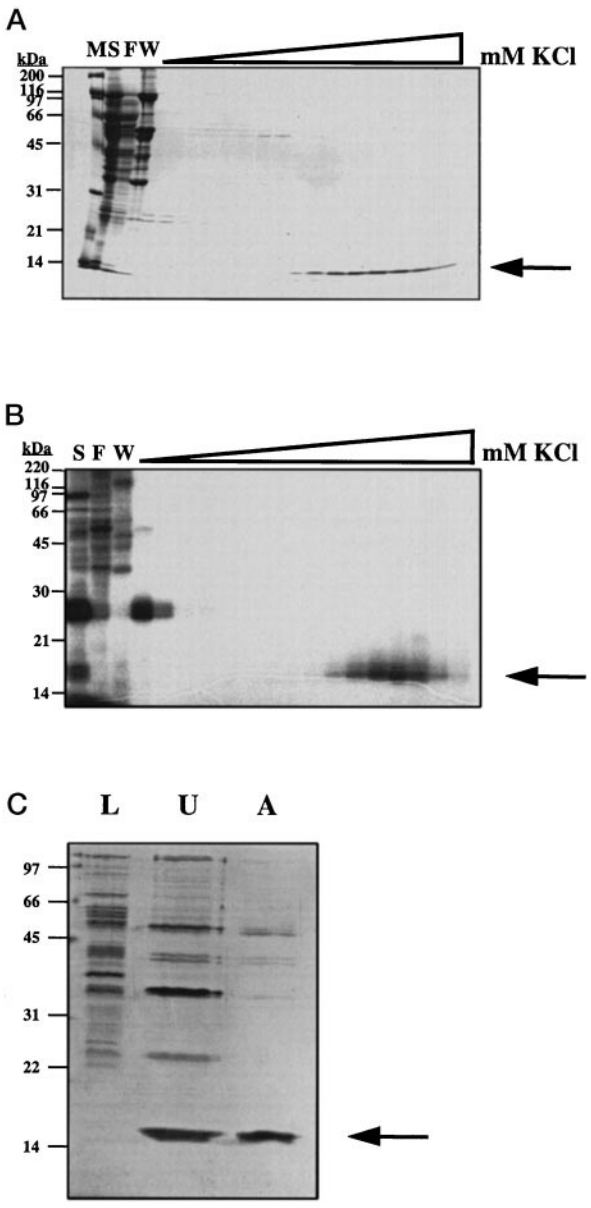

FIG. 1. **Purification of a gRNA-binding protein by affinity chromatography on poly(U)- and poly(A)-Sepharose.** *T. brucei* mitochondrial extract was loaded onto a poly(U)-Sepharose column, and the column was washed with loading buffer containing 300 mM KCl. The column was then eluted with a linear 300–800 mM KCl gradient in loading buffer. *A,* 0.5% of the starting material (*S*), 0.5% of the flow-through (*F*), 10% of the 300 mM wash (*W*), and 10% of eluted fractions were separated by SDS-PAGE on a 12.5% gel and stained with Coomassie Brilliant Blue. Molecular mass standards (*M*) are shown on the *left*. An *arrow* marks the position of an abundant 16-kDa protein (RBP16). *B,* fractions were assayed for gRNA-binding proteins by UV cross-linking to radiolabeled gA6[14]. Ten fmol of gA6[14] was incubated with 4 μl each of total mitochondrial extract (*S*), poly(U)-Sepharose flow-through (*F*), 300 mM KCl wash (*W*), and eluted poly(U)-Sepharose fractions. UV cross-linking proteins were resolved by SDS-PAGE on a 15% gel and detected by autoradiography. The positions of molecular mass markers are shown on the *left*. The position of gRNA UV cross-linking activity that corresponds in size and elution pattern to RBP16 is indicated with an *arrow*. *C,* 1 μg each of mitochondrial lysate (*L*), poly(U)-purified RBP16 (*U*), and the subsequent poly(A)-Sepharose flow-through (*A*) were separated by SDS-PAGE on a 15% gel. Proteins were detected by staining with silver.

Sequence comparison of RBP16 to proteins in several data bases revealed significant homology of the N-terminal half of mature RBP16 to a motif known as the cold shock domain (CSD). The CSD is a well conserved nucleic acid binding do-

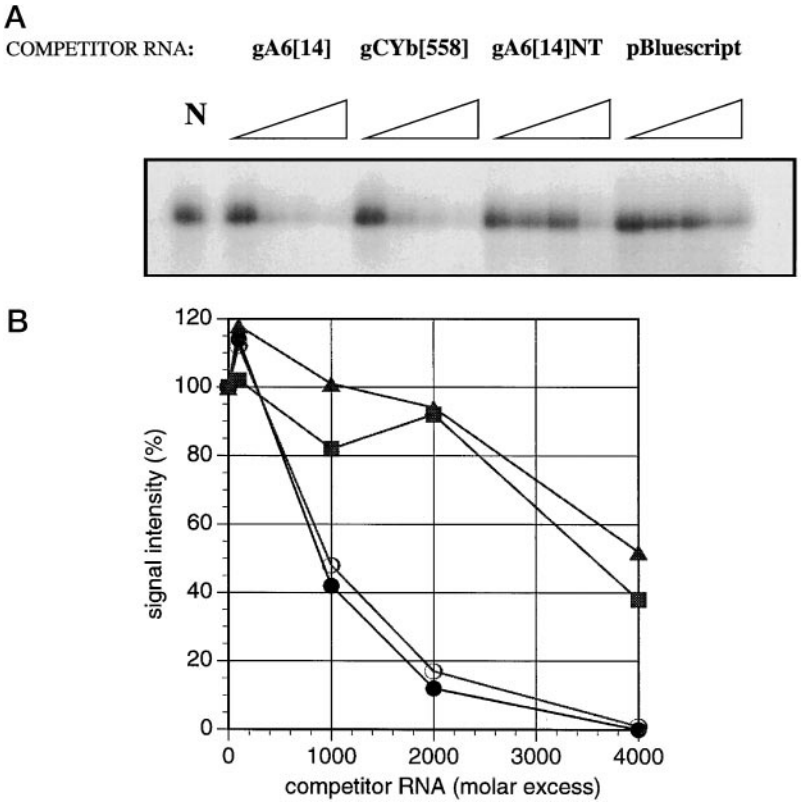

FIG. 2. **gRNA specificity of RBP16 binding.** *A,* 100 ng of protein from the poly(A)-Sepharose flow through was UV cross-linked to radiolabeled gA6[14] in the presence of no competitor (*N*), increasing amounts of unlabeled gA6[14], gCYb[558], gA6[14]NT, or a similarly sized transcript from a Bluescript plasmid. Competition reactions contained either 100-, 1000-, 2000-, or 4000-fold molar excess competitor RNA as compared with the labeled RNA substrate. *B,* densitometer analysis of the UV cross-linking signal in *A*. Competitor levels are plotted on the *x* axis against signal intensity. The UV cross-linking signal in the presence of no competitor is defined as 100%. ●, gA6[14]; ○, gCYb[558]; ■, gA6[14]NT; ▲, pBluescript transcript.

main that is homologous to the bacterial cold shock proteins and is a component of the eukaryotic Y-box proteins (reviewed in Ref. 30). Bacterial cold shock proteins consist entirely of one CSD, whereas the eukaryotic Y-box proteins consist of one or more CSDs usually with auxiliary domains. The CSD of RBP16 presumably participates in gRNA interactions because it contains the RNP1 RNA binding motif, conserved in all CSDs (31) (Fig. 3*B, box*). Fig. 3*B* shows the CSD of RBP16 aligned with CspA of *E. coli* and the CSDs of three eukaryotic Y-box proteins. The cold shock domain of RBP16 exhibits somewhat higher homology to prokaryotic cold shock proteins (43–46% identity) than to eukaryotic CSDs (33–38% identity). At the C terminus of RBP16 is a region that is rich in arginine (14%) and glycine (32%) and may also be capable of interacting with RNA based on an RGG type RNA binding motif (32). It is worth noting that this domain contains an amino acid sequence (amino acids 107–113 of the preprotein; amino acids 90–96 of the mature protein) that is consistent with the preferred recognition motif reported for arginine methylation of hnRNP A1 (33).

Bacterial Expression of MBP-RBP16 Fusion Protein—For bacterial expression of RBP16, the mature open reading frame beginning with asparagine 18 was cloned into the pMal-C2 expression vector. Upon induction with isopropyl-1-thio-β-D-galactopyranoside, an abundant protein corresponding to the expected size of a MBP-RBP16 fusion protein (apparent molecular mass, 59 kDa), was produced (Fig. 4*A, lane In*). This protein was not present in extracts of uninduced cells (data not shown). The expressed fusion protein was purified from bacterial cells first based on amylose affinity (Fig. 4*A, lane Am*) and subsequently based on affinity for poly(U) (Fig. 4*A, lane U*). After the two-step purification, no contaminating bands were evident when 13 μg of protein were stained with Coomassie Brilliant Blue.

Characterization of RBP16 Nucleic Acid Binding Properties—UV cross-linking competitions were used to characterize the binding of RBP16 to various nucleic acid substrates and to compare the binding properties of the MBP fusion protein with partially purified RBP16. The partially purified native RBP16 and the MBP fusion protein showed nearly identical preferences for various nucleic acid substrates (Fig. 4, *B–D*). These data demonstrate that the amplified cDNA sequence encodes the RBP16 protein that was purified from trypanosome mitochondrial vesicles. RBP16 shows affinity for poly(U) and, to a lesser extent, poly(G) (Fig. 4*B*). The RBP16 UV cross-linking signal is almost completely abolished by unlabeled poly(U) at 1000-fold mass excess over labeled gRNA substrate and is competed with unlabeled poly(G) to the same extent by approximately 5000-fold mass excess cold competitor. No binding to poly(A) or poly(C) was observed. Unlabeled poly(GU) and poly(CU) heteropolymers competed the UV cross-linking signal as effectively as poly(U) (Fig. 4*C*). AU and AC ribopolymers were ineffective in competing UV cross-linking to gA6(14). The inability of RBP16 to bind poly(AU) maybe due to extensive formation of double-stranded regions in this RNA. Fig. 4*D* demonstrates that RBP16 can bind RNA (poly(U)) and single-stranded DNA (oligo-$(dT)_{20}$), but does not bind double-stranded RNA (poly(A)·(U)). Although binding to oligo-$(dT)_{20}$ appears somewhat stronger than to poly(U), the significance of this result is difficult to interpret. Given equal masses, oligo-$(dT)_{20}$ contains much higher concentration of 5′- and 3′-ends than poly(U), which is hundreds of nucleotides in length. The requirement for 5′- and/or 3′-ends for RBP16 binding is unknown.

Gel retardation assays were used to estimate the affinity of RBP16 for gA6[14]. When internally labeled gA6[14] was incubated with a 1 μM concentration of MBP-RBP16, no shift in the gRNA probe was observed. A single shifted band was observed when MBP-RBP16 was added to a concentration of 2.5 μM (Fig. 5). At MBP-RBP16 concentrations of 5 μM and above, two band shifts were evident (Fig. 5). The appearance of two shifted forms may represent two RBP16 molecules bound to one gRNA molecule, and this possibility is currently being investigated. No shift was seen when MBP was used in the assay (Fig. 5). This experiment demonstrated that MBP-RBP16 forms stable

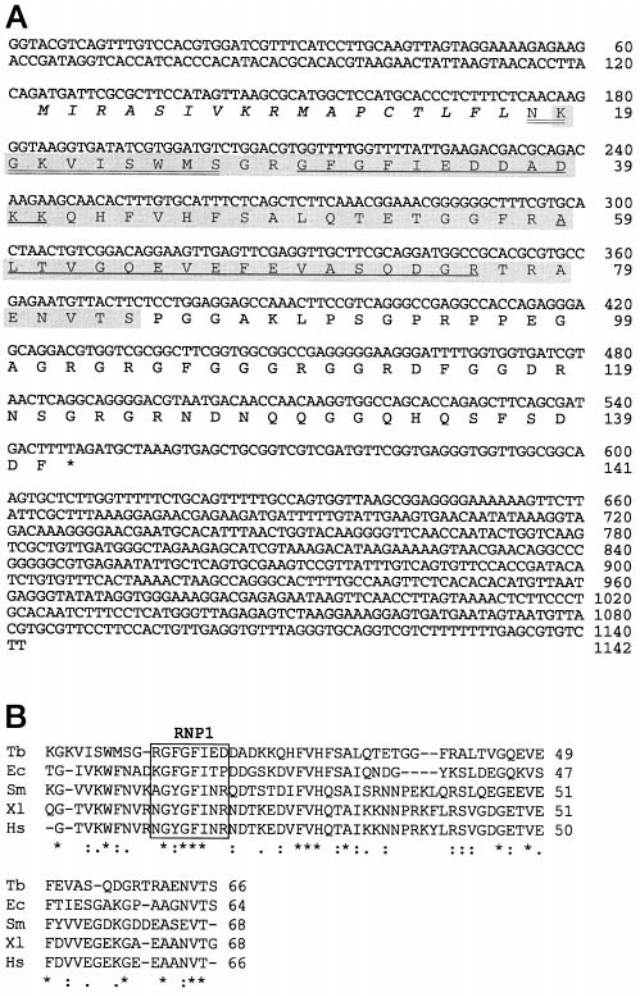

FIG. 3. **RBP16 nucleotide and deduced amino acid sequence.** *A,* the complete 1142-nucleotide cDNA sequence predicts a 141-amino acid preprotein with a predicted molecular mass of 13.9 kDa and a pI of 9.1 and a mature protein with a predicted molecular mass of 13.2 kDa and a pI of 9.3. The N-terminal 17 amino acids that presumably make up a cleaved mitochondrial import sequence are shown in *italics*. N-terminal sequence obtained from the purified RBP16 protein is indicated by a *double underline*. Peptide sequences obtained from trypsin cleavage products are shown with a *single underline*. The N-terminal half of the mature protein is highly homologous to a known nucleic acid binding motif called the CSD (*shaded region*). The C-terminal half of the mature protein is rich in glycine (32%) and arginine (14%) residues. *B,* alignment of the RBP16 CSD (*Tb*) with the *E. coli* CspA (*Ec*) (GenBank™ accession number P15277) and the CSD of eukaryotic Y-box proteins from *Schistosoma mansoni* (*Sm*) (GenBank™ accession number U39883), *Xenopus laevis* (*Xl*) (GenBank™ accession number P21574), and *Homo sapiens* (Hs) (GenBank™ accession number P16991). Identical amino acids are indicated with an *asterisk*. Semiconservative and conservative substitutions are indicated with *one dot* and *two dots,* respectively. The RNP1 RNA binding motif, conserved in all CSDs, is *boxed*.

complexes with gA6[14] at a minimum concentration of 2.5 μM.

Co-immunoprecipitation of gRNA with RBP16—To demonstrate an interaction between RBP16 and gRNA within mitochondrial vesicles, we investigated the ability of antibodies directed against the MBP-RBP16 fusion protein to co-immunoprecipitate gRNA. Anti-RBP16 antibodies were purified from rabbit serum using protein A-Sepharose and did not recognize any proteins other than RBP16 in Western blots of mitochondrial extracts (data not shown). Anti-RBP16 and preimmune IgG were incubated with mitochondrial extract from 3×10^9 trypanosomes. Immune complexes were captured with protein A-Sepharose and purified by centrifugation, and RNA was isolated from reaction supernatants as well as protein A-Sepharose pellets. RNAs were subsequently detected by labeling the 5′-ends with either T4 kinase or guanylyl transferase. RNA was dephosphorylated prior to labeling with T4 kinase; therefore, all RNA species should be labeled by this method. Capping with guanylyl transferase specifically labels 5′ tri- or diphosphates and has been previously shown to label primarily the gRNA component of *T. brucei* mitochondrial RNA (34). Quantification of the resulting autoradiographs indicated that anti-RBP16 antibodies co-immunoprecipitated approximately 30% of gRNA within mitochondrial extracts (Fig. 6*A*). Because immunoprecipitation experiments were performed under conditions where approximately 90% of RBP16 was precipitated (data not shown), we conclude that this number accurately reflects the percentage of gRNA associated with RBP16 within mitochondrial vesicles. Anti-RBP16 antibodies also precipitated approximately 30% of the 9 S and 12 S rRNAs (Fig. 6*A, lanes 1–3*). This association is likely a result of RBP16 binding to the oligo(U) tails also present on the 3′-end of the rRNAs (35) and suggests that RBP16 may play multiple roles in *T. brucei* mitochondrial gene expression. RBP16 was not associated with tRNAs or with unidentified RNAs between the sizes of 120 and 210 nt (Fig. 6*A*). The unidentified RNAs could be rRNA degradation products or previously described tRNA precursors (36). Preimmune IgG did not precipitate any RNA species (Fig. 6*B*). Preliminary results using Northern analysis and reverse transcription-PCR indicate that anti-RBP16 antibodies also precipitate several mRNAs. However, we cannot distinguish a direct interaction between mRNA and RBP16 from an indirect association mediated by gRNA and/or ribosomes. Interestingly, anti-RBP16 antibodies did not precipitate small (<50 nt) RNAs that were capped with guanylyl transferase (Fig. 6*A, lanes 4–6*). The identities of these RNAs are unknown; however, their size and ability to be capped with guanylyl transferase are consistent with the truncated gRNAs lacking correct 3′-ends and oligo(U) tails previously observed (16). Taken together, these data support the *in vitro* observation that RBP16 interacts with gRNA oligo(U) tails.

DISCUSSION

We describe here the identification and initial characterization of RBP16, an abundant protein from *T. brucei* mitochondria with gRNA binding activity. Although RBP16 was initially purified from mitochondrial vesicles, the presence of a spliced leader sequence on RBP16 cDNAs indicates that it is encoded in the nucleus. In addition, the N-terminal 17 amino acids of the predicted protein are absent from the N terminus of the purified protein, indicating that RBP16 is most likely transported into the mitochondrion via a cleaved signal peptide. Mature RBP16 is composed of an N-terminal CSD and a C-terminal arginine- and glycine-rich region. This structure defines RBP16 as a member of the eukaryotic Y-box family. RBP16 is the first member of this family to be identified in protozoans, as well as the first organellar member of this protein family described.

The CSD was first identified as the sole component of the bacterial cold shock proteins (37) and was later recognized as a constituent of several eukaryotic proteins now collectively called the Y-box proteins. The Y-box proteins are composed of a highly conserved N-terminal CSD flanked by a more variable C-terminal domain (reviewed in Ref. 30). Y-box proteins can possess distinct types of C-terminal domains, including the basic and acidic islands present in many vertebrate Y-box proteins, as well as the zinc finger motif, a characteristic of the *Caenorhabditis elegans* developmental regulating protein Lin-28 (38). The C terminus of RBP16 is a basic, 57-amino acid region rich in arginine (14%) and glycine (32%), reminiscent of

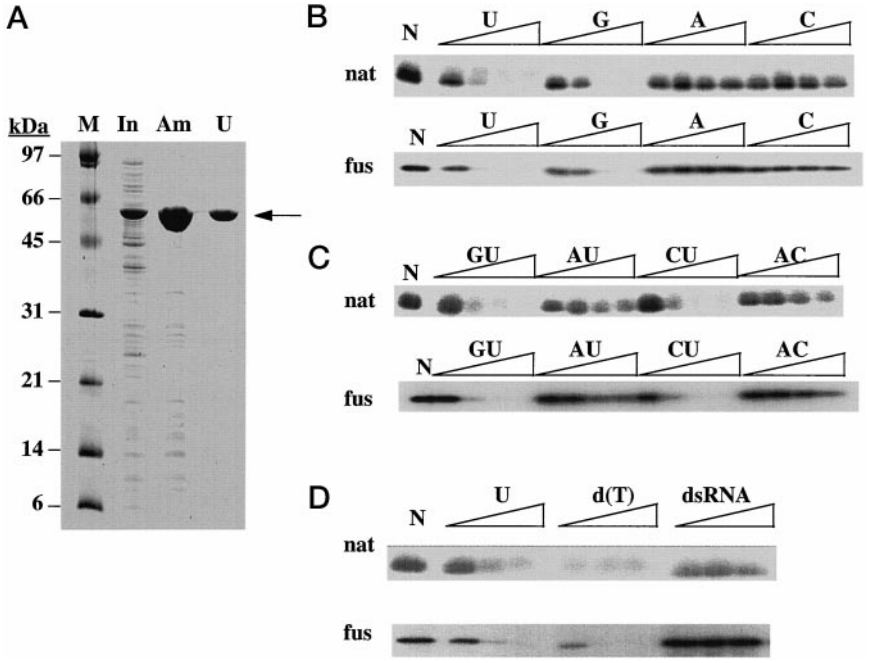

FIG. 4. **Expression of MBP-RBP16 fusion protein in *E. coli* and characterization of RBP16 nucleic acid binding properties.** *A, E. coli* cells harboring the pMal-C2 plasmid with the RBP16 gene in frame with MBP were grown in the presence of 0.3 mM isopropyl-1-thio-β-D-galactopyranoside. Molecular mass markers (*M*), extracts from isopropyl-1-thio-β-D-galactopyranoside-induced cells (*In*), amylose column eluant (*Am*), and subsequent poly(U)-purified protein (*U*) were separated by SDS-PAGE on a 12.5% gel and stained with Coomassie Brilliant Blue. The 59-kDa fusion protein is indicated with an *arrow*. *B,* native RBP16 (*nat*) purified from trypanosome mitochondria by poly(U)- and poly(A)-Sepharose chromatography (100 ng/reaction) and the bacterially expressed MBP-RBP16 fusion (*fus*) (680 ng/reaction) were UV cross-linked to radiolabeled gA6[14] in the presence of no competitor (*N*) or in the presence of 100-, 1000-, 5000-, or 10,000-fold mass excess unlabeled ribonucleotide homopolymer competitors. *C,* UV cross-linking, as in *B* except in the presence of ribonucleotide heteropolymer competitors. *D,* UV cross-linking in the presence of unlabeled poly(U), oligo-$(dT)_{20}$, and double-stranded RNA (poly(A)·(U)).

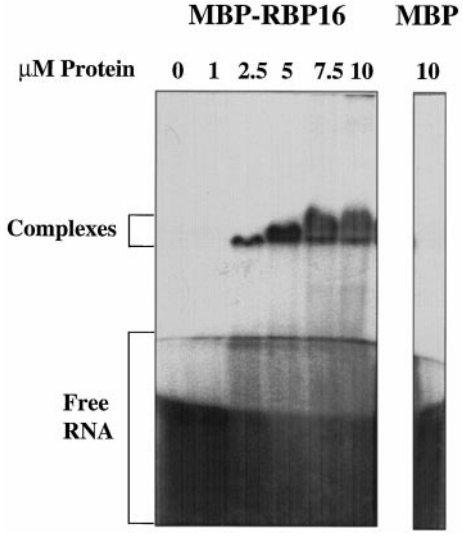

FIG. 5. **Determination of gRNA binding affinity of MBP-RBP16 fusion protein.** Increasing concentrations of MBP-RBP16 or 10 μM MBP were incubated with radiolabeled gA6[14] as in UV cross-linking experiments. Following electrophoresis on a 4% nondenaturing acrylamide gel, RNA-protein complexes were detected by autoradiography. Positions of unbound RNA and MBP-RBP16-gA6[14] complexes are indicated by *brackets*.

the RGG-type RNA binding motif (32). Y-box proteins from several invertebrates having arginine- and glycine-rich C-terminal domains have been reported (39–42), although these proteins have significantly larger arginine- and glycine-rich regions than RBP16. Although the functions of these invertebrate Y-box proteins are unknown, the planarian protein DjY1 is localized at sites of regeneration (39). The CSDs of virtually all Y-box proteins share greater than 90% sequence homology and are approximately 40–45% identical to the bacterial cold shock proteins. The invertebrate Y-box proteins DjY1 (from *Dugesia japonica*) and SMYB1 (from *Schistosoma mansoni*) are exceptions, as their CSDs are 60–64% identical to other eukaryotic CSDs (39, 40). In contrast, the CSD of RBP16 possesses a higher sequence identity to the prokaryotic cold shock proteins (43–46%) than to any eukaryotic CSDs (33–38%). This may reflect the ancient evolutionary position of the kinetoplastids or a mitochondrial ancestry of the RBP16 gene.

The Y-box proteins are a highly conserved family of nucleic acid-binding proteins that interact with both DNA and RNA (reviewed in Ref. 21). The Y-box proteins function to regulate gene expression at the transcriptional as well as posttranscriptional level, although the mechanism by which this is achieved is largely unknown. The regulation of gene expression is most likely accomplished through the ability of the CSD to relieve nucleic acids of secondary structure, whereas the C-terminal domain mediates further molecular interactions (43). The most extensively studied Y-box protein is the FRGY2 protein, which inhibits the translation of maternal mRNA in *Xenopus* oocytes until critical cellular signals are present (reviewed in Ref. 44). The translation of these messages is mediated by a dephosphorylation event that inhibits the nucleic acid binding of FRGY2 (45). Interestingly, translational silencing is dependent on the processing history of the message (46). This raises the possibility that Y-box proteins are important for the coupling of mRNA processing events to translation.

RBP16 binds to both single-stranded RNA and DNA consistent with the nucleic acid binding properties of the Y-box proteins (reviewed in Ref. 21). We provide evidence here that at least one function of RBP16 involves RNA binding, although we cannot omit DNA as an *in vivo* nucleic acid target. Competition studies show that RBP16 binds U- and G-containing RNA polymers, with a 5-fold higher affinity for the former. RBP16

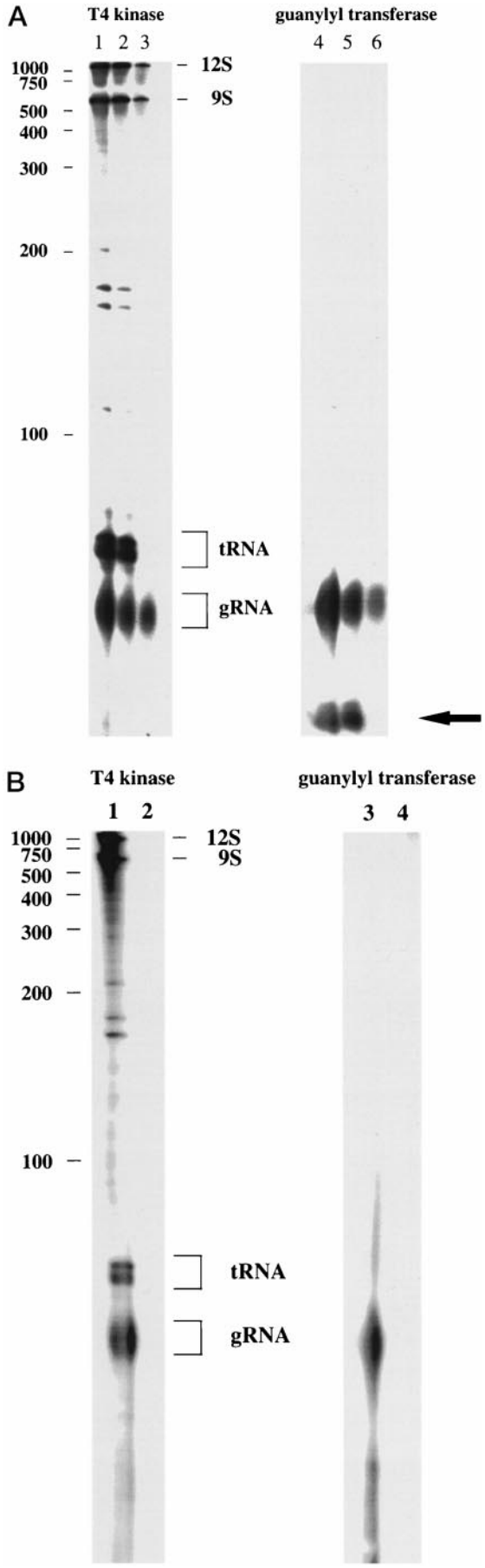

FIG. 6. **Co-immunoprecipitation of RNA with RBP16.** *A,* anti-RBP16 IgG (100 μg) was incubated with mitochondrial extract from 3 × 10^9 trypanosomes. Immune complexes were purified using protein A-Sepharose. Co-immunoprecipitating RNA was extracted and precipitated from protein A-Sepharose pellets, as well as reaction supernatants. RNA was either dephosphorylated and labeled with T4 kinase or capped with guanylyl transferase. The positions of RNA size markers are shown on the *left*. Total RNA from 3 × 10^9 trypanosome mitochondria (*lanes 1* and *4*), unbound RNA (*lanes 2* and *5*) and bound RNA (*lanes 3* and *6*) are shown. RNA in *lanes 1–3* was labeled with T4 kinase. RNA in *lanes 4–6* was labeled with guanylyl transferase. The positions of the 9 S and 12 S rRNAs are indicated. Labeled gRNA and tRNA are shown in *brackets*. Capped RNA that may correspond to the truncated gRNAs observed by Blum and Simpson (16) is marked with an *arrow*. *B,* same as in *A* except that mitochondrial extract was incubated with 100 μg of preimmune IgG. Unbound RNA (*lanes 1* and *3*) and bound RNA (*lanes 2* and *4*) are shown. *Lanes 1* and *2* were labeled with T4 kinase after dephosphorylation. *Lanes 3* and *4* were labeled with guanylyl transferase.

was identified based on its ability to UV cross-link to gRNA, and subsequent experiments with gRNA deletions suggest that the oligo(U) tail is the major determinant for this interaction. The minimum concentration of MBP-RBP16 that is required for stable binding to gRNA in our gel retardation experiments is 2.5 μM. The abundant nature of RBP16 suggests that its cellular concentration may be sufficient for stable gRNA interactions *in vivo*. Further evidence that RBP16 interacts with gRNA is provided by the observation that anti-RBP16 antibodies co-immunoprecipitate approximately 30% of native gRNAs in mitochondrial extracts. An RNA population, characterized by its small size (<50 nt) and ability to be labeled with guanylyl transferase, was not associated with RBP16 in immunoprecipitation experiments. These RNAs most likely correspond to the truncated gRNAs lacking correct 3′-ends and oligo(U) tails observed by Blum and Simpson (16). This is consistent with the *in vitro* data identifying the gRNA oligo(U) tail as the RBP16 binding determinant. The association of RBP16 with gRNA implies that RBP16 may be involved in the process or regulation of RNA editing. This prospect is supported by the recent report that gBP21, a high affinity gRNA-binding protein, is associated with RNA editing activity and a 16-kDa gRNA-binding protein (12). Approximately 30% of the 9 S and 12 S rRNAs were also immunoprecipitated from mitochondrial extracts with anti-RBP16 antibodies, raising the possibility that RBP16 binds the posttranscriptionally added oligo(U) tails on these RNAs as well (35). Preliminary evidence suggests that several mRNAs are also present in the immunoprecipitate. RBP16 may interact with mRNA directly via extensive U-rich regions known to be present in many of the mitochondrial messages (47) or indirectly through interactions with gRNA or ribosomes. The ability of RBP16 to bind to various classes of RNA suggests that it may play multiple roles in *T. brucei* mitochondrial gene expression, as has been hypothesized for Y-box proteins in other systems (21).

It is commonly held that the CSD element of the Y-box proteins destabilizes RNA secondary structure, exemplified by the "RNA chaperone" activity demonstrated *in vitro* for *E. coli* CspA (19). Given the extensive interactions that must occur among nucleic acids and proteins during the process of RNA editing, it is very likely that RNA structure is intricately involved in this process. RBP16 may act as a gRNA chaperone by destabilizing gRNA secondary structure or maintaining a single-stranded conformation. Portions of both the gRNA anchor and guiding regions have been demonstrated to be contained partly in an intramolecular duplex (17, 18). Destabilization of these structures might be necessary for initial gRNA-mRNA interactions, or interactions during uridine insertion and/or deletion. Similarly, it is postulated that partial hydrogen bonding between the oligo(U) tail and the mRNA maintains the proximity of the pre-mRNA 5′ and 3′ cleavage fragments dur-

ing the course of the editing reaction (16). By discouraging intramolecular hydrogen bonds, contact between RBP16 and the gRNA oligo(U) tail could potentially leave these bases accessible for interactions with the pre-mRNA. Extensively U-rich regions present in many of the mitochondrial mRNAs have the potential to form regions of largely double-stranded character through an interaction with the poly(A) tail.[2] The resolution of these structures could be a prerequisite for efficient editing and/or translation. Furthermore, because the procyclic form of *T. brucei* exists at 27 °C, RBP16 may play an important role in the maintenance of RNA structure in this life-cycle stage particularly. In this manner, the function of RBP16 would be similar to that of CspA during cold shock in bacteria.

RBP16 may also play a more regulatory role in *T. brucei* mitochondrial gene expression. For example, it has been hypothesized that life cycle stage-specific editing is regulated through gRNA usage, because gRNA abundance does not correlate to the life cycle stage-specific abundance of edited transcripts (48). gRNA usage could be controlled in a negative manner through the sequestering or "masking" of specific gRNAs from the editing machinery by RBP16. Alternatively, RBP16 could be a required factor for the usage of a subset of gRNAs. Similarly, RBP16 may affect the access of certain mRNAs to the translational or editing machinery, analogous to the role of FRGY2 described above. Because our results indicate that poly(U) is the main binding determinant for RBP16, an association between RPB16 and another, more sequence-specific RNA-binding protein would most likely be required for the recognition of a subset of gRNA or mRNA. Finally, interaction of RBP16 with both rRNA and gRNA suggests a possible manner in which the processes of editing and translation could be coupled through a mutually required factor. We are currently constructing a trypanosome strain harboring deletions of both RBP16 genes, in order to investigate these and other possible roles of RBP16 in *T. brucei* mitochondrial gene expression.

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