CHAPTER FOUR

Isolation of RNA Binding Proteins Involved in Insertion/Deletion Editing

Michel Pelletier,[†] Laurie K. Read,[†] *and* Ruslan Aphasizhev[*]

Contents

[*] Department of Microbiology and Molecular Genetics, University of California, Irvine, California
[†] Department of Microbiology and Immunology, SUNY Buffalo School of Medicine, Buffalo, New York

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Abstract

RNA editing is a collective term referring to a plethora of reactions that ultimately lead to changes in RNA nucleotide sequences apart from splicing, 5′ capping, or 3′ end processing. In the mitochondria of trypanosomatids, insertion and deletion of uridines must occur, often on a massive scale, in order to generate functional messenger RNAs. The current state of knowledge perceives the editing machinery as a dynamic system, in which heterogeneous protein complexes undergo multiple transient RNA–protein interactions in the course of gRNA processing, gRNA–mRNA recognition, and the cascade of nucleolytic and phosphoryl transfer reactions that ultimately change the mRNA sequence. Identification of RNA binding proteins that interact with the mitochondrial RNAs, core editing complex, or contribute to mRNA stability is of critical importance to our understanding of the editing process. This chapter describes purification and characterization of three RNA binding proteins from kinetoplastid mitochondria that have been genetically demonstrated to affect RNA editing.

1. Introduction

Uridine insertion/deletion editing in kinetoplastid protozoa entails massive processing of pre-mRNAs. Many pre-mRNAs undergo hundreds of uridine additions and less frequent deletions, and require the sequence information from dozens of *trans*-acting guide RNA (gRNAs) molecules for the creation of a translatable mRNAs. Precise editing requires that cognate mRNA–gRNA pairs form in the presence of hundreds of potential substrates. The editing reaction then necessitates a vast number of RNA–protein and RNA–RNA interactions and extensive rearrangements as editing progresses along an mRNA. In addition, editing is regulated during the trypanosome life cycle in the face of relatively constant mRNA and gRNA levels, implying that gRNA and/or mRNA usage is regulated in a specific manner (Stuart *et al.*, 2005). All of these scenarios suggest that RNA binding proteins will have a major impact on insertion/deletion editing, as is the case for other complex RNA processing events such as RNA splicing (Sanford *et al.*, 2005). The editing machinery is emerging as a dynamic system, in which heterogeneous

protein complexes undergo multiple transient RNA–protein interactions. Thus, the identification of RNA binding proteins that interact with the editosome and/or impact the editing of specific RNAs is of critical importance to our understanding of the editing process. To date, three RNA binding proteins from kinetoplastid mitochondria have been genetically demonstrated to affect RNA editing. These are the related MRP1 and MRP2 proteins (Vondruskova *et al.*, 2005) and RBP16 (Pelletier and Read, 2003).

1.1. MRP1 and MRP2

MRP1, originally designated as gBP21, is an arginine-rich mitochondrial protein that was identified by UV cross-linking to a synthetic radiolabeled gRNA and purified from *Trypanosoma brucei* (Koller *et al.*, 1997). Purification of the poly(U) binding proteins from *Crithidia fasciculata* (Blom *et al.*, 2001) and proteins cross-linking to a model gRNA–mRNA hybrid in the mitochondrial extracts of *Leishmania tarentolae* (Aphasizhev *et al.*, 2003) independently led to identification of gBP21 orthologs in these organisms, gBP29 and p28. Studies in both *Crithidia* and *Leishmania* also identified a second RNA binding protein, which copurified with gBP21 orthologues. The copurifying polypeptides were termed gBP27 and p26, respectively. Under the current nomenclature, the gBP21 orthologs have been renamed as MRP1 and gBP27(p26) protein, designated as MRP2 (Simpson *et al.*, 2003). The two highly positively charged proteins are homologous, although the sequence similarity is relatively low (18% overall identity) (Blom *et al.*, 2001). MRP1 and MRP2 homologs can be identified in several kinetoplastids, but are apparently absent from other species. *In vivo*, MRP1 and MRP2 associate to form an $\alpha_2\beta_2$ heterotetramer (Aphasizhev *et al.*, 2003; Schumacher *et al.*, 2006). The MRP1/2 complex interacts with mitochondrial gRNAs, preedited mRNAs, and edited mRNAs, as shown by coimmunoprecipitation analysis (Allen *et al.*, 1998; Aphasizhev *et al.*, 2003; Blom *et al.*, 2001). Additional indirect evidence of MRP1/2 involvement in editing came from coimmunoprecipitation and affinity purification experiments demonstrating that some fraction of MRP1 is associated with an *in vitro* editing activity, RNA editing TUTase 1 (RET1), and RNA editing ligase (REL) activities (Allen *et al.*, 1998; Aphasizhev *et al.*, 2003; Lambert *et al.*, 1999). Importantly, all interactions between MRP1/2 and the aforementioned activities appeared to be sensitive to RNase treatment, suggesting an RNA-mediated nature of these contacts.

MRP1 null mutants in the bloodstream from *T. brucei* are viable, but incapable of transforming into procyclic forms (Lambert *et al.*, 1999). Editing is essentially unaffected in the bloodstream from MRP1 knockout cells, although the stabilities of some never edited, preedited, and edited RNAs are substantially decreased. However, a more dramatic phenotype is observed in the procyclic form of trypanosomes in which RNAi-mediated knockdown

of MRP1, MRP2, or both leads to a slow growth phenotype that is especially pronounced in cells with reduced MRP2 or MRP1/2 (Vondruskova *et al.*, 2005). Depletion of MRP2 mRNA causes a reduction in both the MRP2 and MRP1 proteins, presumably because the complex formation is required for MRP1 stabilization. In MRP1/2-depleted cells, editing of the apocytochrome *b* (CYb) RNA is dramatically reduced (96% reduction compared to uninduced levels) and RPS12 RNA editing is moderately decreased (51% reduction). The editing of several other RNAs is unaffected. The transcript-specific effects on editing due to MRP1/2 depletion by RNAi remains a mystery, as the complex binds RNA in a nonspecific fashion *in vitro* (Aphasizhev *et al.*, 2003). Editing complexes enriched from MRP1/2 knockdown cells demonstrate normal *in vitro* editing activity, indicating that MRP1/2 depletion has no effect on core editosome activity. In addition to causing decreased editing of specific RNAs, MRP1/2 knockdown also leads to destabilization of several never-edited RNAs. Therefore, the possibility of the MRP1/2 complex being involved in selective stabilization of some fully edited mRNAs may not be excluded.

The mechanism by which MRP1/2 participates in editing is presumed to be through facilitation of gRNA–mRNA annealing. The recombinant MRP1 stimulates annealing of cognate mRNA–gRNA pairs within the anchor duplex region up to 30-fold compared to spontaneous hybridization (Muller *et al.*, 2001). MRP1 can stimulate annealing of both RNA/RNA and RNA/DNA duplexes, but not DNA/DNA duplexes, with an apparent lack of sequence specificity. The recombinant MRP2, native and reconstituted MRP1/2 complexes were also shown to facilitate annealing of complementary RNAs (Aphasizhev *et al.*, 2003). Biochemical studies on MRP1 suggest a matchmaker type of annealing function in which the protein binds to one of the RNA reactants and converts it to an annealing-active conformation (Muller and Goringer, 2002). Reduction of electrostatic repulsion between the two RNAs is also thought to contribute to formation of the RNA–RNA hybrid. The matchmaker model of MRP1/2 annealing was recently validated by the crystal structure of the MRP1/2 bound to gRNA (Schumacher *et al.*, 2006). The structure reveals that within the heterotetramer, MRP1 and MRP2 both adopt the ''whirly'' conformation previously seen in plant transcription factor p24, which binds to a single-stranded DNA in a sequence-specific manner (Desveaux *et al.*, 2002). The MRP1/2 heterotetramer contacts an extended region of the gRNA, in which MRP2 binds stem loop II of the gRNA, the basic region between MRP1 and MPR2 interacts with nucleotides between the anchor sequence and stem loop II, while MRP1 contacts the anchor sequence that is required for interaction with mRNA. In the absence of MRP1/2, the gRNA anchor sequence forms stem loop I of the gRNA (Hermann *et al.*, 1997). Remarkably, MRP1/2 captures the gRNA in a form in which stem loop I is unfolded, thereby maintaining the anchor sequence in a structure competent for pre-mRNA annealing.

1.2. RBP16

RBP16 was initially identified as a gRNA binding protein from *T. brucei* mitochondria and purified based on its affinity for poly(U) (Hayman and Read, 1999). The protein is highly conserved in both *Leishmania* and *T. cruzi*, although its function has not been analyzed in these species. RBP16 is composed entirely of two RNA binding domains. At its N-terminus, the protein contains a cold shock domain (CSD), the most evolutionarily conserved nucleic acid binding domain (Kohno *et al.*, 2003). The CSD is homologous to the cold shock proteins of prokaryotes and comprises a feature of eukaryotic Y-box family proteins. At its C-terminus, RBP16 contains an RG-rich RNA binding domain (Miller and Read, 2003). MALDI-TOF analysis shows that three arginine residues within the RBP16 C-terminal domain can undergo posttranslational methylation, and suggests that differentially methylated forms of the protein exist *in vivo*, which may contribute to the regulation of RBP16 function (Goulah *et al.*, 2006; Pelletier *et al.*, 2001). Native RBP16 purified from *T. brucei* mitochondria binds gRNAs of different sequences *in vitro*, and oligo(U) tail binding is critical for this interaction (Hayman and Read, 1999). Detailed RNA binding studies are consistent with a model in which CSD–oligo(U) tail interactions provide the primary affinity and specificity for binding, while the RG-rich domain helps stabilize the interaction (Miller and Read, 2003; Pelletier *et al.*, 2001). *In vivo* cross-linking studies demonstrate that RBP16 is associated with gRNA within *T. brucei* mitochondria, and coimmunoprecipitation experiments consistently show that 30–40% of the total gRNA pool is associated with RBP16 *in vivo* (Hayman and Read, 1999; Militello *et al.*, 2000; Pelletier and Read, 2003). In addition, substantial amounts of 9S and 12S rRNAs and some mRNAs are also coimmunoprecipitated with RBP16 from mitochondrial extracts (Goulah *et al.*, 2006; Hayman and Read, 1999). Recent studies have shown that the majority of RBP16 is present within mitochondria in two multicomponent complexes of 5S and 11S (Goulah *et al.*, 2006). The 11S complex is a ribonucleoprotein complex containing RBP16, gRNA, and additional proteins whose identities are not currently known. The 5S complex is an entirely proteinaceous subcomponent of the 11S complex, and may represent a primary functional form of RBP16 that interacts with other cellular components to regulate its activity and specificity. Methylation of RBP16 arginine residues 78 and 85 by the TbPRMT1 protein arginine methyltransferase is required for assembly and/or stability of the 5S and 11S RBP16-containing complexes. RBP16 does not appear to be a stable component of the 20S editosome or the MRP1/2 complex, although transient interactions with these complexes cannot be ruled out.

The demonstration that RBP16 associates with gRNAs suggested that this protein might function in RNA editing *in vivo*. Indeed, targeted depletion of RBP16 by RNAi in procyclic form *T. brucei* leads to cessation of growth as well as a very dramatic and specific effect on the editing of CYb mRNA

(Pelletier and Read, 2003). In the absence of RBP16, CYb editing is decreased by 98%, while editing of other RNAs examined is essentially unaffected. Levels of CYb gRNAs do not change under these conditions, suggesting that RBP16 affects gRNA utilization. In addition, RBP16 knockdown alters the abundance of two never-edited RNAs, COI and ND4. The RBP16 RNAi phenotype and pattern of affected mRNAs in procyclic *T. brucei* are strikingly similar to that of the MRP1/2 knockdown, suggesting that these proteins cooperate or participate in the same pathway to facilitate CYb mRNA editing and never edited RNA stabilization. Finally, in support of *in vivo* studies demonstrating a role for RBP16 in RNA editing, recombinant RBP16 significantly stimulates RNA editing *in vitro* (Miller *et al.*, 2006). Editing stimulation is exerted primarily at, or prior to, the step of pre-mRNA cleavage as evidenced by increased accumulation of 3′ cleavage product intermediates in the presence of increasing RBP16. The mechanism by which RBP16 stimulates editing is not currently known. Interestingly, other CSD-containing proteins have been reported to modulate RNA structure and facilitate RNA annealing (Jiang *et al.*, 1997; Skabkin *et al.*, 2001). Therefore, a possible scenario is that RBP16 acts as an RNA chaperone that affects RNA structure and/or annealing and leads to an increase in the functional association of RNA with the editing machinery and, hence, increased pre-mRNA cleavage.

Here we present methods for conventional and affinity purification of the MRP1/2 complex and assessment of RNA–protein interactions in mitochondrial extracts. Expression and purification of the recombinant MRP1 and MRP2 proteins, reconstitution of the MRP1/2 complex, as well as RNA binding and RNA annealing assays are provided. We also describe methods for purification of RBP16 from procyclic and bloodstream form trypanosomes followed by methods for the isolation of the recombinant protein from *Escherichia coli* and an RNA binding assay.

2. Purification and Characterization of Mitochondrial RNA Binding Proteins 1 and 2 from *Leishmania tarentolae*

Purification of RNA binding proteins from mitochondria of trypanosomes historically has been aimed at the identification of factors involved in uridine insertion/deletion RNA editing. The UV-induced cross-linking of mitochondrial extracts with synthetic RNA molecules, which resemble gRNAs or gRNA–mRNA hybrids, was the primary assay used throughout biochemical fractionations. In addition, a hypothetical affinity of editing complexes for gRNA's universal features, such as the 3′ oligo(U) tail, warranted application of affinity chromatography on poly(U) resins. Notwithstanding the obstacles posed by a presence of a large number of

abundant, nonspecific RNA binding proteins in the mitochondrial extracts (Bringaud *et al.*, 1995, 1997), efforts undertaken by the laboratories of Göringer (Koller *et al.*, 1997), Benne (Blom *et al.*, 2001), and Simpson (Aphasizhev *et al.*, 2003) ultimately led to identification of the stable 100-kDa $(\alpha + \beta)_2$ tetramer complex composed of two homologous proteins, MRP1 and 2. This chapter describes the purification of the MRP1/2 complex from *L. tarentolae* by conventional and affinity techniques, methods for analysis of MRP1/2 interactions in the mitochondrial extracts, reconstitution of the 100-kDa complex from recombinant proteins, and *in vitro* assays that may be relevant to the MRPs' function in mitochondrial RNA editing. As an experimental system, *L. tarentolae* remains an attractive option in kinetoplastid research due to its low cost and robust cultivation combined with the availability of protein expression tools.

3. Purification of the MRP1/2 Complex from *Leishmania tarentolae*

3.1. Overview

The choice between conventional and affinity purification procedures depends on the specific experimental objective and available resources. The conventional protocol does not require plasmid construction, *Leishmania* transfections, and clonal selection; it is highly efficient and produces an essentially pure MRP complex. Exposure to high salt conditions effectively removes bound RNAs and disrupts association with RNA editing components, such as RNA Editing TUTase 1 (Chapter 2, this volume) and the 20S editosome (Chapter 1, this volume). Tandem affinity purification (TAP) can be performed under low ionic strength conditions and is the method of choice for analyzing MRP's transient interactions, including RNA-mediated contacts. This affinity method is particularly suitable for protein mass spectrometry and RNA detection. Finally, a mitochondrial fraction enriched by centrifugation in isopycnic gradients is recommended as a starting material for both methods. If growing sufficient volumes of *Leishmania* cultures is not feasible, the affinity techniques described for purification of the RNA editing TUTase 2 (RET2) from whole-cell lysate of *T. brucei* are applicable (Chapter 2, this volume). Contamination with cytoplasmic proteins, mostly tubulins and ribosomal proteins, may be expected.

3.2. Methods

3.2.1. Isolation of the mitochondrial fraction

L. tarentolae cells (UC strain) are grown in 10-liter batches of brain–heart infusion (BHI) medium (Gibco) supplemented with hemin (0.2 mg/liter) to a late logarithmic growth phase ($\sim 120 \times 10^6$ cells/ml). Roller bottles

revolving at 6–8 rpm or a fermentor vessel can be used. The latter allows for yields of up to 200–250 $\times$ 10^6 cells/ml and requires proportional scaling of the protocol. The mitochondrial fraction is enriched by the modified procedure originally described in Braly *et al.* (1974). All steps are performed at 4°. Cells are collected by centrifugation at 5000$\times g$ for 10 min, washed in 1 liter of ice-cold phosphate-buffered saline (pH 7.6), and resuspended in DTE buffer [1 m*M* Tris–HCl (pH 8), 1 m*M* EDTA] at 1.4 $\times$ 10^9/ml to homogeneity. Carryover of PBS and extended incubation in DTE should be avoided. Addition of one tablet of the Complete protease inhibitor cocktail (Roche) is optional. The cell suspension is pressed out of a stainless-steel, or polycarbonate pressure device (Millipore) through a 26-gauge needle at 90 lb/in^2. In order to stabilize the mitochondria, a sucrose solution (60% w/v) is immediately added to the cell lysate at the ratio of 6 volumes of sucrose to 50 volumes of cell suspension. Upon lysis and mitochondria stabilization, the extract is supplemented with 20 m*M* Tris–HCl (pH 7.6) and 3 m*M* $MgCl_2$. DNase I (Sigma) is added to 20 U/ml and the lysate is incubated for 30 min on ice with occasional mixing. The membrane fraction is collected by centrifugation at 15,000$\times g$ for 20 min, resuspended in 35 ml of 76% Renografin [0.25 *M* sucrose, 0.5 m*M* EDTA dissolved in RenoCal 76 (Bracco Diagnostics)]. The membrane fraction (6–7 ml) is layered underneath density gradients using a syringe fitted with an 18-gauge needle and polyethylene tubing. Six density gradients should be prepared in advance in SW28 tubes (Beckman) as follows. For a single isolation, light and heavy solutions are obtained by dissolving 8.55 g of sucrose in 26.3 or 46.2 ml of RenoCal 76, respectively. The concentration of Tris–HCl (pH 7.6) is adjusted to 20 m*M* and EDTA to 0.1 m*M* in a final volume of 100 ml. The heavy solution is portioned into six tubes, 16 ml/tube, and frozen at –20°. The tubes are then filled with 16 ml of light solution and refrozen. Approximately 36–40 h prior to isolation, frozen tubes are placed at 4° . Extended light exposure should be avoided. Gradients are centrifuged for 2 h at 24,000 rpm (76,221$\times g$) in an SW28 rotor (Beckman). The mitochondria-containing band typically sediments in the middle of the tube and should be clearly visible when placed in front of a light source. The band is extracted by puncturing the tube on the side and under the band with an 18-gauge needle fitted to a 10-ml syringe. The fraction is diluted 4- to 5-fold with isotonic STE buffer [20 m*M* Tris–HCl (pH 7.6), 0.25 *M* sucrose, 0.1 m*M* EDTA] and centrifuged for 20 min at 15,000$\times g$. The pellet is gently resuspended in 50 ml of STE, centrifuged again, and frozen at –80°. The expected yield is approximately 5–6 g of pellet (wet weight), or 0.3–0.5 g of total protein.

3.2.2. Conventional chromatography purification of the MRP1/2 complex

For successful conventional purification, an expeditious handling is essential due to the high sensitivity of the MRP proteins to proteolysis. Ideally, all chromatographic steps should be performed on a protein purification

workstation capable of generating precise gradients. The recommended scheme takes 2 days to accomplish. The amount of starting material may vary between 5 and 8 g of wet weight pellet. On day 1, the enriched mitochondria are extracted in 45 ml of 50 m*M* HEPES (pH 7.6), 300 m*M* KCl, 1 m*M* dithiothreitol (DTT), 1 m*M* EDTA, 1 tablet of Complete protease inhibitors (Roche), and 2 m*M* CHAPS with sonication (five pulses, for 15 sec each, 36 W). The temperature of the extract during sonication should not exceed 10°. The efficiency of lysis may be monitored by fluorescent microscopy after staining with DAPI (4′,6-diamidino-2-phenylindole), and the kinetoplastid DNA, which normally stains as a compact and bright cup-like structure, should be completely dispersed. The extract is centrifuged at 150,000×*g* in Ti 60 rotor (Beckman) for 30 min. Ammonium sulfate (AmS) is gradually added to 40% saturation with constant mixing over an approximately 20-min period, and the centrifugation is repeated in the same mode. The supernatant is recovered and the AmS is added to 60% saturation. After 1 h of gentle mixing, the precipitate is collected by centrifugation as before and dissolved in 25 ml of 50 m*M* HEPES (pH 7.6), 10 m*M* $MgCl_2$, 1 m*M* DTT, 5% glycerol. Conductivity is adjusted to ~15 mSi/cm^2 and the fraction is loaded onto a prepacked 5-ml HiTrap heparin column (GE Healthcare) pre-equilibrated with same buffer plus 150 m*M* KCl. Other heparin resins, e.g., Poros Heparin 20 and self-made columns, may be used with equal success. The column is developed with a 100-ml linear gradient from 150 to 1000 m*M* KCl at 1 ml/min and 1-ml fractions are collected. The MRP1/2 complex (~70% pure) typically elutes at ~600 m*M* as the last discrete peak of optical density at 280 nm. The proteins of interest are detected by SDS–gel electrophoresis as two protein bands of similar intensity migrating at 28 and 26 kDa. The sample can be flash-frozen in liquid nitrogen or left on ice overnight.

Preparations exceeding 90% purity may be obtained by hydrophobic interaction chromatography. On the second day, ammonium sulfate powder is gradually added to the combined fractions from the heparin column (~5–8 ml, 200–300 μg of protein) to 1.4 *M* and centrifuged 10 min at 100,000×*g*. The supernatant is loaded at 0.3 ml/min onto a 1-ml Phenyl Resource column (GE Healthcare), which was preequilibrated with 1.5 *M* AmS in 50 m*M* HEPES (pH 7.6), 0.1 m*M* EDTA. The column is developed with a 20-ml gradient from 1.5 *M* AmS to no salt. The complex elutes at ~1 *M* AmS as a single sharp peak and is readily detectable by SDS gel electrophoresis. Some of the later eluting fractions may contain three or four closely migrating bands, which are proteolysis products. Fractions containing MRP1 and 2 in equal amounts (Fig. 4.1A, I) are dialyzed against 50 m*M* HEPES (pH 7.6), 0.1 m*M* EDTA, 200 m*M* KCl, 10% glycerol, concentrated to 1–2 mg/ml with a 10-kDa cut-off Amicon centrifugal device (Millipore) and flash-frozen in liquid nitrogen in small aliquots. Multiple cycles of freezing and defrosting are not recommended. The expected yield is 50–100 μg of protein.

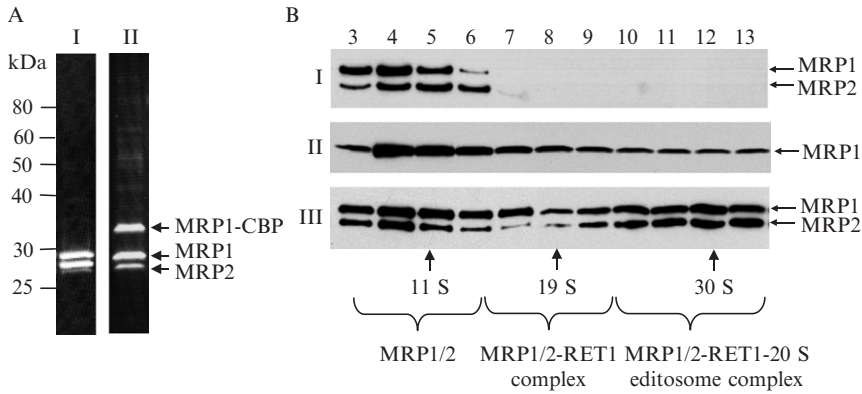

Figure 4.1 Purification and sedimentation analysis of the MRP1/2 complex. (A) The MRP1/2 complex obtained by conventional (I) or affinity (II) purification techniques was separated on 10–20% SDS–polyacrylamide urea gel and stained with Sypro Ruby (Invitrogen). The positions of the MRP proteins and MRP1–calmodulin binding peptide fusion (CBP) are indicated by arrows. (B) Fractions (I) and (II) from (A) and the mitochondrial extract (III) were separated on a 10–30% rate zonal glycerol gradient and analyzed by Western blotting. Polyclonal antibodies against MRP1/2 were used in (I) and (III). (II) was treated with antibodies raised against MRP1. Sedimentation zones of MRP1/2, MRP1/2–RET1, and MRP1/2–RET1–20S editosome particles, as determined by immunoprecipitation analysis of each fraction, are shown by brackets.

3.2.3. Affinity purification of the MRP complex

A construct for expression of the MRP1-TAP C-terminal fusion in *Leishmania* has been described (Aphasizhev *et al.*, 2003) and is available from the author's laboratory. The transfection protocol is adopted from Robinson and Beverley (2003). Briefly, *L. tarentolae* cells (UC strain) are grown to 5–8 $\times$ 10^7 cells/ml in BHI media and harvested by centrifugation at 2000$\times g$ for 10 min. The cell pellet is gently resuspendend in 1/2 of the culture's original volume in the Cytomix electroporation buffer [25 m*M* HEPES (pH 7.6), 120 m*M* KCl, 10 m*M* K_2HPO_4, 5 m*M* $MgCl_2$, 2 m*M* EDTA, 0.15 m*M* $CaCl_2$]. Cells are pelleted again and resuspendend in Cytomix at 1.5 $\times$ 10^8 cells/ml. Typically, 10 μl of plasmid DNA (1 mg/ml) is added into a 4-mm gap electroporation cuvette followed by 0.5 ml of cell suspension. After a 10-min incubation on ice, two pulses (1500 V, 25 μF) are applied in 10-sec intervals with a Gene Pulser apparatus (Bio-Rad). Cells are immediately transferred into 10 ml of BHI media prewarmed to 27°and incubated overnight without antibiotic. Geneticine (G418, Sigma) is added to 50 mg/liter on the next day and to 100 mg/liter the day after. After the culture has been growing for a total of 3 days, 3 ml is centrifuged at 2000$\times g$ for 10 min, resuspendend in 100 μl of residual media, and plated on BHI-agar. Plates are sealed with Parafilm and incubated at 27°

until 2- to 3-mm colonies appear (7–10 days). To prepare the plates, 37 g of BHI, 0.8 g of folic acid, and 8 g of agar are autoclaved in 1 liter of water for 15 min. Heat-inactivated serum (100 ml), hemin (5 g/liter), and 200 mg of G418 are added at ~50° prior to preparing plates. Plates are left to dry for 15–20 h at room temperature with covers on.

Individual colonies appear in 7–10 days. Colonies of 2–3 mm in diameter are transferred into 10-ml cultures and grown in the presence of 100 mg/liter of G418 to late-log phase (~100×10^6 cells/ml). One milliliter of culture is collected, washed with 1 ml of PBS, boiled in 100 μl of 1× SDS loading buffer, and centrifuged for 30 min at 15,000×*g* at room temperature. The supernatant equivalent to 10^7 cells is loaded on SDS–polyacrylamide gel. Blotting analysis with peroxide–antiperoxidase (PAP) reagent (Sigma), which detects the protein A moiety of the TAP cassette, can be used to test protein expression. For isolation, a 10-liter culture is grown in the presence of 100 mg/liter of G418 and the mitochondrial fraction is isolated as described above. The TAP protocol is adapted from Puig *et al.* (2001) with modifications. The mitochondrial fraction is adjusted to 12 ml with TMK buffer (20 m*M* Tris–HCl, pH 7.6, 60 m*M* KCl, 10 m*M* $MgCl_2$) plus 0.4% of NP40. One tablet of Complete protease inhibitors (Roche) is added, the extract is resuspended, gently sonicated (three pulses for 10 sec, 12 W), and incubated on ice for 15 min. After centrifugation at 200,000×*g* for 15 min, the pellet is reextracted with sonication (three pulses for 30 sec, 12 W) in 12 ml of the TMK buffer without detergent and centrifugation is repeated. Supernatants from both extractions are pooled, filtered through 0.45-μm low-protein binding filter, and incubated with 0.3 ml (packed volume) of IgG Sepharose FF (GE Healthcare) for 1 h in a 50-ml conical tube. Suspension is then transferred to a disposable 1-ml column (Pierce) and the extract is reloaded three times. The column is washed sequentially with 50 ml of TMK buffer plus 0.1% of NP40, and 10 ml of TMK, 0.1% of NP40, 1 m*M* DTT. An attempt should be made to resuspend the resin with each wash. TMK buffer plus 0.1% of NP40 and 1 m*M* DTT (1.5 ml) with 200 U of TEV proteinase (Invitrogen) is added to the column, which is sealed and incubated overnight with constant mixing. The IgG resin is drained into a 15-ml conical tube containing 0.3 ml (packed volume) of calmodulin agarose (Stratagene) prewashed with CB buffer (20 m*M* Tris–HCl, pH 7.6, 60 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* imidazole, 10 m*M* 2-mercaptoethanol, 2 m*M* $CaCl_2$, 0.1% NP40). To improve recovery, the IgG column is rinsed with 4.5 ml of CB buffer and the rinse is collected into the tube with calmodulin resin. Calcium chloride is added (5 μl of 1 *M* stock) and the eluted material (~6 ml) is incubated with calmodulin resin for 1 h, transferred to a disposable 1 ml column (Pierce), reloaded three times, and washed with 50 ml of CB buffer. Elution is performed with 5 ml of 20 m*M* Tris–HCl (pH 7.6), 60 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* EGTA, 2 m*M* CHAPS. Fractions of 0.5 ml are collected and, if necessary, are concentrated

with Slide-A-Lyzer solution (Pierce) to 300 μl. A typical protein profile is shown in Fig. 4.1A, II. The expected yield is ~50 μg of protein.

4. Analysis of MRP1/2 Interactions in Mitochondrial Extracts

4.1. Overview

Affinity isolation of the MRP1/2 complex provides an excellent starting point for assessing its apparently numerous interactions in the mitochondrial extract (Aphasizhev *et al.*, 2003) that may be occurring, in part, due to MRP's high affinity for RNA and lack of sequence specificity (Schumacher *et al.*, 2006). The high degree of purity and mild isolation conditions allow for immunochemical detection of interacting partners that are present in sub-stoichiometric amounts and bind to MRP via an RNA component. Rapid isolation also preserves bound gRNAs that can be isolated from the affinity-purified material and selectively radiolabeled. In addition, expression of the TAP-tagged proteins followed by pull-down with IgG-Sepharose provides a highly specific generic alternative to immunoprecipitation analysis. For example, the mitochondrial extract may be subjected to gradient fractionation and IgG-Sepharose used for pull-downs in each fraction. RNA ligase adenylation and *in vitro* editing activity are readily detected in complexes bound to the beads, which may be further analyzed by SDS–gel electrophoresis and Western blotting.

4.2. Methods

4.2.1. Two-dimensional size fractionation of the affinity purified MRP1/2-containing particles

The concentrated calmodulin fraction after affinity purification is loaded on a 10–30% glycerol gradient, which allows for effective separation in the 5–35S range. The 10% and 30% (v/v) glycerol stocks are prepared in 25 m*M* HEPES (pH 7.6), 10 m*M* $MgCl_2$, 60 m*M* KCl, 1 m*M* CHAPS. Rate zonal sedimentation is performed in an SW41 rotor (Beckmann) for 20 h at 30,000 rpm (111,000×*g*). Fractions of 700 μl are collected. Each fraction may be assigned an S value by calibrating the gradient with catalase (11S), thyroglobulin (19S), and small ribosomal subunit from *Escherichia coli* (30S). Excellent resolution and reproducible results can be obtained with gradient maker/fractionator from Biocomp (Fredericton, NB, Canada) following the manufacturer's instructions. Whereas TAP affinity-purified MRP1/2 sediments over a broad range (10–35S), the MRP1/2 complex purified by combination of heparin and hydrophobic interaction chromatography sediments as a discrete peak at 10–15S (Fig. 4.1B). The fraction of MRP1/2 associated with the 20S

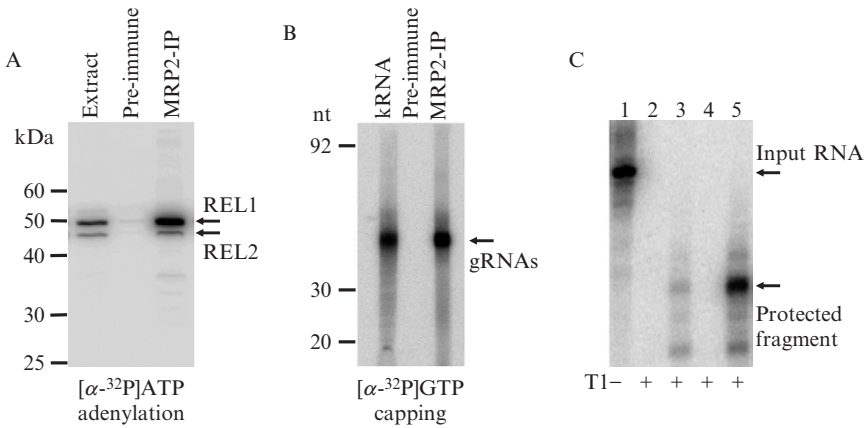

Figure 4.2 MRP1/2 interactions and RNA annealing activity. (A) Self-adenylation of RNA editing ligases, REL1 and REL2, may be used as a sensitive method to detect the 20S editosome in MRP1/2 complexes. Adenylation was performed in mitochondrial extract and material coimmunoprecipitated with preimmune serum and anti-MRP2 polyclonal antibody. (B) Guide RNAs are selectively labeled with vaccinia virus capping enzyme in the presence of [α-^{32}P]GTP. (C) RNA annealing assay. (1) Input RNA; (2) noncomplementary RNA added; (3) self-annealing in the absence of protein; (4) same polarity strand added; (5) annealing in the presence of the MRP1/2 complex.

editosome sediments in a 25–35S region. Two components of the 20S editosome, RNA Editing Ligases 1 and 2, may be radioactively labeled by self-adenylation with [α-^{32}P]ATP in the absence of RNA substrate, which provides a sensitive method for editosome detection (Fig. 4.2A).

Adenylation is performed for each fraction by mixing 0.5 μl of [α-^{32}P]ATP (6000 Ci/mmol) with 10 μl of the gradient fraction and incubating the reaction for 30 min at 27°. The adenylated complexes may be further analyzed under denaturing or native conditions using the 8–16% gradient polyacrylamide precast Tris–glycine gels (Invitrogen). For SDS gel fractionation, 10 μl of the loading buffer (0.25 *M* Tris–HCl, pH 6.8, 2% SDS, 100 m*M* DTT, 0.05% bromophenol blue, no glycerol) is added to the adenylation reaction followed by standard SDS electrophoresis. The proteins are transferred to a nitrocellulose membrane by semidry or tank electroblotting methods. Upon exposure to a phosphor storage screen, RNA ligases are visualized as two closely migrating bands of ~50 and 48 kDa (Fig. 4.2A). For separation under native conditions, 10 μl of 20 m*M* Tris–HCL (pH 7.6), with 0.01% of bromophenol blue is added to the adenylation reaction. Native Tris–glycine buffer (pH 8.2) (Invitrogen) is used for the electrophoresis at 4° (~16 h at 45 V). Native high-molecular-mass protein standards (GE Healthcare) may be run alongside to determine the apparent molecular mass of the adenylated complexes. A refrigerated chamber and a power supply capable of maintaining low current (<2 mA)

are required. The gel is soaked in transfer buffer (10 m*M* Tris, 100 m*M* glycine, 10% methanol) for 30 min in the cold room and subjected to tank electrotransfer at 40 V for 8–10 h at 4°. In order to visualize molecular mass standards, the corresponding lane is cut off from membrane and stained with Ponceau S (Pierce). The membrane may be exposed to a phosphor storage screen to visualize the 20S editosome typically migrating at 1.2–1.6 MDa, or treated with antibodies. Because of MPRs' high isoelectric point, under native condition these proteins enter the gel only if associated with RNA or negatively charged partners.

4.2.2. Pull-down of TAP-tagged MRP1/2 complexes from gradient fractions

In this approach, a mitochondrial fraction is isolated from *L. tarentolae* cells that express TAP-tagged proteins. Rather than carrying out the full-scale TAP purification, the mitochondrial extract is fractionated on a 10–30% glycerol gradient followed by IgG pull down in each fraction. This protocol takes advantage of gradient fractionation as a tool to assess the sedimentation values for complexes that had not been affected by TAP purification, which usually removes weakly associated components. Another consideration that may contribute to the choice of running the gradient fractionation prior to TAP purification is the amount of protein that is being handled. If the TAP procedure produces less than 10 μg of protein, significant losses may be expected during subsequent gradient fractionation due to nonspecific binding.

The mitochondrial pellet (~200 mg wet weight, ~15 mg of total protein) is resuspendend in 0.5 ml of 50 m*M* HEPES (pH 7.6), 60 m*M* KCl, 10 m*M* $MgCl_2$, 1/50 part of Complete protease inhibitor tablet, and 0.4% of NP40. All concentrations are final, adjusted for the mitochondrial pellet's volume. The extract is gently sonicated using a micro tip (three pulses, 5 sec, 6 W) and incubated on ice for 15 min. The membrane fraction is removed by centrifugation for 10 min at 200,000×*g* in a Beckman TL-100 ultracentrifuge and the soluble fraction is recovered. Gradient fractionation is performed using 200 μl of cleared extract. Each fraction may be analyzed in the second dimension by SDS–gel electrophoresis in order to provide references to the sedimentation position of the 20S core editosome, or other complexes of interest. For IgG pull-downs, 5 μl of bead suspension (settled volume) and 20 μl of 50 mg/ml of acetylated bovine serum albumin are added to each fraction and binding is performed for 1 h with constant rocking. The beads are collected by centrifugation at 1000×*g* for 1 min and washed three times for 15 min with 1 ml of buffer. Depending on the experimental objective, buffer composition may vary in KCl concentration from 50 to 500 m*M* of salt, the presence of Mg ions or EDTA, and from 0.1 to 0.5% of nonionic detergent without affecting IgG–protein A binding. Prior to the adenylation reaction, an extra wash with low salt buffer [50 m*M*

HEPES (pH 7.6), 50 m*M* KCl, 10 m*M* $MgCl_2$] is required. The reaction is initiated by adding 1 μCi of [α-^{32}P]ATP to pelleted beads and incubating at 27° for 30 min. The final wash is performed with low salt buffer and 10 μl of 2× SDS loading solution is added to the beads. Bound proteins are eluted from beads by heating to 95° for 2 min and analyzed on SDS–polyacrylamide gel followed by blotting onto a nitrocellulose membrane. If proteomic identification of the coprecipitating components becomes a necessity, the selected gradient fractions may be subjected to a full-scale TAP procedure following the above-described protocol, but downscaled 2-fold in terms of the amount of resin and volumes of washes. No prior treatment is required for gradient fractions.

4.2.3. Analysis of MRP1/2-associated proteins and RNAs

In order to identify proteins copurifying with the MRP1/2 complex through the TAP procedure, the polypeptides are recovered by binding to Strataclean resin (Stratagene) prior to SDS–gel electrophoresis. Typically, 10 μl of bead suspension is added to 1 ml of gradient fraction and incubated at room temperature for 30 min with constant mixing. The suspension is centrifuged at 3000×*g* for 5 min, the supernatant is removed, and 10 μl of 2× SDS loading buffer is added. The sample is boiled for 3 min and the entire mixture is transferred onto a precast gradient SDS–PAGE. Depending on the available mass spectrometry resources, the gel is stained with colloidal Coomassie Blue or Sypro Ruby for further analysis.

Guide RNAs are detected in gradient fractions after phenol/chloroform extraction and ethanol precipitation in the presence of glycogen (20 μg/ml) and 0.5 *M* sodium acetate, pH 5.0. Because gRNAs are primary transcripts bearing diphosphates or triphosphates at the 5′ end (Blum *et al.*, 1990), they are readily detected by incubation with vaccinia virus capping enzyme, a guanylyltransferase (Ambion), in the presence of [α-^{32}P]GTP. Upon separation on a 15% acrylamide/8 *M* urea gel, the radioactively labeled gRNAs migrate as a group of bands corresponding to ~60–65 nucleotides (Fig. 4.2B).

5. Reconstitution of the MRP1/2 Complex from Recombinant Proteins

5.1. Overview

The MRP1 and 2 proteins form a stable complex in mitochondria, which prevents purification of individual proteins for biochemical studies from the native source. Furthermore, it has been shown that the RNA interference-mediated depletion of the MRP2 subunit in *T. brucei* leads to a decrease in abundance of MRP1 without affecting the respective mRNA

(Vondruskova *et al.*, 2005). Therefore, formation of the complex is likely to be essential for stability of these proteins *in vivo*. At the functional level, a certain degree of redundancy may be anticipated, as MRP2 or dual MRP1/2 RNAi knockouts, but not the MRP1 depletion, have been found to inhibit the parasite's growth. A possible explanation for this phenomenon may come from MRP2's ability to form dimers (Aphasizhev *et al.*, 2003), which are apparently the functional units in MRP1/2 complexs (Schumacher *et al.*, 2006). The methods described below allow purification of individual MRP proteins for biochemical studies and reconstitution of the complex. An alternative strategy for reconstitution of the MPP1/2 complex via coexpression of both subunits in *E. coli* has been reported by others (Schumacher *et al.*, 2006).

5.2. Methods

5.2.1. Purification of recombinant MRP1 and 2 from *E. coli*

The experimentally determined mitochondrial localization signals for *L. tarentolae* proteins, position −46 for MRP1 and position −30 for MRP2, were omitted during construction of pET15b-based (Novagen) *E. coli* expression vectors (Aphasizhev *et al.*, 2003). MRP1 forms inclusion bodies upon expression in bacteria and should be purified under denaturing conditions and refolded. The 2 liters of *E. coli* culture, strain BL21(DE3) CodonPlus RIL (Stratagene), are grown in 2YT media in the presence of 100 mg/liter of ampicillin at 37°. Expression is induced at ~0.6 OD_{600} by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 m*M* and cultivation is continued for 3 h. The bacterial pellet is resuspendend in 40 ml of 6 *M* guanidine hydrochloride, 50 m*M* sodium phosphate (pH 7.5), and subjected to sonication (five pulses, 36 W for 30 sec). The extract is centrifuged for 30 min at 200,000×*g*, filtered through a 0.45-μm low-protein binding filter, and loaded onto a 2-ml column with Talon metal affinity resin (Clontech). Followed by washing of the column with 50 ml of 8 *M* urea in 50 m*M* sodium phosphate (pH 7.5), the protein is refolded while still attached to the Talon resin. The 40 ml reverse gradient of urea from 8 to 0 *M* in 20 m*M* sodium phosphate (pH 7.5) 300 m*M* NaCl is run at 20° for 20 h at 2 ml/h. The protein is eluted with 200 m*M* imidazole, and further purified on a 1-ml HiTrap heparin column using the procedure described above for the MRP1/2 complex, but downscaled 5-fold. The expected yield is 1–2 mg of protein.

MRP2 is partially soluble in *E. coli* extracts if the bacterial culture is induced and grown at 20°. The protein is purified at 4° under native conditions by Talon metal affinity and heparin chromatography. The cell pellet is resuspended in 50 ml of 50 m*M* HEPES (pH 8.0), 50 m*M* NaCl and passed through a French pressure cell at 12,000 psi. The sodium chloride is adjusted to 300 m*M* and the extract is centrifuged at 200,000×*g* for 30 min.

The supernatant is loaded into a 2-ml Talon column, which is washed sequentially with 50 ml of 50 m*M* HEPES (pH 8.0), 300 m*M* NaCl, and 20 ml of the same buffer with 10 m*M* imidazole. The MRP2 is step-eluted with 50 m*M* HEPES (pH 8.0), 300 m*M* NaCl, 200 m*M* imidazole, and is typically more than 95% pure. Additional purification by heparin chromatography is required for reconstitution, but not RNA activity assays (below). The fraction obtained from the Talon column is diluted 4-fold with 50 m*M* HEPES (pH 7.6), 10 m*M* $MgCl_2$, 1 m*M* DTT, 5% glycerol, and loaded on a 1-ml HiTrap (GE Healthcare) heparin column. A 40 ml linear gradient (0.1–1 *M*) of sodium chloride is applied and 1-ml fractions are collected. A single protein peak containing 3–5 mg of protein is eluted at ~400 m*M* NaCl.

5.2.2. Reconstitution of the MRP1/2 complex

Purified recombinant MRP1 and 2 are mixed in 200 μl at a 1:2 molar ratio (final protein concentration of 2–4 mg/ml) and dialyzed against 20 m*M* sodium phosphate, 200 m*M* NaCl for 1 h at 25°. The excess of the MRP2 protein is necessary for *in vitro* complex formation, likely due to its ability to form a homodimer. After a 10-min centrifugation at 15,000×*g*, the sample is loaded on a Superose 12 column and eluted in the same buffer at 0.1 ml/min. Fractions of 0.2 ml are collected, separated on 12% acrylamide–SDS gel, and stained with Sypro Ruby (Invitrogen). Fractions eluting at ~11.5 ml that contain protein bands of equal intensity are pooled and concentrated, if necessary, with Slide-A-Lyzer solution (Pierce). The expected yield is 200–300 μg. The protein sample is supplemented with glycerol to 10%, flash-frozen in liquid nitrogen, and stored at –80°.

6. RNA Binding and RNA Annealing Activities of the MRP1/2 Complex

6.1. Overview

Several studies have addressed the specificity and affinity of RNA binding by the MRP1 (gBP21) protein (Koller *et al.*, 1997; Lambert *et al.*, 1999; Muller *et al.*, 2001) and MRP1/2 complex from *T. brucei* (Schumacher *et al.*, 2006) and the MRP1/2 complex from *L. tarentolae* (Aphasizhev *et al.*, 2003). Excellent agreement between results obtained for the complexes (Aphasizhev *et al.*, 2003; Schumacher *et al.*, 2006), combined with structural data (Schumacher *et al.*, 2006), makes it possible to conclude that MRP1/2–RNA interactions are sequence independent and driven by electrostatic interactions of positively charged residues with the phosphodiester backbone. Importantly, single-stranded and double-stranded RNAs are bound by the MRP1/2 complex with high affinity: apparent dissociation constants for both types of molecules fall into the 2–10 n*M* range. An *in vitro* RNA

annealing activity, which was reported for the MRP1 (Muller *et al.*, 2001), MRP2, and MRP1/2 complex (Aphasizhev *et al.*, 2003), remains the most significant, albeit circumstantial, indication of MRP's function in RNA editing. The capacity of RNA binding proteins to promote the formation of a double-stranded RNA from complimentary single-stranded molecules is well documented, but may be occurring by a variety of mechanisms (Rajkowitsch *et al.*, 2005). Although folding of all guide RNAs into a similar secondary and tertiary structure remains to be established, the structure of the MRP1/2 complex with a fragment of the gND7–506 (Schumacher *et al.*, 2006) suggests that the interaction of MRP1/2–gRNA–mRNA presents the gRNA's ''anchor'' region in the unfolded state with bases exposed to the solvent and suitable for hybridization with preedited mRNA.

6.2. Methods

6.2.1. RNA binding

The affinity of RNA binding is measured as an equilibrium dissociation constant, K_d, by fitting nitrocellulose filter binding data from three independent experiments into an equilibrium binding model. The radioactively labeled guide RNA of interest is synthesized by run-off transcription from a linear DNA template using T7 RNA polymerase (Invitrogen). Typically, 1 μg of linearized plasmid DNA is incubated in the supplied buffer with 50 U of RNA polymerase in the presence of 1 m*M* GTP, CTP, and UTP and 50 μM ATP plus 50 μCi of [α-^{32}P]ATP. After a 2-h reaction, 2 U of RNase-free DNase is added and incubation is continued for 30 min. RNA is purified by 15% acrylamide/urea gel electrophoresis, eluted into 0.1 *M* sodium acetate, pH 5.0, 0.1% SDS, 1 m*M* EDTA, and ethanol precipitated. The RNA concentration is determined spectrophotometrically. Double-stranded RNA is assembled from complementary molecules, which are independently synthesized and purified. Prior to binding assays, RNAs are mixed at 10 μM each in a buffer with 10 m*M* HEPES (pH 7.6), 50 m*M* KCl, and 0.1 m*M* EDTA, heated to 90° for 2 min, and cooled to room temperature over a 30-min time period. Individual RNAs are subjected to the same folding procedure. RNA–protein binding is carried out in a 10-μl reaction mixture containing 10 m*M* HEPES (pH 7.6), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.5 m*M* DTT, 6% glycerol, and 0.05 mg/ml of bovine serum albumin (BSA) at 27° for 30 min. The entire reaction is spotted on a 33-mm (0.22-μm) nitrocellulose filter, which is held in a vacuum-driven manifold (Millipore). The filter must be prewetted and washed with binding buffer lacking BSA. Washing volumes required for a low background need to be determined empirically, but typically ~15 ml/filter is sufficient. Filters are dried and exposed to a phosphor storage screen followed by scanning and quantitation. Alternatively, scintillation counting may be used.

With either detection method, the amount of bound RNA is determined by calculating the RNA's specific activity. To obtain this value, the reaction mix is spotted on a separate filter and dried without washing. For apparent K_d determinations, the RNA concentration is kept constant at 5 n*M* and increasing amounts of protein are used. Recommended ranges are 0.1–1000 n*M* for the MRP complex purified from *L. tarentolae*, 0.1–2000 n*M* for the recombinant MRP1, and 1–4000 n*M* for the recombinant MRP2. The percentage of active protein in each preparation must be determined in reciprocal experiments in order to adjust the concentration values used for K_d calculations. This is done with 2 n*M* protein and increasing concentrations of RNA from 0.01 to 2 μM. The MRP1/2 complex purified from *L. tarentolae* by conventional chromatography is typically 60% active, and the recombinant LtMRP1 and LtMRP2 are 25–35% active, depending on the preparation. Prizm3 (GraphPad) or SigmaPlot 8 software packages are suitable for K_d calculation by nonlinear regression analysis.

6.2.2. RNA annealing

Stimulation of annealing of two complementary RNAs by RNA binding proteins may be measured quantitatively by monitoring an increase in the resistance of guanylyl residues to digestion by RNase T1, which cleaves a single-stranded RNA 3′ of these nucleosides. Upon strand hybridization, Gs in the double-stranded region become inaccessible while single-stranded RNA is rapidly digested. Because the RNA annealing activity is nucleotide sequence-independent, substrates of arbitrary sequence or derived from trypanosomal gRNA–mRNA pairs may be utilized. The choice of a particular strand to be synthesized as radiolabeled RNA does not appear to affect the results. Importantly, the radioactively labeled strand should have noncomplimentary termini containing at least one guanosine residue so that the protected fragment can be electrophoretically separated from the input RNA. Fragments of 30–60 bases are synthesized by run-off transcription as for RNA binding assays, except that for the synthesis of nonlabeled RNA concentrations all NTPs are maintained at 2 m*M*. To generate the fully annealed control, 1 pmol of radioactively labeled strand is incubated with 2 pmol of nonlabeled RNAs in a 10-μl reaction at 90° for 2 min in 20 m*M* HEPES (pH 7.5) 50 m*M* KCl, 0.1 m*M* EDTA and cooled to 20° over a 30-min time interval.

The RNA annealing reactions contain 0.5 n*M* labeled mRNA and 0.5–50 n*M* nonradioactive RNA, and the protein concentrations vary from 0 to 5 μM. Routinely, the protein is preincubated with radiolabeled RNA in 10 μl of annealing buffer [20 m*M* HEPES (pH 7.6), 50 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* DTT] at 27° for 10 min. The nonlabeled RNA is treated identically, and the reaction is initiated by combining the two mixtures. After incubation for 0.5, 1, 2, 4, and 10 min, the reaction is stopped by the addition of 1 μl T1 RNase (18 U/μl) and incubated for

10 min at 27°, followed by phenol/chloroform extraction and ethanol precipitation. Products are analyzed on 15% polyacrylamide/urea gels and exposed to a phosphor storage plate (Fig. 4.2C). The time dependence of the signal intensity of the protected fragment at a given complementary RNA concentration is fitted into a single-phase exponential association model to obtain the first-order rate constant.

7. Purification of RBP16 from Procyclic Form *Trypansoma brucei*

Native RBP16 is posttranslationally methylated on arginine residues, and different methylated derivatives of the protein are apparently present in procyclic forms (Pelletier *et al.*, 2001). Methylation modulates some aspects of the RBP16 function *in vivo* (Goulah *et al.*, 2006). Since recombinant RBP16 is unmodified, comparison of native and recombinant proteins will be useful for analyzing the direct effects of methylation on RBP16 interaction with specific RNAs and proteins. It is not yet known whether the methylation state of RBP16 in the bloodstream forms differs from that in procyclic forms, but comparative analysis of RBP16 isolated from both life-cycle stages may reveal different forms of the proteins with different properties.

7.1. Cells and growth medium

Procyclic form *T. brucei* clone IsTAR1 stock EATRO 164 is routinely used in our laboratory. Cells are grown in SDM-79 media supplemented with fetal calf serum (10% final) (Brun and Schonenberger, 1979). Indicated quantities are per liter of media: 7 g of MEM F-14 (Invitrogen), 2 g Medium 199 TC 45 (Sigma-Aldrich), 1 g glucose, 8 g HEPES, 5 g 3-morpholinopropanesulfonic acid (MOPS), 2 g sodium bicarbonate, 200 mg L-alanine, 100 mg L-arginine, 300 mg L-glutamine, 70 mg L-methionine, 80 mg L-phenylalanine, 600 mg L-proline, 60 mg L-serine, 160 mg L-taurine, 350 mg L-threonine, 100 mg L-tyrosine, 10 mg adenosine, 10 mg guanosine, 50 mg glucosamine–HCl, 8 ml MEM amino acids (50×) without glutamine (Invitrogen), 6 ml MEM nonessential amino acids (100×) (Invitrogen), 9.08 ml sodium pyruvate (100×) (Invitrogen), 10 ml vitamins (100×) (Invitrogen), and 5 ml glycerol. Prior to sterile filtration on 0.22-μm filters, the pH of the media is adjusted to 7.3, and the volume is completed to 900 ml with double deionized water. Following filtration, 3 ml of 2.5 mg/ml hemin (in 0.05 *M* NaOH), 100 ml of fetal calf serum (denatured by heating at 55° for 30 min), and 10 ml of penicillin–streptomycin (10,000 units each/ml) (Invitrogen) are sterilely added. Cells are grown in this media at 27° with gentle agitation (100 rpm).

7.2. Purification of mitochondrial vesicles

The following buffers are used during the mitochondria purification procedure (Harris *et al.*, 1990): (1) DTE: 1 m*M* Tris (pH 8.0), 1 m*M* EDTA (pH 8.0); (2) SBG: 20 m*M* glucose, 150 m*M* NaCl, 20 m*M* sodium phosphate buffer (pH 7.9); (3) STM: 250 m*M* sucrose, 20 m*M* Tris (pH 8.0), 2 m*M* $MgCl_2$; and (4) STE: 250 m*M* sucrose, 20 m*M* Tris (pH 8.0), 2 m*M* EDTA (pH 8.0). For the lysis of the purified mitochondrial vesicles, we use a buffer composed of 25 m*M* Tris (pH 8.0), 15 m*M* MgOAc, and 50 m*M* KCl, which we designate Buffer A. All buffers are filter-sterilized.

Routinely, 12 liters of cells are grown up in SDM-79 media to a density of 1–3 $\times$ 10^7 cells/ml. We also have isolated mitochondria from as little as 2 liters and as much as 20 liters of cells at a time (Ryan and Read, 2005). Cells are counted using a hemacytometer and pelleted at 6000$\times g$ for 10 min at 4°. The pellets are then resuspended in 200–300 ml of SBG and combined in one centrifuge bottle. Following a 10-min centrifugation at 6000$\times g$ at 4°, the SBG is carefully removed and the pellet is resuspended in DTE at 1.2 $\times$ 10^9 cells/ml. The suspension is then dounced five times using a Kontes tissue grinder pestle SC40 (catalog no. 885302–0040) and a Wheaton Potter-Elvehjem tissue grinder (catalog no. 358049). The cells are then lysed by passage through a 26-gauge needle into a 60% sucrose solution (filter sterilized). We use a volume of 60% sucrose corresponding to one-seventh of the volume of DTE from the previous step. The membrane fraction is then pelleted by centrifugation at 15,800$\times g$ for 10 min at 4° and resuspended in a volume of STM buffer equal to one-sixth of the original cell lysate plus sucrose. We then add 3 m*M* $MgCl_2$ (final), 0.3 m*M* $CaCl_2$ (final), and 117 units of RNase-free DNase I (Invitrogen) per ml. Following a 30–60 min incubation on ice, an equal volume of STE is added and the crude mitochondrial fraction is pelleted at 15,800$\times g$ for 10 min at 4°. The pellet is resuspended in 75% sterile Percoll (prepared in STE buffer), dispersed as much as possible, and loaded at the bottom of a linear 32 ml 20–35% Percoll gradient (prepared in STE). For every 2 liters of cells, we use 4 ml of 75% Percoll, and this suspension is layered under one gradient. For loading, we use a 10-ml syringe and a 14-gauge, 15-cm-long needle. The gradients are formed in Beckman ultraclear centrifuge tubes (25 $\times$ 89 mm, catalog no. 344058) using a Hoefer SG50 gradient maker. The tubes are balanced with 20% Percoll (in STE) and centrifuged at 103,900$\times g$ for 50 min at 4° in a Beckman SW-28 rotor. The mitochondrial vesicles are present in a broad smear, which is located between two more distinct bands, one near the top of the gradient and a slightly less prominent one near the bottom of the gradient. An 18-gauge, 1.5-in. needle connected to a 30-ml syringe is used to puncture the centrifuge tube just below the smear containing mitochondrial vesicles. The vesicles are collected in the syringe and transferred to a 40-ml Oakridge tube. The tube

is then filled with STE and centrifuged at 32,530×*g* for 15 min at 4° using a slow deceleration setting. About half of the supernatant is discarded, typically using a 10-ml pipette. This step must be performed carefully, so as not to disrupt the initial pellets, which are very soft. The STE washes are repeated three additional times. As the pellets become more solid with every wash, more of the supernatant can be discarding every time until after the final wash, when all the supernatant can be discarded. Finally, the mitochondria are resuspended in 50% glycerol in STE (1 part 100% glycerol, 1 part 2× STE) at a protein concentration of about 1 mg/ml. Typically, 1×10^{10} cells equals 5 mg of mitochondrial protein. Immediately snap freeze this in liquid nitrogen and store at −80°. This procedure usually results in 40–50 1-ml aliquots from 12 liters of culture at $1–3 \times 10^7$ cells/ml.

7.3. Mitochondrial vesicle lysis

Purified mitochondrial vesicles are thawed and centrifuged at 13,000 rpm in a Biofuge centrifuge (Heraeus Instruments) for 15 min at 4°. The pellet is resuspended in Buffer A [containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml pestatin A to minimize proteolysis] at a concentration of 1×10^{11} cells/ml. Vesicles are lysed by the addition of NP-40 to 0.2% (final) and incubation on ice for 5 min. The insoluble material is then cleared from mitochondrial extracts by centrifugation at 13,000×*g* for 10 min at 4°. After addition of $CaCl_2$ to 1 m*M*, the cleared extract is incubated with micrococcal nuclease (Sigma-Aldrich) (100 units/1×10^{11} cells) at 27° for 15 min. Micrococcal nuclease is then inhibited by the addition of ethylene glycol bis(2-aminoethyl ether)-*N,N, N′N′*-tetraacetic acid (EGTA) to 5 m*M*.

7.4. Poly(U) sepharose chromatography

Tris-based buffers containing magnesium chloride, EGTA, and potassium chloride are used. A buffer containing 25 m*M* Tris (pH 8.0), 1.5 m*M* magnesium acetate, 5 m*M* EGTA, 50 m*M* KCl, and 10% glycerol is used as column equilibration and wash buffer [''Poly(U)50 Buffer'']. For the elution of RBP16, the same buffer, except that it contains either 300 m*M* or 800 m*M* KCl, is used to create a linear gradient ranging from 300 to 800 m*M* KCl. To prevent proteolysis, PMSF (1 m*M* final), leupeptin (1 μg/ml final), and pestatin A (1 μg/ml final) are added to all buffers.

Typically, 1-ml of poly(U) Sepharose (GE Healthcare) is sufficient to purify RBP16 from a mitochondrial fraction equivalent to $1–4 \times 10^{11}$ cells. A 1-ml poly(U) Sepharose column is equilibrated with 20 column volumes of wash buffer. The cleared, micrococcal nuclease-treated mitochondrial extract is then loaded onto the column and allowed to flow by gravity at a rate of approximately 1 ml/min. The flow rate is usually controlled

manually using a two-way stopcock. The column is washed with 10 column volumes of wash buffer containing 300 m*M* KCl at a flow rate of approximately 1 ml/min. Bound proteins are eluted with 10 column volumes linear gradient from 300 to 800 m*M* of KCl. To generate the salt gradient, we use a Hoefer SG15 gradient maker connected to a peristaltic pump. Five milliliters of wash buffer containing 800 m*M* KCl is poured into the chamber the farthest from the outlet connector. Five milliliters of wash buffer containing 300 m*M* KCl is then poured into the chamber closest to the outlet connector (the mixing chamber). A small magnetic stir bar is placed in the mixing chamber. The magnetic stirrer is started and the delivery stopcock is then opened. Next, the connector stopcock is opened and the pump is started simultaneously. Twenty 0.5-ml fractions are collected in 1.5-ml Eppendorf tubes. Fractions are analyzed by SDS–PAGE on a 15% acrylamide gel Coommassie staining and and by UV cross-linking to synthetic gRNA gA6[14] internally labeled with [α-^{32}P]UTP. Typically, the bulk of RBP16 is eluted in fractions 10–15 (Fig. 4.3A and B).

7.5. Poly(A) sepharose chromatography

RBP16 can be further purified by chromatography on a poly(A) Sepharose column. The protein is recovered in the flowthrough of the poly(A) Sepharose column, as RBP16 does not bind poly(A). Fractions from the poly(U) Sepharose column that contain RBP16 are pooled and dialyzed twice against 1 liter of wash buffer containing 150 m*M* KCl at 4° for at least 5 h. The dialyzed sample is then applied to a 0.5-ml (bed volume) poly(A) Sepharose column and RBP16 is allowed to flow through at a rate of ~1 ml/min. The presence of RBP16 in the flowthrough is confirmed by SDS–PAGE on a 15% acrylamide gel followed by silver staining (Ansorge, 1985) and by Western hybridization using polyclonal anti-RBP16 antibodies as described (Hayman and Read, 1999). RBP16 is the predominant protein in the flowthrough fraction (Fig. 4.3C). Anti-RBP16 antibodies for detection of the protein in column fractions are available from the author. Using this procedure, mitochondrial vesicles from 12 liters (1–3 $\times$ 10^{11} cells) yield approximately 40 μg of RBP16.

8. Purification of RBP16 from the Bloodstream Forms *Trypansoma brucei*

8.1. Cells and growth medium

T. brucei strain 221 cells are grown in medium HMI-9, which contains (per liter): 714 ml Iscove's modified Dulbecco's medium (IMDM) (contains L-glutamine, 25 m*M* HEPES buffer, 3024 mg/liter sodium bicarbonate,

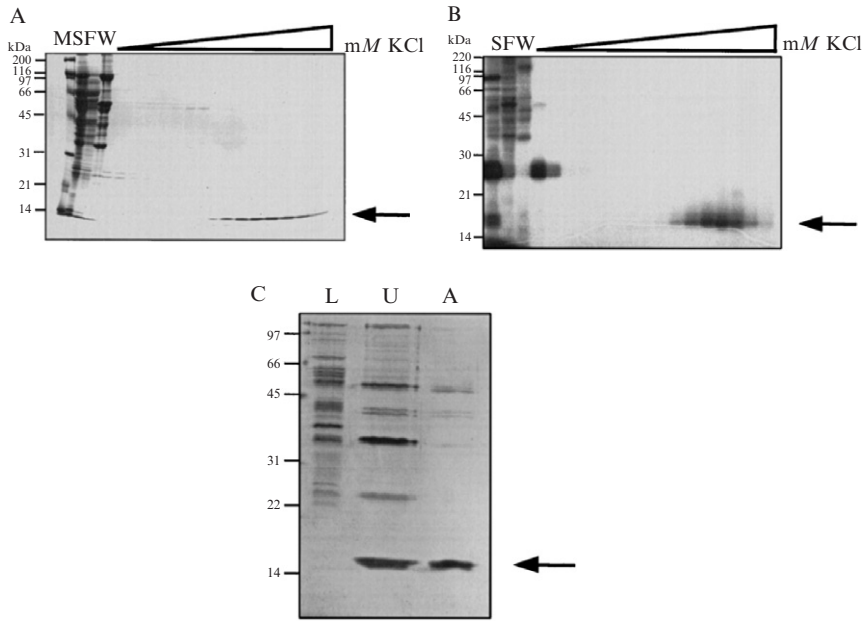

Figure 4.3 Purification of native RBP16 from procyclic form *T. brucei* by affinity chromatography on poly(U) and poly(A) Sepharose. *T. brucei* mitochondrial extract was loaded onto a poly(U) Sepharose column, and the column was washed with loading buffer containing 300 m*M* KCl. The column was then eluted with a linear 300–800 m*M* KCl gradient in loading buffer. (A) 0.5% of the starting material (S), 0.5% of the flow-through (F), 10% of the 300 m*M* 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 shown in (A) were assayed for gRNA-binding activities by UV cross-linking to radiolabeled gA6[14]. Ten femtomoles of gA6[14] was incubated with 4 μl each of total mitochondrial extract (S), poly(U) Sepharose flow-through (F), 300 m*M* KCl wash (W), and eluted poly(U) Sepharose fractions. Proteins were resolved by SDS–PAGE on a 15% gel and UV cross-linking proteins were 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) One microgram each of mitochondrial lysate (L), poly(U)-purified RBP16 (U), and the subsequent poly(A) Sepharose flowthrough (A) were separated by SDS–PAGE on a 15% gel. Proteins were detected by staining with silver. (Reprinted with permission from Hayman and Read, 1999.)

but no α-thioglycerol or 2-mercaptoethanol) (Invitrogen), 12.5 ml 100 m*M* sodium pyruvate (Invitrogen), 136 mg hypoxanthine (dissolved in 20 ml 0.1 *N* NaOH), 28 mg bathocuproine disulfonic acid (in 10 ml water), 182 mg cysteine (in 20 ml water), and 39 mg thymidine (in 10 ml water). The pH is adjusted to 7.6, and the medium is then sterilized by filtration on 0.22-μm filters. Following filtration, 100 ml of fetal calf serum (denatured at 55° for 30 min), 100 ml of Serum Plus (JRH Bioscience), and 10 ml of

penicillin–streptomycin (10,000 units each/ml) (Invitrogen) are sterilely added. Prior to use, 2-mercaptoethanol is added to a final concentration of 0.2 m*M*. We typically prepare a 1000× stock solution (140 μl of 2-mercaptoethanol in 10 ml water). Cells are grown in this media at 37° in the presence of 5% CO_2 (Hirumi and Hirumi, 1989).

8.2. Isolation of mitochondria

Twelve liters of cells in HMI-9 are grown up to a density of 1×10^6 cells/ml. Cells are processed as described for the procyclic cells except that once the cells are resuspended in DTE, sucrose is added to a final concentration of 0.5%.

8.3. Purification of RBP16 from mitochondrial vesicles

RBP16 is purified from the bloodstream form *T. brucei* as described previously for the procyclic form. The typical yield is approximately 1–2 μg of RBP16 from 12 liters ($\sim 1 \times 10^{10}$ cells).

9. Expression and Purification of Recombinant RBP16

Recombinant RBP16 is routinely expressed as a fusion with Maltose Binding Protein at the N-terminus (MBP-RBP16), or as a fusion protein with a C-terminal 6xHis-Tag sequence (His-RBP16) (Hayman and Read, 1999; Hayman *et al.*, 2001). Expression as an MBP fusion protein yields nearly 10-fold more protein than that of the 6xHis-tagged version. However, since MBP is a large tag (42 kDa), production of RBP16 with the smaller 6xHis tag is often preferable. Expression of both fusion proteins is carried out in *Escherichia coli* BL21 pLyS cells (Novagen). Purification of MBP-RBP16 is achieved by chromatography over amylose resin followed by poly(U) Sepharose. Purification of His-RBP16 is carried out by chromatography over nickel-resin followed by poly(U) Sepharose.

9.1. Expression and purification of MBP-RBP16

The buffers used in the purification are (1) amylose column buffer: 20 m*M* Tris (pH 7.5), 200 m*M* NaCl, 1 m*M* EDTA, 10% glycerol and (2) amylose elution buffer: 20 m*M* Tris (pH 7.5), 200 m*M* NaCl, 1 m*M* EDTA, 10% glycerol, 20 m*M* maltose. For poly(U) Sepharose chromatography, we use the same buffers previously described for the purification of native RBP16. PMSF (1 m*M* final), leupeptin (1 μg/ml final), and pestatin A (1 μg/ml final) are added to all buffers.

The full-length RBP16 open reading frame was amplified by PCR from total procyclic cDNA and cloned into the *Bam*HI/*Sal*I sites of pMal-C2 (New England Biolabs) as described (Hayman and Read, 1999). Induction of MBP-RBP16 is performed as follows: *E. coli* BL21 pLyS cells containing pMal-C2-RBP16 are grown overnight in 20 ml of LB broth with 100 μg/ml ampicillin at 37°. The next day, 10 ml of this overnight culture is transferred to 1 liter of LB broth containing 100 μg/ml ampicilin and the cells are allowed to grow at 37° (225 rpm) up to an OD_{600} of 0.5–0.6. IPTG is then added to a final concentration of 0.3 m*M*, and the cells are grown for 2 more hours at 37° (225 rpm). Following induction, cells are harvested by centrifugation at 5000×*g* for 10 min at 4°, and the pellet is resuspended in 1/10 volume (100 ml) of amylose column buffer. The cells are then sonicated for four periods of 30 sec at intensity 5, 50% pulse (settings are for the Sonifier cell disruptor 300 from Branson Ultrasonics), and centrifuged at 14,000×*g* for 20 min at 4°. The supernatant is diluted 1:4 in amylose column buffer, NP-40 is added to 0.1%, and the supernatant is incubated with 2 ml of amylose resin (GE Healthcare) (preequilibrated with amylose column buffer) for 2 h at 4° with gentle rocking. The mixture is then applied to a 1.5-cm-diameter column and allowed to flow through at a rate of ~1 ml/min. The column is washed with 20 column volumes (40 ml) of amylose wash buffer at a flow rate of ~1 ml/min. Bound MBP-RBP16 is eluted with 5 column volumes (10 ml) of elution buffer. Ten 1-ml fractions are collected and electrophoresed on SDS–PAGE, and the presence of MBP-RBP16 determined by Coomassie staining (MBP-RBP16 has an electrophoretic mobility of 58 kDa) (Fig. 4.4). Fractions that contain MBP-RBP16 are pooled, diluted 1:2 in 25 m*M* Tris (pH 8.0), 1.5 m*M* magnesium acetate, 5 m*M* EGTA, 50 m*M* KCl, and 10% glycerol buffer and further purified by poly(U) Sepharose chromatography, as described above, using a column with a 3-ml bed volume. The resulting MBP-RBP16 is essentially pure (Fig. 4.4). The expected yield following these two chromatographic steps is approximately 10–30 mg of purified MBP-RBP16 per liter of *E. coli* cells.

9.2. Expression and purification of His-RBP16

The following buffers are used for the purification of His-RBP16. (1) Lysis buffer: 10 m*M* Tris (pH 6.8), 300 m*M* NaCl, 10 m*M* imidazole, 10% glycerol, 0.1% sodium deoxycholate, 0.01% NP40, 10 m*M* $MgCl_2$, 0.06 mg/ml lysozyme, and 0.06 mg/ml DNase I. (2) Wash buffer: 10 m*M* Tris (pH 6.8), 300 m*M* NaCl, 30 m*M* imidazole. (3) Elution buffer: 10 m*M* (pH 6.8), 300 m*M* NaCl, 250 m*M* imidazole. All buffers contain PMSF (1 m*M* final), leupeptin (1 μg/ml final), and pestatin A (1 μg/ml final).

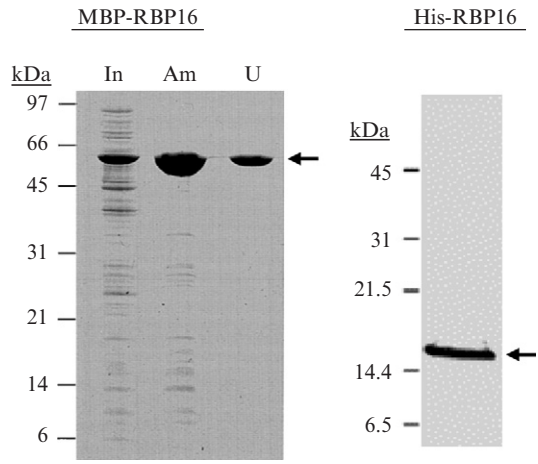

Figure 4.4 Expression and purification of recombinant RBP16 from *E. coli*. Left panel: *E. coli* cells harboring the pMal-C2 plasmid with the RBP16 gene in frame with MBP were grown in the presence of 0.3 m*M* isopropyl-1-thio-*β*-D-galactopyranoside. Input (In), amylose column eluate (Am), and subsequent poly(U)-purified protein (U) were separated by SDS–PAGE on a 12.5% gel and stained with Coomassie Blue. The 59-kDa fusion protein is indicated with an arrow. (Reprinted with permission from Hayman and Read, 1999.) Right panel: *E. coli* cells harboring the pET-21a plasmid with the RBP16 gene in frame with a C-terminal 6xHis-Tag sequence were grown in the presence of 0.1 m*M* isopropyl-1-thio-*β*-D-galactopyranoside. Purified His-RBP16 was electrophoresed on 15% SDS–PAGE and stained with Coomassie Blue. The position of the 20-kDa fusion protein is indicated with an arrow. (Reprinted with permission from Miller and Read, 2003.)

The full-length RBP16 open reading frame was amplified by PCR and cloned into the *Nde*I/*Xho*I sites of pET-21a (Novagen) as described (Miller and Read, 2003). Induction of His-RBP16 is achieved as follows: *E. coli* BL21 pLyS cells containing pET-21a-RBP16 are grown at 37° in 20 ml of LB broth containing 100 μg/ml ampicillin overnight at 37° with vigorous shaking (225 rpm). The next day, 10 ml of this overnight culture is transferred to 1 liter of LB broth containing 100 μg/ml ampicillin and the cells are allowed to grow at 37° (225 rpm) up to an OD_{600} of 0.5–0.6. IPTG is then added to a final concentration of 0.1 m*M*, and the induction of His-RBP16 is allowed to proceed for 2.5 h at 30° (225 rpm). Cells are collected by centrifugation for 10 min at 5000×*g* at 4°. Cells are weighed and resuspended in 3 ml lysis buffer per gram of cells and sonicated for four periods of 30 sec at intensity 5, 50% pulse (settings are for the Sonifier cell disruptor 300 from Branson Ultrasonics). The supernatant fraction is collected after centrifugation at 12,000×*g* for 20 min at 4°. Polyethylenimine is added to the supernatant to a final concentration of 0.05%. The resulting suspension is rocked for 20 min at 4°, and then centrifuged at 12,000×*g* for

10 min at 4° and the supernatant is recovered. To this supernatant is added one-twentieth volume of ProBond resin (Invitrogen), which has been preequilibrated with wash buffer. This mixture is rocked for 1 h at 4° and then poured into a 1.5-cm-diameter column. The flow rate is set at ~1 ml/min, and the solution containing the unbound proteins is allowed to flow through. The column is washed with 20 bed volumes of wash buffer, and the recombinant His-RBP16 eluted from the column in 5 volumes of elution buffer. Fractions containing His-RBP16 are identified by SDS–PAGE and Coomassie staining. These fractions are pooled and dialyzed against 100 volumes Poly(U)50 buffer [see Poly(U) Sepharose chromatography]. His-RBP16 is then subjected to poly(U) Sepharose chromatography essentially as described above, using a column with a 3-ml bed volume, except that the column is first washed with 10 volumes of poly(U) Sepharose wash buffer containing 200 m*M* KCl. Bound His-RBP16 is eluted with 5 volumes of wash buffer containing 800 m*M* KCl. The presence and purity of His-RBP16 are determined by SDS–PAGE Coomassie staining (His-RBP16 has an electrophoretic mobility of ~20 kDa). His-RBP16 is the sole protein visible in the elution (Fig. 4.4). Starting with 1 liter of *E. coli* cells, the typical yield after Probond resin and poly(U) Sepharose chromatography is between 1.5 and 2.0 mg of purified His-RBP16.

9.3. Assay of RBP16 RNA binding activity by UV X-linking to synthetic gRNA

To determine that the purified protein retains RNA binding activity, the ability of RBP16 to bind to gRNA is assayed by UV X-linking to the synthetic gRNA gA6[14] (Fig. 4.3B). For synthesis of body-labeled gA6 [14], we use a construct encoding the gRNA gA6[14] with a 17 nucleotide oligo(U) tail as previously described (Read *et al.*, 1994). The plasmid is digested with *Dra*I (1 unit/μg plasmid) at 37° for at least 1 h. Following extraction with 1 volume phenol/chloroform, the digested plasmid is precipitated with one-tenth volume of ammonium acetate and 2.5 volumes of cold 95% ethanol for 30 min at –20°. The sample is then centrifuged for 30 min at 13,000 rpm and the pellet is washed with 500 μl of cold 70% ethanol, centrifuged again at 13,000 rpm for 5 min, and finally air-dried.

gA6[14] internally labeled with [α-^{32}P]UTP is generated by *in vitro* transcription using the MAXIscript T7/T3 kit (Ambion). A typical 20-μl reaction consists of the following: 1 μg *Dra*I-digested gA6[14] plasmid, 2 μl 10× transcription buffer, 2 μl 0.1 *M* DTT, 1 μl 10 m*M* ATP, 1 μl 10 m*M* CTP, 1 μl 10 m*M* GTP, 1 μl 0.05 m*M* UTP, 5 μl [α-^{32}P]UTP (10 μCi/ μl; 3000 Ci/mmol), and 1 μl RNase inhibitor (2 units/μl). The reaction is initiated by the addition of 2 μl T7 polymerase (15 units/μl) and allowed to proceed for 1 h at room temperature. The reaction is stopped by the addition of 20 μl of 90% formamide (in TBE) and boiled for 3 min.

The ^{32}P-labeled gRNA is gel-purified on a 19-cm tall, 6% acrylamide/7 *M* urea gel. Following electrophoresis in TBE for 1 h at 420 V, the gel is exposed to a film for 30–60 sec. The film is then developed and superimposed on the gel. The band corresponding to the ^{32}P-labeled gA6[14] is excised from the gel using a clean razor blade and incubated in 750 μl of TE buffer (pH 7.5) containing ammonium acetate (0.75 *M* final) overnight at 4° with gentle rocking. The following day, the gRNA is precipitated with an equal volume of cold isopropanol at –20° for 1 h, then centrifuged for 60 min at 13,000 rpm, the pellet washed with 500 μl of cold 70% ethanol, centrifuged again at 13,000 rpm for 5 min, and finally air-dried and resuspended in 20 μl of DEPC-treated water. The concentration and specific activity of the labeled gRNA are determined by scintillation counting 10 μl of a 1:10 dilution.

To assess the RNA binding activity of RBP16 following chromatography, 10 μl of each fraction is incubated with 10 fmol of ^{32}P-labeled gA6[14] for 10 min at room temperature in a 6 m*M* HEPES buffer (pH 7.5) containing 2.1 m*M* $MgCl_2$, 0.5 m*M* DTT, 1.5 m*M* ATP, 5 m*M* creatine phosphate, 0.1 m*M* EDTA, 10 μg/μl torula yeast RNA, and 6% glycerol, in a total volume of 15 μl. Following incubation, samples are transferred to a 96-well plate on ice, positioned 4–5 cm to the light source, and irradiated for 10 min at 254 nm using a UV Stratalinker 2400 (Stratagene). One microgram of RNase A per microliter is then added, and samples are incubated at 37° for 15 min. SDS–PAGE loading buffer is added and the samples are boiled for 5 min before loading on a 15% polyacrylamide gel. The gel is dried and the ^{32}P-labeled proteins are detected by autoradiography or phosphorimager analysis.

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