

RNA Interference Analyses Suggest a Transcript-specific Regulatory Role for Mitochondrial RNA-binding Proteins MRP1 and MRP2 in RNA Editing and Other RNA Processing in *Trypanosoma brucei**

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Mitochondrial RNA-binding proteins MRP1 and MRP2 occur in a heteromeric complex that appears to play a role in U-insertion/deletion editing in trypanosomes. Reduction in the levels of MRP1 (gBP21) and/or MRP2 (gBP25) mRNA by RNA interference in procyclic *Trypanosoma brucei* resulted in severe growth inhibition. It also resulted in the loss of both proteins, even when only one of the MRP mRNAs was reduced, indicating a mutual dependence for stability. Elimination of the MRPs gave rise to substantially reduced levels of edited CyB and RPS12 mRNAs but little or no reduction of the level of edited Cox2, Cox3, and A6 mRNAs as measured by poisoned primer extension analyses. In contrast, edited NADH-dehydrogenase (ND) subunit 7 mRNA was increased 5-fold in MRP1+2 double knock-down cells. Furthermore, MRP elimination resulted in reduced levels of Cox1, ND4, and ND5 mRNAs, which are never edited, whereas mitoribosomal 12 S rRNA levels were not affected. These data indicate that MRP1 and MRP2 are not essential for RNA editing *per se* but, rather, play a regulatory role in the editing of specific transcripts and other RNA processing activities.

Kinetoplastida are early diverged flagellates that differ from other eukaryotes by a number of features. They contain a remarkable single mitochondrion, within which is a large mass of circular DNA molecules that are intercatenated in a unique arrangement (1). Moreover, their mitochondrial RNA processing is also highly unusual. The majority of mitochondrial mRNAs are extensively changed by RNA editing, which is the extensive insertion and less frequent deletion of uridines (Us) at multiple sites. Small guide RNA (gRNA)¹ molecules direct the pattern of U insertions and deletions by base pairing be-

tween the pre-edited mRNA and gRNA. The editing process occurs via a series of “cut-and-paste” steps, and several of the enzymes that catalyze this process, including RNA ligases and terminal uridylyl transferases, have now been identified (for recent reviews see Refs. 2–5).

In *Trypanosoma brucei*, editing enzymes co-sediment in glycerol gradients at ~20 S in a complex (6) that, upon purification, appears to be composed of about 20 stably associated proteins (7–9). In anticipation of further knowledge this complex was termed the “editosome”; its existence has recently been confirmed by a number of approaches including electron microscopy (5, 9). In addition, a complex of similar size and protein composition (termed the ligase-containing complex or L-complex) was identified in *Leishmania tarentolae* (4, 10). Editing activities also sediment at ~40 S, indicating that the 20 S complex may associate less stably with additional proteins or complexes (2, 4, 6, 10, 11).

The low efficiency of the *in vitro* editing assays and the low abundance of editing components have hampered, thus far, a full characterization of the editing machinery. In an attempt to identify other *T. brucei* proteins involved in RNA editing, several proteins have been characterized with affinity for pre-mRNAs and/or gRNAs. The 45-kDa protein REAP1 preferentially binds to poly(G) and single-stranded guanosine-rich regions of pre-mRNAs and has been proposed to bring pre-edited mRNAs into the editing complex (12). The 75-kDa TbRGG1 protein, which is rich in arginines and glycines, binds gRNAs with a preference for poly(U) (13), and the 16-kDa RBP16 protein primarily binds to gRNAs via the oligo(U) tails but also binds to mRNAs and rRNAs (14–16).

Another protein that binds gRNAs is *T. brucei* gBP21, which was identified by specific cross-linking (17) and shown to bind RNA with high affinity (18). More recently, it was demonstrated that gBP21 can act as a matchmaker, promoting the annealing of gRNAs to cognate pre-mRNA *in vitro* (19), after which it dissociates from the RNA duplex (20). Its involvement in editing was suggested by its association with editosomal complexes (21), albeit RNA-mediated and perhaps transient (4, 5). Polyclonal antibodies against gBP21 inhibited editing activity *in vitro* (22), but *in vivo* confirmation of a role in editing was lacking. Bloodstream form *T. brucei* cells in which both gBP21 alleles are knocked-out by homologous recombination are viable, with only minor changes in the levels of some edited and unedited mRNAs. Hence gBP21 is not essential in bloodstream forms. However, these gBP21 null mutants did not transform into the insect forms (22). gBP21 homologues have been identified in *Crithidia fasciculata* (gBP29;23) and *L. tarentolae* (Ltp28; 11).

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¹ The abbreviations used are: gRNA, guide RNA; dsRNA, double-stranded RNA; RNAi, RNA interference; MRP, mitochondrial RNA-binding protein; REL, RNA-editing ligase; PPE, poisoned primer extension; ND, NADH-dehydrogenase; REAP1, RNA editing-associated protein 1.

Importantly, a second gRNA-binding protein gBP27 was found in *C. fasciculata* that co-purified and co-immunoprecipitated with gBP29, the gBP21 homologue (23). A similar tight association was observed for the homologous proteins in *L. tarentolae*, in which Ltp28 and Ltp26, the gBP27 homologue, form a 100-kDa heterotetrameric complex (11). This complex may contain several other as yet unidentified proteins and was shown to bind to gRNA and catalyze the annealing of complementary RNAs (10, 11). This led to the suggestion that it could have a role in the initiation of RNA editing by stimulating gRNA:pre-edited RNA duplex formation (11). A gBP27 homologue, gBP25, has also been identified in *T. brucei* (23). *C. fasciculata* gBP27 and gBP29 have weak but significant similarity (23), which is conserved between the homologous pairs in the other two trypanosomatids. This homology, together with their almost identical hydrophilicity profiles, suggests that these proteins may have at least partially overlapping functions and hence molecular redundancy, a possible explanation for the viability of the gBP21 null bloodstream forms (3, 23). Recently, the observation has been made that these proteins bind RNA rather nonspecifically, and it was suggested to label these proteins mitochondrial RNA-binding proteins (MRPs) (4). For the sake of simplicity, this designation has been used throughout the remainder of this article.

The functions of individual proteins involved in RNA editing *in vivo* have been examined by genetic methods that employ homologous recombination. Knock-out of both alleles has been accomplished when a gene is not essential, as with gBP21 in bloodstream form *T. brucei*, but cannot be used for essential genes, which may be the case with gBP21 procyclic forms. Both essential and non-essential genes have been examined by knock-out of endogenous alleles in cells that contain an ectopic allele under the control of a regulatable promoter. This approach has shown that one of the two RNA-editing ligases (REL), REL1, is essential for editing and cell viability (24). In addition, the knock-out technique identified TbMP44 as critical for the structural integrity of the 20 S editing complex and essential for editing (25).

RNA interference (RNAi) represents a less laborious approach, in which a DNA sequence that generates a double-stranded RNA under the control of a regulatable promoter is inserted in the genome by homologous recombination. For this reason RNAi has become the method of choice for high throughput functional analysis of trypanosomatid proteins. So far, RNAi has been used to study the function of a large number of editing (complex-associated) proteins: the two RNA ligases (26–30); TbMP81 (also called band II), which has a zinc-finger motif and is essential for the presence of REL2 in the editing complex (29, 31); the terminal uridylyl transferases RET1 and RET2 (32, 33); and the RNA-binding protein RBP16 (16).

In the work described here, we have applied the RNAi technology to analyze the function of MRP1 (gBP21) and MRP2 (gBP25) in *T. brucei* procyclic forms. To this end, we have constructed RNAi strains in which the expression of either MRP1 or MRP2, or both, can be blocked inducibly. The results suggest that the MRPs function in regulating the extent of editing of specific transcripts in procyclic trypanosomes, with roles that differentially affect various mRNAs. In addition, these proteins seem to play a role in other aspects of RNA metabolism.

EXPERIMENTAL PROCEDURES

Plasmid Constructs, Transfection, Cloning, and RNAi Induction—The 5' part of the *MRP1* gene spanning nucleotides 1–615 (the A of the AUG initiation codon being number 1) was PCR-amplified using oligonucleotides g21-F (5'-CACCTCGAGATGAT TCGACTCGCATGCC TG-3') and g21-R (5'-CCCAGCTTGGTATCGCGATGTGTC ACTTA-3') (added XhoI and HindIII restriction sites are underlined). The

amplified fragment was cloned in pCR2.1 TOPO® (Invitrogen) and subcloned in pZJM (34), resulting in the construct MRP1-I (Fig. 1A). Similarly, nucleotides 1–675 of the *MRP2* gene were amplified with the g25-F (5'-CACCTCGAGATGCTCCGACTGATCATCAGCCAG-3') and g25-R1 oligonucleotides (5'-CCCAAGCTTGCCACATCAAACTCTACAGTCCAC-3') (added XhoI and HindIII restriction sites are underlined) and cloned using the same vectors, creating the construct MRP2-I (Fig. 1A). The double knock-down construct was prepared as follows. First, a smaller 5'-fragment of the *MRP2* gene (nucleotides 1–480) was amplified with oligonucleotides g25-F and g25-R2 (5'-AAACTGCAGG-ATTCGTGTGAAGGTG GCGTTAG-3') (the PstI restriction site is underlined) and cloned into pZJM, yielding MRP2-II. Next, we ligated a gel-purified, XbaI-PstI MRP2-II fragment into the MRP1-I plasmid linearized with the same enzymes. This generated the MRP1+2 construct containing the gene fragments in a head-to-tail orientation (Fig. 1A). All constructs were checked by sequence analysis.

The procyclic *T. brucei* strain 29-13, transgenic for T7 RNA polymerase and the tetracycline repressor (50), was grown at 27 °C in SDM-79 medium supplemented with 10% fetal bovine serum in the presence of hygromycin (50 µg/ml) and G418 (15 µg/ml). Exponentially growing cultures (about 5×10^7 cells/ml) were used for transfection, following the protocol of Wang *et al.* (34). Electroporation with 10 µg of linearized plasmid DNA was performed in 2-mm cuvettes using a BTX electroporator with the settings of 1500 V, 50 microfarads, and 500 ohms. After transfection, cells were transferred into fresh medium to which 2.5 µg/ml phleomycin was added 24 h after electroporation. After 2 weeks of cultivation, only cells resistant to phleomycin survived, which were cloned using limiting dilution in 96-wells plates at 27 °C in the presence of 5% CO₂. After 3 weeks, several clones were transferred into larger volumes of SDM-79, and the synthesis of dsRNA was induced by the addition of 1 µg/ml tetracycline. The MRP1 and MRP2 protein levels were analyzed in total cell lysates of bloodstream and procyclic stages of *T. brucei* and dyskinetoplastic cells EATRO164Dk (35).

Growth and Morphological Analysis—Following Northern blot analysis, one of three clones was used for further experiments (see "Results"). Growth curves obtained over a period of 11 days after the induction of RNAi were established using the Beckman X2 Cell Counter. One hundred cells from non-induced and tetracycline-induced cultures were measured using the ocular micrometer at several time points upon RNAi induction, and cell morphology was analyzed under the light microscope and by staining with 4',6-diamidino-2-phenylindole.

Southern and Northern Blotting—Total DNA from the RNAi cells was isolated, digested with selected restriction enzymes, subjected to agarose gel electrophoresis, blotted, and hybridized with DNA probes labeled by random priming (Fermentas) with [³²P]dATP (ICN). Total RNA was isolated using TRIzol (Sigma) according to the manufacturer's instructions. Approximately 5 µg of RNA/lane was loaded on a 1% formaldehyde agarose gel, blotted, and cross-linked following standard protocols. After prehybridization in Church-Gilbert solution (0.5 M Na₂HPO₄, pH 7.2, 1 mM EDTA, 7% SDS, 1% bovine serum albumin) for 2 h at 60 °C, hybridization was performed overnight in the same solution at 55 °C. A wash in 2× SSC + 0.1% SDS at room temperature for 20 min was followed by two washes in 0.2× SSC + 0.1% SDS for 20 min each at 55 °C.

Glycerol Gradients—A total of 6×10^8 log-phase procyclic cells were lysed in 1 ml of lysis buffer (40 mM HEPES, pH 7.9, 20 mM Mg(OAc)₂, 100 mM KCl, 2 mM EDTA, pH 8.0, 1 mM dithiothreitol, 0.5% Triton X-100) supplemented with 1 mM pepabloc, 2 µg of leupeptin, and 1 µg of pepstatin. The lysate was loaded on a 10–30% glycerol gradient and centrifuged at 38,000 rpm in a Beckman SW41 rotor for 5 h at 4 °C. Individual 0.5-ml fractions were flash-frozen in liquid nitrogen and stored at –80 °C for further use.

Preparation of Antibodies and Western Blotting—The coding region of MRP1 and MRP2 was PCR-amplified, cloned into the pQE9 vector, and transfected into *E. coli* SG13009 cells, and the resulting clones were verified by sequencing. The cells were grown in the presence of 100 µg/ml ampicillin and 25 µg/ml kanamycin (MRP1) or 50 µg/ml carbenicillin (MRP2) at 27 °C and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. They were resuspended in sonication buffer (20 mM NaPO₄, pH 7.4, 500 mM NaCl) and lysed by the addition of 1 mg/ml lysozyme followed by four cycles of sonication for 30 s at 16 mA. The suspension was centrifuged, and the overexpressed protein was purified from the supernatant by the nickel-nitrilotriacetic acid chelate resin as specified by the manufacturer (recombinant MRP1 and MRP2 were eluted with 200 and 400 mM imidazole, respectively).

Polyclonal antibodies were prepared by immunizing rabbits at 2-week intervals with four subcutaneous injections of 0.5 mg of purified recombinant MRP1 or MRP2 protein emulsified with complete (first

TABLE I
Oligonucleotides used in poisoned primer extension

RNA	Sequence	Coordinates
A6 (ed) ^a	5'-CTTATTGATCTTATTCTATAACTCC-3'	367-392
Cox3 (ed)	5'-AACTTCTACAACTACC-3'	446-463
ND4	5'-ATAGACTTTTCGAAGGCTATC-3'	303-322
ND5	5'-CAAACGCGCTAACACACAAC-3'	1097-1116
ND7 (ed)	5'-CACATAACTTTCTGTACCACGATGC-3'	122-147
RPS12 (ed)	5'-AAAAACATATCTTAT-3'	207-221
α -tubulin	5'-GGGGTCCGAGTTTGTTC-3'	906-922
Cox1	5'-TTCAGTCAAAAACCCACTTACTAAG-3'	342-366
Cox2 (ed)	5'-AAATTATTTTCATTACACCTACCAGG-3'	516-540
CyB (ed)	5'-TATATAACACCTGACATTAAAG-3'	54-78
12 S rRNA	5'-AAATAATATAGTATTGCCCAATC-3'	905-929

^a ed, edited.

injection) and incomplete (following injections) Freund's adjuvant. Sera were collected 7 days after the fourth injection and were tested by Western blotting. Antibodies were affinity-purified on immobilized antigen columns by standard protocols. The antibody-containing fractions were dialyzed against phosphate-buffered saline and stored with 0.02% sodium azide at 4 °C. Cell lysates were prepared from 1×10^7 cells/10 μ l, analyzed on a 12% SDS-polyacrylamide gel, blotted, and probed with polyclonal anti-MRP1 (MRP2) antisera (1:2000). The secondary anti-rabbit IgG antibody (1:2000) coupled to alkaline phosphatase or horseradish peroxidase was visualized according to the manufacturer's protocols using the ECL kit (Amersham Biosciences). The preparation of polyclonal rabbit antisera against REAP1 and RBP16 and mouse monoclonal antibodies against TbMP81, TbMP63, TbMP62, REL1, and α -phosphoglucokinase has been described elsewhere (7, 14, 36-38).

Immunoprecipitation—Immunomagnetic beads (Dynabeads M-280 or M-450, Dynal) were coupled with affinity-purified polyclonal anti-MRP1 or anti-MRP2 antibodies and affinity-purified monoclonal anti-MRP1 antibody MAb56 (21). Coupling was performed in immunoprecipitation buffer containing 200 mM KCl. Immunoprecipitation of mitochondrial proteins was performed by incubating the pooled glycerol gradient fractions 3-7 (see "Glycerol Gradients") of mitochondrial lysates from 29-13 cells generally following the protocol of Allen *et al.* (21). The immunoprecipitated proteins were isolated from the beads and analyzed by SDS-PAGE followed by silver staining or Western blotting.

Poisoned Primer Extension—Two protocols were performed for poisoned primer extension (PPE). For A6, Cox3, ND4, ND5, ND7, RPS12, and α -tubulin, the PPE protocol was according to Estévez *et al.* (39) with 50 μ g of total RNA and 0.1 pmol of 5' ³²P-labeled (ICN) oligonucleotides. The PPE on transcripts of Cox1, Cox2, CyB, and 12 S rRNA was performed generally following the protocol of Lambert *et al.* (22). The oligonucleotides used for RNAs that are edited were derived from unedited regions, enabling the simultaneous analysis of edited and pre-edited RNA versions; the coordinates given refer to the pre-edited and never edited transcripts (Table I). Routinely, PPEs for individual mRNAs were performed at least three times. The amount of signal was measured using a Storm 860 PhosphorImager (Amersham Biosciences). The nuclear-encoded α -tubulin mRNA, which does not undergo RNA editing, was used as an internal control.

In Vitro Editing—The *in vitro* precleaved insertion editing assay was performed with a tripartite RNA substrate with a 5'-fragment (5'CL18), a 3'-fragment (3'CL13pp), and gRNA (gPCA6-2A), which specifies the addition of 2 Us as described previously (40). The precleaved deletion assay was performed with a tripartite RNA substrate with a 5'-fragment (U5-5'CL), a 3'-fragment (U5-3'CL), and gRNA (gA6[14]PC-del), which specifies the removal of 4 Us as described previously (41). Briefly, reactions contained 25 fmol of radiolabeled 5'-fragment, 1 pmol of 3'-fragment, and 0.5 pmol of gRNA with 15 μ l of glycerol gradient purified material in a total volume of 30 μ l in HHE buffer (25 mM HEPES, pH 7.9, 10 mM Mg(OAc)₂, 5 mM CaCl₂, 50 mM KCl, 0.5 mM dithiothreitol) and were incubated for 3 h at 28 °C. The reaction products were phenol-chloroform-extracted, ethanol-precipitated, run on 11% polyacrylamide, 7 M urea gels, and visualized by phosphorimaging. Full-round *in vitro* deletion editing was assayed using the A6short/TAG.1 pre-mRNA substrate and the D33 gRNA, which forms a duplex with pre-mRNA and contains 3 Cs opposite the editing site (42), following the protocol published by Seiwert *et al.* (43).

RESULTS

Loss of MRP1 and/or MRP2 Is Lethal to Procyclic Form *T. brucei*—5'-Fragments of the MRP1 and the MRP2 genes

were cloned into the pZJM RNAi vector, both separately and together, as described under "Experimental Procedures" (see Fig. 1A). Transfection of the constructs into procyclic trypanosomes resulted in their stable integration into the intergenic region of the rRNA gene region (data not shown). The morphology of non-induced cells appeared normal by both light and electron microscopy. Growth of the cloned cell lines was inhibited upon RNAi induction with tetracycline, to a different extent in individual cell lines. Growth of the MRP1 and MRP1+2 knock-downs was inhibited by 51 and 69%, respectively, after 1 week, and the inhibition rose to 97% for both cell lines by 11 days post-induction (Fig. 1B). After 7 days of induction, growth of the MRP1 knock-down was inhibited by about 26%. However, the extent of inhibition increased eventually to 70% and showed no recovery.

After induction of the RNAi response, total RNA was isolated from the different cell lines at different time points and analyzed on Northern blots (Fig. 2). The analysis showed that in all cases the target MRP mRNA was virtually completely eliminated after 3 days of induction, not only in the single but also in the double knock-downs, and remained very low upon further cultivation for at least 9 days post-induction (Fig. 2). Reassuringly, in the single knock-downs the levels of the non-targeted MRP mRNA were not affected, and the levels of GAPDH mRNA, which was used as a control of the specificity of the transfection and RNAi-induction procedures, remained at similar levels in all samples. In addition, no leaky transcription from the pZJM constructs was evident, because dsRNA, which is visible as a partially smeared band, appeared only after the addition of tetracycline. From this we infer that the generation of dsRNA from our constructs results in specific and essentially complete elimination of the intended target MRP mRNAs.

Western analysis with polyclonal antibodies generated against recombinant MRP1 and MRP2 proteins revealed that the ablation of the target protein(s) occurred in parallel with the reduction of mRNA (Fig. 3A). There was a strong decrease in the target proteins in the three MRP knock-down strains after 2 days of induction, followed by almost complete elimination of the proteins at day 4. In contrast to the specificity of target mRNA degradation, elimination of one of the MRP proteins resulted in loss of the other. In the MRP2 knock-down strain, MRP1 and MRP2 were lost at essentially the same rate, whereas in the other two knock-down strains, the decline in MRP2 was somewhat slower than that of MRP1. One explanation for this mutual dependence on stability would be that like their homologues in *L. tarentolae* (3) and *C. fasciculata* (6), *T. brucei* MRP1 and MRP2 are present in a complex that becomes unstable and is degraded if one of the partners is missing (see below).

Editing-associated Proteins Are Present in MRP Knock-downs—The repression of MRP1 and/or MRP2 expression by RNAi did not result in a detectable loss of editosome core proteins TbMP42, TbREL1, TbMP63, and TbMP81 or the potentially editing-associated proteins RBP16 and REAP1, although the amount of REAP1 appeared slightly decreased at later time points (Fig. 3B). These proteins were present in the MRP knock-downs in roughly the same amounts as in wild type cells even after 6 days of induction, when MRP1 and MRP2 were completely absent.

The interdependent stability of the MRP1 and MRP2 proteins in the procyclic stage (see Fig. 3A) and the differential abundance of edited RNAs between life cycle stages led us to assess the expression levels of these proteins in the *T. brucei* bloodstream form. We found both MRPs to be abundant in the mammalian stage, with no significant difference of expression

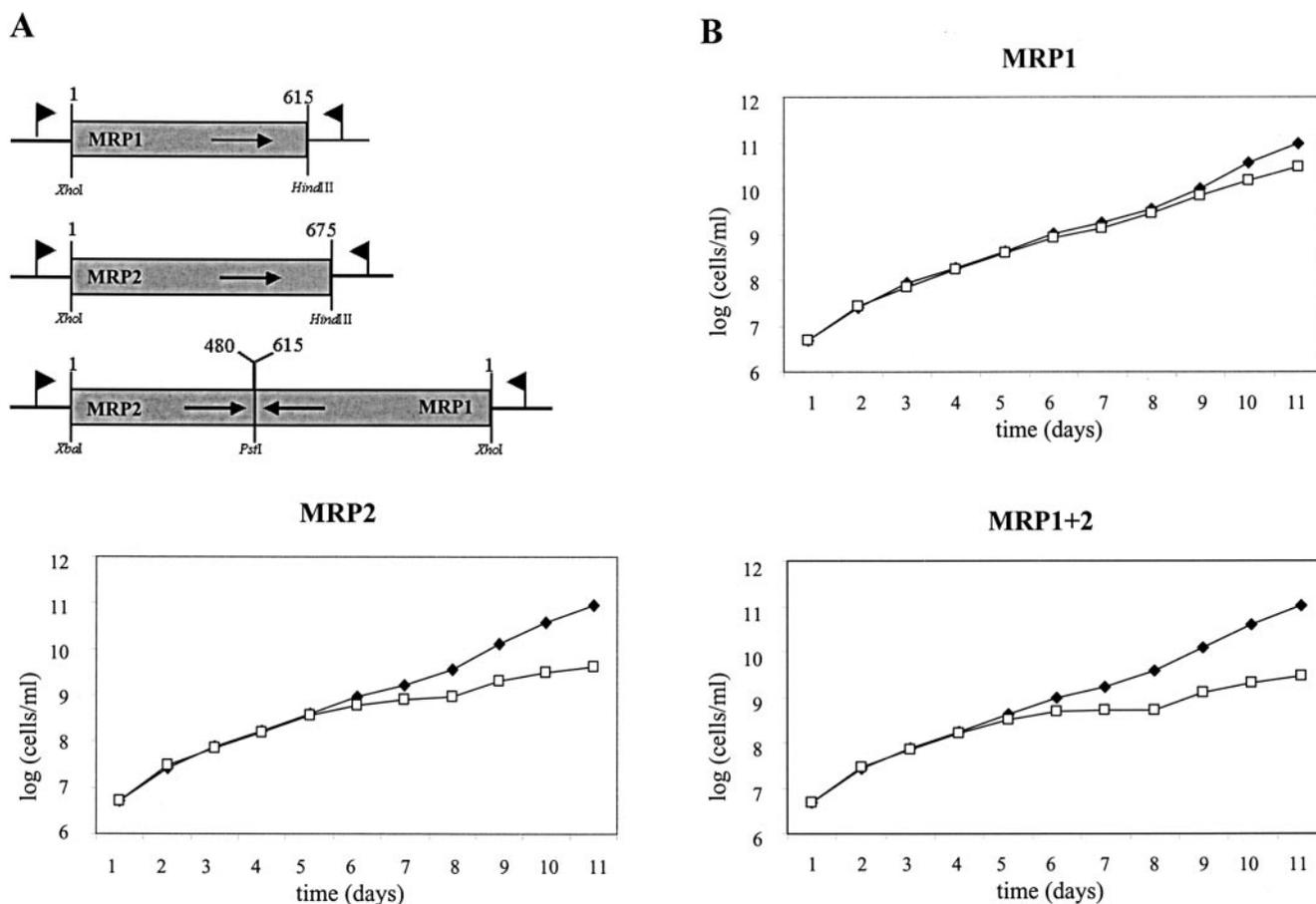


FIG. 1. Effect of MRP1 and MRP2 RNAi on cell growth. *A*, schematic representation of the targeting vectors. *B*, growth inhibition following induction of MRP1 and/or MRP2 dsRNA. Cell numbers were measured using a Coulter Counter Z2. The numbers of non-induced cells (diamonds) and those after RNAi induction by the addition of 1 μ g/ml tetracycline (squares) are indicated. The y axis is labeled by a log scale and represents the product of cell densities measured and total dilution.

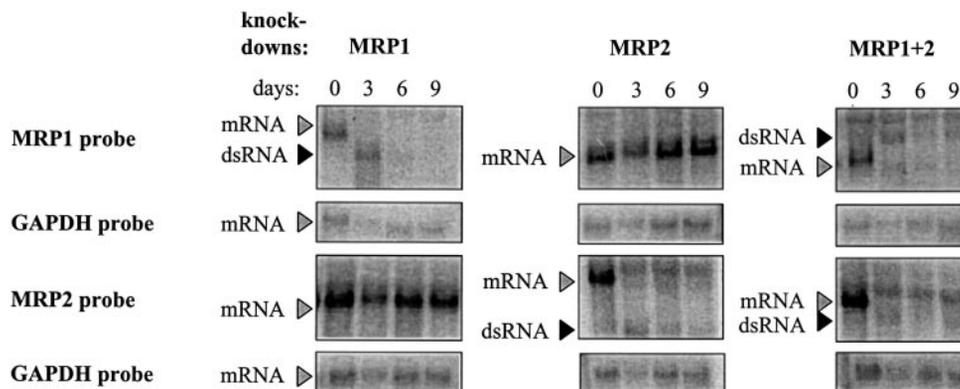


FIG. 2. Effect of MRP RNAi on mRNA levels. MRP1 mRNA (1.1 kb) and MRP2 mRNA (1.8 kb) levels were analyzed by Northern blot analysis in extracts from non-induced cells (day 0) and in extracts isolated 3, 6, or 9 days after induction of single and double MRP1 and MRP2 knock-down cells. The positions of the targeted mRNAs and the dsRNAs synthesized following induction are indicated with gray and black arrowheads, respectively. Note that in the MRP1+2 double knock-down, the dsRNA is longer than the MRP1 mRNA. The size difference between the dsRNA products synthesized in single and double knock-downs is in agreement with their position in the gel. Glycosomal nuclear-encoded glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was used as a loading control.

levels (Fig. 3C). However, both proteins were less abundant in the dyskinetoplastic *T. brucei* strain EATRO164Dk, which is devoid of kinetoplast DNA and therefore lacks both mt mRNA and gRNA but contains complexes that are capable of *in vitro* editing (Fig. 3C) (35).

MRP1 and MRP2 Are Components of a Complex—To study the association between MRP1 and MRP2, mitochondrial lysates from procyclic *T. brucei* were analyzed by glycerol gradient sedimentation. Probing the gradient fractions with anti-MRP1 and anti-MRP2 polyclonal antisera revealed that the

MRP proteins co-sediment in fractions 3–9, with the bulk of these proteins present in fractions 5–7 (Fig. 4A). Probing the same gradient with anti-TbRGG1 showed that this protein has a similar sedimentation pattern. However, RBP16 displayed a different distribution, with most of the protein sedimenting higher up in the gradient, although some of the protein appeared to co-sediment with the MRPs (Fig. 4A). Four of the core editosome proteins, TbMP42, REL1, TbMP63, and TbMP81, showed a sedimentation profile different from that of the MRPs, predominantly sedimenting in fractions 9–17 (Fig. 4A

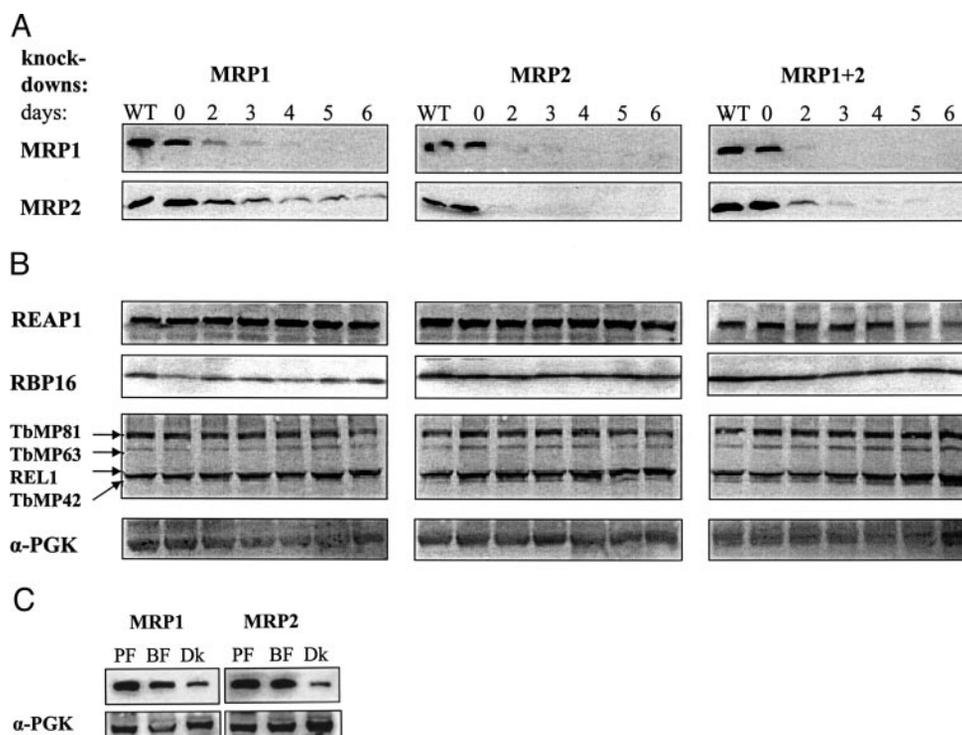


FIG. 3. **Effect of MRP RNAi on protein levels.** A, MRP1 and MRP2 protein levels were analyzed by Western blot analysis in extracts from single and double knock-down cells, both before and after 2–6 days of RNAi induction. Each lane was loaded with protein from 10^7 cells, and blots were immunostained using anti-MRP1 and anti-MRP2 polyclonal rabbit antisera, as described under “Experimental Procedures.” B, Western blot analysis of REAP1, RBP16, TbMP81, TbMP63, TbMP42, and REL1. α -Phosphoglucokinase (α -PGK) was used as a loading control. C, Western blot analysis of MRP1 and MRP2 of extracts from procyclic (PF) and bloodstream (BF) stages of *T. brucei* wild type cells and of dyskinetoplasmic (Dk) EATRO164Dk cells; α -phosphoglucokinase was used as a loading control.

and data not shown; see ref. 44), in agreement with data previously reported for MRP1 (gBP21) (22).

Fractions from glycerol gradients of lysates from MRP1 or MRP2 RNAi knock-down cells harvested 4 days after induction were analyzed. As shown in Fig. 4B, MRP1 was completely absent in the MRP2 knock-down, whereas only minute amounts of MRP2 remained in the MRP1 knock-down, confirming what was observed for total cell lysates (Fig. 3A). In addition, the residual MRP2 was at the top of the gradient, sedimenting at a considerably lower S-value than that observed when MRP1 is present, again demonstrating that both proteins are required for the stability of a MRP-containing complex. In contrast, the sedimentation profiles of TbRGG1, RBP16, and core editosome proteins remained unaltered in the knock-down cells (Fig. 4B and data not shown).

We also examined the interaction between MRP1 and MRP2 by co-immunoprecipitation experiments (Fig. 5). Immunoprecipitates generated with affinity-purified anti-MRP1 or anti-MRP2 polyclonal antibodies invariably contained also the other MRP, both with mitochondrial vesicles (Fig. 5) and lysates of total cells (data not shown) as starting material. Also the use of the monoclonal antibody MAb56, which is specific for MRP1 (21), resulted in immunoprecipitates containing both MRP1 and MRP2 (Fig. 5). In contrast, none of the core editosomal proteins tested (TbMP42, TbMP63, TbMP81, REL1) or RET1 or TbRGG1 was present in the precipitates generated with any of the anti-MRP antibodies (data not shown, see “Discussion”).

Editing of CyB, RPS12, and ND7 mRNAs Is Affected by MRP Elimination—Poisoned primer analysis was performed to assess simultaneously the amount of specific pre-edited and edited mRNAs in the MRP1, MRP2, and MRP1+2 RNAi strains. The wild type levels of individual edited, pre-edited, and never edited mRNAs were obtained by PPE of RNA from the 29-13

cells. To determine the most appropriate time point for the PPE analysis, we analyzed the levels of a number of edited, pre-edited, and never edited RNAs on different days after RNAi induction. Fig. 6 shows the results for two representative RNAs, CyB mRNA, which is edited, and never edited Cox1 mRNA, at 2, 4, and 6 days following RNAi induction. The analysis revealed a steady simultaneous decline of the levels of edited CyB and Cox1 mRNAs in all three knock-down strains. The kinetics of mRNA decay are in agreement with the decrease in MRP proteins (Fig. 3A), suggesting that the decrease in RNA levels is a primary effect of MRP knock-down. The proteins had not completely disappeared 2 days post-induction, particularly in the MRP1 knock-down strain. The MRP1 knock-down contained wild type levels of the mRNAs, whereas the MRP2 and MRP1+2 knock-downs contained 30–70% of the wild type levels. Minimal protein levels appear to be reached 4 days post-induction, typically resulting in the greatest reduction on mRNA level.

Therefore, a large number of RNAs was analyzed by PPE at day 4 post-induction (Fig. 7). As already observed in Fig. 6, a dramatic decrease in edited CyB mRNA was observed in all strains following RNAi induction (Fig. 7D). Although the ratio of pre-edited to edited mRNAs was about 1:1 prior to RNAi induction, at 4 days after RNAi induction this ratio changed to 5:1 in the MRP1 knock-down and to about 20:1 in the other two knock-downs. Thus, editing of this transcript, which is edited by U insertions only, dropped by 95%. The loss of MRP1 protein affected editing of CyB about 10 times less than the loss of MRP2, which may reflect the relatively high amount of MRP2 still present in the MRP1 knock-down cells. In addition, the relative level of the extensively edited ribosomal protein S12 (RPS12) mRNA decreased considerably upon RNAi induction, albeit not to the same extent as edited CyB mRNA (Fig. 7C).

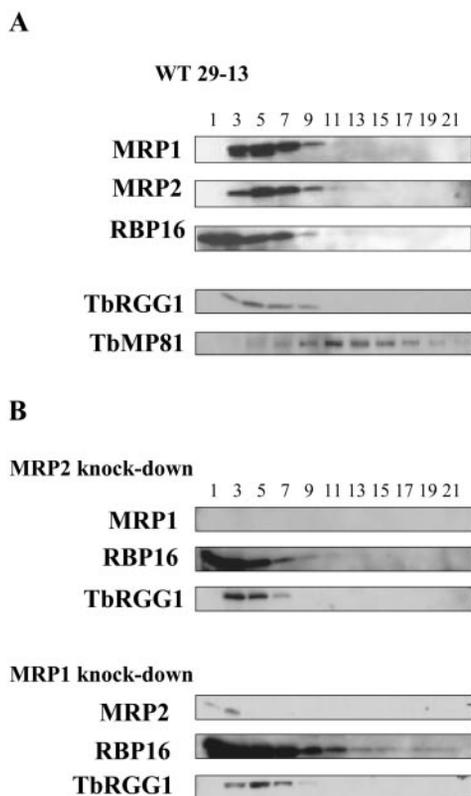


FIG. 4. Sedimentation analysis of MRP1 and MRP2. Western blot analysis of glycerol gradient fractions from lysates of 29-13 cells (A) and MRP1 or MRP2 knock-down cells (B) (fraction 1 is at the top). Blots were immunostained with specific sera as indicated (see "Experimental Procedures"). The knock-down cells were harvested 4 days after RNAi induction when the target proteins were completely eliminated.

In contrast, the editing of cytochrome oxidase (Cox) subunit 2 mRNA, which is edited by insertion of four Us as specified in *cis* by the 3'-untranslated region (45), was unaffected by RNAi induction. The levels of pre-edited and edited Cox2 mRNAs were similar in these cells before and after induction, with only minor variations that were well within the experimental error (Fig. 7E). Similarly the relative levels of edited Cox3 and ATPase subunit 6 (A6) mRNAs, transcripts that are extensively edited by U insertion and deletion as specified by numerous minicircle encoded gRNAs, were not significantly reduced after RNAi induction (Fig. 7, A and B) as determined by PPE. The relative level of edited NADH-dehydrogenase subunit 7 (ND7) mRNA was unaffected in the MRP1 knock-down following RNAi induction (Fig. 7F). In contrast, RNAi induction in the MRP1+2 RNAi cells resulted in about a 5-fold relative increase in edited RNA after 4 days.

Never edited mt mRNAs Are Also Affected—PPE analysis of the levels of never edited mRNAs in the knock-down cells revealed that the highly abundant 12 S rRNA appears largely unaffected by RNAi induction, although a small increase was observed in some cases (Fig. 7I). However, three never edited mRNAs, Cox1 mRNA (Figs. 6 and 7J) and ND4 and ND5 mRNAs were reduced after RNAi induction, especially in the MRP2 and MRP1+2 knock-downs (Fig. 7, G and H). Only about 10–20% of these transcripts was present 4 days after RNAi induction. Northern analysis also revealed a substantial reduction in Cox1 mRNA after 3 days and virtually complete elimination after 6 days of induction (data not shown).

In Vitro Editing Is MRP-independent—Next, we checked the effect of the absence of MRP1 and MRP2 on *in vitro* editing activity. Pooled glycerol gradient fractions containing the peak of editing proteins from MRP knock-down cells that were either

non-induced or induced for 4 and 5 days were assayed. The results reveal that these lysates were fully competent for pre-cleaved deletion (Fig. 8A) and insertion (Fig. 8B) editing. The lysates from the MRP knock-down cells also were fully active in full-round deletion editing assays, as judged from a comparison with lysates from non-induced and 29-13 cells (data not shown). These results demonstrate that the absence of MRP1 and/or MRP2 in the knock-down strains does not affect the capacity of crude or purified protein fractions to perform RNA editing *in vitro*, indicating that all activities needed for the *in vitro* reactions, *i.e.* endonuclease, terminal uridylyl transferase, uridylylate-specific 3'-exoribonuclease, and RNA ligase were present.

DISCUSSION

Most proteins known to function in RNA editing are part of a multiprotein complex termed the editosome (37) or L-complex (4). The composition of this multi-catalyst complex and the functions of its individual proteins are beginning to be elucidated (reviewed in Refs. 2–5). However, evidence is accumulating for the participation in RNA editing of proteins that are not a stable part of the editosome, although their roles are less well understood. In this report, we have used RNA interference as a genetic approach, in combination with a number of biochemical techniques, to assess the function in RNA editing of two such proteins, MRP1 and MRP2. These proteins possess RNA annealing activity (11, 19) and are found in a complex in *L. tarentolae* (11) and *C. fasciculata* (23) and, as we show here, in *T. brucei*. We further show that RNAi-mediated knock-down of MRP1 and/or MRP2 expression resulted in severe growth inhibition (Fig. 1) and reduction of edited CyB and RPS12 mRNAs, whereas the levels of Cox2, Cox3, and A6 mRNAs remained unaltered (Figs. 6 and 7). Surprisingly, however, we observed an increase in another edited RNA (ND7 mRNA) and reduction of RNAs that do not get edited (Cox1, ND4, and ND5 mRNAs). In addition, we found that the stability of the *T. brucei* MRP1 and MRP2 complex requires the simultaneous presence of both MRPs (Figs. 4 and 5).

The decline of edited CyB and Cox1 RNA levels (Fig. 6) closely follows the decrease of the MRP proteins (Fig. 3A). This appears to suggest that the decrease of the levels of both mRNAs is a primary effect of MRP shortage. Importantly, the decline of edited CyB mRNA occurs in parallel with an increase of unedited CyB RNA (Figs. 6 and 7), implying that the effect of MRP deficiency is indeed on CyB RNA editing and not on edited RNA stability. These results provide direct evidence for a role of MRP1 and MRP2 in editing and also suggest that, in addition to an apparent role in regulating the editing of specific transcripts, these proteins may also be involved in other RNA processing activities. Although originally shown to bind gRNAs, MRP1 and MRP2 have recently been shown to have a broader RNA binding specificity and no sequence preference for the RNA annealing activity (4, 11, 19). Thus, although MRP1 and MRP2 may function in gRNA:mRNA duplex formation, they may also function in other types of RNA processing. For example, they may promote secondary structure formations that affect the stability of specific transcripts (*e.g.* Cox1, ND4, and ND5 mRNAs) or processing of maxicircle (46) and/or minicircle (47) polycistronic primary transcripts.

The finding that MRP1 and MRP2 knock-downs affect the levels of specific transcripts differentially (Figs. 6 and 7) implies that MRP1+2 have transcript-specific roles but are not essential for RNA editing/processing *per se*. Therefore, MRP1+2 do not seem to play a role during the catalytic events that are central to the U insertion/deletion process or in maintaining the structure of the complex that performs these reactions. Hence, MRP1+2 are not required for RNA editing

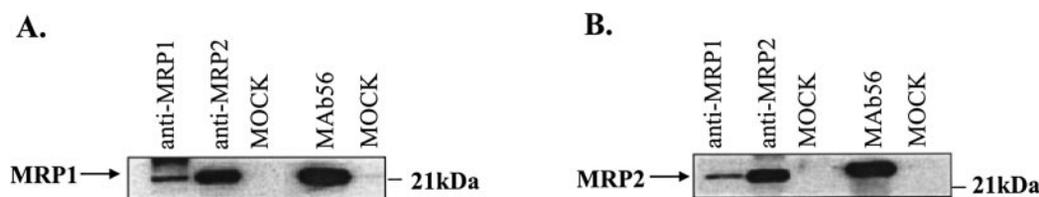


FIG. 5. **Co-immunoprecipitation of MRP1 and MRP2.** Fractions 3, 5, and 7 from glycerol gradients of mitochondrial lysates from 29-13 cells were pooled followed by immunoprecipitation with affinity-purified rabbit polyclonal antibodies specific for MRP1 or MRP2 or with mouse monoclonal antibody MAb56 specific for MRP1; antibodies were coated on sheep anti-rabbit IgG or goat anti-mouse IgG immunomagnetic beads, respectively. Proteins in the collected immunoprecipitates were identified by Western analysis using affinity-purified antisera specific for MRP1 (A) or MRP2 (B). Protein size marker positions are indicated.

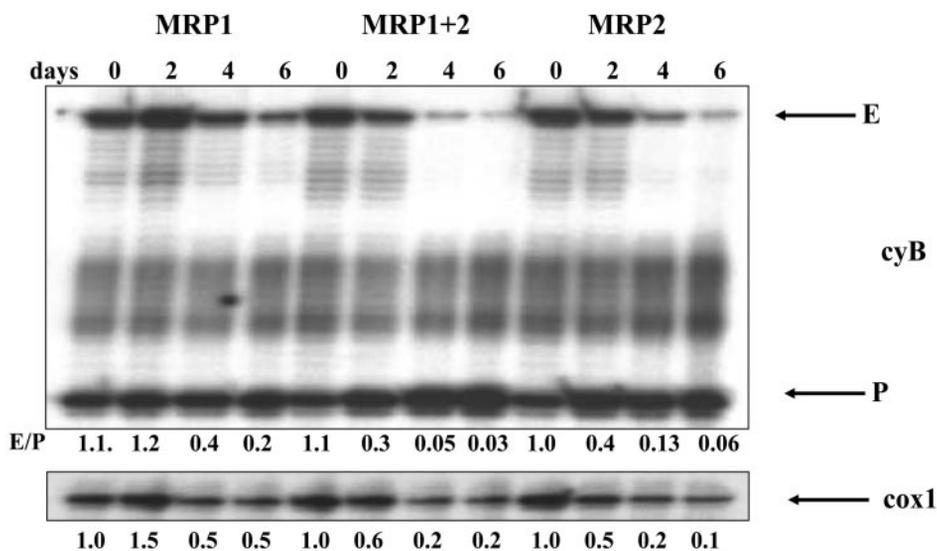


FIG. 6. **Effect of MRP RNAi on CyB and Cox1 RNA levels at different times post-induction.** Levels of edited (E) and pre-edited (P) CyB mRNA and never edited Cox1 mRNA were determined by PPE in total RNA extracted at days 2, 4, and 6 post-induction as described under "Experimental Procedures." For CyB mRNA the ratio between edited and pre-edited mRNA (E/P) is shown below each lane. For Cox1 mRNA, the change relative to the non-induced state (1.0) is given.

in vitro because extracts from MRP knock-down cells (Fig. 8) or purified editosomes, which lack MRPs (3–5), catalyze full rounds of U insertion or deletion. Thus, it seems plausible that the MRPs function in annealing gRNAs and mRNAs during editing. However, their differential effects on editing imply that the MRPs, alone or in concert with other cofactors, play a role in selective editing of different mRNAs (*e.g.* CyB and RPS12), perhaps by affecting specific gRNA utilization. Further analysis is needed to elucidate the differential editing.

The immunoprecipitation/depletion experiments performed by Lambert *et al.* (22) and Allen *et al.* (21) at low stringency (30–50 mM KCl) suggested an interaction between MRP1 (gBP21) and editosomal components. However, such experiments are difficult to interpret because components of the editing machinery could be pulled down aspecifically when bound to the same RNAs to which the MRPs are bound, without a direct interaction between them. Indeed, the association in crude extracts between catalytic core components of the editing machinery and the MRPs appeared to be weak, substoichiometric (11), and RNase-sensitive (11, 21). Accordingly, purified MRP complexes are devoid of editosomal components and vice versa (11, 18, 23) (reviewed in Refs. 3–5).² The different sedimentation profiles of editosomal TbMP81 and the MRPs (Fig. 4A), the unaltered levels of editosomal proteins in our MRP knock-downs (Fig. 3), and their absence in MRP immunoprecipitates generated at high stringency

(200 mM KCl; data not shown) confirm the absence of a direct, strong interaction between the MRPs and editosomal components.

The consequences of the MRP knock-downs closely resemble those of the knock-down of RBP16, which resulted in disruption of the editing of CyB mRNA, but not of A6, Cox2, or MURF2 RNAs, whereas the amounts of never edited Cox1 and ND4 mRNAs were also reduced (16). We speculated that RBP16 may be a regulatory factor that directs editing in the procyclic stage in which CyB is the only procyclic-specific *trans*-edited mRNA. This implies that RBP16 may directly or indirectly functionally interact with MRP1+2. However, our results failed to produce evidence for a direct interaction, in view of the differences in sedimentation profiles in wild type cells between MRP complexes and RBP16 (Fig. 4A) and the lack of change in RBP16 concentration and sedimentation profiles in the MRP knock-downs (Fig. 3B).

MRP1 null mutant bloodstream forms were viable, although they grow more slowly, and edited mRNA levels are only marginally affected; edited ND7 mRNA is increased and edited A6 mRNA and never edited Cox1 mRNA are decreased (22). Because the stability of MRP1 requires MRP2 and vice versa, one would expect MRP2 protein to be reduced or absent in the MRP1 null mutant. This suggests that molecular redundancy is not the explanation for the lack of a large effect on editing in the MRP1 null mutant. Instead, it is likely that both MRPs are dispensable in bloodstream form *T. brucei* but that at least one MRP is important in procyclic forms. This is implied by the inability of the MRP1 null strain to transform into the procyclic

² A. Ziková, K. Stuart, and J. Lukeš, unpublished results.

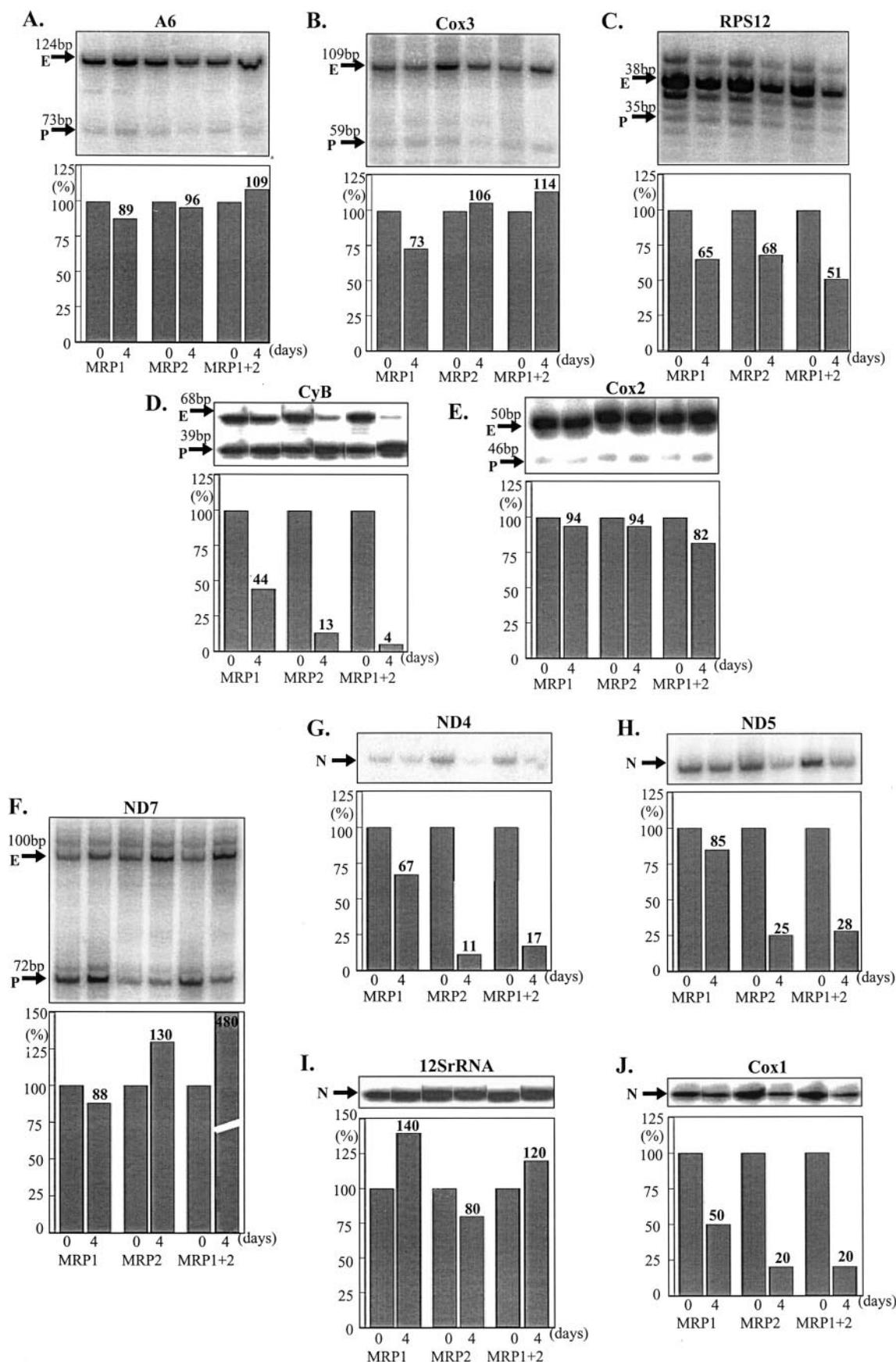


FIG. 7. Effect of MRP RNAi on mRNA abundance at day 4 post-induction. The levels of edited (*E*), pre-edited (*P*), and never edited mRNA (*N*) in total RNA isolated at day 4 post-induction were determined by PPE as described under "Experimental Procedures." Underneath the gels of edited RNAs, diagrams indicate the percent change in *E/E* + *P* (= relative amount of edited RNA) following RNAi induction with *E/E* + *P* levels in non-induced cells set at 100%. *E/E* + *P* levels in non-induced cells are comparable with those obtained previously (16, 49). For never edited mRNAs and rRNA, the percent change in amount following induction is given. Nucleus-encoded, never edited α -tubulin mRNA was used as an internal control (data not shown).

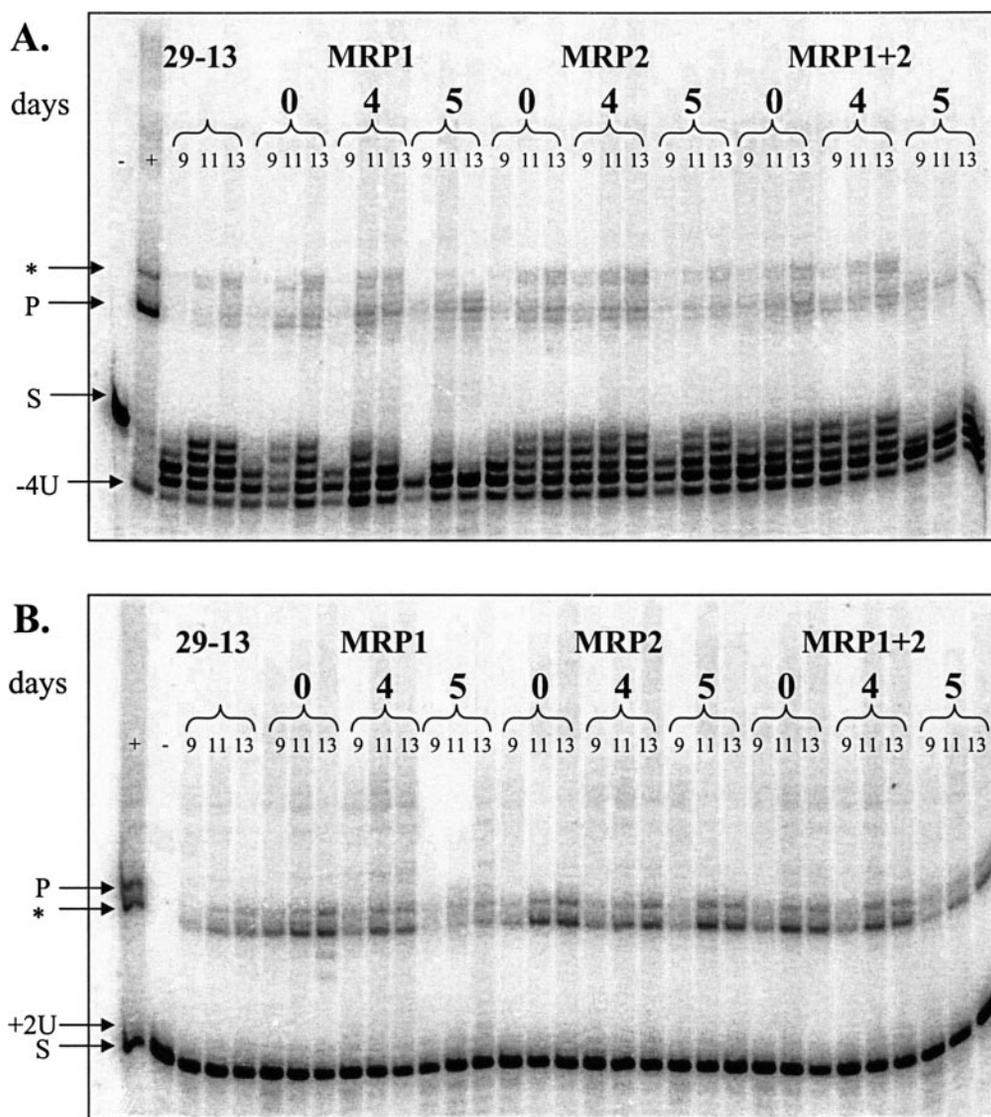


FIG. 8. Effect of MRP RNAi on precleaved deletion and insertion editing *in vitro*. *In vitro* precleaved deletion (A) and insertion (B) editing of the pooled most active glycerol gradient fractions (fractions 9, 11, and 13) of whole cell lysates from wild type 29-13 cells and from knock-down cells before and 4 and 5 days after MRP1, MRP2, and MRP1+2 RNAi induction. The positions of the radiolabeled 5'-CL18 or U5-5'-CL pre-mRNA substrates (S), products with uridines removed (-4U) or with two uridines added (+2U), and the edited product (P) have been indicated. Ligation of the substrate RNA to the 3'-fragment with no uridines removed or added is indicated by an asterisk.

form (22) and the substantial effects on growth and editing in the knock-downs reported here. Thus, the MRPs appear to have more critical roles in the procyclic *versus* the bloodstream stage. This stage specificity could be the basis for the greater abundance of edited ND7 mRNA in the procyclic knock-downs reported here, perhaps by negative regulatory processes that involve the MRPs. It may also account for the greater abundance of edited ND7 mRNA in MRP1 null mutant bloodstream forms compared with the wild type. Indeed, the 3'-domain of ND7 mRNA is only partially edited in procyclics but fully is edited in the bloodstream form *T. brucei* (48). These considerations raise the question of how the transcript-specific and stage-specific effects might be mediated. Both MRP1 and MRP2 are present in similar amounts in both life cycle stages (Fig. 3C). Although specific sequence recognition, possibly mediated by processes such as posttranslational modifications of MRPs (*e.g.* phosphorylation), come to mind, no relevant data appear to exist. Further work is obviously required to shed light on the precise role of the MRP complex in RNA processing and the interplay between the MRPs and other potential editing proteins and complexes.

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