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# Pentatricopeptide Repeat Proteins in *Trypanosoma brucei* Function in Mitochondrial Ribosomes▽†

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**The pentatricopeptide repeat (PPR), a degenerate 35-amino-acid motif, defines a novel eukaryotic protein family. Plants have 400 to 500 distinct PPR proteins, whereas other eukaryotes generally have fewer than 5. The few PPR proteins that have been studied have roles in organellar gene expression, probably via direct interaction with RNA. Here we show that the parasitic protozoan *Trypanosoma brucei* encodes 28 distinct PPR proteins, an extraordinarily high number for a nonplant organism. A comparative analysis shows that seven out of eight selected PPR proteins are mitochondrially localized and essential for oxidative phosphorylation. Six of these are required for the stabilization of mitochondrial rRNAs and, like ribosomes, are associated with the mitochondrial membranes. Furthermore, one of the PPR proteins copurifies with the large subunit rRNA. Finally, ablation of all of the PPR proteins that were tested induces degradation of the other PPR proteins, indicating that they function in concert. Our results show that a significant number of trypanosomal PPR proteins are individually essential for the maintenance and/or biogenesis of mitochondrial rRNAs.**

Sequencing of the *Arabidopsis* genome led to the discovery of a very large protein family that is defined by degenerate 35-amino-acid pentatricopeptide repeat (PPR) motifs. PPR proteins contain 2 to 26 PPR motifs that usually are arranged as tandem arrays (45). The genome of *Arabidopsis* encodes 450 distinct PPR proteins. Experimental and bioinformatic analyses have shown that most of these are targeted to organelles (approximately 75% to mitochondria and 25% to plastids) (22). Inactivation of plant PPR proteins frequently causes embryonic lethality (10, 22), showing that despite the large size of the PPR protein family, there is little functional redundancy. Studies of individual PPR proteins indicate that they function at essentially all levels of organellar gene expression, including transcription, RNA processing and editing, and RNA stability and translation (1, 31, 41). The simplest explanation for these diverse functions is that PPR proteins are sequence-specific RNA binding proteins capable of recruiting effector enzymes to defined sites on organellar RNAs (22). This idea is supported by structural models of the PPR motif and by the fact that a number of PPR proteins are able to bind RNAs in vitro. However, evidence of binding specificity for physiological substrates has been shown in only a few cases (27, 30, 34).

One of the most intriguing aspects of PPR proteins is their phylogenetic distribution. They seem to be absent from the bacterial domain. No PPR proteins have been identified in the genomes of *Escherichia coli*, *Rickettsia prowazekii*, and *Synechocystis* (22), the latter two being the closest extant relatives of mitochondria and plastids, respectively. However, with a few exceptions, PPR proteins are found in all eukaryotes. Interestingly, there is an extraordinary discrepancy between their numbers in plant and nonplant organisms. Whereas plants have several hundred PPR proteins, only five and six putative PPR proteins are encoded by the yeast and human genomes, respectively (22).

A number of nonplant PPR proteins have been studied as well. PET309, the first PPR protein to be investigated, is a yeast protein essential for expression of the mitochondrial *cox1* gene (23). A similar PPR protein in humans also is required for correct expression of COX1, and mutations in this PPR gene are linked to genetic myopathies (29). The picture emerging from these and other studies (reviewed in reference 1) is that, as in plants, PPR proteins are involved in the expression of specific mitochondrial RNAs. However, their mode of action remains very poorly understood.

A recent study identified 23 distinct putative PPR proteins in the genome of the parasitic protozoan *Trypanosoma brucei* (28). Our own analysis of the *T. brucei* genome using different bioinformatic methods detected 28 distinct PPR proteins (Table 1; also see Fig. S1 in the supplemental material). These numbers are much higher than those for any other nonplant organism. The mitochondrial RNA metabolism of *T. brucei* is known to have many unique features. The most prominent ones in the context of this work are RNA editing and aberrant short rRNAs. Most mitochondrial mRNAs in *T. brucei* require extensive RNA editing by multiple uridine insertions and/or deletions. However, unlike the case in plant organelles, where the specificity of editing is likely determined by PPR proteins (20, 41), in trypanosomatids the specificity of RNA editing is mediated by short RNA transcripts, termed guide RNAs. The 12S large subunit (LSU) and 9S small subunit (SSU) rRNAs of trypanosomatid mitochondria are among the smallest found in nature (11, 12); as a consequence, the mitochondrial ribosomes

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TABLE 1. Genomic analysis of PPR proteins of *T. brucei*

| Protein investigated in this study | *T. brucei* | | | | | *L. major* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Open reading frame | PPR probability (%)[a] | No. of PPRs | Targeting prediction[b] | Identified previously[c] | Open reading frame | PPR probability (%)[a] | No. of PPRs | Identified previously[d] |
| | Tb927.8.6040 | 100 | 4 | Cyto. | Y | LmjF24.2200 | 100 | 3 | |
| | Tb927.8.4860 | 100 | 9 | Mito. | Y | LmjF16.1560 | 99.3 | 3 | |
| | Tb927.6.4190 | 100 | 10 | Cyto. | Y | LmjF30.2880 | 100 | 8 | |
| TbPPR1 | Tb927.2.3180 | 100 | 15 | Mito. | Y | LmjF18.0010 | 100 | 20 | |
| TbPPR2 | Tb927.1.2990 | 100 | 8 | Mito. | N | LmjF20.1580 | 100 | 8 | |
| TbPPR3 | Tb927.1.1160 | 100 | 3 | Mito. | Y | LmjF20.0480 | 100 | 4 | |
| TbPPR8 | Tb11.01.6040 | 100 | 15 | Mito. | Y | LmjF32.1210 | 100 | 9 | |
| | Tb10.70.7360 | 100 | 3 | Mito. | Y | | | | |
| | Tb10.70.5780 | 100 | 8 | Mito. | Y | LmjF21.0260 | 100 | 8 | |
| | Tb10.6k15.0120 | 100 | 6 | Mito. | Y | LmjF36.4810 | 100 | 6 | |
| TbPPR4 | Tb10.389.0260 | 100 | 6 | Mito. | Y | LmjF18.0910 | 98.6 | 5 | |
| | Tb09.211.3720 | 100 | 13 | Mito. | Y | LmjF35.2950 | 100 | 13 | |
| | Tb927.7.1350 | 100 | 2 | Mito. | Y | LmjF26.0610 | 100 | 2 | |
| TbPPR7 | Tb927.4.4720 | 100 | 4 | Mito. | Y | LmjF31.1700 | 22.2 | 3 | |
| | Tb10.61.2890 | 100 | 4 | Mito. | Y | LmjF18.0500 | 94.6 | 3 | |
| | Tb11.02.5120 | 100 | 3 | Mito. | Y | LmjF28.0040 | 12.2 | 3 | |
| TbPPR6 | Tb927.3.4550 | 99.9 | 4 | Mito. | N | LmjF29.2010 | 100 | 5 | |
| | Tb927.6.4400 | 99.4 | 5 | Mito. | Y | LmjF30.3100 | 99.8 | 6 | |
| TbPPR5 | Tb10.70.7960 | 98.4 | 2 | Mito. | N | LmjF21.1620 | 99.9 | 2 | Y |
| | Tb11.01.7210 | 96.5 | 5 | Cyto. | N | | | | |
| | Tb11.01.5980 | 95.5 | 2 | Mito. | N | | | | |
| | Tb11.03.0440 | 86.1 | 2 | Mito. | N | LmjF25.0630 | 69.8 | 2 | |
| | Tb927.6.4450 | 85.6 | 4 | Mito. | Y | LmjF30.3100 | 99.8 | 6 | |
| | Tb927.4.2790 | 75.2 | 3 | Mito. | N | LmjF34.1460 | 99.3 | 2 | |
| | Tb927.8.3170 | 62.1 | 1 | Mito. | N | LmjF23.1240 | 90.6 | 1 | |
| | Tb927.3.5240 | 36.5 | 2 | Mito. | Y | LmjF29.0430 | 72.3 | 3 | Y |
| | Tb927.5.1710 | 30.6 | 2 | Mito. | N | LmjF15.0280 | 86.0 | 3 | |
| | Tb11.02.3180 | 28.3 | 3 | Mito. | N | LmjF24.0830 | 40.0 | 2 | Y |

[a] Score obtained by the TPRpred tool for identifying proteins carrying repeated helical repeats (18).
[b] Predicted subcellular localization by Predotar (44) and Mitoprot (9). Cyto., cytosolic; Mito., mitochondrial.
[c] Y indicates a PPR protein identified previously by Mingler et al. (28).
[d] Y indicates a PPR protein identified in the proteomics study of *L. tarentolae* mitoribosomal particles by Maslov et al. (25).

(mitoribosomes) of *T. brucei* are expected to have a higher protein content than other ribosomes (24, 25).

The fact that the PPR protein family of *T. brucei* is expanded by approximately an order of magnitude compared to those of other eukaryotes makes trypanosomes an excellent system to study the function of nonplant PPR proteins. Here we present a comparative analysis of eight putative PPR proteins of *T. brucei*.

## MATERIALS AND METHODS

**Bioinformatic analysis.** Predicted *T. brucei* protein sequences were obtained from the version 4 release of the genome annotation (3). The *T. brucei* proteome was screened for PPR proteins using TPRpred (18). Proteins with a TPRpred probability score of over 25% were retained. PPR motifs are characteristically found in tandem arrays, and therefore all proteins not identified as containing tandem arrays of motifs were investigated further using BLAST searches against the total nonredundant protein database at NCBI. Twelve proteins lacking obvious matches to multiple known PPR proteins across a region of over 70 amino acids (indicating at least two motifs in tandem) were eliminated from the study. The resulting set of 28 *T. brucei* proteins was used to recover putative *Leishmania major* orthologues from GeneDB (16). Putative PPR motifs in the *L. major* proteins then were detected as described above. The complete set of 53 putative trypanosomatid PPR proteins was aligned with ClustalW.

**Epitope tagging.** To localize the eight PPR proteins, we produced transgenic cell lines expressing protein variants containing a carboxy-terminal Ty-1 tag (for TbPPR2, TbPPR4, TbPPR6, TbPPR7, and TbPPR8) or a hemagglutinin (HA) tag (for TbPPR1, TbPPR3, and TbPPR5), respectively. Both tags are routinely used with *T. brucei* (2, 40). The Ty-1 tag is detected by the monoclonal BB2 antibody, and the HA tag is visualized by the monoclonal antibody HA11 (Covance Research Products). Localization was analyzed either by immunofluorescence or by immunoblotting. Cell fractionation was done by digitonin extraction as described previously (7). For the experiment shown in Fig. 7B, we used TbMRPL21 (Tb927.7.4140), a mitochondrial ribosomal protein of the LSU, containing three copies of the c-*myc* tag at its C terminus. The genes encoding the tagged TbPPR1, TbPPR3, TbPPR5, and TbMRPL21 were integrated into their own genomic regions to achieve levels of expression that were as close as possible to natural levels (32, 40). The tagged TbPPR2, TbPPR4, TbPPR6, TbPPR7, and TbPPR8 were cloned into a derivative of the plasmid pLew-100 (47), which allows tetracycline-inducible overexpression of the tagged proteins (2). None of the cell lines expressing tagged PPR proteins exhibited a growth phenotype in SDM-79 medium.

**Production and analysis of RNAi cell lines.** RNA interference (RNAi) was performed using stem-loop constructs containing the puromycin resistance gene as described previously (5). As inserts, we used the sequences of the following open reading frames (nucleotide positions are indicated in parentheses): TbPPR1, Tb927.2.3180 (639 to 1190); TbPPR2, Tb927.1.2990 (446 to 1003); TbPPR3, Tb927.1.1160 (385 to 863); TbPPR4, Tb10.389.0260 (555 to 1123); TbPPR5, Tb10.70.7960 (399 to 988); TbPPR6, Tb927.3.4550 (130 to 596); TbPPR7, Tb927.4.4720 (487 to 979); and TbPPR8, Tb11.01.6040 (567 to 1087). Transfection of *T. brucei*, selection with antibiotics, cloning, and induction with tetracycline were done as described previously (26). All transgenic cell lines used in this study are based on *T. brucei* strain 29-13, which was grown in SDM-79 medium supplemented with 15% fetal calf serum (FCS), 50 μg/ml hygromycin, and 15 μg/ml G-418. Growth of RNAi cells also was analyzed in SDM-80 medium, a modification of SDM-79 that lacks glucose and that is supplemented with 10% dialyzed FCS, as described previously (21), both in the presence and in the absence of 1 μg/ml of tetracycline.

**Northern blots.** Total RNA (5 μg each) was separated on 1% (wt/vol) agarose gels containing 0.2 M formaldehyde and was electrophoresed in 20 mM morpholinepropanesulfonic acid-NaOH, pH 7.0, and 0.2 M formaldehyde. After being transferred, the GeneScreen Plus membranes were UV cross-linked and baked. As probes, we used random hexamer [α-$^{32}$P]dCTP-labeled double-stranded DNA fragments corresponding to the following regions (nucleotide positions are indicated in parentheses) of the indicated mitochondrion-encoded proteins: COX1 (18 to 1526), COX2 (113 to 620), CYTB (87 to 1032), unedited RPS12 (2 to 221), and unedited A6 (182 to 400). Furthermore, DNA fragments corresponding to the indicated region of two edited mRNAs, RPS12 (2 to 325) and A6 (374 to 819), were used. Finally, detection of the mitochondrial rRNAs was done by the following $^{32}$P-kinase oligonucleotides: 9S rRNA, TTGGTTAA ATCAGCACTTAAC, and 12S rRNA, CTTGTTAACCTGCTCGAACC.

For the Northern blot analysis shown in Fig. 4, RNA was isolated (i) 24 h before the growth arrest, (ii) at the time the growth arrest became apparent, and

(iii) 24 h after the growth arrest. In all cases, essentially identical results were obtained. Thus, for the statistical analysis (see Fig. 4B), all data points within this 48-h time period were combined.

**Subfractionation of mitochondria.** Mitochondria were isolated using the hypotonic procedure and were resuspended in 20 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 2 mM EDTA (37). Next, 15 μl (approximately 300 μg proteins) of the mitochondrial fraction was diluted with 135 μl of 10 mM $MgCl_2$ and was subjected to 10 successive cycles of flash freezing in liquid $N_2$ and thawing at 25°C. The resulting suspension was centrifuged for 5 min at 10,000 × *g* at 4°C, yielding a supernatant (matrix fraction) and a pellet (membrane fraction) that were resuspended in 150 μl of 10 mM $MgCl_2$. A volume of 12.5 (supernatant) and 100 μl (pellet) of each fraction was analyzed by immunoblotting and, after RNA extraction, by Northern blotting, respectively.

**Immunoprecipitation.** Twenty-five microliters (approximately 500 μg total protein) of hypotonically isolated mitochondria was lysed at 4°C using 500 μl of lysis buffer (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.5% Triton X-100). The suspension was cleared by centrifugation (10 min at 10,000 × *g*), and the resulting supernatant, corresponding to the total fraction, was combined with 50 μl of anti-HA affinity matrix (Roche Applied Science) and incubated on a rotator for 3 h at 4°C. The beads were reisolated, yielding a supernatant corresponding to the unbound fraction. The pellet was washed thrice using lysis buffer containing 0.1% Triton X-100. Half of each of the washed pellets representing the bound fraction were boiled in sodium dodecyl sulfate sample buffer to analyze the bound proteins or were subjected to RNA extraction to analyze the bound RNAs.

**Miscellaneous notes.** All RNA isolations were done by the acid guanidinium method (8). ATP production assays using digitonin-purified mitochondria from RNAi cell lines were done as described previously (5, 36). Assays were done at the time when the growth arrest became apparent. Carbonate extraction of mitochondrial membranes was done as described previously (35).

## RESULTS

**The *T. brucei* genome encodes 28 putative PPR proteins.** To identify PPR proteins in trypanosomatids, we screened the predicted *T. brucei* proteome with TPRpred (18), a new, sensitive tool for identifying proteins carrying repeated helical repeats such as PPR and TPR motifs. In this manner, we could increase the already unexpectedly large number of putative PPR proteins known in this group of organisms (Table 1). Mingler et al. (28) reported 23 PPR proteins in *T. brucei*, whereas our analysis identifies 28, of which 10 were unique to our study (Table 1). Conversely, five of the proteins identified in the previous analysis did not pass the criteria we used to define PPR proteins. Figure S1 in the supplemental material shows the distance tree obtained by aligning the putative PPR proteins of *T. brucei* and *L. major* together with schematic views of each protein, including the positions of the identified PPR motifs. The number of formally detected PPR motifs in these proteins ranges from 1 to 20, but in virtually all cases the sequence alignments and secondary structure predictions (data not shown) strongly suggest that structurally similar motifs adjoin, or are interspersed with, the motifs shown in Fig. S1 in the supplemental material. Thus, the number of motifs indicated represents an underestimation, and the total number of PPR proteins in the *T. brucei* proteome still may be an underestimate. The likely subcellular localization of the candidate PPR proteins was predicted using Predotar (44) and Mitoprot (9). Both programs strongly predicted a mitochondrial localization for almost all of these proteins. The exceptions are the clusters related to Tb927.6.4190, Tb927.8.6040, and Tb11.01.7210 (Table 1). The predicted mitochondrial localization is consistent with the current understanding of PPR protein function. The *L. major* genome (17) contains putative orthologues for 25 of 28 *T. brucei* PPR proteins (discounting one recently duplicated *T. brucei* gene). This is a greater proportion than that for the genome as a whole; overall, only 77% of *T. brucei* genes have clear orthologues in *L. major* (13). Thus, PPR genes are particularly well conserved in trypanosomatids and are likely to have conserved functions. To begin to delineate what these functions might be, we selected eight putative PPR proteins of *T. brucei*, termed TbPPR1 to TbPPR8, for further analysis.

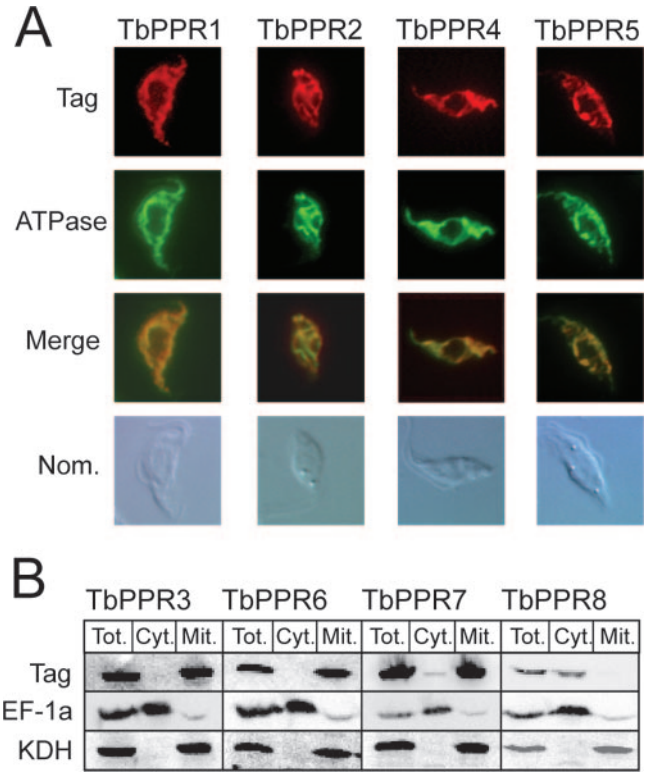


FIG. 1. Localization of epitope-tagged TbPPR1 to TbPPR8. (A) Double immunofluorescence analysis of *T. brucei* cell lines expressing the Ty-1 tag or the HA tag at their carboxy termini. The cells were double stained with monoclonal antitag antibodies (Tag) and a polyclonal antiserum directed against a subunit of the mitochondrial ATPase (ATPase). A merged picture of the antitag antibody and the ATPase staining (Merge) as well as the Nomarski picture (Nom.) is shown in the bottom two panels. (B) Immunoblot analysis of $0.3 \times 10^7$ cell equivalents each of total cellular (Tot.), crude cytosolic (Cyt.), and crude mitochondrial (Mit.) extracts for the presence of the indicated tagged PPR proteins (top panels). Only the relevant regions of the blots are shown. Comparison to molecular size markers showed that the sizes of the tagged proteins were consistent with the predictions (data not shown). Elongation factor 1a (EF-1a) served as a cytosolic marker (middle panel), and KDH served as a mitochondrial marker (bottom panel).

**Seven out of eight selected PPR proteins are mitochondrial.** All selected PPR proteins are predicted to have mitochondrial targeting signals. In order to determine their localization experimentally, we prepared transgenic cell lines allowing expression of the eight PPR proteins carrying either the Ty-1 peptide or the HA tag at their carboxy termini. Immunofluorescence analysis using antitag antibodies showed a colocalization of tagged TbPPR1, TbPPR2, TbPPR4, and TbPPR5 with the mitochondrial marker (Fig. 1A).

The other four cell lines did not give a signal in immunofluorescence and therefore were subjected to a more sensitive

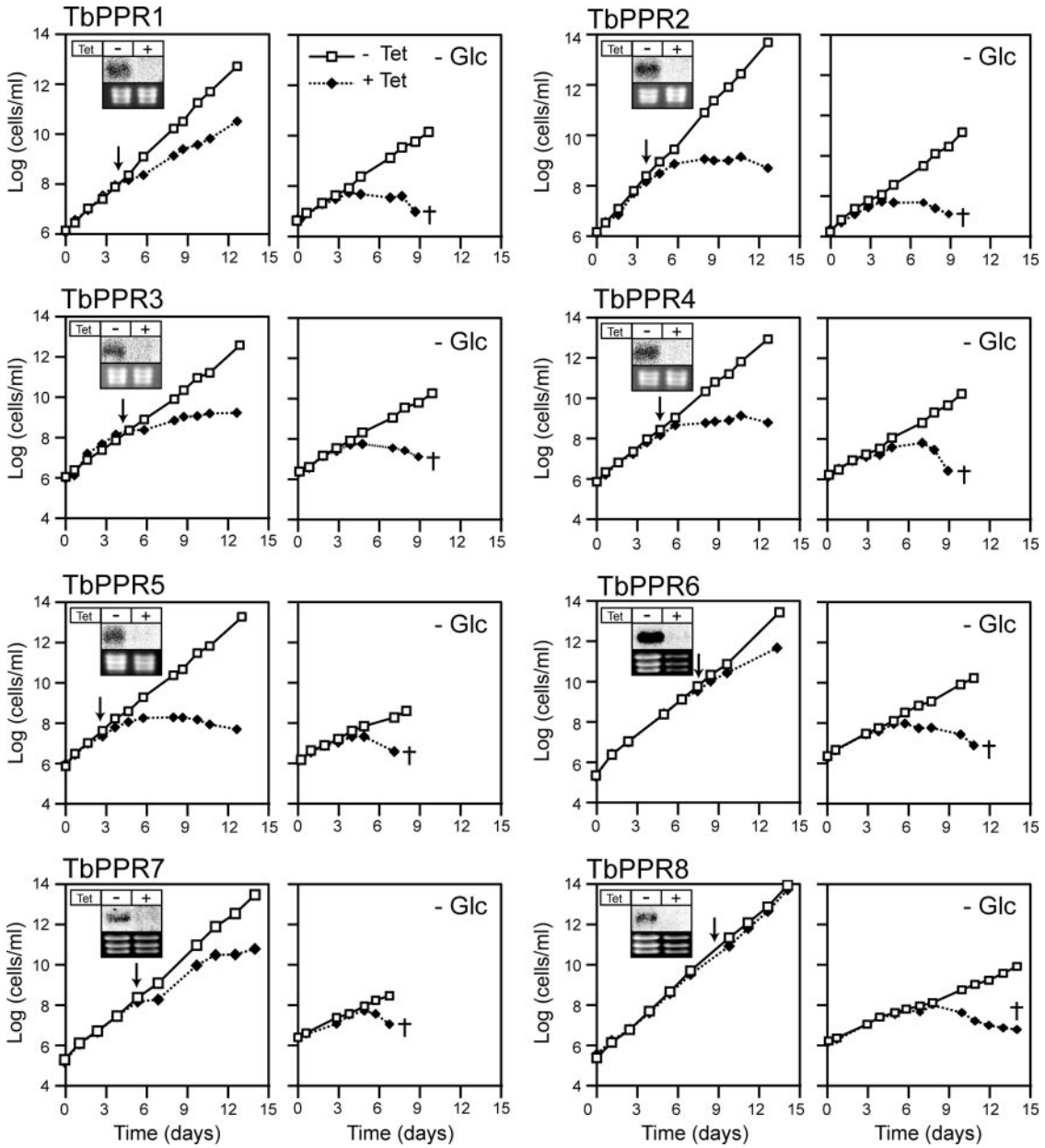


FIG. 2. TbPPR1 to TbPPR8 are required for growth and survival in glucose-free culture medium. Shown are representative growth curves of uninduced (− Tet) and induced (+ Tet) representative clonal *T. brucei* TbPPR1 to TbPPR8 RNAi cell lines in standard culture medium SDM-79 (left graphs) and in culture medium SDM-80 that lacks glucose (−Glc) (right graphs). The crosses indicate that further incubation led to the death of the whole population. Insets depict Northern blots of the corresponding TbPPR mRNAs. The RNA from induced cells was isolated at the time of the growth arrest (arrows). The rRNAs in the lower panel serve as loading controls.

biochemical analysis. Digitonin extractions were used to prepare mitochondrial and cytosolic fractions that could be analyzed by immunoblotting (7). The results showed that the tagged TbPPR3, TbPPR6, and TbPPR7 copurify with the mitochondrial marker (Fig. 1B).

Tagged TbPPR8 is predominantly localized in the cytosol, even though it is predicted to have a mitochondrial import signal (Table 1). However, while most of TbPPR8 is cytosolic, it cannot be excluded that a small fraction of it is imported into the mitochondrion.

In summary, except for TbPPR8, all selected PPR proteins are exclusively localized in mitochondria.

**All tested PPR proteins are required for normal growth.** To study the function of the selected PPR proteins, we established stable transgenic cell lines that allow inducible RNAi-mediated ablation of each of the eight proteins. For each cell line, the efficiency of RNAi was verified by Northern analysis (Fig. 2, insets). Furthermore, even though PPR proteins belong to the same family, their nucleotide sequences show little similarity. Off-target effects of the RNAi can therefore be excluded.

Growth of uninduced and induced RNAi cell lines was tested in both the standard culture medium SDM-79 (6) (Fig. 2, left graphs), which contains proline and glucose as the major energy sources, and in SDM-80, a modified version of SDM-79 that lacks glucose (21) (Fig. 2, right graphs, −Glc). Three distinct phenotypes were observed in SDM-79. Ablation of TbPPR2, TbPPR3, TbPPR4, and TbPPR5 caused growth arrest, whereas induced TbPPR1, TbPPR6, and TbPPR7 RNAi cell lines kept growing, although at a much lower rate. Finally, growth of cells downregulated for TbPPR8 was not or was only marginally affected.

Interestingly, however, in SDM-80 all induced RNAi cell lines showed the same phenotype: they stopped growing, and a few days later they started to die. Thus, all eight tested PPR proteins are essential for survival in SDM-80.

In procyclic *T. brucei*, glucose (after conversion into pyruvate) is used for mitochondrial substrate-level phosphorylation (SUBPHOS) in the trypanosome-specific acetate:succinate coenzyme A (CoA) transferase/succinyl-CoA synthetase cycle (33). When grown in SDM-79, where glucose is available, the energy needs of *T. brucei* can be fulfilled by substrate-level phosphorylation alone (5, 21). In glucose-free SDM-80, however, the sole energy source is proline that can be utilized only by oxidative phosporylation (OXPHOS) (21). Growth in SDM-80 therefore selects for cells capable of performing efficient OXPHOS.

The mitochondrial gene products of *T. brucei* include cytochrome oxidase subunits (COX1 to COX3) and cytochrome *b* (CYTB), subunit 6 of the ATPase (A6), a ribosomal protein (RPS12), and the SSU and LSU rRNAs (9S and 12S rRNA). These gene products either function directly in OXPHOS or are components of the mitochondrial translation machinery, the function of which is to produce components of the OXPHOS complexes. Based on results with plants, PPR proteins are expected to be involved in posttranscriptional processes required for mitochondrial gene expression. Should this also be the case for *T. brucei*, we predict that the lack of PPR proteins ultimately will affect OXPHOS. The fact that RNAi-mediated ablation of all tested trypanosomal PPR proteins causes cell death in glucose-free SDM-80 medium but much milder phenotypes in SDM-79 medium supports this prediction. In conclusion, the results show that all eight PPR proteins analyzed perform nonredundant functions that ultimately are required for OXPHOS.

**All tested PPR proteins are required for efficient OXPHOS.** We have recently established an assay that allows us to quantify the different modes of ATP production in isolated mitochondria of *T. brucei* (5). This assay enables us to confirm whether the lack of growth of induced RNAi cell lines on glucose-free medium is indeed due to deficient OXPHOS. Besides OXPHOS, *T. brucei* mitochondria can produce ATP either by SUBPHOS in the citric acid cycle or in the trypanosome-specific acetate:succinate CoA transferase/succinyl-CoA synthetase cycle (5, 33). To measure OXPHOS, mitochondria are incubated with ADP and succinate. To measure SUBPHOS in the citric acid cycle, α-ketoglutarate is used as a substrate, whereas measuring SUBPHOS in the acetate:succinate CoA transferase/succinyl-CoA synthetase cycle requires the addition of pyruvate as well as the cosubstrate succinate (5). Atractyloside treatment prevents mitochondrial import of the added ADP and thus will inhibit all forms of mitochondrial ATP production. OXPHOS, in contrast to either form of SUBPHOS, is antimycin sensitive.

We tested the ability of mitochondria from all RNAi knockdown cell lines to perform either OXPHOS or SUBPHOS using both methods.

Figure 3 shows that ablation of TbPPR1 to TbPPR7 selectively knocks down OXPHOS (induced by succinate) but does not interfere with either of the two forms of SUBPHOS (induced by α-ketoglutarate or pyruvate). Whereas OXPHOS was completely inhibited in TbPPR1 to TbPPR5 and TbPPR7 RNAi cell lines, it was only approximately 50% reduced in TbPPR6 RNAi cells. This is consistent with the relatively weak growth phenotype that ablation of TbPPR6 causes on SDM-79 medium (Fig. 2), and it indicates that ablation of TbPPR6 only slightly interferes with the level of OXPHOS activity required for normal growth in SDM-79 medium.

Furthermore, we also tested TbPPR8 (Fig. 3B). It is required for growth in glucose-free SDM-80 medium, despite being mainly or even only extramitochondrially localized (Fig. 1B). SDM-80 medium selects for cells having maximal OXPHOS activity, suggesting that TbPPR8 function is at least indirectly connected to OXPHOS. This is supported by the ATP production assays presented in Fig. 3B, which show that ablation of TbPPR8 causes a partial reduction of OXPHOS to approximately 60%. Thus, these results explain why the lack of TbPPR8 does not affect growth in SDM-79 medium that allows cells to grow even if their OXPHOS activity is suboptimal. It is possible that TbPPR8 stabilizes a cytosolic mRNA encoding a mitochondrial protein that is required for efficient OXPHOS. However, we did not further study TbPPR8, since the focus of the present work was on the mitochondrially localized PPR proteins.

**Lack of six out of eight PPR proteins affects mitochondrial rRNAs.** Total RNA from uninduced and induced RNAi cell lines, isolated at the time of the first apparent growth phenotype, was analyzed by Northern hybridization to determine the steady-state levels of mitochondrially encoded RNAs. Blots were probed for COX1, COX2, and CYTB (for TbPPR1 to TbPPR8) as well as for RPS12 and A6 mRNAs (for TbPPR1 to TbPPR5). COX2 and CYTB transcripts are edited in a small domain only. The hybridization probes therefore detected both edited and unedited RNAs. RPS12 and A6 transcripts, on the other hand, are extensively edited. Thus, two probes were used: one that detects unedited and minimally edited RNAs, and another one that recognizes extensively and fully edited molecules. Finally, the levels of 9S and 12S rRNAs were analyzed in all eight RNAi cell lines.

As expected based on previous analyses, most mRNAs in the uninduced cell lines showed a doublet of closely spaced bands (4) (Fig. 4). These correspond to two mRNA populations having poly(A) tails of different lengths (approximately 20 nucleotides and 200 nucleotides). In six out of the eight induced cell lines, the two bands converged into a single, more intense band corresponding in size to the population having a short poly(A) tail. The presence of a short poly(A) tail on the COX1 mRNA in induced TbPPR2 cells was experimentally verified by circular reverse transcription-PCR (data not shown). Furthermore, comigration of oligo(dT)/Rnase H-treated CYTB mRNA in induced TbPPR2 with the corresponding untreated mRNA is

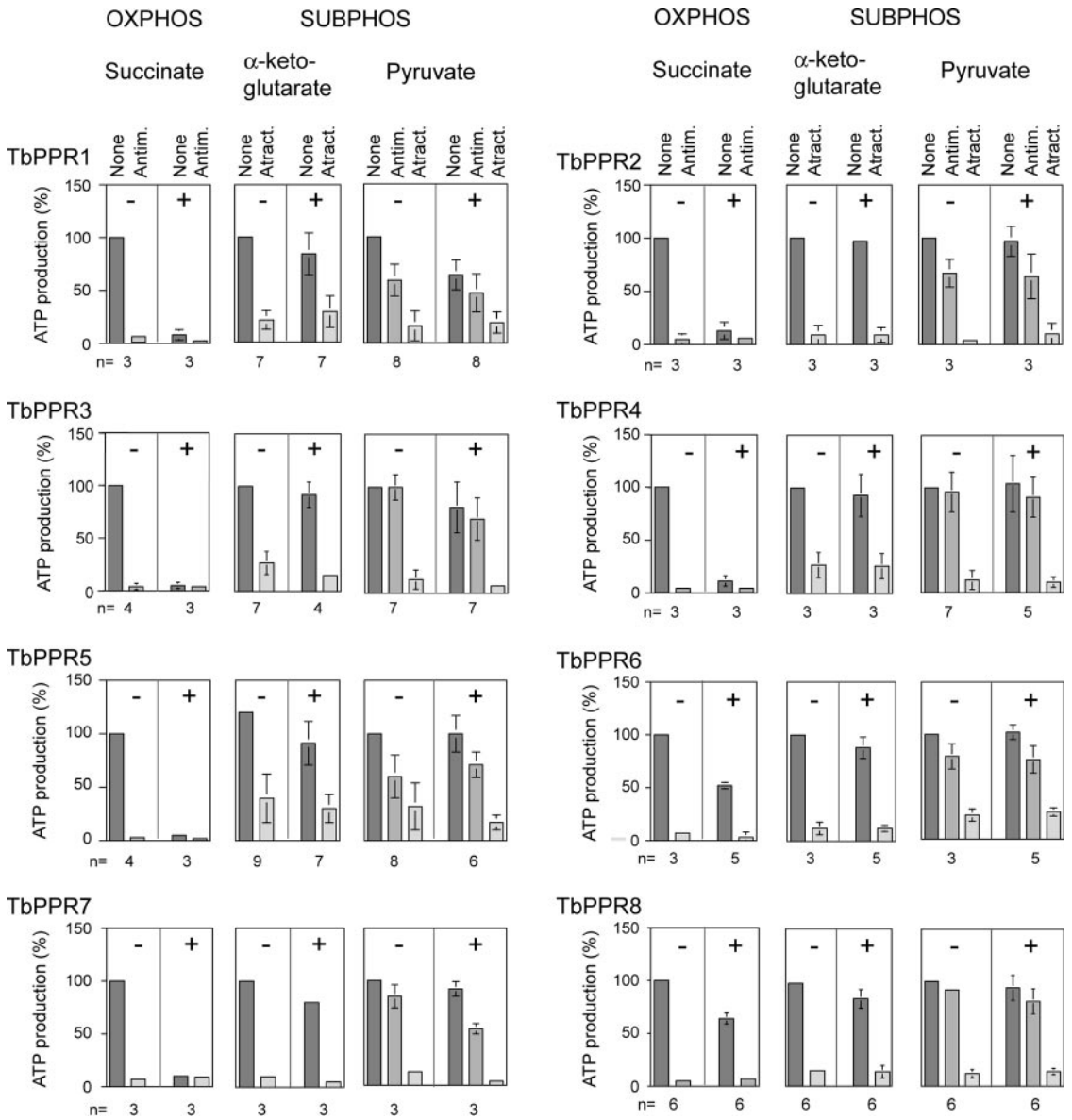


FIG. 3. Ablation of TbPPR1 to TbPPR8 selectively abolishes OXPHOS. Succinate-, α-ketoglutarate-, and pyruvate-induced mitochondrial ATP production in crude mitochondrial fractions from uninduced (−) and induced cells (+) is shown for TbPPR1 to TbPPR8 RNAi cell lines. The substrates tested and the additions of antimycin (Antim.) and atractyloside (Atract.) are indicated at the top. ATP production in mitochondria isolated from uninduced cells tested without antimycin or atractyloside is set to 100%. The bars represent means expressed as percentages. Standard errors and the number of independent replicates (n) are indicated.

consistent with the absence of the long poly(A) tail population in the knockdown cells (Fig. 5A). Shortening of the poly(A) tail was seen for all mature mRNAs, at least at later times of induction (in Fig. 4 this is best visible for COX2, CYTB, and edited RPS12). This suggests that it is an indirect effect of PPR protein depletion caused by the reduced ATP levels due to the loss of OXPHOS. In order to test this possibility, we performed Northern analyses for COX1, COX2, and CYTB mRNAs in RNAi cell lines ablated for cytosolic and mitochondrial tryptophanyl-tRNA synthetases (TrpRS) (Fig. 5B). These two proteins are essential for the normal growth of procyclic *T. brucei*, as was the case for TbPPR1 to TbPPR7 (7). Furthermore, ablation of the mitochondrial TrpRS shows a selective inhibition of OXPHOS identical to that observed in the induced TbPPR1 to TbPPR5 RNAi cell lines (Fig. 3). Figure 5 shows that no qualitative differences of COX1, COX2, and CYTB mRNAs were observed in cells ablated for cytosolic TrpRS. However, in cells ablated for the mitochondrial enzyme, only the lower band was detected. Thus, ablation of the mitochondrial TrpRS had the same effect on mitochondrial polyadenylation as knockdown of six of the eight tested PPR proteins. Moreover, the lack of accumulation of the short poly(A) tails in the TbPPR6 and TbPPR8 knockdown cell lines is consistent with the observation that these two cell lines grow essentially normally on SDM-79 medium (Fig. 2)

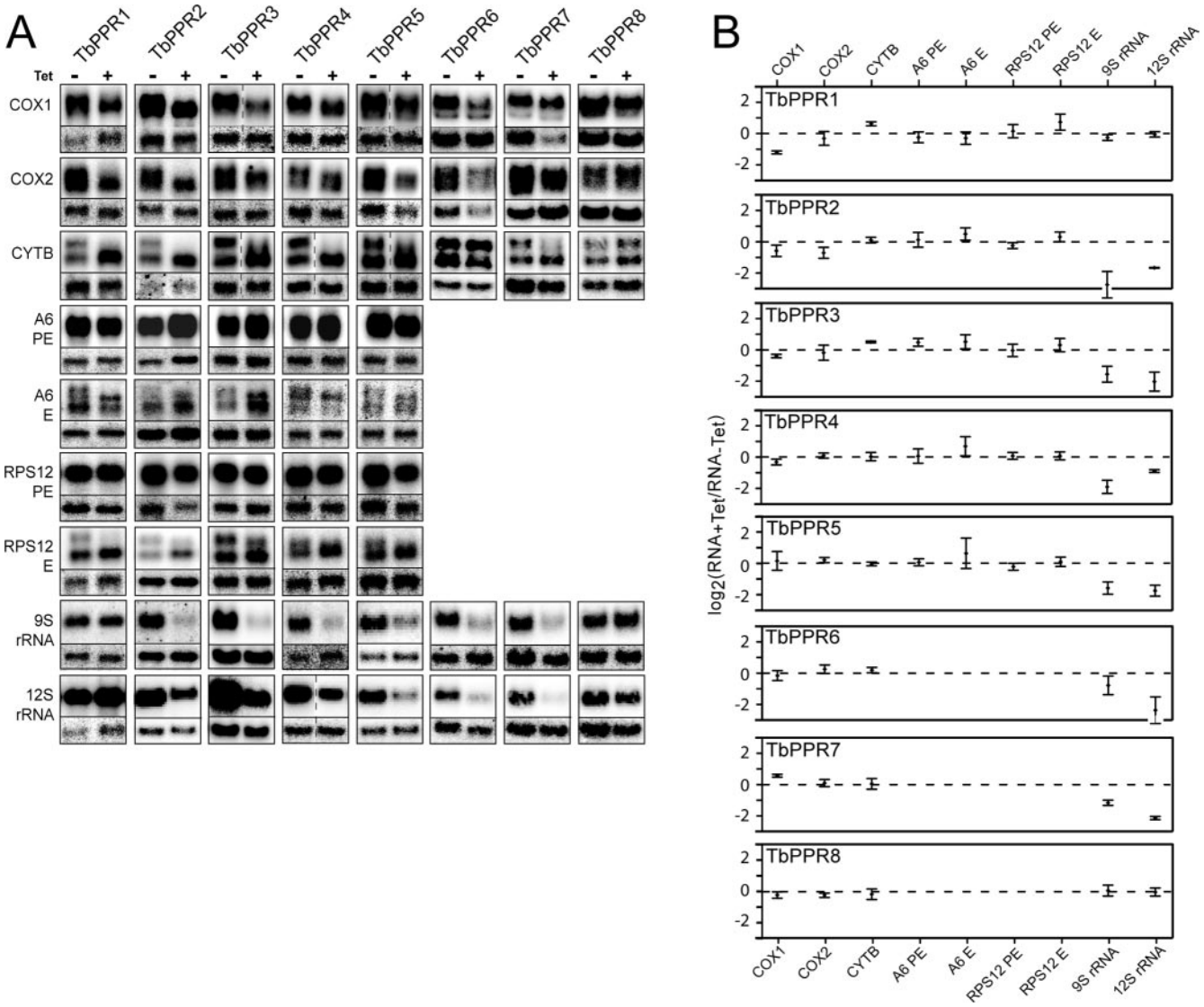


FIG. 4. Lack of six out of eight PPR proteins affects mitochondrial rRNAs. (A) Total RNA from the indicated uninduced (−Tet) and induced (+Tet) RNAi cell lines was analyzed for the levels of COX1, COX2, CYTB, preedited (PE) A6 and edited (E) A6, preedited and edited RPS12 RNAs, and 9S and 12S rRNAs using Northern hybridization. To normalize for loading differences, each filter was reprobed for the mRNA of the cytosolic TrpRSs (lower part of each panel). (B) Graph showing the means of $\log_2$-transformed normalized levels of each RNA species relative to that in uninduced cells [$\log_2(\mathrm{RNA}_{+\mathrm{Tet}}/\mathrm{RNA}_{-\mathrm{Tet}})$] obtained from three to six independent RNAi experiments. The 95% confidence intervals (means ± 2 standard errors) are indicated.

and with the only partial inhibition of OXPHOS observed in the TbPPR8 cell lines (Fig. 3B).

To obtain accurate quantitative information on mitochondrial RNA levels, we performed replicate Northern blots ($n$ = 3 to 6) for each of the tested RNA species in all eight RNAi cell lines. The levels of the tested mRNAs were normalized using the mRNA of cytosolic TrpRS as an internal standard. Figure 4B shows the means of the $\log_2$-transformed relative levels of each individually normalized RNA species of induced cells relative to the normalized one in uninduced cells.

This quantitative analysis revealed three distinct phenotypes: (i) induced TbPPR1 cell lines showed a specific 2.3-fold reduction of the COX1 mRNA level, (ii) ablation of the putative cytosolically localized TbPPR8 did not affect the level of any of the tested mitochondrial RNAs, and (iii) knockdown of TbPPR2 to TbPPR7 caused a three- to eight-fold reduction of at least one mitochondrial rRNA. These results suggest that the mitochondrial rRNAs are the targets of six out of the seven tested mitochondrial PPR proteins. This is very different from the plant PPR proteins that are generally found to affect processing or expression of mRNAs. Furthermore, even though TbPPR2 to TbPPR7 all specifically affect rRNAs, there is no functional redundancy among these proteins, since all are individually essential for efficient OXPHOS (Fig. 2 and 3). It should be noted, though, that it is possible that the phenotypes of the RNAi cell lines might be more complex, since we did not test all mitochondrially encoded RNAs.

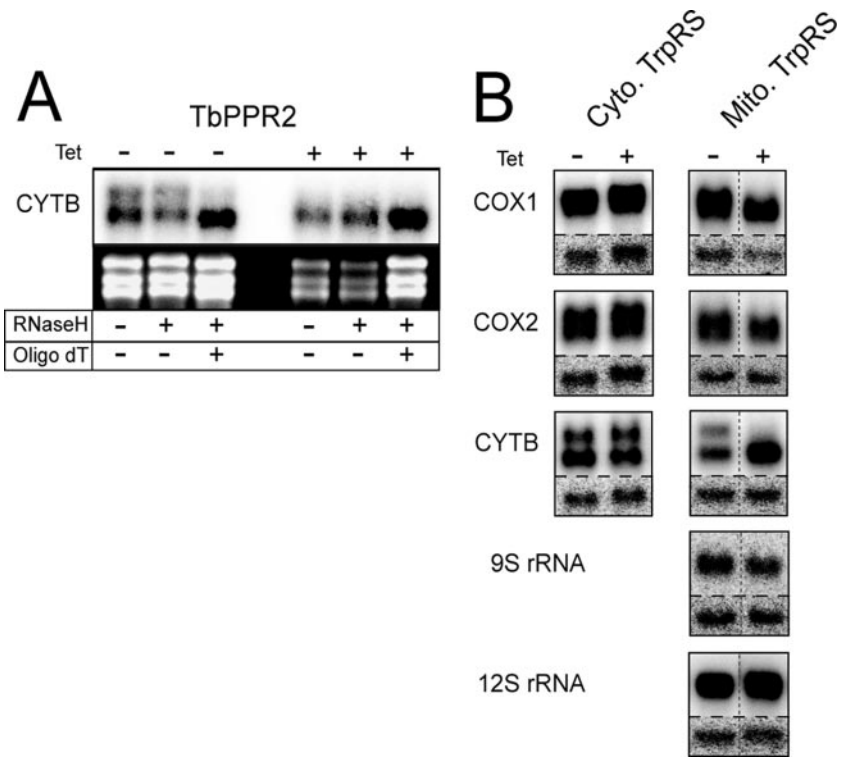


FIG. 5. Short poly(A) tail is a consequence of the lack of OXPHOS. (A) Northern blot analysis of the poly(A) tail length of CYTB mRNAs in uninduced (−Tet) and induced (+Tet) TbPPR2 cells by oligo(dT)-induced RNase H digestion. Additions of RNase H and oligo(dT) are indicated. (B) Total RNA isolated from uninduced and induced cytosolic (Cyto.) or mitochondrial (Mito.) TrpRS RNAi cell lines was analyzed for COX1, COX2, and CYTB mRNAs (and for 9S and 12S rRNA in the case of the mitochondrial TrpRS) using Northern hybridization. As a loading control, filters from the cytosolic TrpRS RNAi cell line were reprobed for the mRNA of mitochondrial TrpRS and vice versa (lower part of each panel).

**Kinetics of mitochondrial rRNA depletion.** In the next series of experiments, we analyzed the kinetics of 9S and 12S rRNA depletion in the TbPPR2 to TbPPR7 RNAi cell lines relative to each other and to the appearance of the growth phenotype. Figure 6 shows that the loss of the mitochondrial rRNAs is very rapid. In all cell lines, the level of the two rRNAs already reached less than 50% for at least one rRNA species 24 h after induction of RNAi. This is long before the appearance of the growth phenotypes and suggests that rRNA depletion is a direct consequence of the ablation of the various PPR proteins.

The rRNA depletion is specific to the TbPPR2 to TbPPR7 RNAi cell lines. It does not occur in the TbPPR1 RNAi cell line (Fig. 6B) and therefore is a general consequence of neither the growth arrest nor the inhibition of OXPHOS. Furthermore, degradation of rRNAs also does not occur in cells that are ablated for TbPPR8 (Fig. 4) or for mitochondrial TrpRS (Fig. 5B), an essential mitochondrial protein.

In all but the TbPPR4 RNAi cell line, degradation of the 12S rRNA is more extensive than degradation of the 9S rRNA. These results suggest that the selected PPR proteins, with the exception of TbPPR4, may act primarily on the 12S rRNA. However, it is also clear that eventually both rRNAs are affected in all cell lines, indicating that the levels of the two rRNAs are coregulated.

**PPR proteins affecting rRNAs are membrane associated.** To investigate the physical connection of PPR proteins with organellar ribosomes, we purified mitochondria from cell lines expressing tagged TbPPR2 to TbPPR5 and fractionated them into membrane and matrix fractions. Mitoribosomes generally are associated with the mitochondrial inner membrane. This also applies for *T. brucei*, as seen in Fig. 7A. Both 9S and 12S rRNAs as well as a tagged ribosomal protein of the LSU (Fig. 7B) are quantitatively associated with the membrane fractions. tRNAs and α-ketoglutarate dehydrogenase (KDH), however, mainly are recovered in the matrix fraction, as expected. Tagged TbPPR2 to TbPPR5 cofractionate with the membrane fraction and thus with the ribosomal markers.

Except for Tb927.7.1350 and Tb11.03.0440, as well as their orthologues LmjF26.0610 and LmjF25.0630, which likely contain a single transmembrane segment, none of the trypanosomatidal PPR proteins is predicted to be an integral membrane protein. In line with this, all tested membrane-associated PPR proteins are recovered in the supernatant fraction after carbonate extraction, whereas most of the integral membrane protein cytochrome oxidase subunit 4 remains in the pellet (Fig. 7A, bottom panels). In summary, these results are consistent with an association of the tested PPR proteins with mitochondrial rRNAs.

Interestingly, however, tagged TbPPR1, whose function is not linked to mitochondrial rRNAs but to the COX1 mRNA, is at least partially recovered in the matrix fraction (Fig. 7C). Thus, these results are consistent with the idea that TbPPR2 to

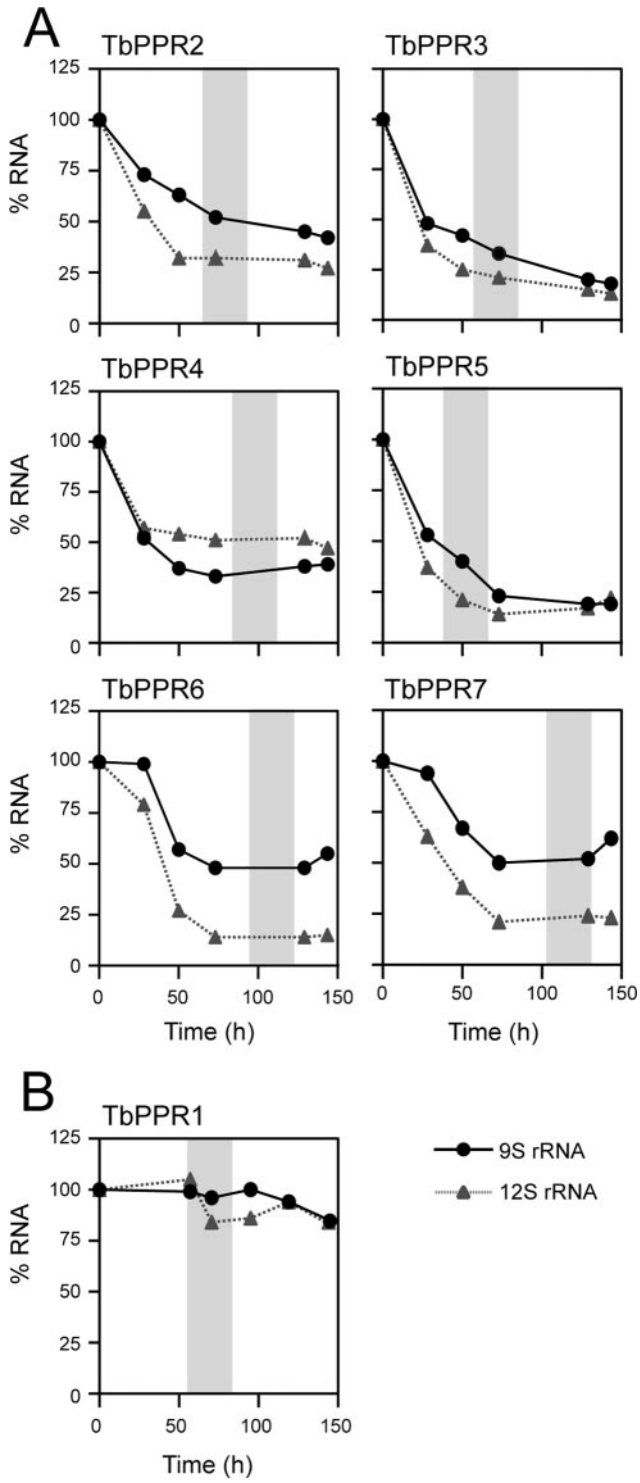


FIG. 6. Kinetics of mitochondrial rRNA depletion. (A) Graph showing the relative changes in the levels of mitochondrial rRNAs during ablation of the indicated PPR proteins. The level of rRNAs in uninduced cells was set to 100%. All levels of rRNAs were normalized by reprobing the same blots with cytosolic TrpRS. For each cell line, the growth phenotype was monitored in parallel. The gray bar indicates the time interval at which the growth phenotype became apparent. The interval encompasses 24 h before and 24 h after the first appearance of the growth phenotype. Each experiment was repeated at least twice, and the variation between the same time points in the two experiments was less than 10%. (B) Kinetics of mitochondrial rRNA depletion in the TbPPR1 RNAi cell line.

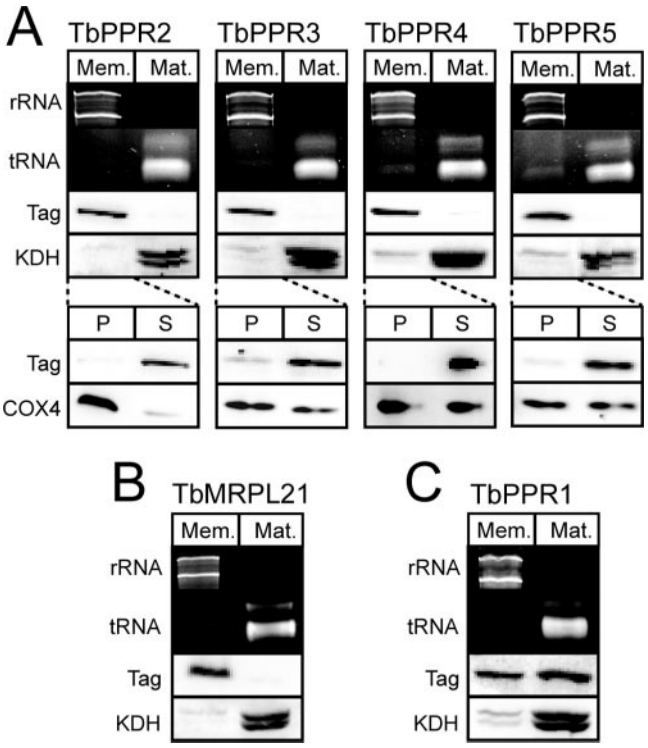


FIG. 7. PPR proteins required for rRNA accumulation are membrane associated. (A) Mitochondria of cell lines expressing the indicated tagged PPR proteins were fractionated into membrane (Mem.) and matrix (Mat.) fractions. The top two panels show the ethidium bromide staining of RNA isolated from the two fractions; only the regions corresponding to the mitochondrial rRNAs and tRNAs are shown. The middle two panels show immunoblot analyses for the tagged PPR proteins and the mitochondrial marker KDH. The bottom two panels show immunoblot analyses for the tagged PPR proteins and the integral membrane protein COX4 of the pellet (P) and supernatant (S) fractions from carbonate-extracted mitochondrial membranes. The images in panels B and C are similar to those in panel A, but cell lines expressing a tagged mitochondrial LSU protein (TbMRPL21) or tagged TbPPR1 were analyzed. The membrane and matrix fractions that are compared correspond to equal cell equivalents.

TbPPR5 and the mitochondrial rRNAs are part of the same macromolecular complexes.

**TbPPR5 is associated with 12S rRNA.** Purification of mitoribosomes of trypanosomatids is complicated by the lack of a functional assay as well as by the presence of additional rRNA-containing particles, and therefore the purification is technically very challenging (24, 25, 42). As an alternative, we performed immunoprecipitations from mitochondrial extracts that originate from cell lines expressing tagged TbPPR2, TbPPR3, and TbPPR5 using anti-HA tag antibodies. While we were not able to precipitate tagged TbPPR2 and TbPPR3, presumably because the proteins are in tight (perhaps ribosomal) complexes that hide the epitope from the antibody, a significant fraction of the tagged TbPPR5 was recovered from the bound fraction (Fig. 8B). Most interestingly, some of the 12S rRNA coprecipitated with the tagged protein, whereas this was not the case for the 9S rRNA or tRNAs. Thus, this experiment directly shows that TbPPR5 is directly or indirectly associated with the LSU of the mitoribosome.

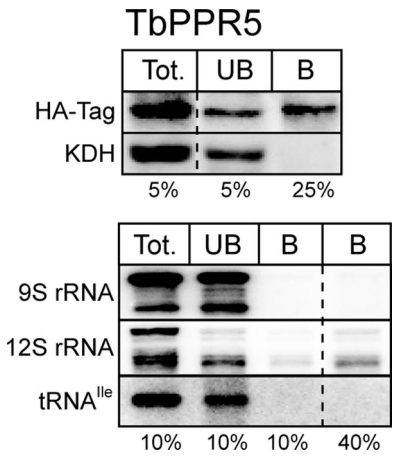


FIG. 8. TbPPR5 is associated with 12S rRNA. Mitochondrial extract of a cell line expressing HA-tagged TbPPR5 was subjected to immunoprecipitation using anti-HA antibody. The total extract (Tot.), the unbound fraction (UB), and the bound fraction (B) were analyzed for the presence of HA-tagged TbPPR5 or KDH (top panel) using immunoblots and were analyzed for mitochondrial rRNAs and $tRNA^{Ile}$ using Northern blotting. The percentages of the total samples that were analyzed in the different lanes are indicated at the bottom.

**rRNA-affecting PPR proteins and mitochondrial rRNAs are coregulated.** If the rRNA-affecting PPR proteins are in the same particle and/or are bound to the same RNAs, we might expect that ablation of one would lead to degradation of the other. To test this, we prepared RNAi cell lines allowing RNAi-induced ablation of TbPPR4 or TbPPR5 with simultaneous inducible expression of tagged TbPPR2 or/and TbPPR6, respectively (Fig. 9). For all these cell lines we monitored the kinetics of rRNA depletion and the level of the tagged proteins during induction of RNAi. The results show that ablation of one set of PPR proteins leads to the degradation of tagged PPR proteins that are not targeted by RNAi. There are two main interpretations of these results: (i) the selected PPR proteins directly interact with each other and form a protein complex that is destabilized by ablation of one of its members, and (ii) the degradation of the tagged PPR proteins in the RNAi cell lines is caused indirectly by the depletion of the rRNAs. The observation that the degradation of tagged TbPPR2 in the TbPPR4 RNAi cell line is less extensive than the degradation of the rRNAs suggests that depletion of the tagged proteins might, in this case, mainly be triggered by the lack of protein binding partners rather than by the altered levels of the rRNAs. In the TbPPR4 RNAi cell line, however, both the rRNA and the tagged TbPPR6 are degraded to approximately the same extent, consistent with the idea that it is the ablation of the rRNA that causes the disappearance of the tagged protein. These two explanations, that degradation of the tagged PPR proteins is caused by either missing protein-protein or missing protein-RNA interactions, clearly are not mutually exclusive. Most importantly, both imply that the tested PPR proteins are functionally linked to rRNAs.

**Overexpression of PPR proteins slows down RNAi-induced phenotypes.** Unexpectedly, expression of the tagged PPR proteins delayed and reduced the extent of depletion of the mitochondrial rRNAs compared to the amount of depletion in the parent RNAi cell lines. This is probably due to the fact that the tagged PPR proteins were overexpressed compared to the expression of endogenous protein (Fig. 9, middle panels). Causing a delay of rRNA degradation appears to be a general effect of PPR protein overexpression; it was seen in all combinations of tagged proteins and RNAi cell lines tested. In line with their presumed association with the 12S rRNA, the main effect of overexpressing tagged TbPPR2 and TbPPR6, when tested in TbPPR4 RNAi cell lines, was a slowing down of the degradation of the 12S rRNA. The 9S rRNA was affected only for 24 h. However, when tagged TbPPR6 was overexpressed in TbPPR5 RNAi cells, it slowed down the degradation of both 9S and 12S rRNAs. Moreover, in all cell lines, overexpression of tagged PPR proteins suppressed the growth phenotype caused by the RNAi by 24 h (data not shown). Finally, in a TbPPR4 RNAi cell line that simultaneously overexpresses both tagged TbPPR2 and TbPPR6, the complementation effect was cumulative to the one seen in the cell line expressing the tagged proteins individually (Fig. 9B). Thus, the degradation of the 12S rRNA essentially was abolished, and the growth arrest appeared 48 h later than that in the parent RNAi cell line.

PPR proteins are hypothesized to bind to specific RNA sequences. In agreement with this model, all mitochondrial PPR proteins we investigated were individually essential for OXPHOS (Fig. 2 and 3). Thus, it is unlikely that overexpression of a PPR protein allows it to bind to an RNA sequence that normally is recognized by the ablated PPR protein. However, there might be some redundancy in the putative protein-protein interactions. Thus, overexpression of a PPR protein might indeed allow it to stabilize a protein complex that lacks the ablated PPR protein, even though if it were not expressed at high levels it would not bind to it. However, full complementation would require both correct protein-protein and correct RNA-protein interactions and therefore cannot be achieved by overexpression of heterologous PPR proteins. In summary, even though the underlying mechanism of how overexpression of one set of PPR proteins delays the RNAi-induced phenotypes of another set is unknown, the results underscore that rRNA-affecting PPR proteins are intricately connected to each other and to the mitochondrial rRNAs.

## DISCUSSION

The genome of *T. brucei* encodes 28 distinct PPR proteins, which is approximately five times more PPR proteins than most other nonplant eukaryotes. Eight of these proteins (TbPPR1 to TbPPR8) were chosen for experimental analysis. Epitope tagging has shown that TbPPR1 to TbPPR7 are localized in mitochondria (Fig. 1). Moreover, they all are essential for OXPHOS and thus for growth in medium lacking glucose (Fig. 2 and 3). Surprisingly, six out of the seven mitochondrial PPR proteins (TbPPR2 to TbPPR7) are functionally linked to mitoribosomes.

A recent proteomics study of an abundant mitochondrial ribonucleoprotein complex of *Leishmania* that contains the 9S rRNA and many SSU ribosomal proteins but no 12S rRNA identified three PPR proteins (25). Homologues of two of them also were detected in our bioinformatic analysis of the *T. brucei* genome but were not investigated experimentally (Table

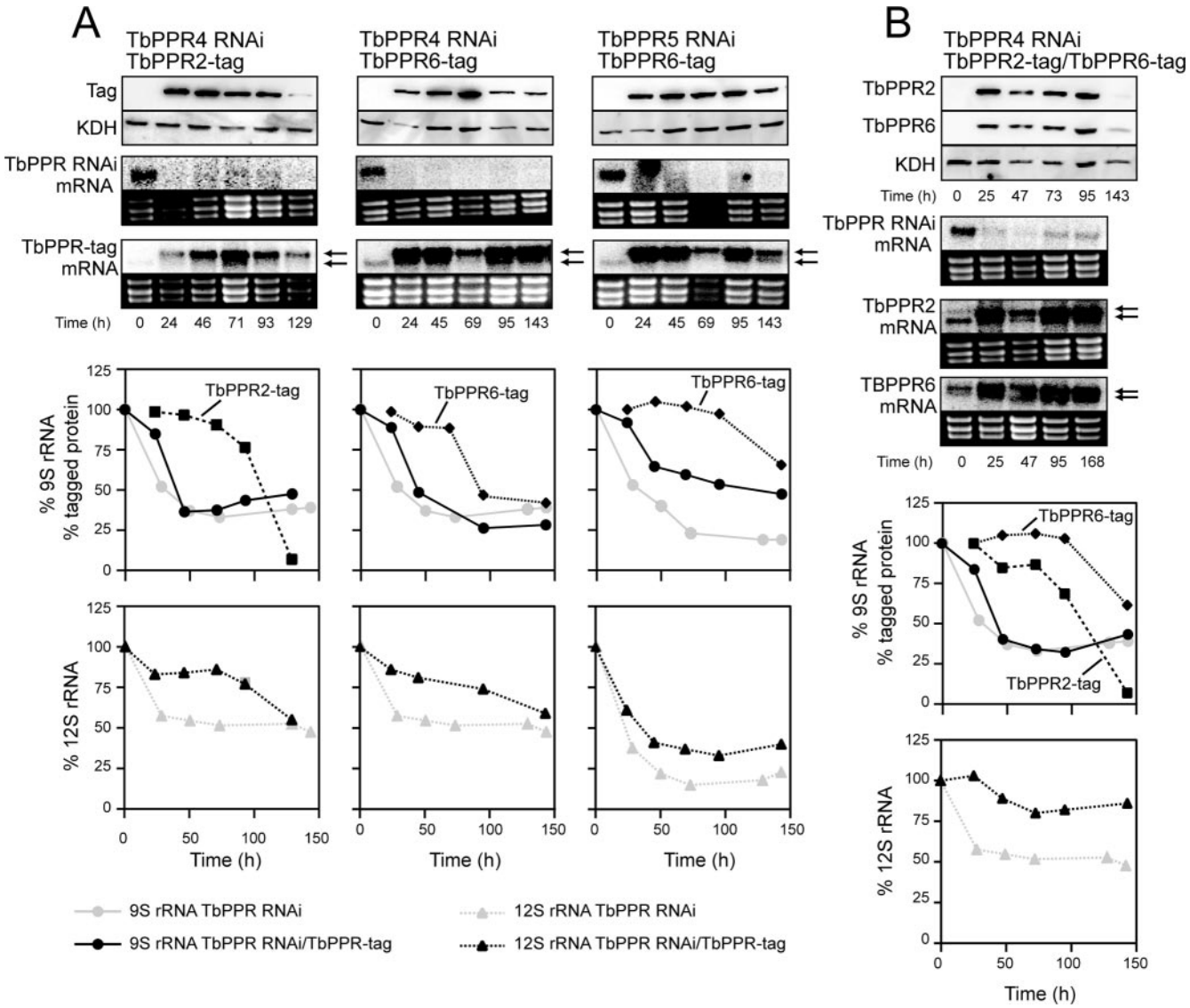


FIG. 9. Fate and effect of tagged PPR proteins in RNAi cell lines ablated for other PPR proteins. (A) For the left column, a cell line allowing inducible ablation of TbPPR4 with simultaneous inducible expression of tagged TbPPR2 was tested for the kinetics of mitochondrial rRNA depletion as well as for depletion of tagged TbPPR2. The top panels show immunoblot analyses for the tagged TbPPR2 and KDH as a loading control, respectively. The middle panels show Northern blot analyses of the RNAi-targeted TbPPR4 mRNA and the corresponding ethidium bromide stain of the cytosolic rRNAs as a loading control, respectively. The bottom panels show Northern blot analyses of the epitope-tagged TbPPR2 encoding mRNA and the corresponding ethidium bromide stain of the cytosolic rRNAs as a loading control, respectively. The lower and upper arrows indicate the position of the wild-type TbPPR2 mRNA and the mRNA-encoding epitope-tagged TbPPR2, respectively. The upper graph shows the relative changes in the levels of mitochondrial 9S rRNA and of tagged TbPPR2 during RNAi-induced ablation of TbPPR4. The graph at the bottom shows the same for the 12S rRNA. In both graphs the rRNA depletion kinetics in the parent TbPPR4 RNAi cell line that does not express the tagged PPR protein are indicated in light gray. These curves are identical to the ones shown in Fig. 6. The middle column is similar to the first column, but the analysis was done for a TbPPR4 RNAi cell line expressing tagged TbPPR6. The right column is similar to the other two columns, but the analysis was done for a TbPPR5 RNAi cell line expressing tagged TbPPR6. The images in panel B are similar to those in panel A, but analysis was done for a TbPPR4 RNAi cell line expressing both tagged TbPPR2 and tagged TbPPR6, respectively. All curves were determined twice in two independent experiments, yielding very similar results.

1). Furthermore, the complex also contained at least a small amount of the leishmanial orthologue of TbPPR5 (25).

Thus, in *T. brucei* at least eight distinct PPR proteins are functionally or structurally connected to mitoribosomes. TbPPR1 to TbPPR8, which were selected for experimental analysis, were chosen essentially randomly, the only condition being that they contained a putative mitochondrial targeting signal. This is true for 25 out of the 28 trypanosomal PPR proteins (Table 1). Thus, finding that six (75%) out of eight randomly selected PPR proteins were linked to the mitochondrial rRNAs, together with the two distinct 9S rRNA-associated PPR proteins identified in *Leishmania* (25), predicts that many more of the remaining 21 trypanosomal PPR proteins will target the mitochondrial rRNAs.

What role could TbPPR2 to TbPPR7 play in ribosome function? We think that TbPPR2 to TbPPR7 are either required for ribosome biogenesis or bona fide components of the mitoribosomes. In the former case, they may be associated with the rRNA-containing particles but not or only transiently with mature ribosomes. In the latter case, they are expected to be stably and permanently associated with the mature ribosomes.

It is not possible at present to distinguish between these two scenarios due to the difficulties of purifying mitoribosomes of trypanosomatids. Furthermore, two recent studies have shown that there are at least six stable rRNA-containing particles that can be isolated from trypanosomatid mitochondria (24, 25). The 9S rRNA was recovered in four distinct ribonucleoprotein complexes that had sedimentation constants of 30, 45, 50, and 65S. A similar situation was seen for the 12S rRNAs, which were found in 40, 50, and 65S particles. The 50S particle contained both rRNA and SSU and LSU ribosomal proteins and thus probably corresponds to the fully assembled ribosome. The 40S complex contained 12S rRNA and LSU ribosomal proteins and therefore probably represents the LSU of the ribosome. The role of all other particles is unclear.

TbPPR5 is stably associated with the 12S rRNA (Fig. 8) and thus is a good candidate for a bona fide component of the LSU of the ribosome. The facts that the rRNA-affecting trypanosomal PPR proteins are membrane bound and are coregulated with the rRNAs argue for a stable association with the rRNAs. Moreover, the fact that ablation of all rRNA-affecting PPR proteins, except for TbPPR4, first and most extensively affects the 12S rRNA suggests that they are associated with the 12S rather than the 9S rRNA. However, since we extrapolate that the function of a large fraction of the mitochondrial PPR proteins of *T. brucei* is connected to ribosomes, it is reasonable to assume that some are actual ribosomal proteins, whereas others might be required only for the biogenesis of the ribosomes and thus at steady state might not be associated with mitoribosomes.

Mitoribosomes are of the bacterial type, and with the exception of plants, there is an evolutionary trend to reduce the length of their rRNAs (38). Thus, whereas the 16S and 23S rRNAs in *E. coli* are 1,542 and 2,904 nucleotides in length, respectively, the rRNAs in human mitochondria have been reduced to a length of 953 and 1,555 nucleotides. In trypanosomatids (e.g., *T. brucei*), this reduction is taken much further, and the 9S and 12S rRNA are only 611 and 1,150 nucleotides long (11, 12, 14, 43). This makes them the shortest rRNAs known to date. However, even in the mitochondrial rRNAs of trypanosomatids, most domains of bacterial rRNAs have been retained, though some stems and loops have been drastically reduced or completely eliminated. There is evidence that the length reduction of the rRNAs in mitoribosomes of mammals is compensated for by having more numerous and larger ribosomal proteins (39). Since the mitochondrial rRNAs in trypanosomatids are even shorter, it is tempting to speculate that one of the main functions of PPR proteins in trypanosomatids is to functionally and structurally compensate for the lacking parts of the truncated rRNAs.

In summary, our results are in agreement with the postulated role of PPR proteins as sequence-specific RNA binding proteins and suggest that the global function of the PPR protein family-mediating organellar gene expression is conserved within eukaryotes. On the other hand, the fact that, in *T. brucei*, a large fraction of the trypanosomal PPR proteins act on rRNAs was unexpected. However, association of PPR proteins with organellar ribosomes is not restricted to trypanosomatids. Data obtained from maize have shown that a null mutant for a plastid PPR protein is devoid of plastid ribosomes (46). Moreover, single PPR proteins associated with the SSU of the mitoribosome recently have been described for yeast (15) and mammals (19). Thus, while it is most prominent in *T. brucei*, a role in rRNA maintenance and thus in ribosome function is a conserved function of PPR proteins in all species. This leads to the interesting situation in which eukaryote-specific PPR proteins have become essential components of bacterial-type organellar ribosomes.

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