The Pentatricopeptide Repeats Present in Pet309 Are Necessary for Translation but Not for Stability of the Mitochondrial COX1 mRNA in Yeast*

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Pet309 is a protein essential for respiratory growth. It is involved in translation of the yeast mitochondrial COX1 gene, which encodes subunit I of the cytochrome c oxidase. Pet309 is also involved in stabilization of the COX1 mRNA. Mutations in a similar human protein, Lrp130, are associated with Leigh syndrome, where cytochrome c oxidase activity is affected. The sequence of Pet309 reveals the presence of at least seven pentatricopeptide repeats (PPRs) located in tandem in the central portion of the protein. Proteins containing PPR motifs are present in mitochondria and chloroplasts and are in general involved in RNA metabolism. Despite the increasing number of proteins from this family found to play essential roles in mitochondria and chloroplasts, little is understood about the mechanism of action of the PPR domains present in these proteins. In a series of in vivo analyses we constructed a pet309 mutant lacking the PPR motifs. Although the stability of the COX1 mRNA was not affected, synthesis of Cox1 was abolished. The deletion of one PPR motif at a time showed that all the PPR motifs are required for COX1 mRNA translation and respiratory growth. Mutations of basic residues in PPR3 caused reduced respiratory growth. According to a molecular model, these residues are facing a central cavity that could be involved in mRNA-binding activity, forming a possible path for this molecule on Pet309. Our results show that the RNA metabolism function of Pet309 is found in at least two separate domains of the protein.

Biogenesis of the mitochondrial cytochrome c oxidase (COX) complex depends on a large set of proteins. In the yeast Saccharomyces cerevisiae more than 20 nuclear genes have been found to be necessary for assembly and maintenance of the functional COX (1–3). The enzyme in mammals and yeast is composed of 13 and 12 subunits, respectively. The core of the enzyme is formed by subunits Cox1, Cox2, and Cox3, which are encoded in the mitochondrial DNA. Expression of the mitochondrial-encoded subunits is highly regulated by proteins involved in transcription, transcript stability and processing, translation, and assembly into the mitochondrial inner membrane (3–5).

In humans, deficiency in COX assembly is associated with mitochondrial disorders. The majority of these are caused by autosomal recessive mutations that affect COX assembly factors (6, 7). An example of such a factor is the mRNA-binding protein Lrp130 (8, 9). Mutations in the LRPI30 gene have been associated with the neurodegenerative disorder Leigh syndrome of the French Canadian type (10). These patients lack fully functional COX activity, associated with defects in the COX1 and COX3 transcripts (9).

It has been proposed that PET309 is the yeast homologue of LRPI30 (10), with 37% of similarity over 300 amino acids. Both genes seem to participate in mRNA processing and may have similar functions in mitochondria. Pet309 is a translational activator necessary for Cox1 synthesis. It specifically acts on the 5′-UTR of the COX1 mRNA to activate translation. In addition, it is required to stabilize the pre-COX1 transcript (11). It has been observed that translational activators specific for the COX1, COX2, and COX3 mRNAs interact with each other and with the mitochondrial inner membrane (12–15), suggesting that the activators promote that translation initiation takes place close to the insertion and assembly sites of the three COX subunits in the mitochondrial inner membrane (15, 16).

Both Lrp130 and Pet309 contain several pentatricopeptide repeats (PPRs). These repeats belong to a protein family that is very large in plants, with at least 442 members in Arabidopsis thaliana. However, there are fewer examples of these proteins in fungi, animals, and protists (17, 18). Pet309 is the only yeast translational activator that has been found to contain PPR motifs. In general, PPR proteins are usually found to localize in mitochondria and chloroplasts. It is known from the small set of PPR proteins studied to date that they participate mostly in different steps of sequence-specific RNA metabolism. They are implicated in precursor transcript stability and processing (which includes splicing and editing) (19–23), as well as in translation (11, 19, 24, 25). However, in a few examples specific RNA-binding activity or their natural RNA targets has been demonstrated (21, 26). These proteins play essential roles in plant embryogenesis, cytoplasmic male sterility restoration.

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2 The abbreviations used are: COX, cytochrome c oxidase; UTR, untranslated region; PPR, pentatricopeptide repeat; TPR, tetratricopeptide repeat; HA, hemagglutinin.
and chloroplast to nucleus retrograde signaling (for examples see Refs. 17, 27, 28).

PPRs are degenerated 35-amino acid motifs proposed to consist of two antiparallel α helices. There is no structural information about PPR proteins, but models based on the closely related TPR (tetratricopeptide repeat) proteins suggest that the tandem repeats of these domains form a solenoid-like structure with a hydrophilic cavity where the phosphate skeletons of RNA might interact (29).

Despite the growing number of PPR proteins discovered and characterized to date, very little is understood about the specific role of the PPR motifs present in these proteins. Yeast Pet309 provides a useful model of a PPR protein to elucidate the mechanism of action of the PPR motifs. Pet309 is predicted to contain at least seven PPR motifs located in the central portion of the protein. To test the function of the repeats present in Pet309, a set of deletions of the PPR motifs was constructed and analyzed. A model of the Pet309 PPR region was generated, and site-directed mutagenesis was carried out on residues that are predicted to be necessary for mRNA binding. It was shown that all the seven PPR repeats present in Pet309 are necessary for COX1 mRNA translation, and that mutation of basic residues that could be facing the inner cavity of the PPR structure decrease Pet309 activity. Surprisingly, the COX1 mRNA levels were not affected by the PPR deletions, showing that the mRNA stability function of Pet309 is independent of the PPR domains.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Genetic Methods**—The *S. cerevisiae* strains used in this study are listed in Table 1. All strains are derived from strain D273-10B. Genetic manipulation and standard media recipes were as previously described (30). Yeast were cultured in complete fermentable media (1% yeast extract, 2% Bacto-peptone) or synthetic complete media (0.67% yeast nitrogen base, supplemented with the appropriate amino acids), containing 2% glucose, 2% raffinose or 3% ethanol-3% glycerol. The *pet309Δ::LEU2* deletion construct was obtained by PCR. Strains XPM201 and XPM10b were transformed with the PCR product (31), and correct integration of the *pet309Δ::LEU2* construct was confirmed by PCR.

**Plasmid Constructs**—Total DNA from the strain SB5 was used to amplify the *PET309::HA* sequence, including 310 and 205 bp of the *PET309* 5’- and 3’-UTR, respectively. This product was ligated into XbaI-Xhol sites of pBlueScript to generate plasmid pXP96. In addition, the product was subcloned into the XbaI-Xhol sites of yeast expression vectors pRS416 (32) and pYe352 (33) to generate pXP97 and pXP104, respectively. All pet309 mutant sequences were generated by fusion PCR (34), using Accusyme DNA polymerase (Bioline) and pXP96 as the DNA template. The PCR products obtained from the PPR region of pet309 were ligated into PstI-EcoRI pXP96. After sequencing the constructs the complete pet309 genes were subcloned into XbaI-Xhol pXP97 or pXP104 to generate yeast expression plasmids.

**Analysis of Mitochondrial Proteins**—Mitochondria were isolated from late logarithmic phase cells grown on synthetic complete media without uracil, containing 2% raffinose. Crude mitochondria were isolated and purified by centrifugation on 5–25% Nycodenz gradients (35).

Mitochondria separation into membrane and soluble fractions, and alkaline carbonate extractions of membranes were as described (36–38). Mitoplasting and proteinase K treatment were carried out as previously described (38). Total cellular extracts were isolated from cells grown to mid-log phase on synthetic complete media without uracil, containing 2% raffinose (39).

Proteins were separated by SDS-PAGE on a 12.5% gel (40). Western blots were probed with anti-HA-horseradish peroxidase (Roche Biochemicals), anti-Cox1 (Molecular probes), anti-citrate synthase, anti-Arg8p, anti-Yme1p (the three provided by T. D. Fox), anti-cytochrome c1 (provided by D. Gonzalez-Halphen) or anti-glucose-6-phosphate dehydrogenase (Sigma) antibodies. Secondary goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Sigma or Bio-Rad) was detected with the ECL or ECL + kits (GE Healthcare).

**Synthesis of Mitochondrial Proteins**—Translation in isolated mitochondria in the presence of [35S]methionine was performed as described (41). After translation, mitochondria were washed with 0.6 M sorbitol, 20 mM HEPES, pH 7.4, and the radiolabeled proteins were separated on a SDS-PAGE gel, blotted into protran nitrocellulose membrane, and analyzed with a Typhoon 8600 PhosphorImager (Amersham Biosciences).

**Northern Blot Analyses**—Total RNA was prepared using the TRIzol reagent (Invitrogen) from yeast cultures grown to late log phase on raffinose-synthetic complete media lacking uracil. RNA was blotted to Hybond XL membrane (GE Healthcare). Blots were probed sequentially with the radioactively labeled COX1 exon 4, COX2 and with the 15S rRNA gene (42) to standardize the loading. Blots were analyzed with a Typhoon 8600 PhosphorImager and quantitated with ImageQuaNT 5.1 software.

**Modeling for the PPR Region in Pet309**—A search using the TPRpred server (43) against the whole Pet309 sequence revealed the presence of 11 putative PPR motifs located between residues 312 and 759. The six motifs with the lowest p value (<1e-07-1e-06, which indicates 1 × 10^-7 to 1 × 10^-6) correspond to one segment of the protein comprising residues 347–560. Using this fragment of the sequence, the HHpred server (44) revealed an alignment with the six tandem TPRs of

**Table 1**

| Strain name | Nuclear (mitochondrial) genotype | Reference |
|-------------|---------------------------------|-----------|
| XPM232 | *Mat a* ura3-52 leu2-3,112 lys2 arg8::hisG pet309Δ::LEU2 (p+, ΔΣai) | This study |
| XPM231 | *Mat a* ura3-52 leu2-3,112 lys2 arg8::hisG pet309Δ::LEU2 (p+, cox1Δ::ARG8*) | This study |
| XPM201 | *Mat a* ura3-52 leu2-3,112 lys2 arg8::hisG pet309Δ::LEU2 (p+, ΔΣai) | This study |
| XPM10b | *Mat a* ura3-52 leu2-3,112 lys2 arg8::hisG pet309Δ::LEU2 (p+, cox1Δ::ARG8*) | 52 |
| SB5 | *Mat a* ade2 PET309::3xHA (p+) | S. A. Broadley |
the crystal structure of PilF (45) (PDB code 2ho1) from Pseudomonas aeruginosa, with 10% identity extending over 213 residues. Only five residue insertions of one residue each occur in the alignment, all at the level of the junctions between the repeats. The three-dimensional model of the PPR repeats was constructed using SWISSMODEL (46) alignment interface mode. Fig. 6 was created with PyMOL, and the electrostatic potentials were calculated with the program APBS (47).

RESULTS

The PPR Region of Pet309 Is Necessary for Respiratory Growth—A comparison of the Pet309 sequence against the TPRpred server reveals the presence of 11 PPR motifs in the central portion of the protein. To understand the role of the PPR domain we created a deletion from residues 347 to 632 (pet309Δppr), which corresponds to the 7 most strongly predicted PPR motifs (having the lowest p values) (Fig. 1A). To facilitate detection of Pet309, the protein was tagged at its C terminus with three tandem copies of an HA epitope. The presence of the triple-epitope in wild-type Pet309 did not interfere with the respiratory growth of cells as judged by the ability of PET309-HA to fully complement the pet− phenotype of a pet309Δ::LEU2 mutant. Both the wild-type PET309-HA and the pet309Δppr-HA genes were cloned in the vectors pRS416 and YEp352 to allow expression in yeast in low copy or multiple copy plasmids, respectively. The plasmids were transformed into a yeast pet309Δ::LEU2 mutant, and the respiratory growth of the resulting strains was examined (Fig. 1B). The wild-type PET309-HA supported normal growth on non-fermentable carbon sources, whereas the pet309Δppr-HA strain could not grow on a non-fermentable carbon source. A similar phenotype was observed in cells expressing the single copy or multiple copy expression plasmids, suggesting that overexpression of the mutant protein did not compensate for the absence of the PPR domain of Pet309.

To investigate the basis of the non-respiratory phenotype of the pet309Δppr-HA strain we first looked to see if the mutant protein was localized in mitochondria. Mitochondrial and post-mitochondrial supernatant fractions were obtained from strains transformed with the low copy and high copy plasmids bearing the wild-type PET309-HA or the mutant pet309Δppr-HA genes (Fig. 2A). The protein Pet309 was specifically recognized by the anti-HA antibody as a 118-kDa band for the wild-type PET309-HA or 85-kDa band for the pet309Δppr-HA strain. These polypeptides were not detectable in mitochondria bearing the untagged PET309 gene or the empty plasmids.
PPR Domains of Pet309 Are Required for COX1 Translation

A PET309-HA pet309Δppr-HA

HA  Low High Low High

Cox1  118 KDa 85 KDa

CS

B

Low High Low High

pet309Δ

var1

cox1

cox2

cytb

cox3

Atp6

C

+Arg -Arg

FIGURE 3. The PPR domains present in Pet309 are required for COX1 mRNA translation. A, mitochondria from strains carrying the wild-type PET309-HA, the pet309Δppr-HA, or empty vector (pet309Δ), in low copy (Low) or high copy plasmids (High) were separated by SDS-PAGE. Western blot was probed with antibodies anti-HA to detect Pet309, anti-Cox1, and anti-citrate synthase (CS) as loading control. B, translation in mitochondria (100 mg/ml) in the presence of [35S]methionine was performed at 30 °C for 20 min. Translation products were analyzed as described under “Experimental Procedures.” Cytochrome c oxidase subunit 1, Cox1; subunit 2, Cox2; subunit 3, Cox3; cytochrome b; Cytb; subunit 6 of ATPase, Atp6; ribosomal protein, Var1. C, growth phenotypes of strains carrying the Cox1Δ::ARG8m mitochondrial gene. Cells from strain XPM231 bearing the high copy plasmids were grown on liquid minimal media lacking uracil and spotted on glucose minimal media lacking uracil (+Arg) or arginine (−Arg), and incubated for 3 days at 30 °C.

(data not shown). Greater accumulation of the Pet309 polypeptides was observed under multiple copy expression. These polypeptides were not detectable in the post-mitochondrial supernatant fractions, indicating that the Pet309Δppr-HA protein co-purified with mitochondria.

Next, we investigated whether the Pet309Δppr-HA protein was membrane-bound or soluble. Mitochondria from strains bearing the PET309-HA or the pet309Δppr-HA, low copy plasmids were sonicated and centrifuged. Both the wild-type Pet309-HA and the mutant Pet309Δppr-HA proteins were present in the membrane pellet (Fig. 2B) and absent from the soluble supernatant. Alkaline Na2CO3 extraction of the mitochondrial membranes solubilized the wild-type Pet309-HA and the Pet309Δppr-HA proteins (Fig. 2C), indicating that both behave as peripheral membrane proteins.

To examine the submitochondrial location of the Pet309 proteins, purified mitochondria were converted to mitoplasts by osmotic shock treatment and were subjected to protease digestion. Both Pet309-HA and Pet309Δppr-HA proteins were protected from proteinase K treatment in mitochondria and in mitoplasts (Fig. 2D). This result indicates that both proteins are facing the matrix side of the inner membrane. These results are different from what was observed by Manthey (13), who reported that Pet309-c-Myc was an integral inner membrane protein. However, in that work, a high copy plasmid was used to overexpress the Pet309-c-Myc protein. Overexpression of translational activators has been associated with problems in mitochondrial gene expression (48, 49) and could affect their interaction with the mitochondrial inner membrane. For this reason we analyzed the Pet309-HA proteins expressed from low copy plasmids.

Taken together, these results indicate that the respiratory defect of pet309Δppr-HA mutants is not due to a mitochondrial mislocation of the protein. The association of the mutant protein to the mitochondrial inner membrane and its submitochondrial localization were not altered by the absence of the PPR domains. These observations, together with the capacity of the mutated protein to stabilize the COX1 mRNA (see below) strongly suggest that the Pet309Δppr-HA protein is not misfolded.

The PPR Motifs in Pet309 Are Required for Translation of the COX1 mRNA—Pet309 had been previously demonstrated to be necessary for the translation and stability of the COX1 mRNA (11). We investigated the effect of the PPR domain deletion on expression of the COX1 gene. Western blot analysis of mitochondrial protein extracts showed no accumulation of the Cox1 protein in the pet309Δppr-HA mutant (Fig. 3A). The mutant did not accumulate Cox1 even in pet309Δppr-HA high copy expression. Interestingly, overexpression of the wild-type Pet309-HA led to a substantial decrease in the Cox1 accumulation (3.5-fold). This observation is in agreement with the idea that overexpression of translational activators can lead to defects on the biogenesis of their target genes (49). Overexpression of Pet309 could lead to formation of inactive Pet309 aggregates that could affect accumulation of Cox1 (48).

To investigate the effect of the pet309Δppr-HA mutation on COX1 translation, we first analyzed [35S]methionine-labeled proteins from mitochondria carrying the pet309Δppr-HA mutation in low copy or high copy expression plasmids (Fig. 3B). Labeling of Cox1 was reduced to undetectable levels by the pet309Δppr-HA mutation even in overexpression conditions. As expected, labeling of Cox1 in strains with the wild-type PET309-HA was normal, whereas a null mutation (pet309Δ) completely prevented Cox1 labeling. These results suggest that
the PPR domain of Pet309 is necessary for the COX1 mRNA translation. To corroborate this, we created a pet309Δ::LEU2 strain in which the mitochondrial reporter gene ARG8<sup>+</sup> replaced the COX1 coding sequence (cox1Δ::ARG8<sup>+</sup>). The ARG8<sup>+</sup> product is a matrix-soluble protein involved in arginine biosynthesis (50). This reporter has been widely used to analyze translation of mitochondrial genes (49, 51–53). Translation of cox1Δ::ARG8<sup>+</sup> has been demonstrated to be dependent upon Pet309 (52). In cells carrying the wild-type PET309-HA, the cox1Δ::ARG8<sup>+</sup> gene supported growth in Arg− medium (Fig. 3C). In contrast, cells bearing the high copy or low copy (data not shown) pet309Δ<sup>ppr-HA</sup> gene required arginine to grow, confirming that the PPR domain present in Pet309 is necessary for the COX1 mRNA translation.

The PPR Motifs in Pet309 Are Not Required for Stabilization of the COX1 mRNA—Pet309 is also involved in the COX1 mRNA stability, as null mutants show a reduced accumulation of the mature COX1 mRNA (11). This effect is particularly strong when the COX1 gene has introns, but it is also observed with the intronless COX1 gene (11). We analyzed whether deletion of the PPR repeats present in Pet309 could affect the COX1 mRNA accumulation.

Levels of the COX1 mRNA in cells bearing the intronless COX1 gene were analyzed by Northern blot and normalized to the mitochondrial 15S rRNA (Fig. 4). In wild-type cells expressing the high copy PET309-HA gene, the COX1 mRNA signal was increased 2-fold as compared with the low copy PET309-HA cells. A similar pattern was obtained for the pet309Δ<sup>ppr-HA</sup> cells. This effect was specific for COX1, because the COX2 mRNA levels were not affected in any sample. It has been suggested that high levels of translational activators could stabilize their target mRNAs (49). This result indicates that Pet309 lacking the PPR repeats still has the capacity to stabilize the COX1 mRNA. In contrast, the null pet309 mutant showed a reduced accumulation of the COX1 mRNA as compared with the PET309-HA or the pet309Δ<sup>ppr-HA</sup> cells.

We conclude that the PPR domains present in Pet309 are necessary for translation of the COX1 mRNA. However, the absence of these repeats does not affect the COX1 mRNA stability. Moreover, high expression of the pet309Δ<sup>ppr-HA</sup> protein caused accumulation of the COX1 mRNA, as observed for the wild-type PET309-HA protein.

Each One of the Seven PPR Repeats of Pet309 Is Necessary for Cox1 Synthesis—We next asked whether deletion of single PPR repeats could affect translation of the COX1 mRNA. A pet309Δ::LEU2 strain was transformed with high copy plasmids carrying single deletions of each PPR (pet309Δ<sup>ppr<sub>1–7-HA</sub></sup>). Western blot analysis of total cell extracts revealed the presence of a 114-kDa band whose migration in our SDS-PAGE system was indistinguishable from the wild-type Pet309-HA protein (Fig. 5A).

None of the seven PPR mutants were able to grow on the non-fermentable carbon source ethanol/glycerol (Fig. 5B), suggesting that translation of the COX1 mRNA was affected. To evaluate this hypothesis we tested the Arg growth of cells carrying the cox1Δ::ARG8<sup>+</sup> gene in the mitochondrial DNA (Fig. 5C). None of the seven mutants supported Arg growth in a media lacking arginine, indicating that each one of the PPR repeats present in Pet309 is necessary for COX1 mRNA translation.

Similar Pet<sup>+</sup> and Arg<sup>+</sup> phenotypes were obtained with cells carrying the low copy plasmids (data not shown). Deletion of each PPR did not affect COX1 mRNA levels, whereas overexpression of the mutant proteins led to increased accumulation of the COX1 mRNA (data not shown). This strongly suggests that the translational activation and the mRNA stabilization activities of Pet309 are located on different functional regions of the protein.

**Mutagenesis of Basic Residues Inside the PPR Central Groove Affect the COX1 mRNA Translation**—Based on similarities with the TPR motifs, PPR motifs are predicted to consist of two α helices (named A and B). Tandem PPR motifs are predicted to form a superhelix enclosing a groove, which is positively charged. This charge could be involved in nucleic acid binding. The side chains that face the inner groove are predicted to come
The mutated residues are indicated by arrows. A, total cell extracts were obtained. A sample of 50 μg of proteins was analyzed by SDS-PAGE and immunoblotting. Western blot was probed with anti-HA antibody to detect Pet309-HA and with anti-glucose-6-phosphate dehydrogenase antibody as loading control. B, cells were grown on liquid synthetic complete medium lacking uracil and spotted in the same medium with either glucose or ethanol/glycerol, and incubated for 3 days at 30 °C. C, cells carrying the cox1Δ::ARG8mut mitochondrial gene were spotted on synthetic complete medium lacking uracil (+ Arg) or lacking arginine (−Arg), and incubated for 4 days at 30 °C.

from helix A (29). Based on the crystal structure of the TPR protein PilF (45), a model for six PPR repeats present in Pet309 was generated (Fig. 6). The model suggested that several side chains of basic residues are facing the central groove. This contributes to the calculated groove’s highly positive electrostatic potential (data not shown) and is in agreement with the proposed structural model of PPR proteins (29). If the prediction is correct, lowering the positive charges within the central groove of Pet309 should affect the function of the protein.

To test this hypothesis, selected arginines or lysines from PPR3 were mutagenized to alanines (Fig. 6). The mutations were: K424A, R427A, and K424A/R427A. All these residues are presumably present in helix A of the PPR3 and are facing the inner groove of the PPR region. The pet309-HA mutants were cloned in single copy or high copy expression vectors and transformed into a pet309Δ::LEU2 strain. When expressed on low copy plasmids, the mutants K424A and R427A failed to grow robustly on non-fermentable carbon sources (Fig. 7A); however, under high copy expression the mutants showed normal respiratory growth compared with the wild-type PET309-HA strain. Thus, overexpression of the mutant proteins could compensate for the respiratory defect observed under low expression of the Pet309 mutants. As expected, the double mutant K424A/R427A showed a stronger respiratory defect, compared with the single mutants. This defect could not be bypassed by overproduction of the mutant Pet309 protein.

The respiratory growth defect observed for the mutants was related to a defect in the COX1 mRNA translation. Arg growth of the mutants was analyzed in strains bearing the cox1Δ::ARG8mut gene (Fig. 7B). The Arg phenotype of these strains followed the same pattern: the single mutants supported weak growth in media lacking arginine when expressed in single copy plasmids, whereas in high copy plasmids they showed wild-type Arg+ growth. The double mutant showed a weaker Arg growth than the single mutants, and its overexpression didn’t compensate for the Arg growth defect.

These results indicate that basic residues that presumably are facing the PPR inner groove of Pet309 are important for translation of the COX1 mRNA. These residues could directly be involved in the specific interaction of Pet309 with the COX1 mRNA.

DISCUSSION

It is well established that a set of proteins from the PPR family are involved in mRNA translation in chloroplasts (25, 54, 55) and mitochondria (11, 24). Pet309 from yeast was the first PPR protein described to be essential for translation and stability of the COX1 mRNA. In this work, we have demonstrated that the PPR domains present in Pet309 are necessary for translation of the COX1 mRNA. Genetic evidence demonstrates that Pet309 associates with the 5’-untranslated region of the COX1 mRNA to activate translation (11). Although biochemical evidence for this is still lacking, the PPR domains of Pet309 might be involved in this interaction. PPR proteins are predicted to bind RNA sequences (29), and this prediction has been confirmed in several cases (8, 19, 25, 56, 57). The mutant Pet309 lacking seven PPR domains lost the capacity for translation but not the capacity for stabilization of the COX1 mRNA. This protein might have other domains that are important for either direct mRNA binding or RNA interaction through other factors, because Pet309 has been found to be part of a large protein complex in mitochondria (58).

As observed for the wild-type Pet309, the mutant protein was found to be associated with the mitochondrial inner membrane as a peripheral protein, and facing the matrix side. This indicates that deletion of the seven PPRs did not abolish the proper import of Pet309Δppr into mitochondria. As observed for the wild-type Pet309, overexpression of Pet309Δppr led to an increased accumulation of the COX1 mRNA. Together these observations indicate that the mutant Pet309 protein conserves...
some of its native properties and suggest that Pet309 can behave as a modular protein.

A series of deletions of one PPR motif at a time were generated. We found that in vivo each one of the seven PPR domains present in the central portion of Pet309 was necessary for COX1 mRNA translation. This is not the case for other PPR proteins. The Arabidopsis HCF152 protein is composed of 12 PPR motifs. Only two PPR domains were found to be required for RNA binding but had low affinity. The affinity increased in the presence of more PPR motifs, and the highest affinity was obtained with the full-length protein (19). The human protein LRP130 was found to preferentially bind polypyrimidines, and this RNA-binding activity required only 2 of the 11 predicted PPR motifs (8). It should be noted, however, that these experiments were made in vitro conditions and in the absence of the physiological RNA substrate. It is not known whether HCF152 or LRP130 require the complete set of PPR motifs to be active in vivo.

Deletion of any of the PPR motifs in Pet309 abolished respiratory growth even when the mutant proteins were overproduced. This suggests that in the absence of any of the PPR domains no residual activity is present that could be compensated for by overexpression of the mutant Pet309 proteins. PPR proteins belong to a large family of helical repeat proteins that include the RNA-binding protein Pumilio (29, 59, 60). The Puf domain of Pumilio binds RNA in an extended single-stranded conformation (59, 61). A similar model for the PPR domains was suggested, where RNA would be bound as an extended strand inside the cavity formed by the PPR motifs in tandem (29). Deletion of one of the PPR domains in Pet309 might interfere with the affinity and strength of binding for the rest of PPRs. Each PPR motif could bind a specific sequence of nucleotides in the COX1 mRNA. Deletion of one of these motifs would leave a portion of the extended RNA “naked.” This could affect the structure of the PPR-RNA complexes surrounding the deletion, leading to failed PPR-RNA interactions.

A structural model for PPR 1–6 of Pet309 was generated. In this model the repeats formed a superhelix enclosing a groove.

The majority of residues projecting into this cavity are hydrophilic, with positively charged amino acids facing the bottom of each repeat. These residues could be involved in RNA binding. The Pet309 model is in agreement with the proposed model for PPR proteins (29). When two basic residues from PPR 3 that are facing the inner groove were mutated to alanines we found that efficiency of translation of COX1 was considerably affected. This effect was more pronounced in the double mutant. It is possible that the loss of these positive charges at the bottom of the PPR 3 could interfere with the strength of binding of the RNA phosphate backbone. A similar effect was observed when basic amino acids facing the concave surface of the Puf domain of Pumilio where mutated (59). In this work, a single point mutation (R1127A) abolished RNA binding. In addition to basic residues within the inner cavity of the Puf domain, side chains from polar and hydrophobic residues are facing the groove. Some of the polar residues at conserved positions interact with specific RNA bases, and solvent-exposed hydrophobic and basic residues stack against the bases in RNA (61). Similarly, the model of the PPR domains of Pet309 suggests that some polar and aromatic amino acids are facing the inner groove and could also be involved in the RNA sequence-specific binding of Pet309.

A common feature of proteins from the PPR family studied so far is that they are sequence-specific. This applies to Pet309, which functionally interacts with the COX1 5’-UTR and does not affect other mitochondrial mRNAs (11). The target for Pet309 in the COX1 5’-UTR remains to be elucidated. The sequence and length among the mitochondrial mRNAs are not conserved. This has hampered the identification of shared sequence or structural features that translational activators could recognize.

In addition to the PPR motifs, Pet309 has N-terminal and C-terminal regions of 346 and 333 amino acids, respectively. We propose that the PPR motifs present in Pet309 are necessary for sequence-specific binding to the 5’-untranslated leader of COX1, whereas other portions of the protein could be involved in the regulation of COX1 translation. This regulation could be achieved by interaction with the mitochondrial ribosomes, because some PPR proteins in kinetoplasts have been found to be associated with ribosomes (18, 62). Alternatively, the N- and/or C-terminal ends of Pet309 could interact with other factors involved in the translation regulation of COX1. This could be the case for Mss51p, which acts on the COX1 5’-UTR, but in contrast to Pet309, it also interacts with newly made Cox1 (52, 63).

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PPR Domains of Pet309 Are Required for COX1 Translation

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