Pet111p, an Inner Membrane-bound Translational Activator That Limits Expression of the \textit{Saccharomyces cerevisiae} Mitochondrial Gene \textit{COX2}*

The protein specified by the \textit{Saccharomyces cerevisiae} nuclear gene \textit{PET111} specifically activates translation of the mitochondrially coded mRNA for cytochrome \textit{c} oxidase subunit II (Cox2p). We found Pet111p specifically in mitochondria of both wild-type cells and cells expressing a chromosomal form for a functional epitope-tagged form of Pet111p. Pet111p was associated with mitochondrial membranes and was highly resistant to extraction with alkaline carbonate. Pet111p was protected from proteolytic digestion by the mitochondrial inner membrane. Thus, it is exposed only on the matrix side, where it could participate directly in organelar translation and localize Cox2p synthesis by virtue of its functional interaction with the \textit{COX2} mRNA 5′-untranslated leader. We also found that Pet111p is present at levels limiting the synthesis of Cox2p by examining the effect of altered \textit{PET111} gene dosage in the nucleus on expression of a reporter gene, \textit{cox2::ARG8}*, that was inserted into mitochondrial DNA. The level of the reporter protein, Arg8p, was one-half that of wild type in a diploid strain heterozygous for a \textit{pet111} deletion mutation, whereas it was increased 2.8-fold in a strain bearing extra copies of \textit{PET111} on a high-copy plasmid. Thus, Pet111p could play dual roles in both membrane localization and regulation of Cox2p synthesis within mitochondria.

The key products of mitochondrial gene expression are integral membrane proteins that are assembled with proteins encoded by nuclear genes to form energy-transducing complexes in the inner membrane (1–3). Thus, the activity of mitochondrial genetic systems must be coordinated with cellular gene expression and adapted for efficient delivery of hydrophobic proteins to the inner membrane. Translation of at least five of the seven major \textit{Saccharomyces cerevisiae} mitochondrially coded mRNAs is under the control of mRNA-specific activator proteins encoded by nuclear genes (4). Genetic analysis has revealed that the activator proteins functionally interact with the 5′-UTL\(^1\) of their target mRNAs and probably mediate productive interactions between those mRNAs and ribosomes (4, 5). As discussed below, several of the activator proteins have been found to be associated with mitochondrial membranes, suggesting that they could function to localize the synthesis of hydrophobic mitochondrial gene products to their sites of insertion in the inner membrane (3, 4, 6, 7). This hypothesis is strongly supported by the fact that the untranslated regions of the \textit{COX2} and \textit{COX3} mRNAs contain information that facilitates correct targeting of the Cox2p and Cox3p cytochrome oxidase subunits (8).

The translational activator proteins themselves are present at low levels (9) and have therefore been difficult to study. In no case have they been shown to be localized within mitochondria isolated from wild-type (nonoverproducing) cells such that they could participate directly in organelar protein synthesis. Cbs1p and Cbs2p, activators required for translation of the cytochrome \textit{o} mRNA (10), were detectable at normal levels in wild-type cells. They were found associated with the total mitochondrial membrane fraction. Cbs1p behaved like an integral membrane protein, whereas Cbs2p was peripherally associated, but they were not further localized within the organelle (6). Pet309p, required for Cox1p translation (11), could only be detected as an epitope-tagged species in cells overproducing the protein (12). Under these conditions it fractionated as an integral inner membrane protein, partially exposed on the outer surface (intermembrane space side). Mas51p, also required for Cox1p synthesis, was peripherally associated with mitoplast (mitochondria with ruptured outer membranes) membranes when expressed from a low-copy plasmid (13). Synthesis of Cox3p requires three interacting proteins, Pet54p, Pet122p, and Pet494p (14, 15). Only Pet54p could be detected immunologically in wild-type cells and was found to be peripherally associated with the inner membrane (16). Pet122p and Pet494p, when overproduced, fractionated as integral inner membrane proteins (16).

The mRNA-specific activators could regulate expression of mitochondrial genes in addition to targeting synthesis on the membrane if they were present at levels limiting translation. Alternatively, the activators could be present in excess, with regulation of mitochondrial gene expression controlled at some other step. This has been examined to date only in the case of \textit{COX3} expression using the synthetic reporter gene \textit{ARG8}** inserted into mtDNA at \textit{COX3} (17). Reduction of the gene dosage of \textit{PET494} caused reduced expression of the mitochondrial reporter, although increased dosage of \textit{PET494} caused only a modest increase in reporter expression.

In this study we have focussed on Pet111p, the only known translational activator for the \textit{COX2} mRNA (18, 19). Pet111p is known to be mitochondrially located (20) and to interact functionally with the \textit{COX2} mRNA 5′-UTL (21). Although the amino acid sequence of Pet111p is not highly conserved among...
budding yeasts, its function in specifically activating translation of the COX2 mRNA has been conserved, indicating an important role in the production of cytochrome c oxidase (22). Here we study the submitochondrial localization of Pet111p in cells expressing the PET111 gene at normal levels and examine the effect of altered PET111 gene dosage on expression of the mitochondrial COX2 gene. Our results are consistent with a direct role for Pet111p in localization of mitochondrial translation and demonstrate that it is a rate-limiting factor in the expression of the COX2 gene.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Genetic Methods—**S. cerevisiae strains used in this study are listed in Table I. All strains are congenic to the wild-type strain D273–10B (ATCC 25657) except DFS160rho0, HMD122, and NSG175, which were derived from DBY747 (23). Cells were grown in rich medium (1% yeast extract, 2% bacto-peptone) containing 2% galactose or 2% raffinose or in minimal medium (0.67% yeast nitrogen base) containing 2% raffinose, as indicated in the figure legends. Standard genetic methods were as described (24, 25). Strains deleted for PET111 carry either the pet111–14 deletion (26) or the pet111–9 deletion with LEU2 in its place (27).

**Epitope Tagging of Pet111p—**The PET111 reading frame was modified to encode three copies of the influenza virus HA epitope (28) at its 3′ end using the plasmid pCS124 (obtained from C. Shamu and J. Nunnari), an integrative plasmid carrying three copies of the HA sequence. The TRP1 gene. The downstream 556 base pairs of the TRP1 gene. The downstream 556 base pairs of mitochondrial DNA upstream of the PET111 gene were inserted into the EcoRI site for cloning purposes. In addition, PET111 was transformed into an *H. polymorpha* strain (34), purified on a DEAE Affi-Gel Blue column (Bio-Rad) and treated with acetone powder from *H. polymorpha* yeast strain. After scanning, blots were stripped with 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.4 mM thiourea in 6 M urea at 37 °C for 1 h and then washed in Tris-buffered saline. Protein blots were reprobed with anti-Arg8p antibody. Blots were then reprobed with anti-glucose-6-phosphate dehydrogenase polyclonal antiserum (Visatia) in Tris-buffered saline, pH 7.6, containing 0.1% Tween 20, washed in Tris-buffered saline, and then incubated with the primary antibody in Tris-buffered saline, pH 7.6, containing 0.1% Tween 20 either at 25 °C for 1 h or overnight at 4 °C. The filters were next washed in Tris-buffered saline and then incubated with secondary antibody in Tris-buffered saline, pH 7.6, containing 0.1% Tween 20 for 1 h at 57 °C. The secondary antibody was Alexa Fluor 594-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Life Technologies, Inc.) second antibody and the enhanced chemiluminescence system (Amerham Pharmacia Biotech).

For quantitative Western analysis, cells were harvested at mid-log phase, and total protein was isolated as described (37). Samples were subjected to SDS-electrophoresis in 12% polyacrylamide and then transferred to Immobilon-P (Millipore). Membranes were blocked in 5% skim milk (34) derived from an *H. polymorpha* yeast strain. After scanning, blots were stripped with 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.4 mM thiourea in 6 M urea at 37 °C for 1 h and then washed in Tris-buffered saline. Protein blots were reprobed with anti-Arg8p antibody. Blots were then reprobed with anti-glucose-6-phosphate dehydrogenase polyclonal antiserum (Sigma) diluted 1:250.

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**TABLE I**

| Strain         | Genotype                          | Source                  |
|----------------|-----------------------------------|-------------------------|
| PTY11          | MATa ura3–52 trp1–14              | P. E. Thorsness         |
| NB39–16D       | MATa ura3 trp1 pet111–9           | N. Bonney               |
| NSG91          | MATa ura3–52 trp1–14 PET111-HATrp1 | This study              |
| HMD22          | MATa lys2 leu2–3,12 ura3–52 his3 arg8:hisG [p' cox2:ARG8m] | This study              |
| NB40–20D       | MATa lys2 arg8:hisG [p' cox2:ARG8m] | This study              |
| GW341hr0       | MATa ade2 ura3–52 leu2–112 arg8:hisG [p'] | G. Wiesenberger        |
| NSG176         | MATa ade2 ura3–52 leu2–112 arg8:hisG [p' cox2:ARG8m] | This study              |
| NSG170hr0      | MATa ade2 lys2 arg8:hisG [p']     | This study              |
| NSG165hr0      | MATa ade2 lys2 ura3–52 arg8:URA3 pet111–14 [p'] | This study              |
| NSG164hr0      | MATa lys2 leu2–3,12 arg8:hisG pet494–41 [p'] | This study              |
| NSG161hr0      | MATa ade2 lys2 ura3–52 his3 arg8:hisG [p' cox2:ARG8m] | This study              |
| DFS169hr0      | MATa ade2–101 leu25 ura3–52 arg8:URA3 kar1–1 [p'] | (17)                    |
| HMD122         | MATa ade2–101 leu25 ura3–52 arg8:URA3 kar1–1 [p' cox2:ARG8m] | This study              |
| NSG175         | MATa ade2–101 ura3–52 kar1–1 [p' cox2:ARG8m] | This study              |

Membrane-bound Activator Limits Translation with an arg8 strain (34). The mouse monoclonal antibody 12CA5 against the HA epitope was purchased from Berkeley Antibody Corp. (Berkeley, CA) and was used at 1:3000. Monoclonal anti-Cox2p (CC06) was a gift from T. L. Mason. Polyclonal anti-cytochrome b6 and anti-α-ketoglutarate dehydrogenase were gifts from B. Glick and G. Schatz. Preparation of antiserum against Arg8p (17) and Yme1p (1) was described previously. For the experiments of Figs. 1, 2, and 3, antigen-antibody complexes were visualized on Western blots using horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Life Technologies, Inc.) second antibody and the enhanced chemiluminescence system (Amerham Pharmacia Biotech).

For quantitative Western analysis, cells were harvested at mid-log phase, and total protein was isolated as described (37). Samples were subjected to SDS-gel electrophoresis in 12% polyacrylamide and then transferred to Immobilon-P (Millipore). Membranes were blocked in 5% skim milk (34) derived from an *H. polymorpha* yeast strain. After scanning, blots were stripped with 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.4 mM thiourea in 6 M urea at 37 °C for 1 h and then washed in Tris-buffered saline. Protein blots were reprobed with anti-Arg8p antibody. Blots were then reprobed with anti-glucose-6-phosphate dehydrogenase polyclonal antiserum (Sigma) diluted 1:250. Scanned images were analyzed using ImageQuant version 1.1 software from Molecular Dynamics. Area, the summation of pixel intensity times the number of pixels, was quantitated for the desired bands. Each Arg8p signal was normalized to the corresponding G6PD signal.

**Construction of the cocx2–ARG8m Reporter Gene—**The plasmid pH66 was constructed in the vector pZTS18u (Bio-Rad). It contains the ARG8m sequence (17) (see GenBankTM accession U31093) flanked by 0.57 kilobases of mitochondrial DNA upstream of the COX2 reading frame and 0.81 kilobases of mitochondrial DNA downstream of the COX2 reading frame. The sequence immediately downstream of the ARG8m stop codon contains an artificial BamHI site for cloning purposes. In addition, pH66 contains a 0.75-kilobase PacI–MboI COX3 fragment inserted between the BamHI and HindIII site of the polylinker to serve as an
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RESULTS

Detection of Pet111p, Expressed at Normal Levels, in Purified Mitochondria—Pet111p is expressed at very low levels (9), and mito-
chondrial transformants were identified by their ability to marker
rescue the \( \text{cox3–10} \) mutation (40). One such transformant,
HMD122, was isolated and used to transfer pHID6 into \( \rho^0 \) mitochondria by mating to appropriate strains and selecting Arg\(^{+} \) haploid cytoductants.

A new rabbit polyclonal antiserum was raised against a
fusion protein bearing the C-terminal 542 Pet111p amino acid residues and glutathione S-transferase, expressed in \( E. coli \) (“Experimental Procedures”). This serum was used to probe subcellular fractions from a wild-type strain (PTY11) on a Western blot. It reacted with a protein of the size expected for Pet111p (94 kDa) that was highly enriched in gradient-purified mitochondria and absent from post-mitochondrial supematant (Fig. 1A). This protein was not detected in fractions isolated from a pet111 deletion mutant (Fig. 1) nor in wild-type fractions probed with pre-immune serum (not shown). Furthermore, the level of this protein was increased severalfold relative to total mitochondrial protein in the wild-type strain containing the multicopy plasmid pJM20, which carries a genomic fragment including the \( PET111 \) promoter and tran-
scription terminator (26) (Fig. 1). Cross-reacting species of \( \sim 56 \) kDa, which may include Pet111p degradation products, were also detected (not shown).

In addition, we were able to detect Pet111p as a tagged protein bearing three copies of the HA epitope, expressed from its chromosomal locus. Such strains were fully Pet\(^{+} \), demonstrating that Pet111p-HA is a functional protein. Mitochondria were purified from a strain expressing Pet111p-HA and from a wild-type strain and were probed with an anti-HA monoclonal antibody and with anti-Pet111p (Fig. 1B). The anti-HA antibody reacted with a protein of the appropriate size in the tagged mitochondria but not in the corresponding wild-type mitochondria. The anti-Pet111p reacted with the same protein in both samples, confirming that the species in question is indeed Pet111p.

Pet111p Is Firmly Bound to the Inner Membrane, on the Matrix Side—To determine the location of Pet111p within the mitochondria, submitochondrial fractions were prepared from a strain expressing Pet111p-HA (NSG91). Purified mitochondria were disrupted by osmotic shock and sonication, then fractionated by centrifugation to separate soluble and membrane-associated proteins. The membrane fraction was then extracted with alkaline sodium carbonate to separate peripheral membrane proteins from integral membrane proteins. The resulting fractions were then analyzed by Western blots probed with anti-HA to determine the distribution of Pet111p-HA. Most of the Pet111p-HA was recovered in the membrane pellet after sonication (Fig. 2). Most of the membrane-associated Pet111p-HA remained with the integral membrane protein fraction after alkaline extraction, indicating that Pet111p is firmly membrane-bound. The known integral membrane protein Cox2p was completely associated with membranes in this experiment, whereas the soluble matrix protein Arg8p was not.

Protease protection experiments were used to determine the submitochondrial location of Pet111p-HA (Fig. 3). Proteinase K treatment of mitochondria solubilized by detergent eliminated detectable Pet111p-HA, showing that the epitope is not protected by a stable protein complex. However, Pet111p-HA in both whole mitochondria and in mitoplasts (osmotically shocked to rupture the outer membrane only) was protected from digestion by proteinase K. Thus, the C-terminal epitope appears to be on the inside of the inner membrane. Furthermore, since no shorter species were detected, it appears that the entire Pet111p-HA protein is protected from protease by

\(^2\) C. A. Strick and T. D. Fox, unpublished data.

FIG. 1. Detection of Pet111p in purified mitochondria. A, mitochondria were prepared from cells of \( PET111 \) wild-type (PTY11), pet111 (NB39–16D), and wild-type containing \( PET111 \) on a high-copy (H-C) plasmid, \( PET111 \) (PTY11 [pJM20]), grown to late logarithmic phase in complete medium containing galactose, and purified by equilibrium density gradient centrifugation (“Experimental Procedures”). Approximately 50 \( \mu \)g of protein from total cell extracts (\( T \), post-mitochondrial supernatant, \( S \)), and purified mitochondria (\( M \)) were analyzed by Western blot, probed with the polyclonal anti-Pet111p antiserum. The arrow indicates the Pet111p-specific band of \( \sim 94 \) kDa. A cross-reacting band of \( \sim 55 \) kDa was also evident (not shown). B, mitochondria were prepared as described above from \( PET111 \) wild-type (PTY11) and a \( PET111 \)-HA strain, whose chromosomal gene encodes HA epitope tags at the 3’ end (NSG91). Approximately 50 \( \mu \)g of purified mitochondria from each strain were subjected to Western blotting in duplicate. One blot was probed with a monoclonal anti-HA antibody, the other with polyclonal anti-Pet111p antibody, as indicated.

Additional mitochondrial genetic marker. The sequence of pHID6 is available upon request.

pHID6 was introduced into the \( \rho^0 \) mitochondria of strain DFS160ho0 (17) by microprojectile bombardment as described (38, 39), and mitochondrial transformants were identified by their ability to rescue the \( \text{cox3–10} \) mutation (40). One such transformant, HMD122, was isolated and used to transfer pHID6 into \( \rho^0 \) mitochondria by mating to appropriate strains and selecting Arg\(^{+} \) haploid cytoductants.

**Fig. 2.** Pet111p is firmly bound to mitochondrial membranes. Mitochondria were purified from cells with the chromosomally integrated \( PET111 \)-HA gene (NSG91), grown to late logarithmic phase in complete galactose medium (“Experimental Procedures”) and 50-\( \mu \)g aliquots of total mitochondrial protein (\( T \)), and submitochondrial fractions were analyzed by Western blotting. Mitochondria were separated into soluble (\( S \)) and membrane (\( M \)) fractions (“Experimental Procedures”). Membranes were further extracted with alkaline carbonate (“Experimental Procedures”) to separate peripheral membrane proteins (PM) from integral membrane proteins (IM). The Western blots were probed with anti-HA to detect Pet111p-HA as well as antisera against inner membrane-bound Cox2p and the soluble matrix enzyme Arg8p (encoded by the wild-type nuclear ARG8 gene in this strain).

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FIG. 3. Pet111p is an inner membrane protein facing the matrix. Mitochondria (M) were purified from the PET111-HA strain NSG91 and converted to mitoplasts (MP) by osmotic shock in the absence or presence of proteinase K, as indicated for each lane (“Experimental Procedures”). Mitochondria were also treated with proteinase K in the presence of the detergent octyl glucoside (1%) to solubilize membranes. Treated samples were analyzed by Western blots probed with anti-HA to detect Pet111p-HA as well as antisera against the soluble matrix enzyme α-ketoglutarate dehydrogenase (αKDH), the inner membrane-bound protein Yme1p exposed on the outer surface, and the soluble intermembrane space protein cytochrome (cyt) b2.

the inner membrane. The same behavior was observed for the soluble matrix marker α-ketoglutarate dehydrogenase (Fig. 3). Yme1p is an integral inner membrane protein exposed on the outer surface of the inner membrane (41) and is not protected from protease by mitoplasts, as expected (Fig. 3). Taken together, these data indicate that Pet111p is tightly associated with the inner surface of the inner mitochondrial membrane.

mRNA-specific Translational Activation by Pet111p Is Rate-limiting for Expression of the Mitochondrial Reporter Gene. The expression of the mitochondrial COX2 gene could be limited at the level of translation by the activity of Pet111p. To examine this possibility, we constructed a cox2::ARG8m reporter gene that had the second codon of ARG8m fused to the COX2 translation initiation codon and lacked all other COX2 codons (“Experimental Procedures”). This reporter was inserted into mitochondrial DNA, cleanly replacing the COX2 open reading frame with ARG8m. The resulting chimeric gene specifies an mRNA with the Pet111p target in the COX2 5′-UTL, upstream of the ARG8m-coding sequence, followed by the COX2 3′-untranslated region. Expression of this mitochondrial reporter gene fully complemented the Arg8p requirement caused by a nuclear arg8 mutation. This complementation was abolished by a pet111 nuclear mutation, demonstrating that cox2::ARG8m expression is Pet111p-dependent. As expected, the pet111 mutation prevented accumulation of immunologically detectable Arg8p encoded by the mitochondrial reporter (not shown).

To ask whether the level of cox2::ARG8m mRNA translation was limited by the level of Pet111p, we constructed strains containing the mitochondrial reporter with different dosages of the PET111 nuclear gene. We then measured the relative steady-state levels of Arg8p in whole cell extracts by quantititative Western blotting (“Experimental Procedures”). Measurements of Arg8p were internally controlled by normalization to the similarly measured steady-state level of the cytoplasmic enzyme glucose-6-phosphate dehydrogenase. Expression of the gene encoding glucose-6-phosphate dehydrogenase, ZWF1, appears to be relatively constant under different growth conditions (42, 43).

First, the dosage of PET111 was halved by constructing a diploid strain heterozygous for a pet111 mutation. A representative Western blot of the indicated amounts of total protein probed with antisera against Arg8p and glucose-6-phosphate dehydrogenase (G6PD) is shown. The Arg8p detected here is encoded by the mitochondrial cox2::ARG8m reporter gene in diploids whose relevant nuclear genotypes are wild type, pet494/PET494, and pet111/PET111. A diploid lacking any active ARG8 gene (arg8) is included as a control. B, four such blots were analyzed quantitatively (“Experimental Procedures”), normalizing Arg8p levels to glucose-6-phosphate dehydrogenase, and averaged. Error bars indicate the S.D. The diploids used in this experiment were constructed by mating the following haploids (Table I): arg8, GW241rho0 and NB40–20D; wild-type, NSG176 and NSG170rho0; pet111/PET111, NSG176 and NSG165rho0; pet494/PET494, NSG176 and NSG164rho0. The heterozygous diploids grew as well as the homozygous wild-type diploid on medium lacking arginine (not shown).
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FIG. 5. Expression of the mitochondrial reporter gene \textit{cox2::ARG8} \textsuperscript{m} is increased in a wild-type diploid containing extra plasmid-borne copies of \textit{PET111}. \textbf{A}, total protein was extracted from homozygous wild-type cells transformed with either the empty vector YEp352 (44) or the \textit{PET111} plasmid pJM20 (26) and grown in minimal medium containing raffinose. A diploid lacking any active \textit{ARG8} gene (arg8) is included as a control. Proteins were analyzed as described in the legend to \textbf{Fig. 4}. Bars, three such blots were quantitatively analyzed as described in the legend to \textbf{Fig. 4}. \textit{Bars} indicate S.D. The strains used in this experiment were constructed by mating the following haploids (Table I): \textit{arg8}, GW241rho0 and NB40–20D; wild-type, NSG176 and NSG178. The multicopy PET111 gene dosage on the expression of the \textit{cox2::ARG8} \textsuperscript{m} reporter gene varied (Table I), total protein was extracted from homozygous wild-type cells transformed with either the empty vector YEp352 (44) or the \textit{PET111} plasmid pJM20 (26) and grown in minimal medium containing raffinose. A diploid lacking any active \textit{ARG8} gene (arg8) is included as a control. Proteins were analyzed as described in the legend to \textbf{Fig. 4}. Bars, three such blots were quantitatively analyzed as described in the legend to \textbf{Fig. 4}. \textit{Bars} indicate S.D. The strains used in this experiment were constructed by mating the following haploids (Table I): \textit{arg8}, GW241rho0 and NB40–20D; wild-type, NSG176 and NSG178.

\begin{table}[h]
\centering
\begin{tabular}{ccc}
\hline
          & YEp352 & pJM20  \\
\hline
\textit{arg8} & 100    & 75     & 100   \\
50            & 50     & 75     & 100   \\
75            & 50     & 75     & 100   \\
100           & 50     & 75     & 100   \\
\hline
\end{tabular}
\caption{Expression of the mitochondrial reporter gene \textit{cox2::ARG8} \textsuperscript{m} in wild-type and \textit{pet494Δ/PET494} heterozygous diploid relative to the homozygous wild-type strain but instead appeared to be slightly increased (Fig. 4).}

We next examined the effect of increasing \textit{PET111} gene dosage on the expression of the \textit{cox2::ARG8} \textsuperscript{m} reporter gene. The multicopy \textit{PET111} plasmid pJM20 (26) was transformed into a homozygous wild-type diploid strain. The steady-state level of Arg8p present in this transformant was determined relative to that of the same strain transformed with the empty vector YEp352 (44). The presence of extra copies of \textit{PET111} caused the steady-state level of Arg8p to increase by \textsuperscript{−2.8-fold} (Fig. 5), confirming that Pet111p activity is a limiting factor in the expression of the mitochondrial \textit{COX2} gene.

\textbf{DISCUSSION}

\textit{S. cerevisiae} Pet111p is synthesized in the cytoplasm as an 800-amino acid protein, which may be processed upon entry into mitochondria. It is largely hydrophilic and has a net positive charge. Previous genetic data have argued for a close functional interaction between Pet111p and the \textit{COX2} mRNA in the mitochondrial matrix, where translation occurs. Here, we have detected Pet111p in mitochondria of cells expressing it at normal levels and demonstrated that it is tightly associated with the inner membrane. Pet111p is protected by the inner membrane from added protease, indicating that its hydrophilic domains must be exposed on the matrix side where they could interact with the \textit{COX2} mRNA and components of the organelar translation system. Thus, Pet111p is very likely to be the membrane component that recognizes targeting information in the untranslated portions of the \textit{COX2} mRNA, promoting efficient cytochrome \textit{c} oxidase assembly (8).

Pet111p was largely resistant to removal from mitochondrial membranes by extraction with alkaline carbonate, suggesting that it is an integral membrane protein (32). However, it was not as firmly bound as the known integral membrane protein Cox2p. Although analysis of the \textit{S. cerevisiae} Pet111p sequence using the algorithm TMAP suggests two possible transmembrane helical domains (125 - 146 and 548–564), the more robust analysis, with the same program, of aligned Pet111p homologs from three budding yeasts (22) does not suggest any such domains (45, 46). The Pet122p subunit of the \textit{COX3} mRNA-specific activator behaves as an integral membrane protein both when overproduced (16) and when expressed from a single chromosomal gene.\textsuperscript{3} Yet Pet122p also lacks apparent transmembrane helical domains and residues exposed to proteolysis on the outer surface of the inner membrane.\textsuperscript{3} This behavior contrasts with that of overproduced Pet309p, the \textit{COX1} mRNA-specific activator, that spans the inner membrane and is accessible to protease from the outside (12).

The amount of Pet111p appears to limit expression of the mitochondrial gene \textit{COX2} at the level of translation, since the level of Arg8p encoded by a \textit{cox2::ARG8} \textsuperscript{m} reporter gene varied with \textit{PET111} gene dosage. The reduction of Arg8p was clearly proportional to \textit{PET111} dosage when we reduced the number of \textit{PET111} genes by half. The Arg8p level increased 2.8-fold when \textit{PET111} was introduced into cells on a multicopy vector that caused an approximately similar increase in the level of Pet111p. This result is consistent with a previous report that the \textit{COX2} mRNA level is not limiting for gene expression since overexpression of that mRNA does not lead to increased synthesis of Cox2p (47). We conclude that modulation of the level of Pet111p and/or its translational activation activity could regulate the level of Cox2p synthesis within mitochondria.

Increased activity of a limiting translational activator will increase translation of the target mitochondrial mRNA up to the point that some other component (mRNA, ribosomes, etc.) becomes limiting. We cannot be certain whether this point was reached in our overproduction experiments. However, we did not greatly exceed it since the increase in Pet111p levels in mitochondria from cells with increased \textit{PET111} dosage was approximately similar to the 2.8-fold increase in reporter gene expression. In a previous study of \textit{cox3::ARG8} \textsuperscript{m} expression we found that overproduction of the limiting activator subunit, Pet494p, increased the mitochondrial reporter expression only by \textsuperscript{\textendash}65% (17). The possibility of competition between translational activators for low levels of some general translation component is suggested by the apparent slight increase in \textit{cox2::ARG8} \textsuperscript{m} expression in a diploid heterozygous for a \textit{pet494} deletion, but this effect may not be significant.

The \textit{PET111} mRNA has an unusually long 5'-UTL of 470 nucleotides that contains four overlapping open reading frames upstream of the Pet111p-coding sequence (20), suggesting the possibility that \textit{PET111} expression itself might be controlled at

\textsuperscript{3} C. A. Butler and T. D. Fox, unpublished data.

\(\text{\textsuperscript{arg8}, GW241rho0 and NB40–20D; wild-type, NSG176 and NSG178.}\)
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the level of cytoplasmic translation (48). Such an mRNA structure could serve either as a target for regulation that limits gene expression independently of mRNA levels via feedback, or it could simply reduce the translational efficiency of the PET111 mRNA. The fact that increased copy number of the PET111 gene causes an increase in Pet111p levels and activity demonstrates Pet111p synthesis is not translationally limited independently of the mRNA level. However, we have not established whether the protein and mRNA levels vary proportionately.

Translational feedback regulatory loops that couple synthesis of specific components to the assembly of a chloroplast membrane complex in Chlamydomonas (49, 50) and of the basal body-hook structure of Caulobacter (51) have been described. Our results show that Pet111p is very likely to play roles in both regulating the rate of Cox2p synthesis and localizing that synthesis on the surface of the inner membrane. Thus, Pet111p could participate in a feedback mechanism coupling Cox2p translation with its assembly into the cytochrome c oxidase complex.

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