

# A Novel Function of Pet54 in Regulation of Cox1 Synthesis in *Saccharomyces cerevisiae* Mitochondria\*

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Cytochrome *c* oxidase assembly requires the synthesis of the mitochondria-encoded core subunits, Cox1, Cox2, and Cox3. In yeast, Pet54 protein is required to activate translation of the *COX3* mRNA and to process the *a15β* intron on the *COX1* transcript. Here we report a third, novel function of Pet54 on Cox1 synthesis. We observed that Pet54 is necessary to achieve an efficient Cox1 synthesis. Translation of the *COX1* mRNA is coupled to the assembly of cytochrome *c* oxidase by a mechanism that involves Mss51. This protein activates translation of the *COX1* mRNA by acting on the *COX1* 5'-UTR, and, in addition, it interacts with the newly synthesized Cox1 protein in high molecular weight complexes that include the factors Coa3 and Cox14. Deletion of Pet54 decreased Cox1 synthesis, and, in contrast to what is commonly observed for other assembly mutants, double deletion of *cox14* or *coa3* did not recover Cox1 synthesis. Our results show that Pet54 is a positive regulator of Cox1 synthesis that renders Mss51 competent as a translational activator of the *COX1* mRNA and that this role is independent of the assembly feedback regulatory loop of Cox1 synthesis. Pet54 may play a role in Mss51 hemylation/conformational change necessary for translational activity. Moreover, Pet54 physically interacts with the *COX1* mRNA, and this binding was independent of the presence of Mss51.

Cytochrome *c* oxidase (CcO)<sup>3</sup> is the last electron acceptor of the mitochondrial respiratory chain. In the yeast *Saccharomyces cerevisiae*, this enzyme contains 12 subunits, three of which (Cox1, Cox2, and Cox3) are encoded by the mitochondrial

DNA. Assembly of CcO is a complex process regulated by more than 25 factors and chaperones (for reviews, see Ref. 1). The first steps of CcO biogenesis involve the translational activation of the mitochondria-encoded mRNAs *COX1*, *COX2*, and *COX3* by mRNA-specific proteins. Translational activation of the *COX1* mRNA depends on Pet309 and Mss51 (2, 3), whereas *COX2* translation depends on Pet111 (4, 5), and *COX3* mRNA translation depends on Pet54, Pet122, and Pet494 (6–9). These proteins act on the target mRNA 5'-UTRs to allow translation by the mitochondrial ribosomes. They interact with each other and with the mitochondrial inner membrane and are thought to tether translation initiation close to the assembly sites of CcO in the membrane (for a review, see Ref. 10) (11). The mitochondria-encoded Cox1, Cox2, and Cox3 subunits are proposed to assemble from three different modules, each containing a specific subset of nucleus-encoded subunits (12–14).

Cox1, the largest subunit of the CcO, carries the heme *aa*<sub>3</sub>-Cu<sub>B</sub> center to reduce oxygen. Synthesis of Cox1 inside mitochondria is highly regulated. If CcO assembly is blocked by mutations on either integral subunits or accessory chaperones, Cox1 synthesis is down-regulated (15, 16). It is proposed that by this mechanism, mitochondria avoids accumulation of pro-oxidant Cox1 intermediates (17). In addition to its role as translational activator of *COX1* mRNA, Mss51 also physically interacts with Cox1 protein to form the first high molecular weight intermediaries (COA complexes) that include the chaperones Cox14 and Coa3 (15, 18–20). The current model (reviewed in Refs. 1 and 21) states that if CcO assembly is defective, then Mss51 is sequestered on COA intermediaries to reduce the effective concentration of Mss51 as a translational activator of the *COX1* mRNA, resulting in a decrease of Cox1 synthesis. In this context, Cox14 and Coa3 are negative regulators, because their deletion restores Cox1 synthesis when assembly of CcO is deficient (15, 18, 19). The C-terminal end of Cox1 is also a negative regulator of Cox1 synthesis. Deletion of the last 11–15 residues of the Cox1 C-terminal end results in normal Cox1 synthesis even if CcO is not assembled (16). In addition to the first intermediates formed by Cox1, Mss51, Cox14, and Coa3, subunit 1 forms subsequent intermediates (12, 22) that include proteins like Coa1 (23, 24), Shy1 (23, 25, 26), subunits Cox5/Cox6 (19), Coa2 (25), and Cox15 (26). Moreover, the Hsp70 chaperone Ssc1 is associated with both Mss51 and Mss51-containing complexes and regulates Cox1 synthesis as well (27). Mss51 contains two heme-regulating motifs. These motifs are

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<sup>3</sup> The abbreviations used are: CcO, cytochrome *c* oxidase; ARG8<sup>™</sup>, mitochondria-encoded acetylornithine aminotransferase; RRM, RNA recognition motif; Mss51<sup>TE</sup>, translation-effective Mss51; Mss51<sup>TI</sup>, translational inactive Mss51; BN-PAGE, blue native PAGE; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; COA, cytochrome oxidase assembly.

## Pet54 Regulates COX1 Translation

important for hemylation of the protein and are involved in Cox1 regulation by Mss51 (28).

During the investigation of the role of the Cox1 C-terminal end in the Cox1 synthesis regulatory loop, we observed that Pet54 showed an unusual pattern. Deletion of *PET54* down-regulated Cox1 synthesis; however, in contrast to what was observed for most assembly mutants, deletion of the Cox1 C-terminal end did not recover Cox1 synthesis (16), indicating that Pet54 plays an additional role in this process.

Pet54 is a nuclear encoded, RNA recognition motif (RRM) protein located on the matrix face of the inner mitochondrial membrane as a peripheral protein (29). In addition to its role as translational activator of the *COX3* mRNA (7), Pet54 is required for splicing of the *a15 $\beta$*  intron on the *COX1* pre-mRNA (30). *In vitro* experiments demonstrated that Pet54 binds with the *COX1* pre-mRNA *a15 $\beta$*  intron and the *COX3* mRNA 5'-UTR, both regions sharing 56% identity (31). In the present work, we demonstrated that Pet54 plays a third, novel role in Cox1 synthesis regulation that is independent of Cox14, Coa3, and the Cox1 C-terminal end. We also provide data showing that Pet54 is necessary for activation of translational competent Mss51, probably by binding to the *COX1* mRNA and by modulating the hemylation state and/or conformation of Mss51.

### Experimental Procedures

**Yeast Strains and Genetic Methods**—*S. cerevisiae* strains used in this study are listed in Table 1. Genetic manipulation methods and media were as described previously (32). Strains were cultured in a complete fermentable medium, YPD or YPGal (1% yeast extract, 2% Bacto-peptone, and 2% glucose or 2% galactose), or synthetic complete medium (0.67% yeast nitrogen base, 2% glucose) lacking the indicated amino acids. Nuclear deletion constructs with *URA3*, *LEU2*, or *KanMX4* cassettes were made by PCR. In all cases, correct integration of the different constructs into the nuclear genome was confirmed by PCR. For two-hybrid experiments, the plasmids used were as follows: Pet54-AD, pNGB8 (33); Pet54-BD, pNGB67 (29); and Pet122-AD, pNGB11 (33). Mss51-AD and Mss51-BD were cloned onto the *Cl*I site of pGAD-C1 and pGBDU-C1, respectively (34). Constructs were transformed in the yeast strain Pj69-4a (34), and double-transformed cells were selected on medium lacking leucine and tryptophan or leucine and uracil. Protein interactions were tested by plating on medium lacking histidine supplemented with 3 mM amino-1,2,4-triazole or lacking adenine.

**Analysis of Mitochondrial Proteins**—Cells were grown in YPGal medium until late log phase. Mitochondria were isolated by disruption of cells with glass beads or by zymolyase 20T treatment as described (35). Proteins were resolved by SDS-PAGE on 12% gels (36) and detected by immunoblotting with antibodies to HA (Roche Applied Science), c-Myc (Roche Applied Science), Cox1, Cox2, Cox3, cytochrome *b*, Coa3, Tom40, or citrate synthase. Secondary goat IgG anti-mouse or anti-rabbit (Sigma) conjugated to horseradish peroxidase was detected with the ECL (GE Healthcare) or Immobilon<sup>TM</sup> Western Chemiluminescent HRP Substrate (Millipore).

**Synthesis of Mitochondrial Proteins**—*In vivo* labeling of cells in the presence of [<sup>35</sup>S]methionine was performed as described previously (20). After 20 min of pulse labeling, cells were chilled on ice and disrupted by vortexing with glass beads to obtain mitochondria by centrifugation. Mitochondrial proteins were resolved on a 16% polyacrylamide gel, transferred to a polyvinylidene fluoride membrane, and analyzed with a Typhoon 8600 PhosphorImager (GE Healthcare).

**Blue Native PAGE (BN-PAGE)**—BN-PAGE was performed as described previously (37). Samples (100  $\mu$ g) of mitochondrial protein were washed with 250 mM sorbitol, 50 mM BisTris and lysed with 750 mM aminocaproic acid, 50 mM BisTris, and 1% digitonin for 30 min on ice. Mitochondrial extracts were cleared at 13,200 rpm for 12 min, and the supernatants were mixed with 2.5  $\mu$ l of 5% Coomassie solution (750 mM aminocaproic acid, 50 mM BisTris). Extracts were loaded on a 5–13% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. Proteins were detected by immunoblotting with the respective antibodies.

**RNA Immunoprecipitation Assay**—This technique was performed as described previously (38). Briefly, mitochondria (1 mg) were lysed with 0.7% *n*-dodecyl- $\beta$ -D-maltoside, 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, RNaseOUT (Invitrogen), and Minicomplete protease inhibitors (Roche Applied Science). After a clarifying spin, the solubilized fractions were incubated with an anti-Myc antibody coupled to protein A-agarose (Santa Cruz Biotechnology, Inc.). After centrifugation, immunoprecipitates were washed twice with 500  $\mu$ l of lysis buffer and twice with 1 ml of 20 mM HEPES-KOH, pH 7.4, and then resuspended in 150  $\mu$ l of the same buffer. One-fourth of the precipitate fractions were saved for Western blotting analysis, and the remainder were used for RNA extraction. RNA from total and immunoprecipitated fractions was extracted by incubation with TRIzol<sup>®</sup> reagent (Invitrogen). 20 ng of RNA were treated with 1 unit of DNase I (Invitrogen). The first strand of cDNAs were prepared by the addition of primers for *COX1*, *COX3*, or *VARI* in the presence of SuperScript III reverse transcriptase (Invitrogen). The resulting cDNAs were used as template for PCRs to amplify *COX1*, *COX3*, or *VARI* 5'-UTRs. Note that under these conditions, RT-PCRs are not quantitative.

### Results

**Pet54 Regulates Synthesis of Cox1**—We have previously demonstrated that the C-terminal end of Cox1 is involved in the regulation of its own synthesis. In mutants that block CcO assembly, synthesis of Cox1 is down-regulated; however, deletion of the last 15 residues of Cox1 (Cox1 $\Delta$ C15) recovered normal levels of Cox1 synthesis (16). This pattern was observed in many different mutants on either CcO subunits or chaperones that participate in enzyme assembly. The only exception was a deletion of *pet54*. In this mutant, synthesis of Cox1 is down-regulated, but deletion of the C-terminal end of Cox1 does not recover synthesis (16).

Pet54 activates translation of the mitochondrially coded *COX3* mRNA (7) and also promotes splicing of an intron found in the *COX1* transcript of most yeast strains (39). However, Pet54 was not thought to affect expression of *COX1* genes lacking this intron (39). In order to investigate whether or not Pet54



TABLE 1

## Strains used in this study

All of these strains are congenic or isogenic to D273-10B. Mitochondrial genotypes are shown in parenthesis.  $\Delta\Sigma ai$  represents an intronless *COX1* gene. *cox1Δ::ARG8<sup>m</sup>-1* has a replacement of the *COX1* 3'-UTR by the *COX2* 3'-UTR. *cox1Δ::ARG8<sup>m</sup>-2* has a replacement of the *COX1* 5'-UTR by the *COX2* 5'-UTR (40).

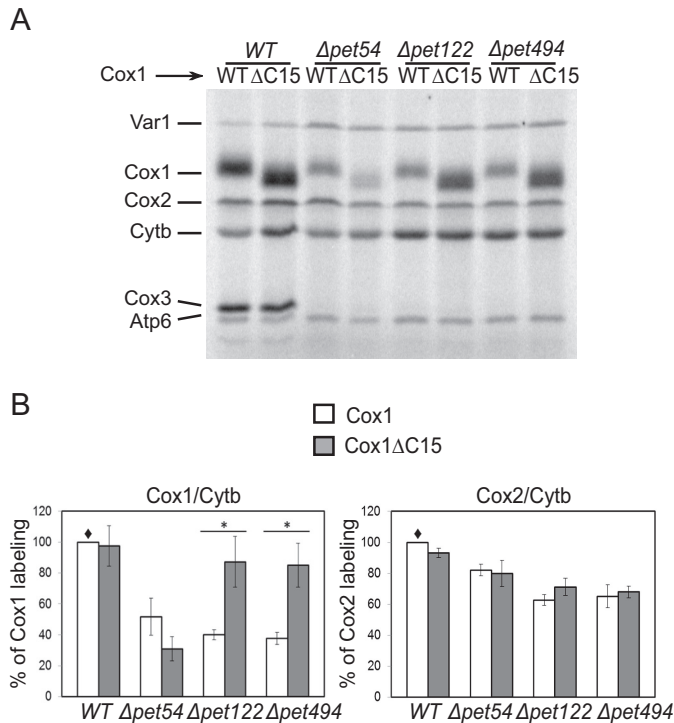
Strain	Nuclear (mitochondrial) genotype	Reference/Source
XPM10b	Mata, <i>arg8::hisG, leu2-3,112, lys2, ura3-52</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup></i> )	Ref. 20
XPM11	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112</i> ( $\rho^+$ , <i>COX1(1-512)::ARG8<sup>m</sup></i> )	Ref. 20
XPM48	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δmss51::LEU2</i> ( $\rho^+$ , <i>COX1(1-512)::ARG8<sup>m</sup></i> )	Ref. 20
XPM171	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>, cox2Δ::COX1, COX2<sup>st</sup></i> )	Ref. 20
XPM182	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet111::URA3</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>, cox2Δ::COX1, COX2<sup>st</sup></i> )	Ref. 20
XPM201	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	Ref. 16
XPM209	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	Ref. 16
XPM295	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	Ref. 16
XPM298	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	Ref. 16
XPM315	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4</i> ( $\rho^+$ , <i>COX1(1-512)::ARG8<sup>m</sup></i> )	Ref. 16
XPM316	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	Ref. 16
XPM317	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	Ref. 16
YC61	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δcox6::KANMX4</i> ( $\rho^+$ , <i>COX1(1-512)::ARG8<sup>m</sup></i> )	Ref. 16
YC75	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δcox14::URA3</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	Ref. 16
YC76	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δcox14::URA3</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	Ref. 16
YC77	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai, \Delta Cox14::URA3$ )	This work
YC78	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4, cox14Δ::URA3</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	Ref. 16
YC100	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, Δpet54::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
YC102	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet494::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
YC 103	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet494::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	This work
YC104	Mata, <i>arg8::hisG, leu2-3,112, lys2, ura3-52, Δpet494::KANMX4</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup></i> )	This work
YC105	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet494::KANMX4</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>, cox2Δ::COX1, COX2<sup>st</sup></i> )	This work
YC106	Mata, <i>arg8::hisG, leu2-3,112, lys2, ura3-52, Δpet122::KANMX4</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup></i> )	This work
YC109	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, PET54-3xMyc</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
YC112	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δcoa3::LEU2</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
YC113	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δcoa3::LEU2</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	This work
YC114	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4, Δcoa3::LEU2</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	This work
YC118	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4, Δcoa3::LEU2</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
LSR13	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet122::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	Ref. 16
LSR28	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet122::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	Ref. 16
LSR33	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>, cox2Δ::COX1, COX2<sup>st</sup></i> )	This work
LSR39	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δcox14::URA3</i> ( $\rho^+$ , <i>COX1(1-512)::ARG8<sup>m</sup></i> )	This work
LSR43	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δcox6::KANMX4</i> ( $\rho^+$ , <i>COX1(1-512)::ARG8<sup>m</sup></i> )	Ref. 16
LSR44	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet122::KANMX4</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>, cox2Δ::COX1, COX2<sup>st</sup></i> )	This work
SB5	Mata, <i>ade2, lys2, Δura3, PET309::3xHA</i> ( $\rho^+$ )	Ref. 60
JPM18	Mata, <i>arg8::hisG, leu2-3,112, lys2, ura3-52, Δpet54::KANMX4</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup></i> )	This work
JPM21	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet309::URA3</i> ( $\rho^+$ , <i>COX1(1-512)::ARG8<sup>m</sup></i> )	This work
JPM23	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δcox14::URA3, Δpet54::KANMX4</i> ( $\rho^+$ , <i>COX1(1-512)::ARG8<sup>m</sup></i> )	This work
JPM30	Mata, <i>ade2, lys2, Δura3, PET309-3xHA, Δpet54::KANMX4</i> ( $\rho^+$ )	This work
JPM40	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, Δcox14::URA3</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
JPM42	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, Δpet54::KANMX4, Δcox14::URA3</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
JPM43	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet111::URA3, Δpet54::KANMX4</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>, cox2Δ::COX1, COX2<sup>st</sup></i> )	This work
JPM44	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, PET54-3xMyc, Δmss51::LEU2</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
JPM49	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, Δpet122::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
JPM50	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, Δcox11::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
JPM52	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, PET54-3xMyc, Δpet494::URA3</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
JPM53	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, PET54-3xMyc, Δpet309::URA3</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
JPM55	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, Δpet122::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
JPM56	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, Δpet54::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	This work
JPM57	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, MSS51-3xHA, Δpet122::KANMX4</i> ( $\rho^+$ , $\Delta\Sigma ai, COX1\Delta C15$ )	This work
XPM330	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>-1, cox2Δ::COX1, COX2<sup>st</sup></i> )	This work
RGV125	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>-1, cox2Δ::COX1, COX2<sup>st</sup></i> )	This work
XPM329	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>-2, cox2Δ::COX1, COX2<sup>st</sup></i> )	This work
RGV124	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4</i> ( $\rho^+$ , <i>cox1Δ::ARG8<sup>m</sup>-2, cox2Δ::COX1, COX2<sup>st</sup></i> )	This work
AGG81	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, LEU2::YIplac128-mss51<sup>F199I</sup></i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work
AGG82	Mata, <i>lys2, arg8::hisG, ura3-52, leu2-3, 112, Δpet54::KANMX4 LEU2::YIplac128-mss51<sup>F199I</sup></i> ( $\rho^+$ , $\Delta\Sigma ai$ )	This work

<sup>a</sup> Ectopic insertion of the chimeric *COX1* gene upstream of the *COX2* gene.

played an additional role in Cox1 synthesis, we analyzed mitochondrial translation products by *in vivo* labeling with [<sup>35</sup>S]methionine in the presence of cycloheximide to inhibit cytoplasmic translation. We observed that absence of Pet54 reduced by 50% the labeling of wild-type Cox1 encoded by the intronless *COX1* gene used here (Fig. 1). This was the only mutant analyzed whose Cox1 labeling was not increased by C-terminal truncation of Cox1, suggesting that this effect might not be due simply to the lack of CcO assembly. Indeed, deletion of Pet122 or Pet494, the other *COX3* mRNA translational activators, resulted in a Cox1 labeling pattern similar to that observed for general CcO assembly factor mutants reported previously (16). In CcO assembly mutants, synthesis of Cox1 is down-regulated;

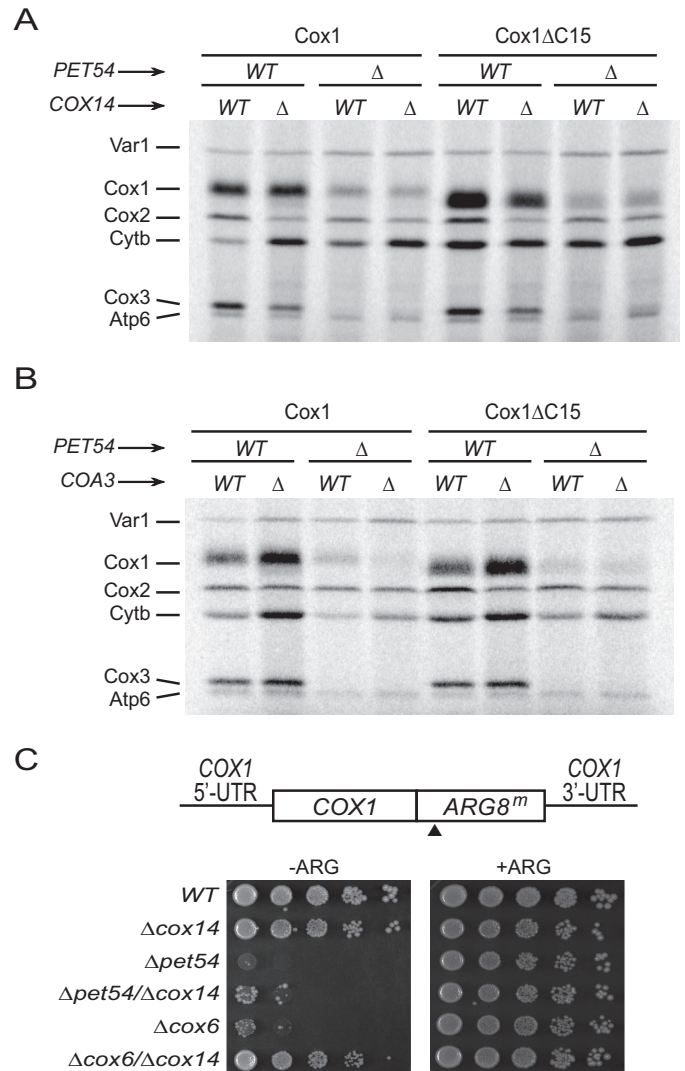
however, additional elimination of *COX14* or *COA3* recovers Cox1 synthesis, even if CcO assembly is impaired (15, 18, 19). To investigate whether Pet54 might be directly promoting Cox1 synthesis, we eliminated the assembly-mediated regulation of Cox1 by removing Cox14 or Coa3. The reduced labeling of Cox1 and Cox1ΔC15 in the double mutants ( $\Delta pet54/\Delta cox14$  and  $\Delta pet54/\Delta coa3$ ) showed a bypass of Cox14 and Coa3 synthesis control (Fig. 2, A and B), in contrast to what has been observed for a  $\Delta cox6$  mutant (16) or other mutants affecting Cox1 assembly (15, 18, 19). A similar result was observed with strains containing introns in *COX1* (data not shown), strongly suggesting that the role of Pet54 in translation of the *COX1* mRNA is independent of the role in *COX1* intron splicing.

## Pet54 Regulates COX1 Translation



**FIGURE 1. Down-regulation of Cox1 synthesis in  $\Delta$ pet54 mutants is independent of the Cox1 C-terminal end.** *A*, mitochondrial translation products from strains carrying the wild-type Cox1 (WT) or the Cox1 lacking the last 15 residues of the C-terminal end ( $\Delta$ C15) were pulse-labeled with [ $^{35}$ S]methionine in the presence of cycloheximide and analyzed by SDS-PAGE and autoradiography. The  $\Delta$ pet54,  $\Delta$ pet122, and  $\Delta$ pet494 mutants were introduced as indicated. Cytb, cytochrome *b*; Atp6, subunit 6 of ATPase; Var1, ribosomal protein. *B*, the intensity of the Cox1 labeling in *A* was quantified using the ImageJ software and normalized to the cytochrome *b* signal. It was expressed as a percentage of the wild-type Cox1 signal ( $\blacklozenge$ ). Error bars, S.D. values from three independent experiments. We also compared the signals of Cox2 with the cytochrome *b* signal. In these cases, no significant difference was observed. The relevant significant differences between strains (\*) were determined by Student's *t* test. A *p* value of  $<0.01$  was considered statistically significant.

To confirm that reduction of Cox1 labeling after elimination of assembly-mediated regulation was due to a defect in translation, we used the *COX1(1–512)::ARG8<sup>m</sup>* reporter inserted at the *COX1* locus. This reporter has been extensively used to differentiate between translation and post-translational defects in Cox1 (16, 20, 25, 28). In this construct, the reporter *ARG8<sup>m</sup>*, which codes for an enzyme involved in arginine biosynthesis, is fused in frame to the C-terminal end of Cox1. Because it codes for a mitochondrial targeting and processing sequence, accumulation of mature Arg8 should not be affected by the stability of Cox1. The Cox1 moiety encoded by *COX1(1–512)::ARG8<sup>m</sup>* is assembled into active CcO complexes, supporting normal respiratory growth (20). Thus, we combined the  $\Delta$ pet54 and  $\Delta$ cox14 mutations with the *COX1(1–512)::ARG8<sup>m</sup>* construct. As observed previously (16), the  $\Delta$ pet54 strain showed reduced growth in medium lacking arginine as compared with wild-type or  $\Delta$ cox14 cells (Fig. 2C). In agreement with the [ $^{35}$ S]methionine labeling pattern of Cox1, growth of the double mutant  $\Delta$ pet54/ $\Delta$ cox14 on medium lacking arginine was only slightly improved as compared with the single  $\Delta$ pet54 mutant. In contrast, and as previously observed (16), a  $\Delta$ cox6 mutant showed reduced growth on medium lacking arginine, but the double



**FIGURE 2. Down-regulation of Cox1 synthesis in  $\Delta$ pet54 mutants is independent of Cox14 and Coa3.** Cox1 or Cox1 $\Delta$ C15 cells with a deletion in *PET54*, *COX14* (*A*), or *COA3* (*B*), as indicated, were pulse-labeled with [ $^{35}$ S]methionine in the presence of cycloheximide. The mitochondrial products were analyzed by SDS-PAGE and autoradiography. *C*, growth phenotype of strains bearing the *COX1(1–512)::ARG8<sup>m</sup>* mitochondrial construct. The *ARG8<sup>m</sup>* gene was fused in-frame to the complete *COX1* codons, and the black triangle indicates the processing site for the pre-Arg8 matrix-targeting signal. Mutation  $\Delta$ pet54,  $\Delta$ cox14, or  $\Delta$ cox6 was introduced as indicated. Cells were spotted as serial dilutions on medium with arginine (+ARG) or lacking arginine (–ARG) and grown for 2 days at 30 °C.

deletion  $\Delta$ cox6/ $\Delta$ cox14 recovered normal growth levels. These observations show that the CcO assembly defect caused by the loss of Pet54 reduced synthesis of the reporter fused to Cox1 and that this reduction is not recovered on  $\Delta$ cox14 cells.

Taken together, our data indicate that deletion of Pet54 reduced Cox1 synthesis and that this reduction was not dependent on the assembly-regulatory C-terminal end of Cox1 or Cox14/Coa3 regulatory factors, as observed for most CcO assembly mutants. Our results strongly support the idea that Pet54 has a previously undetected direct positive role in promoting Cox1 synthesis.

*Pet54 Acts on the 5'-Untranslated Region of COX1 mRNA*—Pet54 could regulate synthesis of Cox1 at two different sites. (i) It could have a positive role in *COX1* mRNA untranslated

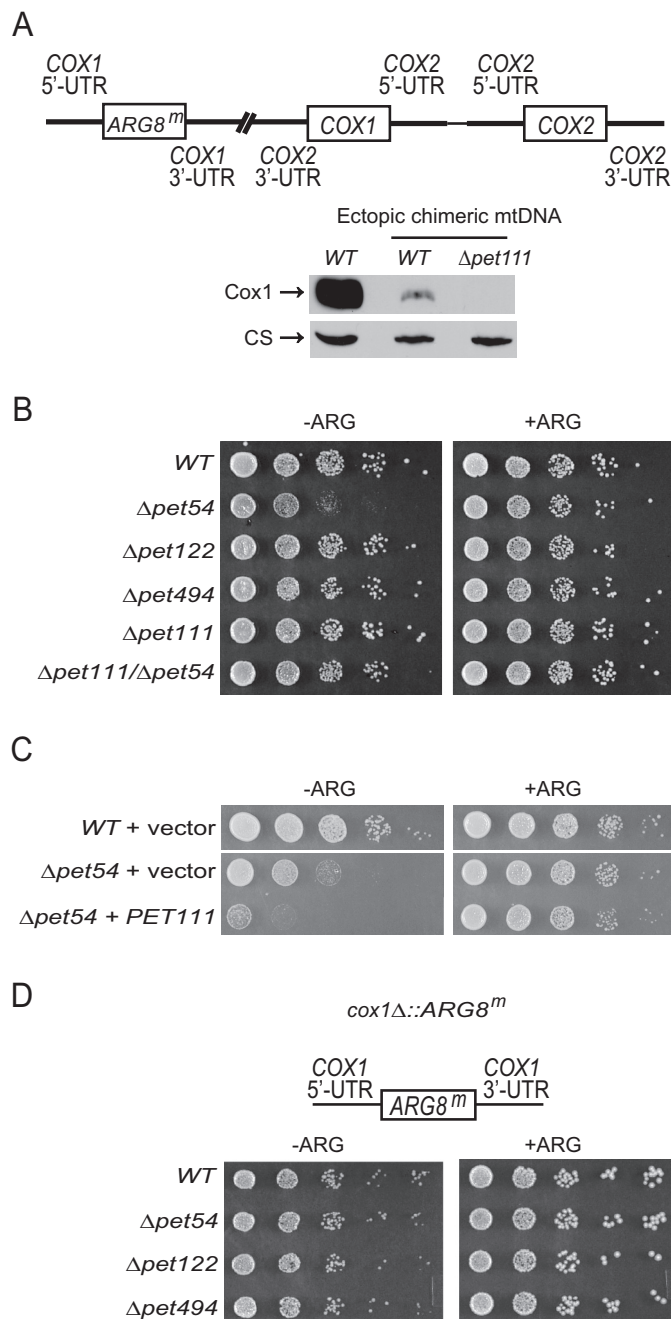


regions; this activity could be either directly or indirectly related to Mss51 and Pet309, which are translational activators of *COX1* mRNA. (ii) It could act on the COA complexes, where it could contribute to Mss51 release and recycling for translational activation. However, this activity should be independent of both the Cox1 C-terminal end and the Cox14/Coa3-mediated feedback assembly regulatory loop.

To test whether Pet54 regulates Cox1 synthesis by acting through the untranslated regions of the *COX1* mRNA, we examined the growth on medium lacking arginine of a strain whose *COX1* coding sequence was replaced by the *ARG8<sup>m</sup>* gene (*cox1Δ::ARG8<sup>m</sup>*). In addition, this strain has the *COX1* coding region flanked by both *COX2* UTRs inserted at an ectopic site on the mtDNA. In this mtDNA, the levels of Cox1 protein are low as compared with a strain with wild-type mitochondrial DNA (Fig. 3A), and its synthesis depends on the translational activator Pet111 (20). We deleted *PET54* as well as the *PET122*, *PET494*, and *PET111* genes and examined the growth of cells in medium lacking arginine. We observed that  $\Delta pet122$ ,  $\Delta pet494$ , and  $\Delta pet111$  mutants showed a similar growth as compared with a wild-type strain with the *cox1Δ::ARG8<sup>m</sup>* construct (Fig. 3B). This is consistent with the idea that in low levels or in the absence of Cox1, Mss51 is not sequestered and therefore is available to support normal *COX1* 5'-UTR translational activation despite the absence of CcO assembly (15). In contrast, expression of the *cox1Δ::ARG8<sup>m</sup>* reporter was reduced by  $\Delta pet54$  mutation, suggesting that the site of action of Pet54 is in the untranslated region of the mRNA, probably the *COX1* 5'-UTR. Surprisingly, in the double mutant  $\Delta pet54/\Delta pet111$ , where Cox1 is not synthesized, expression of the *cox1Δ::ARG8<sup>m</sup>* reporter was similar to wild-type levels, suggesting that the effect of Pet54 on the *COX1* mRNA untranslated regions depends on the presence of Cox1 protein. To confirm this idea, we first overexpressed *PET111*, and we observed a decreased growth on medium lacking arginine after *pet54* deletion (Fig. 3C). Second, we eliminated *PET54* on a *cox1Δ::ARG8<sup>m</sup>* strain where the *COX1* coding region was absent. In this case, the  $\Delta pet54$  mutant showed growth levels on medium lacking arginine similar to those of the wild-type,  $\Delta pet122$ , and  $\Delta pet494$  cells (Fig. 3D).

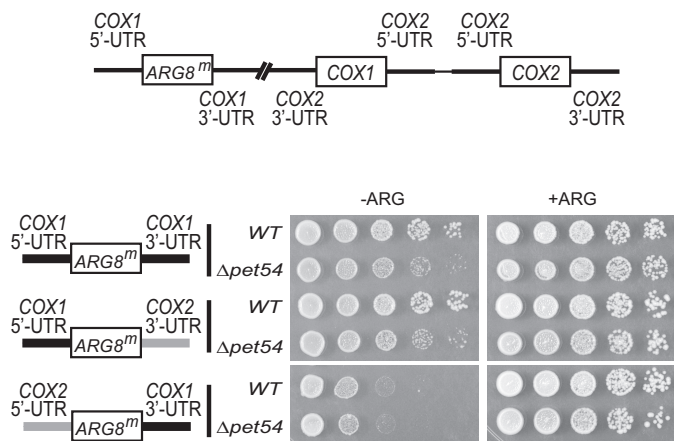
To determine whether the *COX1* 5'-UTR or 3'-UTR are targets *in vivo* for Pet54, we created strains with mitochondrial constructs similar to the one indicated in Fig. 3A, except that either the *COX1* 5'- or 3'-UTR flanking the *ARG8<sup>m</sup>* reporter was exchanged with the *COX2* 5'- or 3'-UTR, respectively. Strains with a wild-type nuclear genome supported growth on medium lacking arginine (Fig. 4), although cells with the substituted *COX1* 5'-UTR had a weaker growth, probably because in these mitochondria, three copies of the *COX2* 5'-UTR are present, and therefore Pet111 is limiting for *ARG8<sup>m</sup>* expression. Deletion of *pet54* did not affect growth in arginine of the strain bearing the *COX2* 5'-UTR, suggesting that the target of Pet54 was lost in this construct. In contrast, the  $\Delta pet54$  strain containing the *COX2* 3'-UTR showed reduced growth in medium lacking arginine. Thus, our data show that the target of Pet54 is localized to the *COX1* 5'-UTR.

Mss51 and Pet309 act on the *COX1* mRNA 5'-UTR (2, 40), and both proteins are limiting for translation (40). We investi-



**FIGURE 3. Pet54 plays a role in *COX1* mRNA untranslated regions that depends on the presence of the Cox1 protein.** A, the *cox1Δ::ARG8<sup>m</sup>* construct was inserted at the *COX1* locus, where the *ARG8<sup>m</sup>* reporter replaced the coding sequence of *COX1*. In addition, the *COX1* coding sequence flanked by the *COX2* UTRs was inserted at an ectopic site, 295 bp upstream of *COX2* on the mtDNA (20). Mitochondria from cells carrying the wild-type mtDNA or the ectopic chimeric mtDNA were analyzed by SDS-PAGE and Western blotting. The membrane was probed with an antibody for Cox1 and afterward for citrate synthase (CS) as a loading control. The  $\Delta pet111$  mutation was introduced as indicated. B, serial dilutions of the indicated mutants bearing the ectopic chimeric mtDNA were spotted on medium lacking (–ARG) or containing (+ARG) arginine and were grown for 2 days at 30 °C. C, cells carrying the ectopic chimeric mtDNA and either an empty plasmid or a plasmid expressing a high copy number of *PET111* were grown on medium lacking (–ARG) or containing (+ARG) arginine. Serial dilutions were grown for 2 days at 30 °C. D, the *COX1* coding region was replaced by the reporter gene *ARG8<sup>m</sup>* (*cox1Δ::ARG8<sup>m</sup>*); however, in this construct, no chimeric *COX1* construct was inserted. The indicated mutants bearing this mtDNA were grown on serial dilutions as in B.

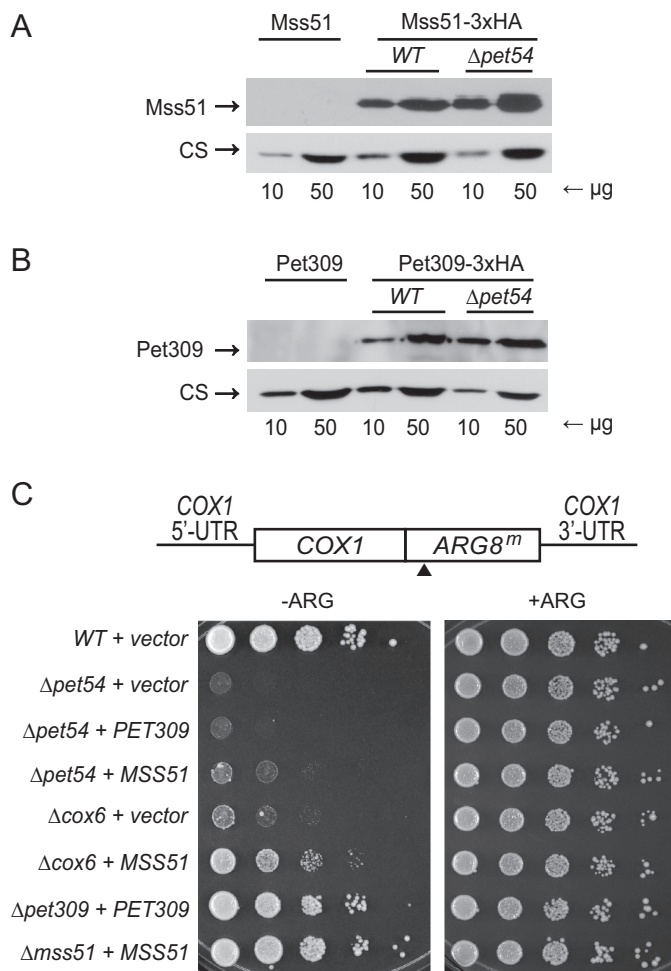
## Pet54 Regulates COX1 Translation



**FIGURE 4. Pet54 acts on the COX1 5'-UTR.** Cells containing wild-type Pet54 or the  $\Delta pet54$  mutation were spotted on serial dilutions on medium lacking (–ARG) or containing (+ARG) arginine and incubated for 2–4 days at 30 °C. The cells contained similar mitochondrial genomes as the one shown in Fig. 3A, except that the  $cox1\Delta::ARG8^m$  gene was flanked by native COX1 5'- and 3'-UTRs (top construct), flanked by the native COX1 5'-UTR and the COX2 3'-UTR (middle), or flanked by the COX2 5'-UTR and the native COX1 3'-UTR (bottom). Black bars, COX1 untranslated regions; gray bars, COX2 untranslated regions. For COX1 3'-UTR replacement, 525 bp of the COX1 downstream sequence were replaced by 118 bp of the COX2 downstream sequence. For COX1 5'-UTR replacement, 505 bp of the COX1 upstream sequence were replaced by 73 bp of the COX2 upstream sequence (40).

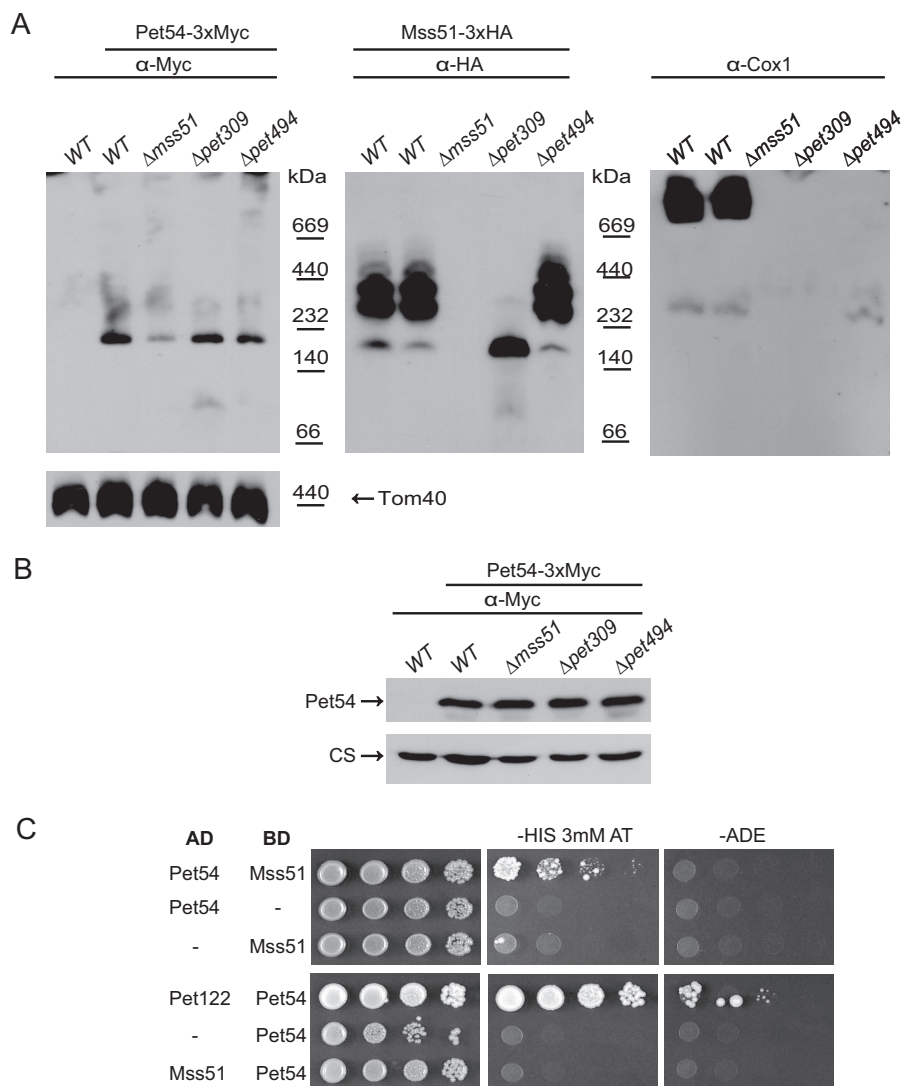
gated whether the steady state levels of these proteins could be affected by deletion of Pet54. Mitochondria from strains with triple hemagglutinin tags fused to the C-terminal end of Pet309 or Mss51 were isolated. These strains did not show any respiratory growth deficiency due to the presence of the tags (data not shown). Western blotting analyses of Mss51–3xHA or Pet309–3xHA revealed that protein levels were not affected by the absence of Pet54 (Fig. 5, A and B). In addition, we asked whether Pet54 might show any genetic interaction with Pet309 or Mss51. As previously observed, the growth in medium lacking arginine of a  $\Delta pet54$ ,  $COX1(1-512)::ARG8^m$  strain was highly reduced (Fig. 2C). We asked whether this phenotype could be alleviated by overexpression of *PET309* or *MSS51*. Each gene was cloned into a 2  $\mu$  plasmid, and they were used to transform  $\Delta pet54$ ,  $COX1(1-512)::ARG8^m$  cells. Overexpression of *PET309* had no compensatory effect, whereas overexpression of *MSS51* showed only a very mild effect (Fig. 5C). In contrast, overexpression of *MSS51* in a  $\Delta cox6$  mutant recovered growth on medium lacking arginine, consistent with the observation that the assembly-feedback regulation of Cox1 synthesis can be overcome by expression of additional copies of *MSS51* (15).

Our results suggest that Pet54 acts on the COX1 mRNA 5'-UTR to promote expression. This effect was observed only if Cox1 protein is present, even in small amounts, suggesting that Mss51 could be related to Pet54 function. The absence of Pet54 does not affect steady state levels of the translational activators Pet309 and Mss51, whose activities are limiting for Cox1 synthesis. Moreover, a mild genetic interaction between *PET54* and *MSS51* was detected because overexpression of the translational activator slightly compensated the growth phenotype of the  $COX1(1-512)::ARG8^m$  reporter in the absence of Pet54.



**FIGURE 5. Pet54 does not affect the levels of Pet309 and Mss51 proteins and shows a mild genetic interaction with Mss51.** A sample of 10 and 50  $\mu$ g of mitochondrial protein from strains expressing the Mss51–3xHA (A) or Pet309–3xHA (B) as well as the untagged versions of these genes were separated by SDS-PAGE and analyzed by Western blotting. The membranes were probed with antibodies anti-HA and anti-citrate synthase (CS) as a loading control. C, cells carrying the  $COX1(1512)::ARG8^m$  construct were transformed with empty plasmid (vector) or with the indicated genes cloned on YEp352 plasmid. The transformants were spotted on synthetic complete medium lacking (–ARG) or containing (+ARG) arginine. 10-Fold serial dilutions were grown for 3 days at 30 °C. The black arrow indicates the processing site for the pre-Arg8 matrix-targeting signal.

*Pet54 Could Promote Mss51 Recycling for Translational Activation*—So far, our results have suggested that Pet54 promotes efficient Cox1 synthesis through a mechanism independent of Cox14/Coa3 and the Cox1 C-terminal end. This role involves the COX1 mRNA 5'-UTR. Because the effect of Pet54 on the COX1 5'-UTR was observable only in the presence of the Cox1 protein, there was a possibility that Pet54 could affect either Mss51 or the COA complexes. COA complexes depend on the presence of Cox1 protein, and Mss51 activity in translation activation depends on how much of this activator is released from the COA complexes (1, 15, 19, 21). Therefore, we first analyzed by BN-PAGE whether Mss51 and Pet54 co-migrate. Mss51 has been observed in at least two different complexes, one of ~120–180 kDa, which has been associated with the translational activator form of Mss51 (translation-effective Mss51, Mss51<sup>TE</sup>), and two or three complexes from ~250 to 450 kDa that form the first assembly intermediates of the CcO



**FIGURE 6. Pet54 migrates into a high molecular weight complex that is different from that of the Mss51 complexes.** *A*, a sample of 100  $\mu$ g of mitochondrial proteins from cells carrying the Mss51-3xHA and Pet54-3xMyc or the untagged proteins was solubilized with 1% digitonin and separated on a 5–13% acrylamide blue native gel. Western blotting was performed with the indicated antibodies after consecutive antibody-stripping treatments. An antibody against Tom40 was used as a loading control. *B*, mitochondria from the strains in *A* were analyzed by SDS-PAGE and Western blotting using antibodies against the c-Myc epitope (to detect Pet54-3xMyc) and citrate synthase as a loading control (CS). *C*, yeast two-hybrid plasmids containing the Pet54, Mss51, and Pet122 coding regions fused in frame with the activation domain (AD) or binding domain (BD) of *Gal4* were co-transformed into the yeast two-hybrid strain Pj69-4a (34) as indicated. The double transformants were selected on medium lacking leucine and uracil or leucine and tryptophan. Growth was tested on medium lacking histidine (in the presence of 3 mM 3-aminotriazole) and medium lacking adenine. 10-Fold serial dilutions were grown at 30 °C for 7 days. Growth of cells containing Pet122-AD and Pet54-BD was used as a positive control of two proteins previously demonstrated to interact (29, 33).

and represent the COA complexes (19, 24, 27). To detect Pet54, we attached a triple Myc epitope to the C terminus of Pet54. The respiratory competence of the Pet54-3xMyc strain was comparable with wild-type levels, indicating that the tagged protein was functional (data not shown). After BN-PAGE of purified mitochondria, transferring to a PVDF membrane, and Western blotting, Pet54-3xMyc was detected as a ~190 kDa band that did not co-migrate with any of the complexes observed for Mss51-3xHA (Fig. 6A). Deletion of Mss51 did not affect Pet54 migration; however, we observed a decrease in the band intensity, suggesting that the Pet54 population in the ~190 kDa band decreased in the absence of Mss51. This decrease was not due to a reduction in the steady state levels of Pet54 on a  $\Delta$ mss51 mutant, as observed in SDS-PAGE and Western blotting (Fig. 6B). Pet54 is associated with the other

COX3 translational activators, namely Pet122 and Pet494 (33), and with Pet309 (29). However, the migration of the ~190 kDa band of Pet54 was not modified after the deletion of Pet494 or Pet309, indicating that this is a different complex (Fig. 6A). As expected, deletion of Pet309 produced only the ~180-kDa complex of the translational competent Mss51 (19), whereas deletion of Pet494, which blocks CcO assembly, stalled Mss51 on COA complexes. Western blotting experiments of the same membrane using an antibody against Cox1 revealed that super-complexes were only observed on WT cells, whereas on  $\Delta$ pet494, only assembly intermediaries were detected. We also investigated whether a physical interaction between Mss51 and Pet54 exists. Yeast two-hybrid experiments showed a weak interaction between Pet54-AD and Mss51-BD (Fig. 6C). This interaction was only observable by growth on medium lacking



## Pet54 Regulates COX1 Translation

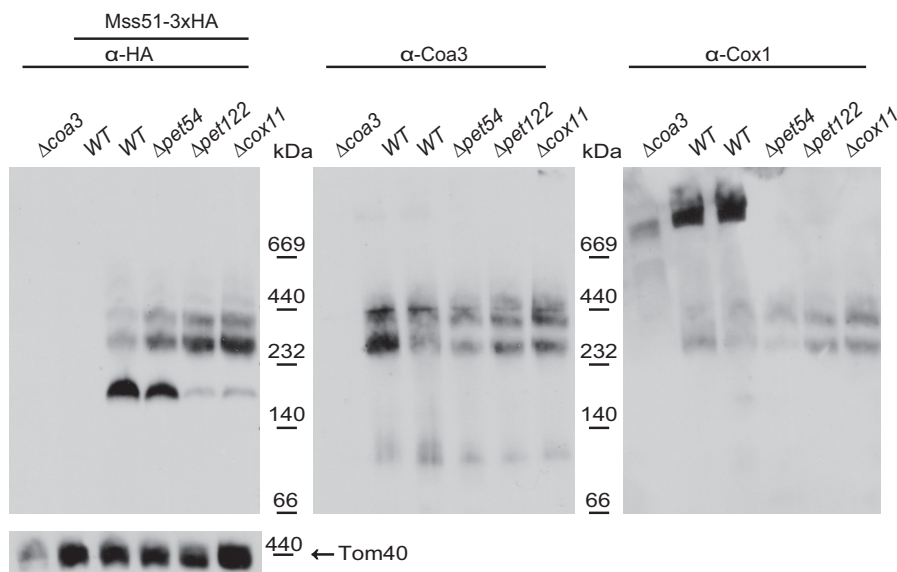


FIGURE 7. **Absence of Pet54 has a wild-type migration pattern of Mss51 complexes on blue native PAGE.** Mitochondria bearing the untagged Mss51 or the Mss51-3xHA proteins with the indicated mutations  $\Delta pet54$ ,  $\Delta pet122$ , or  $\Delta cox11$  were separated by BN-PAGE. The membrane was probed with antibodies against the HA epitope, Coa3, Cox1, and Tom40 antibodies.

histidine and was undetectable on the more stringent medium lacking adenine. These observations are consistent with a rapid, transient or weak but biologically significant protein-protein interaction, as has been described for different proteins (41, 42).

We next asked how Mss51 complexes are affected when Pet54 is deleted. Surprisingly, on a  $\Delta pet54$  mutant, the  $\sim 180$ -kDa complex made up by the translational activator form of Mss51 (Mss51<sup>TE</sup>) was present in similar intensity as compared with the observed levels on wild-type cells (Fig. 7). This is in contrast to the migration of Mss51 on  $\Delta pet122$  or  $\Delta cox11$  mutants, where Cox1 synthesis is regulated by the feedback assembly loop (15, 16, 27). In this case, the majority of Mss51 is present on the high molecular mass COA complexes ( $\sim 250$ – $450$  kDa) and therefore unavailable to promote efficient COX1 mRNA translational activation. This suggests that, in contrast to other mutants blocking CcO assembly, deletion of Pet54 did not trap the most Mss51 on the COA complexes. The same membrane was next decorated with antibodies against Coa3 and Cox1. The migration of Coa3 on COA complexes on BN-PAGE showed a similar pattern in the mutants and in wild-type mitochondria. As reported (19), Cox1 migrated in supercomplexes, together with a population of Coa3 on wild-type cells, whereas in the mutants, only Cox1 assembly intermediaries were observed. In agreement with previous reports, Cox1 migrated with a diffused pattern in the  $\Delta coa3$  mutant (22).

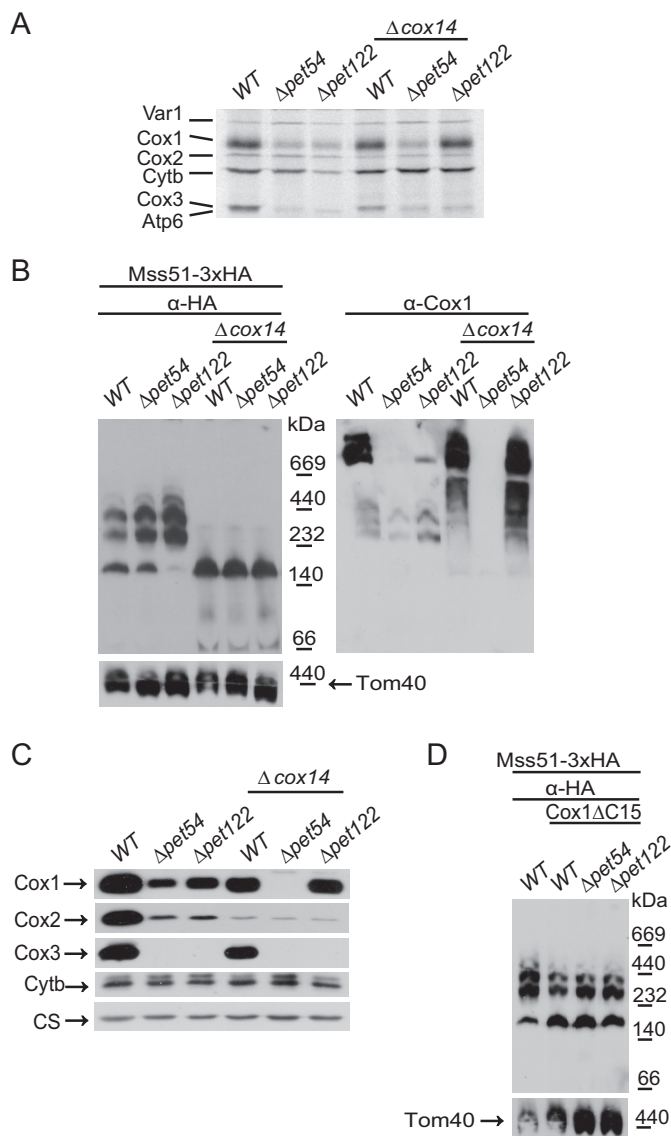
According to the assembly feedback regulatory model, in the absence of Coa3 or Cox14, Mss51 forms the translational active complex. As consequence, the levels of Cox1 synthesis become normal, even if CcO assembly is blocked. An example of this phenotype is observed with the  $\Delta pet122$  mutant, where synthesis of Cox1 decreased, and after elimination of Cox14, labeling of Cox1 showed normal levels (Fig. 8A). However, as previously demonstrated, an exception was the  $\Delta pet54$  mutant, where synthesis of Cox1 was not recovered after elimination of Coa3 or Cox14. Thus, we investigated how Mss51 was distributed between the translational active complex and the COA com-

plexes in the double mutant  $\Delta pet54/\Delta cox14$ . Purified mitochondria from single  $\Delta pet54$  and  $\Delta pet122$  mutants, or combined with the  $\Delta cox14$  deletion were analyzed by BN-PAGE and Western blotting. In the  $\Delta cox14$  cells as well as in the double  $\Delta pet54/\Delta cox14$  and  $\Delta pet122/\Delta cox14$  mutants, Mss51 was present as the  $\sim 180$ -kDa complex representing the translational activator form (Fig. 8B), demonstrating that even when the double mutant  $\Delta pet54/\Delta cox14$  renders Mss51 in the  $\sim 180$ -kDa form, this protein is not efficiently translating the COX1 mRNA.

The same membrane was next decorated with an antibody against Cox1. As expected, whereas supercomplexes were only present on the wild-type strains, intermediary assembly complexes of Cox1 were observed in the mutants lacking Pet54 and Pet122. In the  $\Delta cox14$  and the  $\Delta pet122/\Delta cox14$  mutants, Cox1 was present as diffuse bands corresponding to aggregation of Cox1 protein with other mitochondrial gene products (22). Interestingly, in the  $\Delta pet54/\Delta cox14$  double mutant, no Cox1 signal was detected. This was confirmed by analyzing the steady-state levels of Cox1 by SDS-PAGE and Western blotting experiments. In this case, whereas a reduction of Cox1 levels was observed for the  $\Delta pet54$ ,  $\Delta pet122$ , and  $\Delta pet122/\Delta cox14$  mutants, no signal of Cox1 was detected in the double deletion of Pet54 and Cox14 (Fig. 8C), even when Cox1 was synthesized at similar levels compared with the single  $\Delta pet54$  mutant (Fig. 8A).

The assembly feedback regulatory loop of Cox1 synthesis can be interrupted by deletion of Cox14 (15) or the Cox1 C-terminal end (16); however, in the absence of Pet54, synthesis of Cox1 cannot recover. In the Cox1 $\Delta C15$  mitochondria, Mss51 was enriched on the  $\sim 180$ -kDa complex as compared with a strain bearing wild-type Cox1, although some Mss51 is present on the  $\sim 250$ – $450$ -kDa complexes (43) (Fig. 8D). A  $\Delta pet54$  mutant carrying Cox1 $\Delta C15$  showed a similar enrichment of Mss51 on the  $\sim 180$ -kDa complex (Fig. 8D), supporting the idea that even

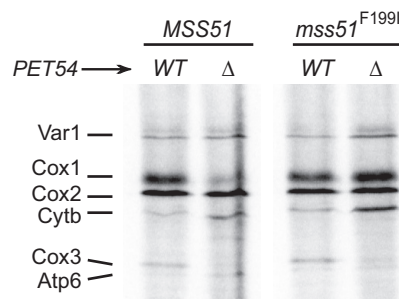




**FIGURE 8. Mss51 is present in the translational active complex but is not competent for efficient synthesis of Cox1 in the absence of Pet54.** *A*, wild type,  $\Delta pet54$ ,  $\Delta pet122$ , or the combined mutants with  $\Delta cox14$  were pulse-labeled with [ $^{35}$ S]methionine in the presence of cycloheximide. The mitochondrial products were analyzed by SDS-PAGE and autoradiography. *B*, mitochondria from the strains in *A* were separated by BN-PAGE and analyzed by Western blotting using antibodies against the HA epitope (to detect Mss51), Cox1, and Tom40 (as a loading control). *C*, a sample of 25  $\mu$ g of mitochondrial proteins was analyzed by SDS-PAGE and Western blotting using antibodies against Cox1, Cox2, Cox3, cytochrome *b* (Cytb), and citrate synthase as loading control (CS). *D*, mitochondria from the indicated strains were analyzed by BN-PAGE as in *B*.

if Mss51 is more available for translational activation, Pet54 is necessary to fully activate Mss51.

Mss51 binds heme b, and hemylation is linked to the role of Mss51 in regulation of COX1 mRNA translation. If hemylation of Mss51 is affected by mutation of two heme-binding CPX motifs or by depletion of heme, then synthesis of Cox1 is decreased (28). However, the *mss51*<sup>T167R</sup> and *mss51*<sup>F199I</sup> variants, originally identified as respiratory suppressors of  $\Delta shy1$  mutants (44), can bypass the need for Mss51 hemylation. In this case, synthesis of Cox1 has wild-type levels even if hemylation is hampered (28). The binding of hemes to Mss51 can induce

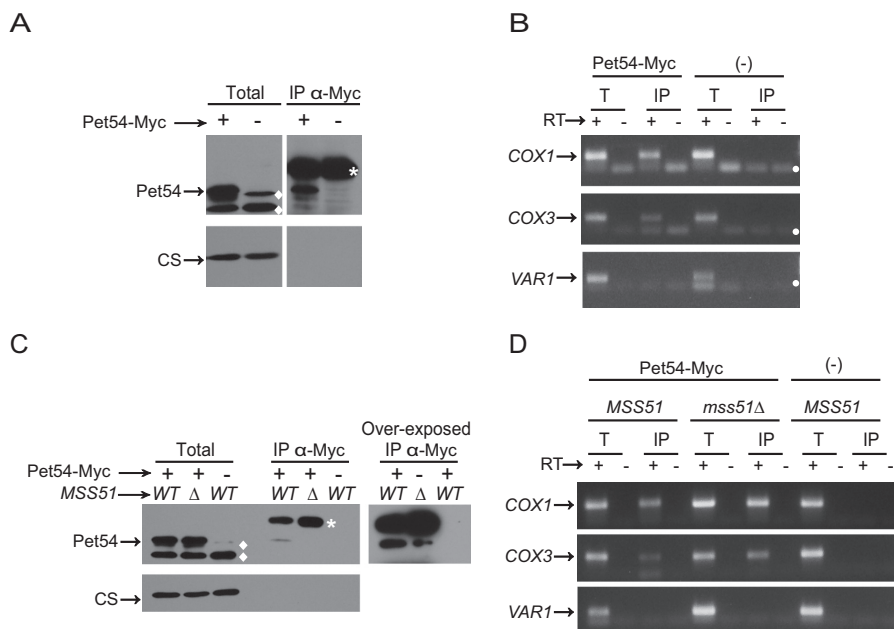


**FIGURE 9. Pet54 might modulate the conformation and/or hemylation of Mss51.** Wild-type or  $\Delta pet54$  cells carrying either the wild-type Mss51 or the *mss51*<sup>F199I</sup> variant were pulse-labeled with [ $^{35}$ S]methionine in the presence of cycloheximide. The mitochondrial products were resolved by SDS-PAGE and subjected to autoradiography as in Fig. 1.

conformational changes necessary for function (45). We sought to investigate whether Pet54 could be linked to Mss51 hemylation. We analyzed whether synthesis of Cox1 in the presence of the *mss51*<sup>F199I</sup> variant depended on Pet54. *in vivo* mitochondrial translation assays showed that in cells carrying *mss51*<sup>F199I</sup>, synthesis of Cox1 is similar in both wild-type and  $\Delta pet54$  cells (Fig. 9). These data suggest that the *mss51*<sup>F199I</sup> mutation bypassed the need for Pet54 to efficiently translate COX1 mRNA and that Pet54 function might be related to Mss51 hemylation and/or conformational changes.

*Pet54 Physically Interacts with the Intronless COX1 mRNA*—Because Pet54 is an RRM protein, it is possible that the fully translational active form of Mss51 requires binding of Pet54 to the COX1 5'-UTR. To test this idea, we analyzed whether Pet54 could physically interact with the intronless COX1 transcript. Mitochondria carrying the Pet54–3xMyc protein were solubilized with dodecyl maltoside; next, Pet54 was immunoprecipitated with anti-Myc antibodies, and RNA from the immunoprecipitate was isolated and analyzed by reverse transcription and PCR. This technique was used previously to study the Pet309–COX1 mRNA interaction (38). Western blotting analysis showed that Pet54–3xMyc immunoprecipitated. In contrast, the unrelated mitochondrial citrate synthase (negative control) was absent from the immunoprecipitate fraction, indicating that the immunoprecipitation was specific (Fig. 10A). To test whether the COX1 mRNA was associated with Pet54, RNA was purified from the immunoprecipitated Pet54–Myc fraction. Next, it was analyzed by reverse transcription using primers for the COX1 5'-UTR, and the cDNA was amplified by PCR. As a positive control, we analyzed whether COX3 mRNA was also amplified because it was previously reported by *in vitro* experiments that Pet54 interacts with the COX3 5'-UTR (31). As a negative control, we also amplified *VARI*, because translation of this mRNA is independent of Pet54. COX1 mRNA was present in the Pet54–3xMyc immunoprecipitation fraction as well as in the total fraction (Fig. 10B); however, it was absent from the immunoprecipitation fraction from untagged Pet54 mitochondria. As expected, COX3 mRNA was also amplified from the Pet54–3xMyc immunoprecipitate, whereas *VARI* mRNA was absent from the immunoprecipitate. We next asked whether the interaction of Pet54–3xMyc with COX1 mRNA is affected by Mss51. To address this question, Pet54–3xMyc was immunoprecipitated from mitochondria carrying an  $\Delta mss51$

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**FIGURE 10. Pet54 interacts with COX1 mRNA, and this interaction is independent of Mss51.** *A*, mitochondria were solubilized with dodecyl maltoside, and Pet54-3xMyc or untagged Pet54 (-) was subjected to immunoprecipitation with antibody anti-Myc. One-fourth of the immunoprecipitate (IP) was resolved by SDS-PAGE and transferred to a PVDF membrane for Western blotting. The membrane was probed with anti-Myc antibody and with anti-citrate synthase antibody (CS) as a negative control for interaction. The total fraction represents 5% of the mitochondrial extract used for immunoprecipitation. \*, nonspecific bands from the immunoglobulin heavy chain used for immunoprecipitation. ♦, nonspecific bands when the anti-Myc antibody was used. *B*, RNA was extracted from the total (T) and immunoprecipitate fractions. Each fraction was divided in two, and cDNA was prepared in the presence (+) or absence (-) of reverse transcriptase (RT) using primers for the COX1 and VAR1 5'-UTRs. The (-) RT lanes represent a negative control for DNA contamination. The PCR products were run on an agarose gel. ●, bands due to primer dimers. *C* and *D*, mitochondria from cells carrying Pet54-3xMyc or the untagged Pet54 and either the wild type MSS51 or the  $\Delta mss51$  deletion were treated and analyzed as in *A* and *B*.

mutation. RNA analysis from this fraction showed that the COX1 mRNA was still present in the immunoprecipitate, indicating that Pet54 binds to the COX1 mRNA even in the absence of Mss51 (Fig. 10, *C* and *D*).

Together, our data suggest that in the absence of Pet54, Mss51 is enriched for the translational activator form. However, the protein is not competent to promote efficient translation of COX1 mRNA. Because Pet54 interacts with the COX1 mRNA, this interaction could be important for the activation of Mss51. In addition, the complex formed by the assembly feedback regulator Coa3 was not altered by the  $\Delta pet54$  mutation. This is not surprising because the phenotype caused by elimination of Pet54 was independent of Coa3/Cox14. Moreover, it was observed that newly made Cox1 is highly unstable after the combined deletion of Pet54 and Cox14.

### Discussion

Pet54 was initially described as a translational activator specific for COX3 mRNA (7) and as a factor necessary for COX1 intron *a15 $\beta$*  splicing (39). Here we demonstrated that Pet54 plays a third role that is independent of the previous ones. Although this protein is not absolutely required, it promotes efficient Cox1 synthesis.

It is well established that COX1 mRNA translation is reduced in mutants that block CcO assembly (for reviews, see Refs. 18 and 19) and that this regulation can be suppressed by deletion of Cox14 (15) or elimination of the last 15 residues from the C-terminal end of Cox1 (Cox1 $\Delta$ C15) (16). The third function of Pet54 was discovered because, in contrast to what is observed for most CcO assembly mutants (including the  $\Delta pet122$  and

$\Delta pet494$  mutants that, as  $\Delta pet54$ , also impair COX3 mRNA translation), a deletion of Pet54 did not recover Cox1 $\Delta$ C15 synthesis. This observation suggested that Pet54 could be a positive regulator of Cox1 synthesis, whose function is independent of the Cox1 C-terminal end. Similarly, the function of Pet54 seems to be independent of Cox14 and Coa3 because a double deletion  $\Delta pet54, \Delta cox14$  or  $\Delta pet54, \Delta coa3$  did not recover Cox1 synthesis to normal levels.

We reasoned that Pet54 might act at two different levels of the Cox1 synthesis pathway. (i) Pet54 could affect the formation of the COA complexes by promoting an efficient release of Mss51 from these complexes to be active for COX1 mRNA translation. In the absence of Pet54, Mss51 would be more stably associated with Cox1 and the COA complexes and therefore not accessible as translational activator. However, this possibility is unlikely because we observed in blue native gel electrophoresis that Mss51 is present on the COA complexes similarly in the presence or absence of Pet54. (ii) A possible site of action of Pet54 could involve COX1 5'-UTR, the target for translational activation, which is driven by Pet309 and Mss51 (2, 20, 40). One possibility was that in the absence of Pet54, Mss51 and Pet309 levels decreased, and because these factors are limiting for Cox1 synthesis (40), this would affect the efficiency of COX1 mRNA translation. Alternatively, COX1 mRNA levels could be reduced in the absence of Pet54, decreasing the efficiency of translation. However, none of these possibilities was the case (Fig. 6) (data not shown), indicating that Pet54 did not affect stability of the COX1 translational activators or COX1 transcript. We observed that deletion of Pet54 reduced expression

of the reporter *ARG8<sup>m</sup>* inserted instead of the *COX1* codons. This suggests that Pet54 function is related to the *COX1* mRNA UTRs. Indeed, we demonstrated that the function of Pet54 mapped to the *COX1* 5'-UTR, which is the site of action of Pet309 and Mss51 (2, 40). This effect is observed only in the presence of Cox1 protein, suggesting that Mss51 could be the target for Pet54 function because Mss51 is regulated as a translational activator through the interaction with Cox1 protein and the COA complexes. In addition, we observed a weak genetic interaction between Pet54 and Mss51 rather than with Pet309; overexpression of Mss51 slightly compensated the growth deficiency on medium lacking arginine of  $\Delta$ *pet54* mutants with *ARG8<sup>m</sup>* located at the *COX1* locus.

In the present study, we concluded that Pet54 is necessary for activation of Mss51 as a translational activator and that this function is not related to the role of Mss51 as part of COA complexes. On BN-PAGE and sucrose gradient centrifugation experiments, Mss51 was detected as two different forms: one of high molecular mass (250–450 kDa) that is associated with the COA complexes in early Cox1 assembly intermediates and one of smaller size (around 120–180 kDa) corresponding to a translation-effective Mss51 (Mss51<sup>TE</sup>), where Mss51 promotes translational activation of the *COX1* mRNA (19, 27). In this work, we observed that the sizes and distribution of both Mss51 populations were not affected by the absence of Pet54, indicating that the levels of Mss51<sup>TE</sup> were similar to wild-type levels. This is in contrast to what is reported for other mutants, like  $\Delta$ *pet122* and  $\Delta$ *cox11*, where the Mss51 population associated with COA complexes is enriched (present work) (27). To confirm our model in which Pet54 positively affects Cox1 synthesis via a mechanism that does not involve assembly intermediates, we observed that when the assembly-feedback regulatory loop is disrupted by a combined deletion of Pet54 and Cox14, Mss51 was exclusively present as the Mss51<sup>TE</sup> form, yet it is not competent to activate translation, thereby reducing Cox1 synthesis. Moreover, deficiency of Cox1 synthesis in the absence of Pet54 was not compensated by overexpression of *PET309* and was slightly compensated by overexpression of *MSS51*, suggesting that this effect is not only due to a decreased affinity of the translational machinery components Pet309 and Mss51 for the *COX1* mRNA. Altogether, our results suggest that Pet54 is necessary to recycle/reactivate Mss51 from the COA state to the “translation-committed” form.

Two examples of similar phenotypes of decreased Cox1 synthesis, even in the presence of  $\Delta$ *cox14* or Cox1 $\Delta$ C15, are reported. The first example is a mutation on the general mitochondrial insertase Oxa1, where Mss51 was observed mainly as the “translation effective” form (15, 27). However, it is unlikely that Pet54 function is related to Oxa1 because in  $\Delta$ *pet54* mutants, Mss51 and Cox1 were on COA complexes, which are thought to be inserted in the membrane (27) (present study). The second example includes the point mutations C85S and C96S on Mss51 that alter two CPX motifs. These heme-binding motifs are important for Mss51 function (28). Affinity of heme binding to Mss51 can be modulated by the redox state. Upon oxidation, Mss51 introduces a disulfide bond between Cys-85 and Cys-96 that decreases Mss51 heme affinity (45). In addition,

binding of hemes to Mss51 induces conformational changes that may be necessary for function (45).

Pet54 could play a role in the heme addition to Mss51 or could induce a conformational change in Mss51 necessary for translational activation. This idea is supported by the fact that deficiency of *COX1* mRNA translation in  $\Delta$ *pet54* mutants is bypassed by the presence of the allele *mss51<sup>F199I</sup>*. This variant suppressed defects on Cox1 synthesis due to lack of Mss51 hemylation (28).

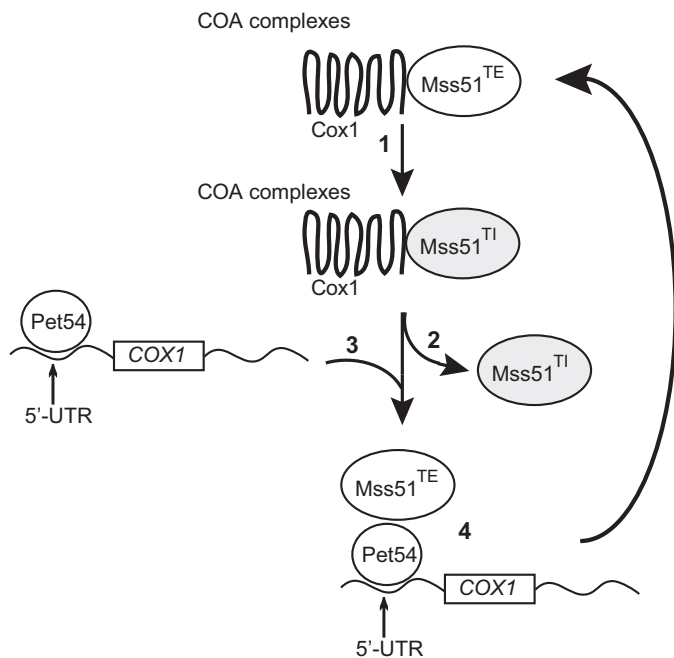
Pet54 could directly interact with the *COX1* mRNA to mediate a more efficient or competent interaction of Mss51 with the translational machinery. In the present work, we have demonstrated that Pet54 physically interacts with the *COX1* mRNA. The detected interaction could be either direct or mediated by additional, unknown proteins. However, a direct interaction is more plausible because Pet54 is an RRM protein that directly interacts with both *COX3* 5'-UTR and the *COX1* *al5 $\beta$*  intron (31). We observed that the Pet54-*COX1* mRNA interaction was not affected by the absence of Mss51, suggesting that the binding of Pet54 to the mRNA is independent of Mss51, probably occurring before Mss51 activates translation.

The role of Pet54 in enhancing Cox1 synthesis does not seem to be related to the generation of mp15, an aberrant peptide derived from the *COX1* transcript that is detectable on  $\Delta$ *mss51* mutants. This peptide is proposed to be the result of a suboptimal translation initiation by Pet309, maybe by positioning the ribosome on a different translation initiation site on the *COX1* transcript (38, 46). In our hands, mp15 was not detectable on  $\Delta$ *pet54* cells (not shown), indicating that in the absence of Pet54, translation initiation of *COX1* starts on the correct AUG codon.

Our data support the notion that binding of translation-effective Mss51 (Mss51<sup>TE</sup>) to newly synthesized Cox1 forms the COA complex. Formation of COA complex in turn renders Mss51<sup>TE</sup> translational inactive (hereby termed Mss51<sup>TI</sup>) (Fig. 11, *step 1*). The existence of Mss51<sup>TI</sup> was proposed previously (19, 21). Mss51 inactivation could be mediated by heme modifications (45) and/or by chaperone Coa1 (19). Assembly of CcO triggers Mss51<sup>TI</sup> release (Fig. 11, *step 2*). Because interaction of Pet54 with *COX1* mRNA does not require Mss51 (present work), and a physical interaction between Mss51 and *COX1* mRNA has not been detected (38), Pet54 might bridge an interaction between Mss51 and *COX1* mRNA (Fig. 11, *step 3*). Binding of Pet54 to the *COX1* 5'-UTR might be necessary to further activate Mss51<sup>TI</sup> into its active form (*i.e.* Mss51<sup>TE</sup>). Thus, Pet54 might reactivate Mss51<sup>TE</sup>, probably by mediating hemylation/conformational changes in Mss51 (Fig. 11, *step 4*). As shown previously, many RRM proteins bind RNA along with other proteins for activity (*e.g.* see Refs. 47 and 48), making it possible that both, Mss51 and the *COX1* mRNA are direct targets of Pet54. In the absence of Pet54, Mss51 remains in its inactive form, resulting in a very low *COX1* translation. This explains the decreased synthesis of Cox1 observed in the double mutants  $\Delta$ *cox14/\Delta*pet54*,  $\Delta$ *coa3/\Delta*pet54*, and *COX1* $\Delta$ C15/ $\Delta$ *pet54*, where Mss51 is free but in an inactive state. This model also explains why the presence of the Cox1 protein is necessary to detect a phenotype on  $\Delta$ *pet54* mutants, because if Cox1 is absent, then Mss51<sup>TE</sup> is not inactivated; thus, Pet54 is not**



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**FIGURE 11. Model of the role of Pet54 on regulation of Cox1 synthesis.** Interaction between translational-effective Mss51<sup>TE</sup> and Cox1 renders Mss51 inactive (Mss51<sup>TI</sup>, depicted in gray). This might happen in a heme- and/or conformation-dependent manner (step 1). Upon CcO assembly, Mss51<sup>TI</sup> is released from COA complex (step 2). Pet54 interacts with the COX1 mRNA 5'-UTR (step 3). Via its interaction with Mss51<sup>TI</sup>, Pet54 may drive Mss51<sup>TI</sup> reactivation, thus bridging an interaction of Mss51<sup>TE</sup> with COX1 mRNA (step 4). In the absence of Pet54, Mss51<sup>TI</sup> is released from the COA complex but is unable to be recycled toward a Mss51<sup>TE</sup> conformation.

required to induce a hemylation-dependent conformational change on Mss51<sup>TE</sup>.

Pet54 is a multifunctional protein with at least three functions. This is not the first case of multifunctional proteins inside mitochondria. Cytochrome *c* is part of the respiratory chain and also participates in apoptosis (49). Aconitase is involved in mitochondrial DNA maintenance and in the tricarboxylic acid cycle (50, 51). Suv3 is part of the mitochondrial RNA degradosome and is also involved in mitochondrial DNA maintenance and COX1 *al5β* intron splicing (52–54). (NAD<sup>+</sup>)-dependent isocitrate dehydrogenase is known to bind *in vitro* to mitochondrial mRNAs and probably can modulate translation (55–58). In addition to the three described roles for Pet54, it could be possible that this protein has some function in the assembly of the CcO because it has been detected in supercomplexes (59). As in the case of isocitrate dehydrogenase, we cannot discard the possibility that Pet54 has a broader function and modulates translation of all of the mitochondrial mRNAs.

**Author Contributions**—X. P.-M. conceived and coordinated the study, analyzed the results, and wrote the manuscript. J. P. M. performed and analyzed the experiments shown in Figs. 1–7 together with M. S.-V. (Fig. 1–3). Y. C.-V. constructed yeast strains, participated in experiments shown in Figs. 1–3, and performed experiments shown in Fig. 10. R. G.-V. created strains and carried out the experiments shown in Fig. 4 and discussed ideas; A. E. G.-G. performed experiments shown in Fig. 9 and discussed ideas; A. Z.-O. performed Northern blotting experiments and discussed ideas; G. H. discussed ideas and wrote parts of the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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