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Alteration of the *Saccharomyces cerevisiae COX2* mRNA 5′-untranslated Leader by Mitochondrial Gene Replacement and Functional Interaction with the Translational Activator Protein PET111

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The ability to replace wild-type mitochondrial DNA sequences in yeast with in vitro-generated mutations has been exploited to study the mechanism by which the nuclearly encoded PET111 protein specifically activates translation of the mitochondrially coded *COX2* mRNA. We have generated three mutations in vitro that alter the *COX2* mRNA 5′-untranslated leader (UTL) and introduced them into the mitochondrial genome, replacing the wild-type sequence. None of the mutations significantly affected the steady-state level of *COX2* mRNA. Deletion of a single base at position −24 (relative to the translation initiation codon) in the 5′-UTL (*cox2-11*) reduced *COX2* mRNA translation and respiratory growth, whereas insertion of four bases in place of the deleted base (*cox2-12*) and deletion of bases −30 to −2 (*cox2-13*) completely blocked both. Six spontaneous nuclear mutations were selected as suppressors of the single-base 5′-UTL deletion, *cox2-11*. One of these mapped to *PET111* and was shown to be a missense mutation that changed residue 652 from Ala to Thr. This suppressor, *PET111-20*, failed to suppress the 29-base deletion, *cox2-13*, but very weakly suppressed the insertion mutation, *cox2-12*. *PET111-20* also enhanced translation of a partially functional *COX2* mRNA with a wild-type 5′-UTL but a mutant initiation codon. Although overexpression of the wild-type PET111 protein caused weak suppression of the single-base deletion, *cox2-11*, the *PET111-20* suppressor mutation did not function simply by increasing the level of the protein. These results demonstrate an intimate functional interaction between the translational activator protein and the mRNA 5′-UTL and suggest that they may interact directly.

INTRODUCTION

Expression of at least five of the eight major yeast mitochondrial genes is controlled at the level of translation by mRNA-specific activator proteins encoded by nuclear genes (reviewed in Costanzo and Fox, 1990; Ackerman *et al.*, 1991; Hinnebusch and Liebman, 1991; Pon and Schatz, 1991; Bolotin-Fukuhara and Grivell, 1992). To date, it has not been possible to analyze yeast mitochondrial translation initiation in vitro (Dekker *et al.*, 1993). However, it is possible to manipulate the mitochondrial genetic system in vivo by taking advantage of recently developed methods for making targeted alterations in yeast mitochondrial DNA (mtDNA) (Anziano and Butow, 1991; Folley and Fox, 1991; Fox *et al.*, 1991; Costanzo and Fox, 1993; Mittelmeier and Dieckmann, 1993; Mulero and Fox, 1993b; Thorsness and Fox, 1993).

Translation of the mRNA specified by the mitochondrial *COX2* gene, encoding the cytochrome *c* oxidase subunit II (coxII) precursor protein (Cabral *et al.*, 1978; Sevarino and Poyton, 1980; Pratje *et al.*, 1983), specifically requires activity of the *PET111* nuclear gene (Cabral and Schatz, 1978; Poutre and Fox, 1987). *PET111* encodes a protein targeted to mitochondria (Strick and Fox, 1987) whose site of action has been mapped ge-

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netically to the *COX2* mRNA 5′-untranslated leader (5′-UTL) (Mulero and Fox, 1993a). In the two other well studied cases, translation of the *COX3* and *COB* mRNAs is similarly activated specifically through their 5′-UTLs by membrane-associated mitochondrially located proteins (Costanzo and Fox, 1986, 1988, 1993; Rödel and Fox, 1987; Michaelis *et al.*, 1991; McMullin and Fox, 1993). The reported data are most easily explained by models in which the translational activator proteins bind directly to their targets in mRNA 5′-UTLs. Although most mitochondrial mRNAs have 5′-UTLs of at least several hundred bases (Grivell, 1989), the *COX2* mRNA 5′-UTL is only 54 bases in length (Bordonné *et al.*, 1988), making it an especially attractive target for functional analysis.

In this study, we have taken advantage of gene replacement to generate a series of mutations in the *COX2* 5′-UTL that reduce or block its translation. We have also selected nuclear mutations that suppress one of these *cox2* mutations and identified one suppressor as a missense mutation in *PET111*. Taken together, these results point to a region of the *COX2* mRNA 5′-UTL that is likely to be important for translational activation and suggest a close functional interaction between the PET111 protein and the mRNA.

MATERIALS AND METHODS

Yeast Strains, Media, and Genetic Methods

The yeast strains and their genotypes are listed in Table 1. All strains are isogenic or congenic to D273-10B (ATCC25627) except MCC123rho° and M4611. The previously published mating type for strain DUL2 was incorrect; it is *MAT*a as shown. Cells were grown on YP medium (1% yeast extract, 2% bacto-peptone) with either 2% glucose (YPD), 2% galactose (YPGal), 2% raffinose (YPR), or 3% glycerol plus 3% ethanol (YPEG). Standard genetic methods and minimal media were as described (Sherman *et al.*, 1986). Yeast nuclear transformation was carried out as described (Elble, 1992). Mitochondrial transformation of the strain MCC123rho° was performed by high velocity microprojectile bombardment (Fox *et al.*, 1988; Johnston *et al.*, 1988) in a Biolistics PDS-1000 (DuPont Biolistic Particle Delivery System, Wilmington, DE) driven by a pressured helium acceleration system. The bombardments were carried out as described (Fox *et al.*, 1991) with an equimolar mixture of the mitochondrially targeted plasmid and the *URA3* shuttle vector YEP352 (Hill *et al.*, 1986). Mitochondrial genetic methods were as described (Fox *et al.*, 1991). Transfer of mitochondrial genomes to different nuclear backgrounds was accomplished by cytoduction (Conde and Fink, 1976). Cytoductants were identified genetically after efficient mating of the parental strains (Rogers and Bussey, 1978).

Nucleic Acid Analysis

Standard DNA manipulations were carried out as described (Sambrook *et al.*, 1989). DNA sequence analysis was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Total yeast DNA and RNA were prepared as described (Rose *et al.*, 1988). The probes used for RNA hybridization consisted of the *COX2* 0.45-kilobase (kb) *Rsa* I restriction fragment (Coruzzi and Tzagoloff, 1979) and the 15S rRNA gene in the Bluescript plasmid (Stratagene, La Jolla) (Shen and Fox, 1989).

Site-directed Mutagenesis of COX2 *mRNA 5′ UTL-coding DNA*

The plasmid pJM2 (Figure 1) was constructed by cloning the *COX2* gene of strain D273-10B (ATCC 25627) into pTZ18u (BioRad, Richmond, CA) as a 2.5-kb *Sal* I-*Hin*dIII fragment with the *Sal* I site closest to the 5′ end of the gene (this fragment corresponds to the 2.45-kb *Hpa* II fragment of mtDNA [Fox, 1979] flanked by polylinker sequences). The construction of the *cox2-11* mutation, a deletion of a single G nucleotide at position −24 in the *COX2* mRNA leader (Figure

Table 1. Yeast strains used in this study

Strain	Genotype	Source
DAU2	*MAT***a** *ade2-101 ura3-52* [*rho*$^+$]	Costanzo and Fox (1988)
DL1	*MATα lys2* [*rho*$^+$]	Folley and Fox (1991)
DUL1	*MATα ura3 lys2* [*rho*$^+$]	This study
DUL2	*MAT***a** *ura3 lys2* [*rho*$^+$]	L. S. Folley
DUL2rho°	*MAT***a** *ura3 lys2* [*rho*^{0}]	L. S. Folley
JJM87	*MAT***a** *ade2-101 ura3-52 pet111-14* [*rho*$^+$]	Mulero and Fox (1993b)
JJM87rho°	*MAT***a** *ade2-101 ura3-52 pet111-14* [*rho*°]	Mulero and Fox (1993b)
JJM120	*MATα lys2* [*rho*$^+$ *cox2-11*]	This study
JJM122	*MATα lys2* [*rho*$^+$ *cox2-12*]	This study
JJM144	*MATα lys2 PET111-20* [*rho*$^+$ *cox2-11*]	This study
JJM150	*MAT***a** *ura3 lys2* [*rho*$^+$ *cox2-11*]	This study
JJM156	*MATα ura3 lys2* [*rho*$^+$ *cox2-11*]	This study
JJM159	*MATα ura3 lys2* [*rho*$^+$ *cox2-12*]	This study
JJM179	*MAT***a** *ura3 lys2 PET111-20* [*rho*$^+$ *cox2-11*]	This study
JJM179rho°	*MAT***a** *ura3 lys2 PET111-20* [*rho*°]	This study
JJM180	*MATα ade2 ura3 lys2 PET111-20* [*rho*$^+$ *cox2-11*]	This study
JJM196	*MATα ade2 ura3 lys2* [*rho*$^+$ *cox2-10*]	Mulero and Fox (1993b)
JJM230	*MATα lys2 ura3* [*rho*$^+$ *cox2-13*]	This study
JJM270	*MAT***a** *ade2-101 ura3-52 kar1-1* [*rho*$^-$ pJM2]	This study
MCC123rho°	*MAT***a** *ade2-101 ura3-52 kar1-1* [*rho*°]	M. C. Costanzo
M4611	*MATα ade1 op1* [*rho*$^+$ *cox2-M4611*]	Fox and Staempfli (1982)
TF145	*MATα ade2 ura3* [*rho*$^+$ *cox2-17*]	Fox et al. (1988)

1), was accomplished by oligonucleotide-directed mutagenesis (Mutagene kit, Bio-Rad) of the plasmid pJM2 with the oligonucleotide 5' CCTTTAGATCTTTTG 3' to produce pJM45. The *cox2-11* allele generates a unique *Bgl* II restriction site in the plasmid. The *cox2-12* insertion allele (Figure 1) on plasmid pJM46 was obtained by cutting pJM45 with *Bgl* II, filling the ends with the large fragment of DNA polymerase (Klenow), and religating. This procedure generated a unique *Cla* I site marking *cox2-12*. The deletion allele *cox2-13* (Figure 1) was obtained by cleaving pJM45 with *Bgl* II and treating with Exonuclease III (BRL, Gaithersburg, MD) and S1 nuclease. The resulting plasmid, pJM50, contains a deletion of 29 nucleotides from −30 to −2 within the region coding for the *COX2* 5'-UTL. Sequence analysis of the region corresponding to the 5'-UTL of pJM2 and the mutants pJM45, pJM46, and pJM50 verified the mutations. Restriction enzyme analysis did not detect any gross alterations in these plasmids.

Molecular Cloning and Identification of the PET111-20 Mutation

The *PET111-20* allele was cloned by gap repair (Orr-Weaver and Szostak, 1983). The plasmid pJM21 was constructed for this purpose from pJM20 [YEP352 (Hill *et al.*, 1986), carrying *PET111* on a 7.3-kb fragment (Mulero and Fox, 1993b)]. pMJ20 was cleaved at a unique *Spl* I site at position +1142 (Strick and Fox, 1987) within the *PET111* structural gene, and a bidirectional deletion was generated by treatment with exonuclease III and S1 nuclease. *Mlu* I linkers were attached at the blunt ends of the deletion, which starts at position −200 and extends to position +2298. To isolate the *PET111-20* allele, pJM21 was cut with *Mlu* I and used to transform a *ura3 PET111-20* strain (JJM180). Repaired plasmids were recovered from Ura$^+$ transformants by using their DNA to transform the *Escherichia coli* strain *DH5α*. A repaired plasmid, pJM55, carrying the *PET111-20* suppressor allele was identified by its ability to suppress the respiratory growth defect of a *cox2-12* mutant (JJM159). Although a single chromosomal copy of *PET111-20* suppresses *cox2-12* only very weakly (≥3 wk of incubation at 30°C are needed; see Figure 6), the same gene carried on the high copy plasmid pJM55 causes much stronger suppression (readily visible after 8 d).

To map the suppressor mutation within pJM55, a 2.7-kb *Hin*dIII fragment, encompassing most of the *PET111* gene (Poutre and Fox, 1987; Strick and Fox, 1987), was subcloned into the YEP352 vector (Hill *et al.*, 1986) generating pJM57. As expected, pJM57 was capable of suppressing the *cox2-12* mutation. Further subcloning was accomplished by swapping the 0.49-kb *Bgl* II-*Hpa* I fragments among the *PET111-20* containing plasmid, pJM57, and the *PET111*-YEP352 plasmid, pJM65. The *Bgl* II-*Hpa* I fragment originating from the pJM57 plasmid provided an otherwise wild-type *PET111* gene with the ability to suppress a *cox2-12* mutation. Conversely, when the *PET111-20* containing plasmid pJM57 acquired the *Bgl* II-*Hpa* I fragment from pJM65, it lost the ability to suppress the *cox2-12* mutation. Thus the suppressor mutation was mapped to 0.49 kb between the *Bgl* II and *Hpa* I sites. All the plasmids that were used in this study were able to complement the respiration deficient phenotype of the *pet111-14* null mutant strain, JJM87.

During the course of our investigations, we uncovered discrepancies with the previously published *PET111* sequence (Strick and Fox, 1987). We found that base 1496 is a C, changing the codon at position 499 from arginine to threonine. Bases 1704–1706 are TGG, changing the codon at position 569 from proline to glycine. An additional T residue follows position 2123 extending the open reading frame (ORF) to predict an 800 amino acid protein. The correct ORF extends beyond the previously published sequence. The corrected *PET111* sequence has been deposited in GenBank with the accession number M17143.

Immunological Methods

A polyclonal rabbit antiserum (5524) directed against residues 172–792 of the PET111 protein was previously prepared (Strick, 1988) using as antigen a trpE-PET111 fusion protein isolated from *E. coli* as described (Dieckmann and Tzagoloff, 1985). Total proteins were extracted from yeast cells grown in YPGal as described (Yaffe, 1991). Approximately 45 μg of each preparation was subjected to electrophoresis on an 8% polyacrylamide-sodium dodecylsulfate (SDS) gel and blotted electrophoretically to a membrane of Immobilon-P (Millipore, Bedford, MA). The polyclonal antisera was used at a 1:100 dilution. Enhanced chemiluminescence (Amersham, Arlington Heights, IL) was used to visualize the anti-PET111 immune reactions using a goat anti-rabbit IgG-horseradish peroxidase conjugate.

RESULTS

Replacement of Wild-Type by cox2 Mutations Affecting the mRNA 5'-UTL

To begin the analysis of functionally important elements in the 54-base *COX2* mRNA 5'-UTL, we generated in vitro three mutations in the DNA encoding it (Figure 1 and MATERIALS AND METHODS). The first mutation, *cox2-11*, deleted the G residue at position −24 in the 5'-UTL, creating a unique *Bgl* II restriction site in the DNA that facilitated the identification of the mutant plasmid (pJM45) and provided an entry point for making further mutations. The second mutation, *cox2-12*, was generated by cleaving this *Bgl* II site, filling in the resulting sticky-ends, and religating. The resulting plasmid (pJM46) thus had a unique *Cla* I site marking the mutation. The third mutation, *cox2-13*, was an exonuclease generated deletion of 29 basepairs (pJM50).

Plasmids bearing wild-type *COX2* and each of the *cox2* mutations were introduced into the mitochondria of a strain lacking mtDNA (MCC123rho°) by microprojectile bombardment (MATERIALS AND METHODS) (Fox *et al.*, 1988). Transformants containing replicating plasmids in their mitochondria were identified by their ability to produce respiring diploids by recombination (marker rescue) when mated to the mutant tester strain, M4611, carrying an ochre allele in the *COX2* coding sequence (Fox and Staempfli, 1982). To determine whether the 5'-UTL mutations affected expression of *COX2*, we tested their ability to complement a complete deletion, *cox2-17* (Fox *et al.*, 1988), by

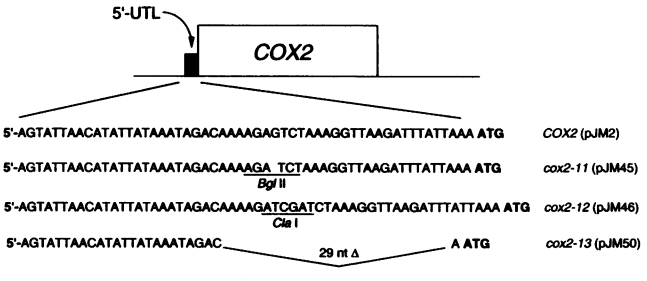

Figure 1. Sequence of the *COX2* 5'-UTL mutants. The small black rectangle represents the DNA sequence encoding the 5'-UTL, and the white rectangle represent the *COX2* structural gene. The bold letters **ATG** represent the translation initiation codon. The uppermost sequence is the wild-type 54-nucleotide-long *COX2* 5'-UTL. The restriction sites created by the mutations are indicated.

mating each transformant to the *rho*$^+$ deletion strain, TF145. The ability of the resulting diploids, heteroplasmic for the *cox2-17*, *rho*$^+$ mtDNA, and each plasmid to grow on nonrespiratory medium (YPEG) was scored. The plasmid-borne wild-type *COX2* gene strongly complemented the complete *cox2* deletion mutation. The plasmid-borne *cox2-11* allele failed to complement strongly, although slowly growing papillae appeared in the patch of respiratory-deficient diploid cells. Both plasmid-borne alleles *cox2-12* and *cox2-13* failed completely to complement the *cox2* deletion.

The wild-type *COX2* gene was replaced with the mutant alleles by homologous double recombination (Folley and Fox, 1991; Fox *et al.*, 1991). Zygotes were produced by mating the initial mitochondrial transformants carrying the plasmid-borne mutations with strains carrying wild-type *rho*$^+$ mtDNA (DL1 and DUL1). The transformants carried the karyogamy-defective mutation *kar1-1*, allowing the isolation of haploid progeny (strains JJM120, JJM122, and JJM230) isogenic to the *rho*$^+$ parents except for the *cox2* mutations. As expected, the integrated mutations produced respiratory defective phenotypes: *cox2-11* caused slow respiratory growth, whereas *cox2-12* and *cox2-13* blocked respiratory growth completely (Figure 2). In each case, the respiratory defect could be rescued by recombination after mating the mutants to a synthetic *rho*$^-$ strain containing the wild-type *COX2* gene (JJM270), confirming that the respiration-deficient phenotype was because of a *cox2* mutation. Furthermore, direct sequence analysis of mtDNA (Fox *et al.*, 1991) confirmed the presence of the mutations.

The 5′-UTL Mutations Decrease Translation of the COX2 mRNA

To determine whether the *cox2* 5′-UTL mutations affected the synthesis of coxII, we examined mitochondrial translational products radioactively labeled in vivo in the presence of cycloheximide (Douglas and Butow, 1976; Mulero and Fox, 1993a). Crude mitochondrial fractions were prepared from labeled cells and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography (Figure 3). As expected the level of coxII protein was strongly reduced in the *cox2-11* mutant (Figure 3, lane 2) relative to wild-type (Figure 3, lane 1), and coxII protein could not be detected in the *cox2-12* and *cox2-13* mutant strains (Figure 3, lanes 3 and 4).

The *cox2* mutations affecting the mRNA 5′-UTL might have reduced coxII synthesis by reducing the steady-state levels of the mRNA. We therefore isolated total cellular RNA from wild-type and mutant strains for hybridization analysis. The RNAs were separated by electrophoresis, blotted to nitrocellulose, and hybridized to labeled probes specific for the *COX2* mRNA and the mitochondrial 15S ribosomal RNA (MATERIALS AND METHODS). The hybridization signals were detected by autoradiography (Figure 4) and quantitated with a Betascope 603 Blot Analyzer (Biogen, Geneva, Switzerland). The relative levels of *COX2* mRNA in the mutants were between one-half and two-thirds of the wild-type level (0.5 ± 0.1 for *cox2-11*, 0.75 ± 0.05 for *cox2-12*, and 0.6 ± 0.1 for *cox2-13*). Thus, the strongly reduced levels of coxII protein in the mutants cannot solely be the result of decreased mRNA levels, indicating that the mutations directly affect translation of the mRNAs.

Suppression of cox2-11 by a Chromosomal Mutation at PET111

To search genetically for factors interacting with the *COX2* mRNA 5′-UTL, we have begun to look for mutations that suppress the defects caused by alterations

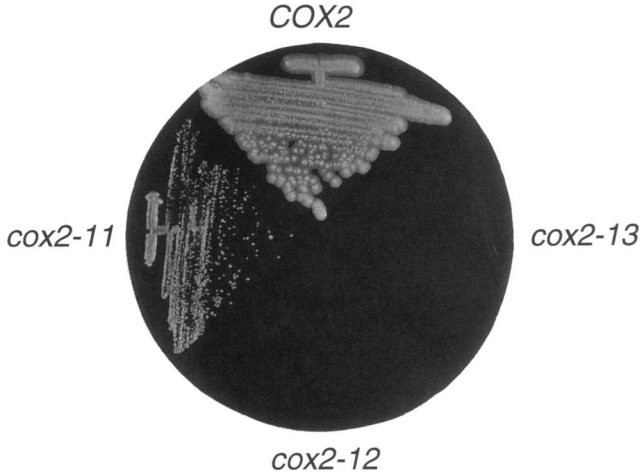

Figure 2. Respiration-dependent growth phenotypes of the strains carrying the 5′-UTL mutations. The isogenic strains DUL2 (*COX2*), JJM120 (*cox2-11*), JJM122 (*cox2-12*), and JJM230 (*cox2-13*) were streaked onto medium containing nonfermentable carbon sources (YPEG) and incubated at 30°C for 6 d.

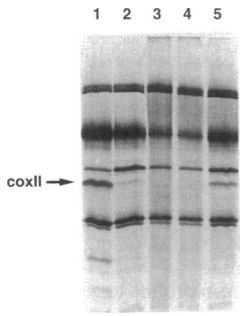

Figure 3. CoxII protein synthesis is affected by the 5′-UTL mutations. Yeast cells were grown on YPGal at 30°C and radioactively labeled with Trans ^{35}S-label (ICN Radiochemicals, Irvine, CA) in the presence of cycloheximide as described (Douglas and Butow, 1976; Fox *et al.*, 1991). Crude mitochondria were prepared and analyzed by SDS-PAGE as described (Mulero and Fox, 1993a). Lane 1, wild-type DUL2; lane 2, the *cox2-11* strain JJM120; lane 3, the *cox2-12* strain JJM122; lane 4, the *cox2-13* strain JJM230; lane 5, the *PET111-20*, [*cox2-11*] strain JJM144.

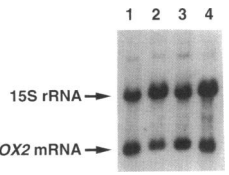

Figure 4. RNA gel-blot-hybridization analysis of the *COX2* 5′-UTL mutants and the wild-type. Total RNA isolated from strains grown at 30°C was fractionated by formaldehyde agarose gel electrophoresis, transferred to nitrocellulose, and probed with *COX2* and 15S rRNA specific sequences (MATERIALS AND METHODS). Lane 1, the wild-type DUL1; lane 2, the *cox2-11* mutant JJM120; lane 3, the *cox2-12* mutant JJM122; lane 4, the *cox2-13* mutant JJM230.

in the 5′-UTL. Strains carrying the *cox2-11* mutation readily produce respiratory-competent (Pet^+) revertants. Seven independent spontaneous Pet^+ revertants were isolated on YPEG medium at 30°C and analyzed genetically for the presence of unlinked suppressor mutations. To determine whether any of the revertants were due to dominant nuclear mutations, they were first grown in the presence of ethidium bromide to produce derivatives lacking mtDNA (Goldring *et al.*, 1970). These rho^0 derivatives were then mated to the rho^+ *cox2-11* mutant strain, JJM150. Six of the resulting diploids grew well on YPEG medium indicating that they contained dominant nuclear suppressor mutations derived from the corresponding revertants. The diploid produced by the rho^0 derivative of the seventh revertant exhibited the *cox2-11* mutant phenotype. This seventh revertant contained a mitochondrial mutation (as opposed to a recessive nuclear suppressor) because mating the original rho^+ revertant to JJM150 produced diploids that grew well on YPEG.

Four of the six revertants carrying nuclear suppressors exhibited temperature-sensitive respiratory growth (Mulero, 1993). To determine whether the suppressor mutations alone caused these defects, the rho^0 derivatives of those four nuclear revertants were crossed to the wild-type rho^+ strain DUL2, and the resulting diploids were sporulated. None of the meiotic progeny of these crosses (all of which contained wild-type mtDNA) were defective for respiratory growth at 16°, 30°, or 37°C. Thus, none of the suppressor mutations caused intrinsically temperature-sensitive respiration; only the combination of the *cox2-11* mutation and the nuclear suppressors produced this phenotype.

Because the *PET111* site of action has been mapped to the 5′-UTL of the *COX2* mRNA (Mulero and Fox, 1993a), we tested whether any of the nuclear suppressors of *cox2-11* were linked to *pet111*. Each nuclear revertant was mated to a rho^0 strain carrying the null mutation *pet111-14*, JJM87rho°. Tetrads were dissected from each cross, and the progeny were scored for growth on YPEG. Although the suppressors present in five of the revertants were unlinked to *pet111* (and were not studied further), the suppressor in the revertant strain JJM144 was tightly linked to *pet111*. All 38 tetrads dissected exhibited the parental ditype segregation of 2 Pet^+:2 Pet^-, where the Pet^+ spores exhibited the suppressed phenotype and the Pet^- spores were completely respiratory deficient. (Residual translation of the mRNA coded by the *cox2-11* allele requires *PET111* activity because the *pet111-14*, [*cox2-11*] spores from this cross were completely deficient for respiratory growth.) This dominant suppressor is a mutation in *PET111*, as shown below, and has been termed *PET111-20*. The ability of this mutation, affecting the *COX2* mRNA-specific translational activator protein PET111, to improve translation of the *cox2-11* mutant mRNA and to suppress the mutant growth defect is illustrated in Figures 3 and 5, respectively.

The allele-specificity of suppression by *PET111-20* was tested by mating a *PET111-20* rho^0 strain (JJM179rho°) to rho^+ strains harboring *cox2* mutations and scoring the respiratory growth of the resulting diploids, heterozygous for the suppressor (Figure 6). After a 2-d incubation on YPEG medium at 30°C, suppression of the 5′-UTL mutation *cox2-11* was readily apparent (Figure 6A). Interestingly, *PET111-20* also suppressed the leaky respiratory defect caused by *cox2-10*, a mutation that changed the *COX2* translation initiation codon from AUG to AUA but did not affect the 5′-UTL (Mulero and Fox, 1993b). No suppression of the other 5′-UTL mutations, *cox2-12* and *cox2-13*, was evident after 2-d incubation. On a similar YPEG plate incubated for 24 d (Figure 6B), very weak suppression of *cox2-12* was evident, although *cox2-13* remained unsuppressed.

Genomic DNA carrying the *PET111-20* allele was cloned by the gap repair method (Orr-Weaver and

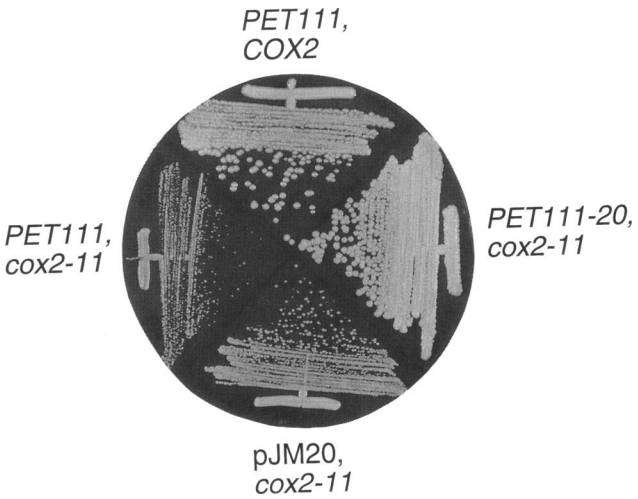

Figure 5. The *PET111-20* allele suppresses the respiration-deficiency of a strain carrying a *cox2-11* mutation. The following strains were streaked on nonfermentable medium (YPEG) and incubated at 30°C for 6 d: the *PET111*, [*COX2*] strain, DUL2; the *PET111*, [*cox2-11*] strain, JJM156; the *PET111*, [*cox2-11*] strain overproducing wild-type PET111, JJM156 (pJM20); the *PET111-20*, [*cox2-11*] strain, JJM179.

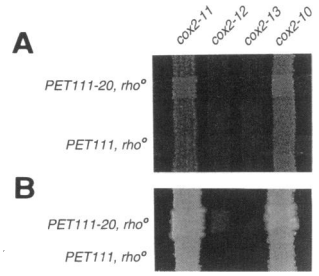

Figure 6. The *PET111-20* mutation readily suppresses the *cox2-11* and *cox2-10* mutations. After 24 d of incubation it weakly suppresses the *cox2-12* mutation. Grids of strains of opposite mating types were mated on YPD for 2 d and then printed to YPEG. The YPEG plate shown in A was incubated at 30°C for 2 d. The YPEG plate shown in B was incubated at 30°C for 24 d. The suppressor *PET111-20, rho°* strain was JJM179rho°. The control *PET111, rho°* strain was DUL2rho°. The mutant strains tested carried the *COX2* 5′-UTL mutations *cox2-11* (JJM156), *cox2-12* (JJM159), and *cox2-13* (JJM230), as well as the *COX2* initiation codon mutation *cox2-10* (JJM196) (Mulero and Fox, 1993b).

Szostak, 1983), and the mutation was mapped by subcloning of DNA fragments to a 0.49-kb *Bgl* II-*Hpa* I fragment within the *PET111* ORF (MATERIALS AND METHODS). DNA sequence analysis of this region identified a unique change of a G to A substitution at position +1954 of the wild-type sequence (GenBank accession number M17143; see MATERIALS AND METHODS) that changed amino acid 652 from alanine to threonine.

Suppression by PET111-20 Is not Because of Overaccumulation of the PET111 Translational Activator Protein

If the respiratory defects caused by the mutations affecting the *COX2* mRNA 5′-UTL were because of weakened interactions between the mRNA and the PET111 protein, then overproduction of PET111 might cause suppression. To test this possibility for the leaky mutation, *cox2-11*, a mutant strain (JJM156), was transformed with a multicopy plasmid carrying the wild-type *PET111* gene (pJM20, MATERIALS AND METHODS). The resulting elevated dosage of *PET111* caused weak suppression of *cox2-11* (Figure 5). However, similar experiments revealed that the more stringent mutations *cox2-12* and *cox2-13* were not detectably suppressed by elevated *PET111* dosage (not shown).

The fact that elevated dosage of the wild-type *PET111* gene caused suppression of *cox2-11*, albeit weakly, raised the possibility that the *PET111-20* suppressor mutation might act by dramatically increasing the steady-state level of PET111 protein (for example by increasing its stability). To test this hypothesis we used an antibody directed against PET111 (MATERIALS AND METHODS) to compare the level of the protein in a *PET111-20* strain with that in a strain carrying the wild-type *PET111* gene on the multicopy plasmid pJM20. Immunoblot analysis of total cellular protein (Figure 7) revealed that the ~94-kDa PET111 protein could be detected, in addition to several unidentified background bands, in the strain containing multiple copies of *PET111* (Figure 7, lane 4). However, PET111 could not be detected in the *PET111-20* mutant (Figure 7, lane 5). Consistent with previous observations using this antiserum (Strick, 1988), PET111 was similarly undetectable in wild-type (Figure 7, lanes 1 and 3). These data show that a strain with multiple copies of the wild-type *PET111* gene contains more PET111 protein than a strain with the chromosomal *PET111-20* mutation. Because *PET111-20* causes far stronger suppression of the *cox2-11* mutation than do multiple copies of the wild-type gene, suppression by *PET111-20* must involve an alteration of the function of the PET111 protein. Obviously these data shed no light on the relative levels of PET111 protein in wild-type and *PET111-20* strains.

DISCUSSION

We have begun to explore translational activation of the *COX2* mRNA by generating mutations in vitro that alter the mitochondrial *COX2* gene and inserting them into the mitochondrial genome in place of wild-type. Here we describe three mutations affecting the mRNA 5′-UTL (Figure 1). All three mutations reduce coxII synthesis and respiratory growth rates without dramatically lowering the steady-state level of *COX2* mRNA, indicating that they directly affect translation.

Our results point to an intimate functional interaction between the 54-base *COX2* mRNA 5′-UTL and the 94-kDa translational activator protein PET111. One of the mutations, a deletion of a single base at position −24 of the 5′-UTL (relative to the start of translation) termed *cox2-11*, clearly interferes with the action of PET111 but does not completely eliminate PET111-dependent translation. The mutation is strongly suppressed at the level of respiratory growth by the nuclear missense mutation *PET111-20*. This observation suggests the possibility that PET111 may directly contact the *COX2* mRNA and that the *PET111-20* mutation could increase the af-

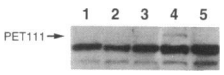

Figure 7. The PET111 protein can be detected immunologically in a strain containing multiple copies of the wild-type *PET111* gene but not in a *PET111-20* mutant. Total yeast protein was isolated, subjected to SDS-PAGE, blotted to a membrane, and reacted with anti-PET111 serum (MATERIALS AND METHODS). Immune complexes were visualized by chemiluminescent film exposure (MATERIALS AND METHODS). Lane 1, the wild-type *PET111* strain DAU2; lane 2, the *pet111-14* (complete deletion) strain JJM87; lane 3, the *PET111*, [*cox2-11*] strain JJM156; lane 4, the *PET111*, [*cox2-11*] strain, JJM156 transformed with the multicopy *PET111* plasmid pJM20; lane 5, the *PET111-20*, [*cox2-11*] strain JJM179.

finity of the protein for the 5′-UTL. Similar genetic interactions have revealed physical RNA-protein interactions (Nishi and Schnier, 1986; Meinnel *et al.*, 1991; Shannon and Guthrie, 1991; Peabody and Ely, 1992). Consistent with the possibility that the *cox2-11* mutation weakens interaction with the PET111 protein, overproduction of the wild-type protein suppressed the mutation, albeit very weakly. Strong suppression by *PET111-20* is not simply because of increased levels of the translational activator protein.

PET111-20 also suppresses a leaky *COX2* translation initiation codon mutation whose phenotype is known to be affected by *PET111* gene dosage (Mulero and Fox, 1993b). Because the initiation codon mutation does not affect the structure of the mRNA 5′-UTL, this observation suggests that *PET111-20* may code for a protein with increased affinity for the wild-type 5′-UTL. Although *PET111-20* has no effect on the respiratory growth of cells containing a fully wild-type *COX2* mRNA, this is not unexpected. The rate of coxII synthesis is apparently not rate-limiting for the growth of wild-type cells on nonfermentable carbon sources, because *PET111-20* fully suppresses the growth phenotype caused by *cox2-11* but only partially restores translation (compare Figures 3 and 5).

A more complex alteration at the same site in the *COX2* mRNA 5′-UTL, termed *cox2-12* (in which 4 new bases were substituted for the single base deleted in *cox2-11*), completely prevents detectable translation and is only very weakly suppressed by *PET111-20*. This suggests that a recognizable site for PET111 action remains in the *cox2-12* mRNA, but it is severely damaged or concealed by aberrant structure. A deletion of over one-half the 5′-UTL *cox2-13* also blocked translation completely but was not detectably suppressed, suggesting that the PET111 site of action may have been removed in this case. These results are particularly interesting in light of a phylogenetic comparison of known or presumed *COX2* mRNA 5′-UTL sequences in the budding yeasts *Saccharomyces cerevisiae, Torulopsis glabrata, Kluyveromyces lactis, Kluveromyces thermotolerans,* and *Hansenula saturnus* (Hardy and Clark-Walker, 1990; Clark-Walker, personal communication). Although these 5′-UTLs are not highly homologous, they all contain the sequence UCUAA at positions between 18 and 37 bases upstream of the translation initiation codon. In *S. cerevisiae* this pentanucleotide sequence comprises bases −19 to −23. Thus, it is immediately downstream of the site altered by the mutations *cox2-11* and *cox2-12* and is deleted by *cox2-13*. Although the significance of this pentanucleotide sequence has yet to be investigated, it is a good candidate for the site of PET111 action. Part of this sequence is bound in vitro by an abundant 40-kDa mitochondrial protein distinct from PET111, the functional significance of which is unknown (Papadopoulou *et al.*, 1990; Dekker *et al.*, 1991, 1992).

The *PET111-20* missense mutation changed amino acid residue 652 from Ala to Thr. Interestingly, this site falls within a region of the PET111 protein previously identified as being similar in sequence to the amino-terminal portion of the coxII precursor protein (16 identities and 10 conservative substitutions in an overlap of 51 amino acids) (Strick and Fox, 1987). We do not know whether this amino acid sequence similarity has any biological significance. Deletion of PET111 amino acids 651–654 (*pet111-12*) blocked *COX2* translation, confirming the importance of this region for function (Mulero, 1993). However, these residues are not critical for activity because a temperature-sensitive revertant of this deletion contained an intragenic suppressor mutation that changed residue 485 from Thr to Pro (Mulero, 1993).

Translation of the *COX3* mRNA in yeast mitochondria is specifically activated by the protein products of three genes, *PET54, PET122,* and *PET494,* which act on a site in the 613 base 5′-UTL (Costanzo and Fox, 1988). These three proteins, which interact with each other (Brown *et al.*, 1994), are located in mitochondria and associated with the inner membrane (Costanzo and Fox, 1986; Ohmen *et al.*, 1988; Costanzo *et al.*, 1989; McMullin and Fox, 1993). PET122 functionally interacts with both the mitochondrial ribosome (Haffter *et al.*, 1990, 1991; McMullin *et al.*, 1990; Haffter and Fox, 1992) and with the *COX3* mRNA 5′-UTL (Costanzo and Fox, 1993), suggesting that the translational activator proteins function to mediate the interaction of ribosomes with the *COX3* mRNA before initiation codon selection (Folley and Fox, 1991; Mulero and Fox, 1993b). PET111, which appears to be membrane bound in cells overproducing the protein (Strick, 1988), could be playing a similar role in translation of the *COX2* mRNA. In this connection it is interesting to speculate that the other, as yet unidentified, nuclear gene(s) detected as suppressors of *cox2-11* may play a direct role in activating translation of the *COX2* mRNA.

There may be parallels between the positive control of translation in yeast mitochondria and gene regulation in chloroplasts of higher plants and *Chlamydomonas.* There is ample evidence that expression of at least some genes in these systems is controlled, both developmentally and in response to light, at the level of translation, and in several cases nuclear genes have been identified that may encode translational activators (Rochaix, 1992; Staub and Maliga, 1993). Furthermore, 5′-UTL sequences have recently been implicated in the translational control of some *Chlamydomonas* mRNAs (Rochaix, 1992; Sakamoto *et al.*, 1993).

The situation in mammalian mitochondria is less clear. Although evidence has been reported indicating that translational control may occur (Whitfield and Jefferson, 1990), mammalian mitochondrial mRNAs typically lack significant 5′-UTLs (Attardi and Schatz, 1988). Although mammalian mitochondrial ribosomes can be associated

with mRNAs in vitro (Denslow *et al.*, 1989; Liao and Spremulli, 1989, 1990), there is very little information on how mitochondrial translation initiates in animal mitochondria.

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