Interactions among Three Proteins That Specifically Activate Translation of the Mitochondrial *COX3* mRNA in *Saccharomyces cerevisiae*

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The PET54, PET122, and PET494 proteins, which are associated with the yeast inner mitochondrial membrane, specifically activate translation of the mitochondrially encoded *COX3* mRNA. We used the two-hybrid system to test whether pairs of these proteins, when fused to either the GAL4 DNA-binding or transcriptional activating domain, can physically associate as measured by the expression of the GAL4-dependent reporter, *lacZ*. PET54 and PET122 interacted in this system, and an amino-terminally truncated PET494 fragment showed an interaction with PET54. We also detected functional interactions between PET54 and PET122 genetically: a *pet54* missense substitution (Phe to Gly at position 244) that caused a severe respiratory defect was suppressed both by a missense substitution affecting PET122 (Gly to Val at position 211) and by overproduction of wild-type PET122. Both Gly and Ala, substituted at PET54 position 244, disrupted the two-hybrid interactions with PET122 and PET494. While Ala at PET54 position 244 caused only a modest respiratory phenotype alone, it caused a severe respiratory defect when combined with a cold-sensitive mitochondrial mutation affecting the *COX3* mRNA 5' leader. This synthetic defect was suppressed by a missense substitution in PET122 and by overproduction of wild-type PET122, indicating functional interactions among PET54, PET122, and the mRNA. Taken together with previous work, these data suggest that a complex containing PET54, PET122, and PET494 mediates the interaction of the *COX3* mRNA with mitochondrial ribosomes at the surface of the inner membrane.

Among the many nuclear gene products required for expression of yeast mitochondrial genes are proteins that activate translation of specific mitochondrially encoded mRNAs (1, 5, 14, 34, 48). Three such proteins, PET54, PET122, and PET494, which are associated with the inner mitochondrial membrane (42), are required for translation of the *COX3* mRNA encoding cytochrome *c* oxidase subunit III (8, 12, 13, 17, 39). These proteins activate translation through a site (or sites) that can be mapped genetically to the upstream half of the 613-base *COX3* mRNA 5' untranslated leader (15, 19, 56).

The yeast mitochondrial translation system has not been amenable to in vitro biochemical analysis (20). However, genetic studies have shed some light on the probable function of mRNA-specific translational activators. The PET122 protein functionally interacts with the mitochondrial ribosome, since mutations that truncate its carboxy terminus can be suppressed by mutations affecting three different proteins of the mitochondrial ribosomal small subunit (29–31, 43). In addition, PET122 interacts functionally with the *COX3* mRNA 5' leader, since a cold-sensitive mutation in the *COX3* mRNA 5' leader can be suppressed by a mutation affecting PET122 (15). The phenotypic similarities of *pet54*, *pet122*, and *pet494* mutants are consistent with the idea that these three proteins act together to carry out the same function. To date, however, no further definitions of the roles of PET54 and PET494 have been possible, nor has any evidence been reported for interactions among these three activator proteins.

In this study, we have used both the well-known genetic methods of suppressor analysis and phenotypic analysis of double mutants (reviewed in reference 35) and a recently developed strategy termed the two-hybrid system (9, 23) to explore interactions among the translational activator proteins and the *COX3* mRNA. The two-hybrid system takes advantage of the fact that the yeast GAL4 protein can activate transcription even when its DNA-binding and transcriptional activation domains are held together by noncovalent interactions. Nuclearly targeted hybrid proteins, consisting of each GAL4 domain fused to other proteins of interest, can be coexpressed in yeast cells. If the proteins of interest interact physically, then GAL4 activity is reconstituted in vivo and a GAL4-dependent reporter gene, in this case *lacZ*, is expressed. Thus, interaction between two proteins can be detected by assays for β -galactosidase activity.

Our results argue strongly for protein-protein interactions between PET54 and PET122 and between PET54 and PET494. In addition, we present genetic evidence for functional interactions among PET54, PET122, and the *COX3* mRNA 5' leader. These findings, taken together with results of earlier studies, support a model in which a membrane-bound complex of translational activator proteins mediates the interaction of mitochondrial ribosomes with the mRNA.

MATERIALS AND METHODS

Strains, media, and genetic methods. The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Most media and genetic methods were as described elsewhere (53). Respiratory growth was assessed on YPEG medium (1% yeast extract, 2% Bacto Peptone, 3% ethanol, 3% glycerol). Yeast cells were transformed by treatment

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TABLE 1. Strains used in this study

Strain	Nuclear [mitochondrial] genotype	Reference or source
DL1	<i>MATα lys2 [rho⁺]</i>	24
DL1rho ⁰	<i>MATα lys2 [rho⁰]</i>	L. S. Folley
DL2	<i>MATα lys2 [rho⁺]</i>	24
GGY1::171 ^a	<i>MATα ade2 leu2 his3 Δgal4 Δgal80 <i>GAL1::lacZ</i></i>	26
JJM196	<i>MATα ade2 lys2 ura3-Δ [rho⁺ cox2-10]</i>	46
MCC87	<i>MATα pet54-5 ura3-52 [rho⁺]</i>	18
MCC135	<i>MATα pet54-A244 ura3-52 [rho⁺]</i>	This study
MCC137	<i>MATα pet54-G244 ura3-52 [rho⁺]</i>	This study
MCC137R5-2	<i>MATα pet54-A244 PET122-V211 ura3-52 [rho⁺]</i>	This study
MCC199	<i>MATα lys2 [rho⁺ cox3-15]</i>	15
MCC213	<i>MATα pet54-A244 lys2 [rho⁺ cox3-15]</i>	This study
MCC215	<i>MATα pet54-A244 ura3 [rho⁺ cox3-15]</i>	This study
MCC215R4-1	<i>MATα pet54-A244 PET122-II75 ura3 [rho⁺ cox3-15]</i>	This study
MCC223	<i>MATα pet54-G244 pet122-6 ade2 ura3 [rho⁺]</i>	This study
MCC225	<i>MATα PET122-V211 ura3 [rho⁺]</i>	This study
MCC246	<i>MATα pet54-A244 pet122-6 ura3 [rho⁺ cox3-15]</i>	This study
MCC247	<i>MATα pet54-G244 ade2 leu2-3 leu2-112 ura3-52 [rho⁺]</i>	This study
MCC248	<i>MATα pet54-A244 ade2 leu2-3 leu2-112 ura3-52 [rho⁺ cox3-15]</i>	This study
MCC258	<i>MATα pet54-A244 ade2 ura3 [rho⁺ cox2-10]</i>	This study

^a Carries additional unknown auxotrophies.

with lithium acetate and polyethylene glycol (22, 36). Transformants of strain GGY1::171 (26) were plated on complete medium containing all amino acids except those being selected and sucrose as a carbon source (GGY1::171 carries unknown auxotrophic mutations in addition to those listed in Table 1). To score *lacZ* expression, these transformants were printed to the same selective medium containing 100 mM phosphate buffer and 40 μ g/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). Selected transformants were propagated from the original transformation plates.

Plasmids. Plasmids were constructed and transformed into *Escherichia coli* DH5 α by using standard techniques (50). The multicopy expression vectors pMA424, encoding the GAL4 DNA-binding domain residues 1 to 147 and bearing *HIS3* (23, 41), and pGAD2F, encoding the GAL4 transcriptional activation domain residues 768 to 881 and bearing *LEU2* (9), have been described elsewhere.

The three translational activator genes were isolated from plasmids by using the PCR (50) with primers containing *Bam*HI sites in order to generate the proper fragments for in-frame cloning into the *Bam*HI sites of pMA424 and pGAD2F. The *PET54* coding region was copied from the cloned gene in M13 mp19 (18), using the primers 5' HBPET54 (5'GGTAAGCTTGGATCCCCATGAAGGCTTCTAGTAAGCT3') and 3'PET54BH (5'GGTAAGCTTGGATCCTCATCTACTAAGATGTTCAAT3'). The amplified DNA fragment was first inserted as a *Hind*III fragment into expression vector pAAH5 (3) to give pTDF3, in order to check that *PET54* was functional. The *PET54* coding region was reisolated from pTDF3 as a *Bam*HI fragment and

inserted into pMA424 and pGAD2F, yielding pNGB7 and pNGB8, respectively. The *pet54-G244* and *pet54-A244* mutant genes were similarly copied from template plasmids pMC267 and pMC266, respectively (see below), and inserted as *Hind*III fragments into pAAH5, creating pNGB37 and pNGB36, respectively. The *pet54-G244* coding sequence was reisolated from pNGB37 as a *Bam*HI fragment and inserted into pMA424 and pGAD2F, yielding pNGB30 and pNGB31, respectively. The *pet54-A244* coding sequence was reisolated from pNGB36 as a *Bam*HI fragment and inserted into pMA424 and pGAD2F, yielding pNGB34 and pNGB35, respectively. The *PET122* coding region was copied from pB122-XB1, using the primers 5' HBPET122 (5'GGTAAGCTTGGATCCTGGTGACCACCGATGTGCGG3') and 3'PET122BH (5'GGTAAGCTTGGATCCTTTATGTGTGATTTCAA3'). This amplified fragment was inserted as a *Hind*III fragment into the vector pBluescript (–) (Stratagene). The *PET122* coding region was reisolated from this plasmid, pNGB9, as a *Bam*HI fragment and inserted into pMA424 and pGAD2F, yielding pNGB10 and pNGB11, respectively. The *PET122-V211* mutant gene clone was similarly amplified from template plasmid pMC286 and inserted as a *Bam*HI fragment directly into pMA424 and pGAD2F, yielding pNGB32 and pNGB33, respectively. The complete *PET494* coding region was amplified from pPHB20 (28), using the primers 5' HBPET494 (5'GGTAAGCTTGGATCCCCGATGCATTTGAAAAAGGGG3') and 3'PET494BH (5'GGTAAGCTTGGATCCTTTATTAAGAAGAGTCGCCC3'), inserted as a *Hind*III fragment into pBluescript (–) (Stratagene), and then subcloned into pAAH5 to give pNGB13. The functional *PET494* coding region was reisolated from pNGB13 as a *Bam*HI fragment and inserted into pMA424 and pGAD2F, yielding pNGB14 and pNGB15, respectively. A DNA fragment encoding the amino-terminally truncated PET494 protein (lacking 146 residues) was similarly amplified, using 5'BPET494-428 (5'GGTGGATCCAGCAACCTGATGTAGCACAT3') as the upstream primer, and directly inserted as a *Bam*HI fragment into pMA424 and pGAD2F, yielding pNGB38 and pNGB39, respectively.

β -Galactosidase assays. Cultures were grown to saturation in sucrose-containing complete medium lacking histidine and leucine, diluted back into the same medium, and grown to an optical density at 600 nm of ~1.0. Cells (1.5 ml) were resuspended in 200 μ l of buffer containing 100 mM Tris-HCl (pH 8), 20% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and lysed by vortexing (five times for 30 s each time) with 0.45-mm-diameter glass beads, and a clear extract was collected after centrifugation in a microcentrifuge at 4°C (49). β -Galactosidase assays were performed by the method of Miller (45), and protein concentrations were determined by the method of Bradford (6).

Immunoblots. Total yeast protein was isolated from overnight cultures as described previously (58), electrophoresed in a sodium dodecyl sulfate polyacrylamide gel, and blotted to an Immobilon-P (Millipore) membrane. The membrane was probed with antiserum against PET54 (18) that had been treated with acetone powder (32) derived from yeast cells lacking PET54. Antibody-antigen complexes were detected by using the Amersham enhanced chemiluminescence system.

Origin and characterization of *pet54* mutant alleles. Site-directed mutagenesis of *PET54* codon 244 was performed by the method of Kunkel et al. (40), using the Muta-Gene in vitro mutagenesis kit (Bio-Rad). The single-stranded DNA template used was the *PET54* gene on a 1.6-kb *Hind*III fragment in phage M13 mp19 (18). The oligonucleotide used

for mutagenesis was 5'CGATTATATCTG(G or C)CGA AAAC3', to change codon 244 to GCC (Ala) or GGC (Gly). Progeny phage were screened for a unique *Bst*BI site present in the parental phage but absent in the mutants. Each mutation was confirmed by nucleotide sequencing (51), and the 1.6-kb *Hind*III fragment carrying each gene was subcloned into pMC216ΔH, a yeast replicating plasmid carrying the *URA3* gene and 1.4 kb of *PET54* flanking sequences but lacking the 1.6-kb *Hind*III fragment. The resulting plasmids were pMC266 (*pet54-A244*) and pMC267 (*pet54-G244*). The mutant genes were integrated into the chromosome at the *PET54* locus by excising them from pMC266 and pMC267 as approximately 3-kb fragments and using the gel-purified fragments to transform the *pet54-5* complete deletion strain MCC87 (18) (Table 1), along with the *URA3* replicating plasmid pCGE137 (47) to provide a selectable marker. Ura⁺ transformants were screened for the ability to grow on YPEG, and integration was checked by DNA gel blot analysis (50).

The following *pet54* mutations were used to characterize the allele specificity of the *PET122-V211* suppressor. *pet54-4* is a frameshift mutation caused by a single-base-pair deletion in the *PET54* coding sequence, and *pet54-5* is a complete deletion of the *PET54* coding sequence (18). *pet54-20* is an uncharacterized mutation causing temperature-sensitive respiration (52). The temperature-sensitive mutation *pet54-21* is a revertant of the *pet54-4* frameshift mutation with a single-base-pair insertion upstream of *pet54-4*, resulting in a *PET54* protein with 30 amino acids of altered sequence (18). The temperature-sensitive mutation *pet54-ts2341* (44) is a T-to-A change at position +23 of the coding sequence, changing codon 8 from isoleucine to asparagine (7).

Selection and analysis of suppressor mutations. Respiring revertants were selected on YPEG medium at 30°C from two mutant strains: a *pet54-G244* mutant (MCC137; Table 1) and a *pet54-A244* [*cox3-15*] (notations in brackets represent mitochondrial genotypes) double-mutant strain (MCC215; Table 1).

The MCC137 revertants were analyzed as follows. First, they were treated with ethidium bromide (25) to remove mitochondrial DNA. The resulting *rho*⁰ derivatives were crossed to a wild-type strain (DL1; Table 1), and the respiratory phenotypes of the meiotic progeny were analyzed to determine whether the suppressor phenotype resulted from a mitochondrial or nuclear mutation and, if nuclear, whether the mutation was linked to *pet54*. Of 26 suppressor strains analyzed, none carried mitochondrial mutations, 6 carried nuclear mutations linked to *pet54*, and the remaining 20 had nuclear mutations unlinked to *pet54*. To determine whether any of those 20 strains carried suppressor mutations linked to *PET122*, they were crossed to the *pet54-G244 pet122-6* mutant strain MCC223 (Table 1), which had been transformed with the *PET54 URA3* replicating plasmid pAF001 (15). (The plasmid was necessary because the respiration of the homozygous *pet54-G244*, heterozygous suppressor strains was not strong enough to promote efficient sporulation.) After dissection, the meiotic progeny were printed twice to medium containing 5-fluoroorotic acid to select against the plasmid (4) and then to YPEG medium to analyze the segregation of respiratory phenotypes. Of the 20 revertants analyzed, one (MCC137R5-2; Table 1) carried a suppressor mutation tightly linked to *pet122-6* (no recombinants in 46 complete tetrads). Eleven of the remaining nineteen revertants were analyzed in a similar manner to test linkage of their suppressor mutations to *PET494*, and none was linked to *PET494*. Of these 19

uncharacterized suppressors, only 3 had intrinsic phenotypes: one caused a very slight temperature-sensitive respiratory defect, and two were temperature-sensitive lethal mutations. At least some of these mutations are likely to be informational suppressors.

Revertants of the *pet54-A244* [*cox3-15*] strain MCC215 were analyzed as follows. They were first crossed to a wild-type strain lacking mitochondrial DNA (DL1rho⁰; Table 1), and the respiratory growth of the meiotic progeny was analyzed to place the suppressor mutations in one of two categories: either mitochondrial or nuclear, at *pet54*, or nuclear and unlinked to *pet54*. One mutation of nine fell into the first group, while the remaining eight were in the second group. To determine whether these suppressor mutations were in *PET122*, the eight revertant strains were crossed to a *pet54-A244 pet122-6* [*cox3-15*] strain (MCC246; Table 1) containing *PET54* on pAF001. After curing of the meiotic progeny of the plasmid on 5-fluoro-orotic acid medium (4), segregation of the suppressor phenotype was scored. Two of the eight revertant strains (MCC215R4-1 and MCC215R7-1; Table 1) carried suppressor mutations tightly linked to *PET122* (no recombinants in 44 and 54 complete tetrads, respectively).

Cloning and analysis of *PET122* suppressor alleles. The *PET122* suppressor mutant genes were isolated from strains MCC137R5-2 (*PET122-V211*), MCC215R4-1 (*PET122-I175*), and MCC215R7-1 (*PET122-I175*), using the PCR as described above. The template was total genomic DNA isolated from 1-ml cultures (53) and digested with *Sal*I. The primers used for amplification were 5'HPET122 (5'GGTA AGCTTATGTTGACTATCACGAAAAG3') and 3'PET122 BH (see above). Amplified fragments were digested with *Hind*III or with *Hind*III and *Bam*HI and inserted into pBluescript (–) (Stratagene). Nucleotide sequences were determined by the dideoxy-chain termination method (51). For strain MCC137R5-2, a partial nucleotide sequence of the total PCR products was determined to verify that the single-nucleotide change observed in the cloned gene was present in the bulk reaction products.

RESULTS

Evidence for interactions among the COX3 mRNA-specific translational activator proteins, using the two-hybrid system. We first sought to test whether interactions between pairs of the translational activator proteins PET54, PET122, and PET494 could bring together the separated DNA-binding and transcriptional activation domains of GAL4 in the nucleus. Plasmids encoding proteins consisting of the translational activators fused to the DNA-binding domain of GAL4 or to the transcriptional activating domain of GAL4 were constructed in the multicopy expression vector pMA424 (23, 41) or pGAD2F (9), respectively (Materials and Methods).

The entire *PET54* coding sequence was used in the fusions, as PET54 is not processed during import into mitochondria (42). Since PET122 is cleaved during import between residues 8 and 9 of the precursor (42), the *PET122* fusions were constructed to begin coding at residue 9 (coincidentally, the fusions reconstituted the eighth codon). Since the site of cleavage of the 56-kDa PET494 precursor protein is unknown, we constructed two pairs of *PET494* fusions. One pair contained the entire *PET494* reading frame. In the other pair of *PET494* fusions, bases 1 through 428 were removed such that the PET494 sequence began at amino acid residue 147. The deleted portion of pre-PET494 included a

TABLE 2. β -Galactosidase production from in vivo PET54, PET122, and PET494 fusion protein interactions

Protein fused to GAL4 domain		β -Galactosidase sp act ^a (U)	Colony color
DNA binding	Activating		
None ^b	None	0.4	White
PET54		0.3	White
PET122		0.3	White
PET494		0	White
NA-PET494 ^c	None	0.1	White
	PET54	0	White
	PET122	0	White
	PET494	0.1	White
None	NA-PET494	0.2	White
PET54	PET54	0.6	White
PET122	PET122	0.5	White
PET494	PET494	0	White
NA-PET494	NA-PET494	0.1	White
PET54	PET122	33.4	Pale blue
PET122	PET54	268.1	Dark blue
PET54	PET494	0	White
PET494	PET54	0	White
PET122	PET494	0	White
PET494	PET122	0.6	White
PET54	NA-PET494	1.2	Pale blue
NA-PET494	PET54	3.2	Pale blue
PET122	NA-PET494	0.1	White
NA-PET494	PET122	0.3	White

^a Average of at least three transformants. A value of 0 indicates no activity above background.

^b Presence of the empty fusion vector.

^c N-terminal truncation of PET494.

33-amino-acid region known to be dispensable for function (16).

To confirm that no inactivating mutations arose in the genes during plasmid construction, we checked whether expression of the fusion proteins would complement the respiratory growth-defective phenotypes of *pet54*, *pet122*, and *pet494* chromosomal mutations. All of the fusion proteins (or proteolytic products derived in vivo from them) functioned in mitochondria by this criterion, although the complementation observed with the PET54 fusions and the amino-terminally truncated PET494 fusions was weak. Thus, despite the absence of mitochondrial targeting signals at their amino termini (and the presence of nuclear targeting signals in the fusions), some active protein entered mitochondria in each case.

An important prerequisite for the detection of interactions with the two-hybrid system is that the individual fusion proteins must not alone activate expression of the GAL4-dependent *lacZ* reporter gene in the host strain GGY1::171. Each of the plasmids encoding fusion proteins was transformed into the host, and β -galactosidase activity in the transformants was analyzed both by colony color on selective medium containing X-Gal and biochemically in cell extracts. None of the fusion proteins was able to activate transcription (Table 2).

To test for interactions, pairs of fusion proteins were expressed together in the host strain, and β -galactosidase activity was analyzed (Table 2). The results indicated protein-protein interactions between PET54 and PET122 and between PET54 and PET494. The highest level of β -galactosidase activity was detected in cells containing the GAL4 DNA-binding domain fused to PET122 and the GAL4 activation domain fused to PET54. While PET54 and PET122

interacted in the reverse orientation as well, the β -galactosidase activity was consistently about eightfold lower. Low but significant β -galactosidase activity was also detected in cells expressing fusions containing PET54 and the amino-terminally truncated form of PET494. None of the other combinations tested, including self-interactions, yielded positive results.

Missense mutations in codon 244 of PET54 affect COX3 translational activation. We obtained further evidence for functional interaction between PET54 and PET122 (as well as between PET54 and the *COX3* mRNA leader) from studies using site-directed *pet54* missense mutations at codon 244, which were suggested by a similarity to the bacteriophage T4 translational repressor protein RegA (55). RegA can be photochemically cross-linked to nucleic acid at Phe-106, suggesting that the region around this residue comes into close contact with mRNA (55) (although determinants for RNA binding specificity may lie elsewhere [37]). A search of protein sequence data bases with the amino acid sequence of this 17-residue region of RegA revealed several proteins with similar regions, among them PET54 (55). Although PET54 and RegA are not similar overall, this short region of PET54 has amino acid sequence similarity to RegA (7 identities in a 17-amino-acid overlap) in the region surrounding Phe-106, including a Phe residue at position 244 that could correspond to Phe-106 of RegA.

To investigate the functional importance of this region, we changed the PET54 Phe codon at 244 to Ala (*pet54-A244*) and to Gly (*pet54-G244*) codons (Materials and Methods). Both alleles, carried on high-copy-number plasmids, supported respiration in a *pet54* deletion strain, demonstrating that neither mutation completely destroyed PET54 activity. To analyze the phenotypes of these mutations in an otherwise wild-type situation, we replaced a complete *pet54* deletion with each missense allele by integrative transformation (Materials and Methods). Both chromosomal mutations reduced respiratory growth, but to different extents. *pet54-A244* had a modest effect on respiration, causing a slight heat-sensitive defect: strain MCC135 (Table 1) grew like the wild type on YPEG medium at 13.5 and 16°C but less well than the wild type at 30 and 37°C. *pet54-G244* had a more severe heat-sensitive phenotype: strain MCC137 (Table 1) grew poorly on YPEG at 13.5 and 16°C, very poorly at 30°C, and not at all at 37°C.

Suppression of *pet54* missense mutations at codon 244 by a PET122 missense mutation. To search for mutations in genes encoding components of the COX3 translational activation complex that interact with PET54, we selected spontaneous revertants of the *pet54-G244* strain MCC137 (Table 1) on YPEG at 30°C. Since this missense mutation altered a putative RNA-binding domain of PET54, we anticipated that suppressor mutations might occur in the COX3 5' untranslated leader region. However, while a *pet54* missense mutation at codon 244 did provide us with some evidence for PET54-COX3 mRNA interaction (see below), none of the *pet54-G244* suppressors proved to be in the mitochondrial genome (Materials and Methods). The revertants were analyzed genetically to determine whether any suppressors were linked to PET122 (Materials and Methods). Of 26 nuclear suppressors analyzed, one (in strain MCC137R5-2; Table 1) was tightly linked to PET122. (None of the suppressors analyzed was linked to PET494 [Materials and Methods].) The PET122 structural gene was copied from genomic DNA of this revertant strain by using the PCR (Materials and Methods), and its nucleotide sequence was determined. One difference from the wild-type sequence was found: a G-to-T

FIG. 1. Suppression of the *pet54-G244* mutation by the *PET122-V211* missense mutation and by overproduction of the wild-type PET122 protein. Strains were streaked on a YPEG plate (see Materials and Methods) and incubated at 30°C for 6 days. (A) A *pet54-G244* strain (MCC137); (B) a *pet54-G244* strain carrying the *PET122-V211* suppressor (MCC137R5-2); (C) wild type (DL2); (D) a strain carrying the *PET122-V211* mutation with wild-type *PET54* (MCC225); (E) a *pet54-G244* strain (MCC247) carrying a plasmid (pEAD122 [42]) overexpressing *PET122*.

change at position +632 of the coding sequence, which would change codon 211 from GGT, encoding Gly, to GTT, encoding Val.

This *PET122* allele *PET122-V211* is a dominant suppressor mutation. It is a moderately strong suppressor, significantly improving the extremely weak respiratory growth of a *pet54-G244* strain at 30°C but not bringing it to a wild-type level (Fig. 1). In an otherwise wild-type strain, the *PET122-V211* mutation had no detectable phenotype on YPEG medium at 13.5, 16, 30 (Fig. 1), or 37°C. We tested the allele specificity of this suppressor by crossing it to several other *pet54* mutant strains and analyzing the respiratory phenotypes of the meiotic progeny. *PET122-V211* failed to suppress two null mutations, *pet54-4* and *pet54-5* (18). It also failed to suppress three temperature-sensitive mutations, *pet54-ts2341* (44) (see Materials and Methods), *pet54-20* (52), and *pet54-21* (18). However, it did suppress the mild phenotype of a *pet54-A244* strain: respiratory growth of a *PET122-V211 pet54-A244* double-mutant strain was indistinguishable from that of the wild type (not shown).

The *pet54-G244* mutation is suppressed by overproduction of the wild-type PET122 protein. Since several lines of evidence suggested that the PET122 and PET54 proteins interact with each other during translational activation, we tested the effect of overproducing the wild-type PET122 protein in a strain (MCC247; Table 1) carrying the *pet54-G244* mutation. Overproduction of the PET122 protein does not suppress *pet54* null mutations, nor does it cause a detectable phenotype in wild-type cells (15). However, transformation of the *pet54-G244* strain MCC247 with a plasmid (pEAD122 [42]) carrying *PET122* under control of the *ADC1* promoter (3) strongly suppressed its respiratory defect, allowing the *pet54-G244* mutant to respire at a nearly wild-type level at 30°C (Fig. 1). In contrast, transformation with a plasmid overproducing PET494 (pMC210 [12]) did not affect its respiratory growth (not shown).

Disruption of two-hybrid interactions by mutations in codon 244 of PET54. If *pet54* mutations affecting codon 244 produce respiratory growth phenotypes by weakening the interaction between the PET54 and PET122 proteins, then these mutations should also affect the interaction in the two-hybrid system. The *pet54-G244* and *pet54-A244* mutations were

TABLE 3. β -Galactosidase production from in vivo *pet54* and PET122 mutant fusion protein interactions

Protein fused to GAL4 domain		β -Galactosidase sp act ^a (U)	Colony color
DNA binding	Activating		
<i>pet54-G244</i>	PET122	0.5	White
PET122	<i>pet54-G244</i>	0.8	White
<i>pet54-A244</i>	PET122	0.2	White
PET122	<i>pet54-A244</i>	0.7	White
<i>pet54-G244</i>	PET122-V211	0.2	White
PET122-V211	<i>pet54-G244</i>	0.1	White
<i>pet54-A244</i>	PET122-V211	0.1	White
PET122-V211	<i>pet54-A244</i>	0	White
PET54	PET122-V211	32.4	Pale blue
PET122-V211	PET54	202.2	Dark blue
<i>pet54-G244</i>	Δ -PET494 ^b	0.1	White
Δ -PET494	<i>pet54-G244</i>	0.2	White
<i>pet54-A244</i>	Δ -PET494	0.1	White
Δ -PET494	<i>pet54-A244</i>	0.2	White

^a Average of at least three transformants. A value of 0 indicates no activity above background.

^b N-terminal truncation of PET494.

inserted into both the DNA-binding domain and transcriptional activating domain GAL4 fusion plasmids (Materials and Methods), and these plasmids were cotransformed into strain GGY1::171 with plasmids encoding PET122 fusion proteins. Both *pet54* mutations disrupted the interaction between PET54 and PET122, as no β -galactosidase was produced in these cells (Table 3). The *pet54* mutations did not cause the PET54 fusion proteins to become unstable in vivo, since immunoblot analysis showed that the mutant proteins accumulated to levels comparable to those of the wild-type PET54 fusions (Fig. 2). Thus, the PET54 wild-type residue Phe-244 is apparently important for the physical interaction between the PET54 and PET122 fusion proteins. The PET122-V211 suppressor protein, which does not affect respiratory growth in otherwise wild-type strains, was also tested in the two-hybrid system. Its interaction with wild-type PET54 was indistinguishable from that of wild-type PET122 protein (Table 3). However, this suppressor protein failed to interact detectably with the mutant PET54 proteins in the two-hybrid system, despite its ability to compensate for the mutant PET54 proteins in mitochondria.

We also tested for interaction of both mutant PET54 proteins with the amino-terminally truncated form of PET494 (Table 3). Both *pet54* mutations disrupted the wild-type interaction, as no β -galactosidase was produced when

FIG. 2. Immunoblot analysis of wild-type and mutant PET54 fusion proteins. All lanes contains the same amount of total yeast protein from strain GGY1::171 carrying various plasmids as follows: lane 1, both empty fusion vectors; lanes 2 to 4, single vector with the GAL4 DNA-binding domain fused to PET54, *pet54-G244*, and *pet54-A244*, respectively; lanes 5 to 7, single vector with the GAL4 activating domain fused to PET54, *pet54-G244*, and *pet54-A244*, respectively. Anti-PET54 antiserum was used in conjunction with the enhanced chemiluminescence system (Materials and Methods).

FIG. 3. Synthetic respiratory defect in a *pet54-A244* [*cox3-15*] strain. Strains were streaked on a YPEG plate (see Materials and Methods) and incubated at 30°C for 7 days. (A) A *PET54* [*cox3-15*] strain (MCC199); (B) *pet54-A244* [*cox3-15*] (MCC213); (C) *pet54-A244* [*COX3*] (MCC135); (D) *PET54* [*COX3*] (wild type; DL2); (E) *PET54* [*cox2-10*] (JJM196); (F) *pet54-A244* [*cox2-10*] (MCC258).

either of the mutant *PET54* fusion proteins was combined with the truncated *PET494* fusion proteins.

The *pet54-A244* missense mutation in combination with a *cox3* 5' untranslated leader mutation causes a synthetic respiratory defect. The nuclear mutation *pet54-A244* causes a very modest reduction in respiratory growth at 30°C (Fig. 3). Since this mutation affected a possible RNA-binding domain of *PET54* (see above), we tested whether it might interact synergistically, in double-mutant strains, with mitochondrial mutations that alter the *COX3* mRNA 5' leader. One such mitochondrial mutation is *cox3-15* (a deletion of nucleotides -437 to -359 and -329 to -1 [15]), which causes cold-sensitive cytochrome *c* oxidase subunit III synthesis but allows moderate respiratory growth at 30°C (Fig. 3). Indeed, a *pet54-A244* [*cox3-15*] double mutant strain was unable to respire at 30°C (Fig. 3) or any other tested temperature. As a control, we constructed a double-mutant strain carrying both *pet54-A244* and the mitochondrial *cox2-10* allele. *cox2-10*, an in vitro-made mutation changing the AUG initiation codon of the *COX2* gene to AUA, causes a leaky respiration-defective phenotype (46). The *pet54-A244* mutation did not interact synergistically with *cox2-10*: respiratory growth of a *pet54-A244* [*cox2-10*] strain was indistinguishable from that of a *PET54* [*cox2-10*] strain (Fig. 3). Thus, the synthetic defect of the *pet54-A244* [*cox3-15*] strain appears to be specific, suggesting that the *PET54* protein interacts functionally with the 5' untranslated leader of the *COX3* mRNA.

The *pet54-A244* [*cox3-15*] synthetic phenotype can be suppressed by a *PET122* missense mutation and by overproduction of the wild-type *PET122* protein. We selected spontaneous respiring revertants of a *pet54-A244* [*cox3-15*] strain, MCC215 (Table 1), at 30°C and analyzed them genetically (Materials and Methods). Two of nine independently isolated revertants (MCC215R4-1 and MCC215R7-1; Table 1 and Fig. 4) had suppressor mutations tightly linked to *PET122*. The *PET122* structural genes from these revertants were copied by the PCR, and their nucleotide sequences were determined. Both genes carried the same single-nucleotide difference from the wild-type *PET122* gene: a G-to-A change at position +525, which would change codon 175 from ATG, encoding methionine, to ATA, encoding isoleucine. We have termed this partially dominant suppressor allele *PET122-I175*.

FIG. 4. Suppression of the synthetic respiratory defect in a *pet54-A244* [*cox3-15*] strain by the *PET122-I175* mutation and by overproduction of the wild-type *PET122* protein. Strains were streaked on a YPEG plate (see Materials and Methods) and incubated at 30°C for 12 days. (A) A *pet54-A244* [*cox3-15*] strain (MCC215); (B) a *pet54-A244* [*cox3-15*] strain carrying the *PET122-I175* suppressor (MCC215R7-1); (C) wild type (DL2); (D) a *pet54-A244* [*cox3-15*] strain (MCC248) carrying a plasmid (pEAD122 [42]) overexpressing *PET122*.

To analyze further the function of the *PET122-I175* allele, we placed it in a wild-type background and in combination with each of the single mutations *pet54-A244* and *cox3-15*. In an otherwise wild-type background, *PET122-I175* had no detectable phenotype: respiratory growth of a *PET122-I175* strain was indistinguishable from that of the wild type at 13.5, 16, 30, or 37°C (not shown). Interestingly, while *PET122-I175* had a significant effect on the respiratory growth of a *pet54-A244* [*cox3-15*] double mutant (Fig. 4), it failed to suppress efficiently either of the single mutations. Respiratory growth of a *PET122-I175* *pet54-A244* [*COX3*] strain was indistinguishable from that of a *PET122* *pet54-A244* [*COX3*] strain at all temperatures tested except 37°C; at this temperature, the *PET122-I175* *pet54-A244* [*COX3*] colonies were slightly larger, indicating very weak suppression (not shown). A *PET122-I175* *PET54* [*cox3-15*] strain grew on YPEG medium like a nuclearly wild-type, [*cox3-15*] control strain at 30 or 37°C (15) but at low temperature (16°C) actually grew more slowly than the control (not shown).

Overproduction of the wild-type *PET122* protein also suppressed the phenotype of a *pet54-A244* [*cox3-15*] double-mutant strain (Fig. 4), although it is known not to affect the respiratory growth of a *PET54* [*cox3-15*] single-mutant strain (15). However, overproduction of *PET494* did not suppress the double-mutant strain (not shown).

DISCUSSION

The primary conclusion of this report is that the three proteins known to activate translation of the mitochondrial *COX3* mRNA interact with each other and thus probably work together to carry out the same function. Our present data indicate physical and functional interactions between *PET54* and *PET122* and physical interaction between *PET54* and *PET494*. They also point to a functional interaction between *PET54* and the *COX3* mRNA 5' leader. Taken together with results of previous work on this system (15, 29-31, 42, 43), our data suggest the picture depicted in Fig. 5, where a complex containing the membrane-bound proteins *PET54*, *PET122*, and *PET494* mediates the interaction of mitochondrial ribosomes with the *COX3* mRNA 5' leader,

FIG. 5. Hypothetical model depicting functional interactions, established here and in previous work, among components involved in translation of the *COX3* mRNA within mitochondria. Data in this report establish interactions between the *COX3* mRNA-specific activator proteins PET54 and PET122 as well as between PET54 and PET494. They also support a functional interaction between PET54 and the *COX3* mRNA 5' untranslated leader. PET122 has been shown previously to interact functionally both with the *COX3* mRNA 5' leader (15) and with proteins of the mitochondrial ribosomal small subunit (29–31, 43). The PET54, PET122, and PET494 proteins have been shown to be associated with the mitochondrial inner membrane (42). Suggestive evidence, from overexpression studies, for interactions between PET494 and PET122 and between PET494 and the *COX3* mRNA 5' leader (15) has not been interpreted in this figure.

thereby activating translation. How mitochondrial ribosomes initiate protein synthesis is not known in any detail. However, they employ a homolog of the *E. coli* initiation factor 2 (54) and apparently identify the start site by recognition of both AUG and some other context information (24, 46).

Our evidence for physical interactions between proteins was obtained by using the two-hybrid system (9, 23), in which the ability of two proteins to bind to each other is revealed by their ability to bring together the separated domains of the transcriptional activator GAL4. We have presented our data on GAL4-activated reporter gene expression (β -galactosidase specific activities) quantitatively. However, differences in the magnitudes of these signals are not determined solely by the affinities of the proteins of interest for each other. We observed an eightfold difference in the signals resulting from PET54 and PET122 interaction, depending on the orientation of these two proteins with respect to the GAL4 DNA-binding and transcriptional activation domains (Tables 2 and 3). Many factors, in addition to the strength of binding between the two proteins of interest, play a role in the activity of the reconstituted GAL4. For example, fusion of some proteins to DNA-binding domains can reduce DNA-binding activity (27). These and other conformational, steric, and stability phenomena must strongly influence the observed reporter gene activity in unpredictable ways. Thus, weak but reproducible signals may be just as meaningful as strong signals. Indeed, it is very important to realize that the absence of a detectable signal cannot be interpreted to mean that two proteins fail to interact.

All of the proteins encoded by our gene fusions had detectable biological activity in mitochondria when highly

expressed: they complemented, at least weakly, the nonrespiratory phenotype of the corresponding chromosomal deletion mutations. Similar GAL4 fusion proteins have previously been shown to function in complementation tests (11). While our results suggest that the translational activator moieties of the fusion proteins adopt normal conformations, it is possible that the biologically active species lack the amino-terminally located GAL4 domains as a result of proteolysis. Import of most proteins into mitochondria is directed by amino-terminal targeting signals that are proteolytically processed (33, 48). While none of our fusion proteins have mitochondrial targeting sequences at their amino termini, the proteolytic processing sites are at least potentially present in the PET122 fusions and the PET494 fusions that contain the entire protein (PET54 is not proteolytically processed during import) (12, 42). In any event, the fact that some fraction of active protein entered mitochondria is not surprising, since high-level expression has previously been shown to drive other proteins lacking amino-terminal targeting signals into mitochondria (10, 21).

Our genetic evidence for functional interactions among PET54, PET122, and the *COX3* mRNA 5' leader is based on site-directed mutations affecting the PET54 codon specifying Phe-244. This Phe residue lies in a short region exhibiting sequence similarity to the phage T4 RegA protein (55), which acts as a translational repressor for a specific subset of T4 mRNAs by binding to their translation initiation regions (2, 38, 57). PET54 Phe-244 appears to correspond to RegA Phe-106, which can be cross-linked to nucleic acid (55).

Substitution of Ala for PET54 Phe-244 (*pet54-A244*) produced a very modest respiratory defect in an otherwise wild-type strain. However, consistent with the notion that this region of PET54 might be in close proximity to RNA, we observed a strong synthetic defective phenotype when *pet54-A244* was combined with a cold-sensitive mitochondrial mutation (*cax3-15*) affecting the *COX3* mRNA 5' leader (Fig. 3). The possibility of intimate interactions among PET54, PET122, and the mRNA is suggested by the fact that this synthetic defect was suppressed both by overexpression of wild-type PET122 and by a chromosomal missense mutation, *PET122-I175* (Fig. 4), which failed to suppress significantly either *pet54-A244* or *cax3-15* as single mutations (indeed, it slightly enhanced the phenotype of the mRNA leader mutation). Substitution of Gly for Phe-244 (*pet54-G244*) produced a severe respiratory defect but did not completely eliminate function. Confirming the idea that PET54 and PET122 interact, *pet54-G244* was allele specifically suppressed by the Gly-to-Val missense mutation *PET122-V211*. *pet54-G244* was also suppressed by overproduction of wild-type PET122 protein. Taken together, these results suggest that the substitutions at PET54 position 244 weakened the protein's ability to interact with PET122 and probably with the *COX3* mRNA 5' leader.

The pairwise protein-protein interactions detected in the nucleus by two-hybrid experiments are apparently more easily disrupted by mutations affecting PET54 residue 244 than are the functional interactions occurring in the putative translational activation complex within mitochondria. While the *pet54-A244* mutation had only a modest effect on respiratory growth (Fig. 3), the same substitution eliminated the interactions with both PET122 and PET494 detectable by the two-hybrid experiment (Table 3) despite the fact that the mutant PET54-containing fusion proteins accumulated at normal steady-state levels (Fig. 2). As expected, the phenotypically more severe *pet54-G244* mutation also disrupted the two-hybrid interaction, but its suppressor, *PET122-*

V211, failed to restore the two-hybrid interaction despite its ability to restore significant translational activation in mitochondria. We hypothesize that in mitochondria, multifaceted interactions among these proteins and the mRNA serve to stabilize complexes containing mutant PET54 proteins. However, residual binding of mutant PET54 proteins to PET122 (both wild-type and suppressor forms) and to PET494 in strictly pairwise combinations is too weak to reconstitute significant GAL4 activity in the two-hybrid system.

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