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Product of *Saccharomyces cerevisiae* Nuclear Gene *PET494* Activates Translation of a Specific Mitochondrial mRNA

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The product of *Saccharomyces cerevisiae* nuclear gene *PET494* is known to be required for a posttranscriptional step in the accumulation of one mitochondrial gene product, subunit III of cytochrome *c* oxidase (coxIII). Here we show that the PET494 protein probably acts in mitochondria by demonstrating that both a PET494–β-galactosidase fusion protein and unmodified PET494 are specifically associated with mitochondria. To define the PET494 site of action, we isolated mutations that suppress a *pet494* deletion. These mutations were rearrangements of the mitochondrial gene *oxi2* that encodes coxIII. The suppressor *oxi2* genes had acquired the 5′-flanking sequences of other mitochondrial genes and gave rise to *oxi2* transcripts carrying the 5′-untranslated leaders of their mRNAs. These results demonstrate that in wild-type cells PET494 specifically promotes coxIII translation, probably by interacting with the 5′-untranslated leader of the *oxi2* mRNA.

Mitochondrial genes are expressed within the mitochondrion by a genetic system distinct from that required for expression of nuclear genes. Only a few of the components of the mitochondrial genetic system of *Saccharomyces cerevisiae* are encoded on the mitochondrial genome (15); most are encoded by nuclear genes and imported posttranslationally into mitochondria. Interestingly, among the known nucleus-encoded elements of the mitochondrial genetic system, several have been found to be necessary for the accumulation of specific mitochondrial gene products. Some are required for processing of particular mitochondrial transcripts (12, 20, 43). Other nuclear gene products are necessary for steps subsequent to the accumulation of specific mitochondrial mRNAs (13, 42, 45; C. G. Poutre and T. D. Fox, manuscript in preparation; M. Costanzo, E. C. Seaver, and T. D. Fox, submitted for publication).

The product of the nuclear gene *PET494* was previously shown to be specifically required for accumulation of the mitochondrially encoded subunit III of cytochrome *c* oxidase (coxIII). *pet494* mutant cells contain all the known mitochondrial gene products except for coxIII (7, 16, 17). However, they contain normal amounts of the mRNA encoding coxIII (42), a transcript of the mitochondrial *oxi2* gene (51). The *oxi2* gene is not interrupted by introns (51), and the *oxi2* mRNA in *pet494* mutant cells is indistinguishable, by Northern blot analysis or by S1 nuclease protection experiments, from that in wild-type cells (42). Therefore, the PET494 protein must be necessary for some posttranscriptional step leading to the accumulation of coxIII.

These previous studies left open two major questions. First, does the PET494 protein act directly in mitochondria or does it act in a more indirect fashion, for example, by promoting the expression of another nuclear gene? Second, is *PET494* activity necessary for translation of the *oxi2* mRNA, or is it required after translation to prevent rapid degradation of the coxIII polypeptide? In this paper we show that the product of the *PET494* gene is indeed a mitochondrial protein, both by immunological detection of the PET494 protein in highly purified mitochondria and by the demonstration that the amino terminus of PET494 directs *Escherichia coli* β-galactosidase to the organelles, implying that it does act directly in mitochondria. We also show that some mitochondrial mutations which suppress a *pet494* deletion are rearrangements of the 5′-flanking region of the *oxi2* gene. These suppressor *oxi2* genes generate mRNAs that encode wild-type coxIII, but whose 5′-untranslated leaders are altered. This result provides strong evidence that the PET494 protein acts at the level of translation rather than protein stability and that it probably activates coxIII translation by interacting with the 5′-untranslated leader of the *oxi2* mRNA.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. The wild-type *S. cerevisiae* strain was DA1 (*MATα ade2*) (42). Strain TF123 is *MATa pet494-41 ura3-52 his3-11 his3-15 leu2-3 leu2-112*. Minimal medium SGal contained 0.67% yeast nitrogen base without amino acids and 2% galactose. Plates containing the β-galactosidase indicator 5-bromo-4-chloro-3-indolyl-β-galactoside were made by the method of Rose et al. (46). Other strains, media, yeast transformations, and genetic methods were as described previously (10).

Preparation of mitochondria and β-galactosidase assays. Yeast cells carrying pBA10Z (the *pet494::lacZ* fusion plasmid) or pLGSD5 (23) were grown to the early logarithmic phase in equal volumes of synthetic minimal medium supplemented with galactose (SGal). Cells were converted to spheroplasts, and mitochondria were isolated as described by Daum et al. (11) except that spheroplasts were lysed by stirring with 0.1-mm glass beads. β-Galactosidase assays were performed by the method of Miller (39), and protein concentrations were determined by the method of Bradford (5).

Plasmids. DNA manipulations and transformation and growth of *E. coli* HB101 were performed by the methods of Maniatis et al. (36). The plasmid pBA10Z was constructed by ligating a 3.0-kilobase (kb) *Bam*HI fragment carrying the *lacZ* gene, isolated from pMC1871 (9), to *Bam*HI-cut pBA10 (10), fusing the first 119 codons of *PET494* in frame to codon 8 of *lacZ*. To construct pORF11, a 777-base-pair (bp) *Xho*I-*Hin*cII fragment was isolated from the *PET494* insert in pM9 (10, 41), the *Xho*I end was filled in with the Klenow fragment of DNA polymerase I, and the fragment was ligated to *Sma*I-cut pORF1 (55). *E. coli* MH3000 and TK1046 were

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used as described previously for the isolation and growth of pORF11 (55).

pMC210, the PET494 expression plasmid, was constructed in two steps. First, pM9, a pBR322 derivative carrying *PET494* on a 1.9-kb insert (10, 41), was linearized with *Sal*I and partially digested with *Ava*I. The partial digestion product, which had been *Ava*I cut only once, at the site 23 bp upstream of the *PET494* initiation codon, was gel purified. The ends were filled with the Klenow fragment of DNA polymerase I and ligated to create the plasmid pMC203. Because joining of the filled-in *Sal*I and *Ava*I sites recreated a *Sal*I site, pMC203 had a unique *Sal*I site just upstream of the *PET494* initiation codon. pMC203 was cleaved with *Sal*I and *Eco*RI, the 1.95-kb fragment carrying *PET494* and 375 bp of pBR322 DNA was isolated, the ends of the fragment were made blunt with the Klenow fragment of DNA polymerase I, and *Hin*dIII linkers (CAAGCTTG; New England BioLabs, Inc., Beverly, Mass.) were ligated to it. After *Hin*dIII cleavage and gel purification, the fragment was ligated to *Hin*dIII-cut pAAH5 (1) such that a *PET494* transcript with a 5'-untranslated leader sequence of minimal length would be synthesized under control of the *ADC1* promoter.

Immunological methods. PET494-related antigens were prepared as follows. To partially purify the OmpF–PET494–β-galactosidase protein synthesized from pORF11, *E. coli* TK1046 carrying pORF11 was grown to the early logarithmic phase at room temperature and then shifted to 37°C for 2 h. Cells were harvested by centrifugation and suspended in electrophoresis sample buffer, boiled for 5 min, and loaded onto 6% sodium dodecyl sulfate (SDS)-polyacrylamide gels (33). The fusion protein was cut from the gel and electroeluted from the gel slices. To prepare the peptide antigen, a synthetic peptide corresponding to the predicted carboxy-terminal 11 residues of PET494 (Lys-Arg-Ile-Ser-Asp-Ile-Gln-Ala-Asp-Ser-Ser) was purchased from the Children's Hospital Corporation Peptide Synthesis Facility (Boston, Mass.). A 10-mg portion of the peptide was mixed with 21 mg of keyhole limpet hemocyanin in 0.5 M Sodium P_i (pH 7)–21 mM glutaraldehyde to couple the peptide and carrier protein (44). The mixture was incubated at room temperature for 24 h and dialyzed.

Rabbits were injected intradermally either with approximately 200 μg of the OmpF–PET494–β-galactosidase fusion protein mixed with an equal volume of Freund complete adjuvant or with 0.5 ml of the peptide-hemocyanin solution (about 20% of the total) mixed with an equal volume of Freund complete adjuvant. Rabbits injected with the OmpF–PET494–β-galactosidase fusion protein were boosted 4 weeks later with 200 μg of antigen mixed with an equal volume of Freund incomplete adjuvant and were terminally bled 5 weeks after the boost. Rabbits injected with the peptide antigen were boosted 5 weeks later with 0.3 ml of the peptide-hemocyanin solution mixed with 0.3 ml of Freund incomplete adjuvant and were terminally bled 2 weeks after the boost. Sera were prepared as described by Daum et al. (11).

The monoclonal antibodies CC06, directed against subunit II of yeast cytochrome *c* oxidase, and IE7IgG3, directed against *E. coli* β-galactosidase, were the gifts of T. Mason.

Western blots were prepared from SDS-polyacrylamide gels (33) and reacted with antisera as described by Towbin et al. (53). Polyclonal antisera were typically used at 1:1,000 dilutions. ^{125}I-labeled protein A (New England Nuclear Corp., Boston, Mass.) was used to visualize anti-PET494 immune reactions; ^{125}I-labeled sheep anti-mouse immunoglobulin G (New England Nuclear Corp.) was used to visualize immune reactions of mouse monoclonal antibodies.

Selection and genetic characterization of mitochondrial *pet494* revertants. Yeast strain MCC3 (*MATa pet494-41 ura3-52* [*rho*+]) (10) was mutagenized by growth in the presence of 2 mM $MnCl_2$ or 50 mM ethidium bromide. Respiring revertants were selected on glycerol-ethanol medium (YPEG) and restreaked on the same medium to check for mitotic instability of the Pet^+ phenotype. Nonrespiring mitotic segregants of MCC3 revertants were mated to DA1 [*rho*^{0}] and MCC8 (*MATα pet494-41 ade2* [*rho*$^+$]) (10), and respiration of the diploids was tested. Segregants carrying a [*rho*$^+$] genome made respiring diploids with DA1 ([*rho*^{0}]) but not with MCC8 ([*rho*$^+$]); segregants carrying a suppressor [*rho*$^-$] genome made respiring diploids with MCC8 ([*rho*$^+$]) but not with DA1 ([*rho*^{0}]). [*rho*^{0}] derivatives were made by prolonged growth in 25 μg ethidium bromide per ml and checked by mating to DA1 ([*rho*^{0}]) and testing for respiration of the diploids. [*rho*^{0}] derivatives of MCC3 revertants were crossed to MCC8 ([*rho*$^+$]), and the resulting diploids were streaked on YPEG to verify that the ability to respire had been lost with the MCC3-revertant mitochondrial genome.

S1 nuclease mapping. Radioactive probes for S1 nuclease mapping of novel *oxi2* transcripts in MCC3 revertants were prepared as follows. Replicative-form DNA of bacteriophage M13 clones carrying the revertant fragments was digested with *Sau*3A. The mitochondrial DNA inserts were isolated and radioactively end labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase (37). The 750-bp *MSU494-2* fragment was then denatured and subjected to electrophoresis to separate the DNA strands as described by Maxam and Gilbert (37). The nucleotide sequences of the strands were determined by the chemical degradation method (37) to identify the strand complementary to *MSU494-2* mRNA. The 2.5-kb *MSU494-6 Sau*3A fragment was digested with *Eco*RI, and a 1.8-kb subfragment whose labeled strand was complementary to *MSU494-6* mRNA was isolated. A uniquely end-labeled 1.7-kb *Sau*3A-*Hin*fI fragment was isolated from the *MSU494-5* insert.

Total RNA was isolated by the method of Sprague et al. (50) from MCC3 mitochondrial revertants grown in YPEG. RNA (100 μg) from the revertant strains or from the wild-type strain DA1 (42) was hybridized with the corresponding probe at 40°C, and the hybrids were treated with S1 nuclease as described previously (48). The digestion products were analyzed on 7 M urea–5% polyacrylamide gels.

RESULTS

The amino terminus of PET494 directs β-galactosidase to mitochondria. It has recently been shown that the signals directing several nucleus-encoded proteins to mitochondria are located near their amino termini and, furthermore, that these signals can direct the mitochondrial import of nonmitochondrial proteins such as β-galactosidase or dihydrofolate reductase (14, 24, 30). To investigate whether PET494 might be a mitochondrial protein, we tested the ability of the amino terminus of PET494 to direct β-galactosidase into mitochondria. A DNA fragment carrying the *E. coli lacZ* gene was ligated into the plasmid pBA10, a YEp13 (6) derivative carrying a small in vitro-made deletion of the *PET494* gene (10), such that approximately 2.6 kb of 5'-flanking DNA and the first 119 codons of *PET494* were joined in frame to *lacZ*. Yeast colonies carrying this plasmid, termed pBA10Z, were blue when grown on plates containing the indicator 5-bromo-4-chloro-3-indolyl-β-galactoside,

TABLE 1. Distribution of β-galactosidase activity in subcellular fractions[a]

Plasmid	Sp act in whole-cell extract (U/mg)	Total activity		% of total activity pelleting with mt
		Post-mt supernatant	Mt pellet	
pBA10Z	26	280	643	70
pLGSD5	1,660	63,000	1,278	2

[a] Yeast cells carrying pBA10Z (the *pet494*::*lacZ* fusion) or pLGSD5 (the unmodified *lacZ* gene under control of a hybrid *GAL10-CYC1* promoter [23]) were grown and fractionated as described in Materials and Methods. mt, Mitochondria.

showing that β-galactosidase was expressed from the plasmid. Cells carrying pBA10Z were fractionated into a mitochondrial pellet and post mitochondrial supernatant, and β-galactosidase activity was assayed in each fraction. Of the total β-galactosidase activity in these cells, 70% pelleted with mitochondria (Table 1). As a control, cells carrying the plasmid pLGSD5 (23), from which unmodified β-galactosidase was expressed, were fractionated in the same way. In this case, 98% of the total β-galactosidase activity was found in the postmitochondrial supernatant, showing that β-galactosidase has no intrinsic affinity for mitochondria. The fact that 30% of the β-galactosidase activity in cells carrying pBA10Z did not pellet with mitochondria probably reflects proteolysis of the PET494–β-galactosidase fusion protein, yielding fragments with β-galactosidase activity but without the PET494 mitochondrial import signal. Degradation products smaller than the fusion protein were in fact seen on Western blots of yeast cells carrying pBA10Z probed with a monoclonal antibody directed against β-galactosidase (data not shown).

To determine whether the PET494–β-galactosidase fusion protein was specifically associated with mitochondria, rather than simply copelleting with the organelles, the mitochondrial pellet from cells carrying pBA10Z was fractionated on sucrose equilibrium density gradients. The location of the mitochondrial peak was verified by subjecting samples from each gradient fraction to SDS-polyacrylamide gel electrophoresis, blotting the gels to nitrocellulose, and probing the blots with antiserum against a mitochondrial marker, subunit II of cytochrome *c* oxidase. β-Galactosidase activity comigrated with mitochondria in the gradients (data not shown), showing that the PET494–β-galactosidase fusion protein was specifically directed to mitochondria.

PET494 is a mitochondrial protein. The fact that the amino-terminal 119 amino acids of PET494 specified the mitochondrial import of the PET494–β-galactosidase fusion protein implied that PET494 is a mitochondrial protein. To confirm this we sought to determine directly the subcellular location of unmodified PET494. As a means of identifying the PET494 protein, antisera were generated against two PET494-related antigens.

The plasmid pORF1 (55), which carries the promoter and 5′ end of the *E. coli ompF* gene joined to the *E. coli lacZ* gene, was employed to prepare one antigen. A 777-bp fragment carrying more than half of the *PET494* gene was inserted into pORF1 such that a trihybrid OmpF–PET494–β-galactosidase protein was produced from the resulting plasmid, pORF11, under inducing conditions (see Materials and Methods). The fusion protein was partially purified from extracts of *E. coli* cells carrying pORF11 by electroelution from SDS-polyacrylamide gels and was used to immunize rabbits. Another PET494-related antigen was generated by preparing a synthetic peptide corresponding to the carboxy-terminal 11 residues of PET494. The peptide was coupled to keyhole limpet hemocyanin as a carrier and was also used to immunize rabbits (see Materials and Methods).

The resulting antisera specifically recognized their rspective PET494-related antigens, as determined by probing nitrocellulose filters upon which the antigens had been immobilized (data not shown). To test whether the antisera would react with the PET494 protein from yeast cells, we used them to probe Western blots of electrophoretically separated mitochondrial proteins from a wild-type strain, a *pet494* deletion mutant, and a strain carrying the *PET494* gene on a multicopy plasmid. Although the antisera reacted with a protein within the expected size range in the *PET494*-carrying strains, we could not be confident that this protein was in fact the *PET494* gene product because of the high background of both preimmune and immune cross-reaction.

To increase the level of the PET494 protein to detect it more reliably, we placed the gene under the control of the strong *ADC1* promoter in the plasmid pAAH5 (1). First, a restriction site was placed 27 bp upstream of the ATG initiation codon of *PET494* (see Materials and Methods). This allowed the placement of the gene immediately downstream of the *ADC1* promoter in pAAH5, so that the resulting *PET494* transcript would have a short 5′-untranslated leader devoid of extra ATG codons. This PET494-expression plasmid, pMC210, conferred the ability to respire upon the *pet494* deletion mutant strain TF123 while the vector, pAAH5, did not, showing that PET494 was indeed expressed from pMC210.

Mitochondria were prepared from TF123 carrying either pAAH5 or pMC210 and subjected to electrophoresis on SDS-polyacrylamide gels. The gels were blotted to nitrocellulose, and the blots were reacted with various preimmune and immune sera (Fig. 1). Both immune sera reacted with a protein of about 44 kilodaltons (kDa) in cells carrying pMC210 which was absent in cells carrying the vector pAAH5. The preimmune sera did not react with this protein (Fig. 1). Since this band represents an immune reaction specific to the presence of the *PET494* gene, we conclude that it is the PET494 protein. Because of the increased level of PET494 in cells carrying pMC210, the background of cross-reaction which nearly overwhelmed the PET494 signal in cells carrying a single wild-type copy of the gene was reduced or eliminated in the Western blots of Fig. 1.

Next, the antiserum used in the experiment of Fig. 1D was used to probe subcellular fractions of TF123 cells carrying pMC210 to establish the intracellular location of native PET494. The PET494 protein was found to be enriched in mitochondria and not detectable in equivalent amounts of protein from the postmitochondrial supernatant (Fig. 2A). To show that PET494 was specifically associated with mitochondria, we fractionated the crude mitochondrial pellet on a sucrose equilibrium density gradient. A Western blot of proteins from each gradient fraction was probed with both anti-PET494 antiserum and a monoclonal antibody against the mitochondrial protein subunit II of cytochrome *c* oxidase (Fig. 2B). PET494 comigrated in the gradient with the mitochondrial marker, establishing that it is a mitochondrial protein.

Mitochondrial second-site revertants that bypass the requirement for PET494. Since the PET494 protein is imported into mitochondria, it is likely to interact with other mitochondrial components involved in the expression or accumulation of coxIII. In an attempt to identify these components genetically, we selected second-site suppressor

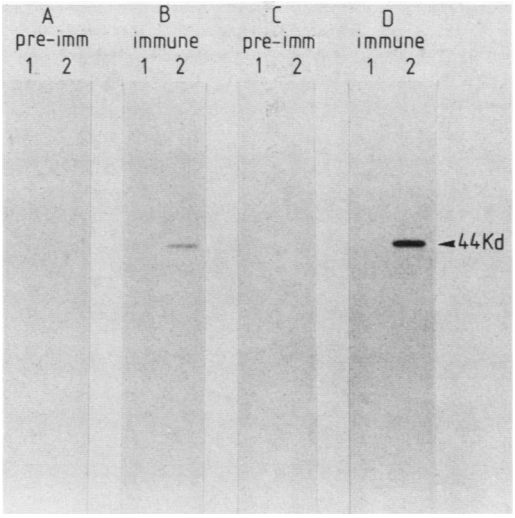

FIG. 1. Specificity of antisera directed against PET494-related antigens. Crude mitochondria were isolated from TF123 (a *pet494* deletion strain) carrying either the unmodified vector pAAH5 (1) or the PET494 expression plasmid pMC210. Mitochondrial proteins (100 μg) from TF123 carrying pAAH5 (lane 1, each panel) or pMC210 (lane 2, each panel) were subjected to electrophoresis on a 10% polyacrylamide-SDS gel, and the gel was blotted to nitrocellulose. Each panel was reacted with a different serum as follows: (A) preimmune serum; (B) immune serum from the same rabbit after immunization with the synthetic peptide described in the text, coupled to keyhole limpet hemocyanin (immune serum was partially purified by the addition of keyhole limpet hemocyanin and centrifugation to remove the resulting precipitate); (C) preimmune serum; (D) immune serum from the same rabbit after immunization with the Ompf–PET494–β-galactosidase fusion protein. Immune reactions were decorated with ^{125}I-labeled protein A and visualized by autoradiography. Each immune serum reacted specifically with a protein of about 44 kDa (plus or minus 4 kDa) in cells carrying pMC210 but not in cells carrying pAAH5.

mutations which made coxIII expression independent of *PET494* and then examined the mutational alterations in detail. Such suppressor mutations can be easily selected, as they allow a *pet494* mutant strain to respire. One such suppressor, *MSU494-1*, was isolated previously from a *pet494-1* (*amber*) mutant strain (42). In this study, to avoid selecting for reversion at the *pet494* gene or *amber* suppressors, respiring revertants were selected from the *pet494* deletion strain MCC3. MCC3 carried the *pet494-41* allele, constructed in vitro, which lacks the promoter and amino-terminal two-thirds of the *PET494* structural gene (10). We isolated 50 respiring revertants of MCC3 which arose either spontaneously or after mutagenesis with $MnCl_2$ or ethidium bromide (see Materials and Methods). To test whether the reversion had occurred in the nuclear or mitochondrial genomes, [*rho*^{0}] derivatives of the revertant strains (lacking mitochondrial DNA) were crossed to the strain MCC8, carrying the *pet494-41* deletion allele and wild-type ([*rho*$^+$]) mitochondrial DNA. None of the resulting diploids respired, suggesting that in all 50 strains the reversion had taken place in the mitochondrial genome.

Like *MSU494-1*, the *pet494-1* revertant isolated previously, all the revertant strains obtained were mitotically unstable, producing nonrespiring mitotic segregants at high frequency. As determined by crosses to tester strains (see Materials and Methods), the nonrespiring mitotic segregants carried either a wild-type ([*rho*$^+$]) mitochondrial genome or a deleted ([*rho*$^-$]) genome capable of suppressing the *pet494-41* deletion mutation when crossed back to a *pet494-41* [*rho*$^+$] strain. (The mitochondrial genome of yeasts can undergo intramolecular recombination to form deletion derivatives termed [*rho*$^-$] genomes [15].) Thus, the revertant strains behaved genetically as if heteroplasmic, carrying both a [*rho*$^+$] and a suppressor [*rho*$^-$] genome.

To locate the sites of the alterations in the suppressor [*rho*$^-$] genomes, we performed Southern blot analysis on total DNA from the revertant strains using either a probe carrying the 5′-end and 5′-flanking sequences of the *oxi2*

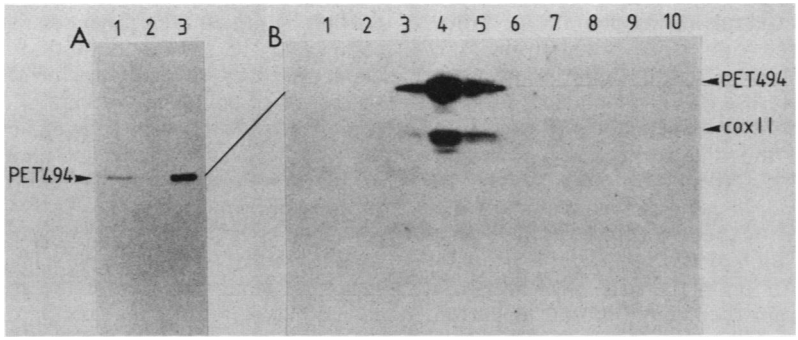

FIG. 2. The PET494 protein is specifically associated with mitochondria. (A) Cells of strain TF123 carrying the PET494 expression plasmid pMC210 were fractionated into a crude mitochondrial pellet and a postmitochondrial supernatant by the procedure of Daum et al. (11), except that spheroplasts were lysed by stirring with 0.1-mm glass beads. Protein concentration in each fraction was measured by the method of Bradford (5). Twenty-microgram samples of the crude lysate, the mitochondrial pellet, and the postmitochondrial supernatant were subjected to electrophoresis on a 10% polyacrylamide-SDS gel, and a Western blot of the gel was reacted with the antiserum directed against the OmpF–PET494–β-galactosidase fusion protein. Immune reactions were visualized with ^{125}I-labeled protein A and autoradiography. Lanes: 1, crude lysate; 2, postmitochondrial supernatant; 3, mitochondrial pellet. (B) The crude mitochondria (2 mg) were applied to a 10-ml sucrose gradient (20 to 70% sucrose in 50 mM Tris [pH 7.5]–0.5 mM phenylmethylsulfonyl fluoride) and centrifuged for 15 h at 20,000 rpm at 4°C in a Beckman SW41 rotor. Ten 1-ml fractions were collected, and 1/10th of each fraction was subjected to electrophoresis on a 15% polyacrylamide-SDS gel. The gel was blotted to nitrocellulose and reacted with anti-PET494 antiserum and ^{125}I-labeled protein A as described above. After autoradiography to identify PET494, the blot was reacted with a mouse monoclonal antibody directed against subunit II of cytochrome *c* oxidase (coxII), and this immune reaction was visualized with ^{125}I-labeled sheep anti-mouse immunoglobulin G and autoradiography. Lanes 1 through 10, Fractions 1 through 10; fraction 1 was the bottom of the gradient and fraction 10, the top. The positions of PET494 and coxII are indicated.

gene or a probe carrying the 3'-end and 3'-flanking sequences (Fig. 3). For each revertant (including the remaining 46 strains not shown in Fig. 3), the 5'-end probe hybridized to two bands: a 2.9-kb fragment comigrating with the wild-type fragment found in [*rho*$^+$] DNA; and another fragment, of different mobility from wild type and differing between revertant strains. In contrast, the 3'-end probe hybridized only to the wild-type 1.17-kb fragment in the seven strains examined in this manner. Therefore the suppressor [*rho*$^-$] genomes of the mitochondrial revertant strains all carried copies of the *oxi2* gene which were altered at or near their 5' ends but, for at least seven strains, not at their 3' ends.

In some revertants the 5' leader sequence of another mitochondrial gene is fused to the *oxi2* gene. In the mitochondrial *pet494* suppressor studied previously, *MSU494-1*, the *oxi2* structural gene was fused in frame to another mitochondrial gene, resulting in the synthesis of a protein immunologically related to coxIII but with lower electrophoretic mobility (42). For reasons discussed in detail below (see Discussion), we were especially interested to learn whether any of the revertants were able to produce wild-type coxIII in a *pet494* nuclear background, owing to alterations outside of the *oxi2* structural gene.

First, the electrophoretic mobility of coxIII produced by the revertants was examined to determine whether any of the strains synthesized a protein comigrating with wild-type coxIII. Mitochondrial translation products were visualized by radioactive labeling of proteins synthesized in the presence of the drug cycloheximide, which inhibits cytoplasmic but not mitochondrial translation, followed by SDS-polyacrylamide gel electrophoresis (data not shown). Like strains carrying *MSU494-1*, many of the revertants synthesized new proteins with lower electrophoretic mobility than wild-type coxIII, probably owing to in-frame fusions of other reading frames to the *oxi2* gene. However, 15 strains synthesized an apparently wild-type coxIII protein, suggesting that the alterations in their suppressor [*rho*$^-$] genomes might have taken place outside of the *oxi2* coding region.

The 5'-end fragments of the altered *oxi2* genes from five of the revertant strains that produced apparently wild-type coxIII were cloned, and their nucleotide sequences were determined to examine the nature of the alterations. Mitochondrial DNA was prepared from the revertant strains, the DNA was digested with *Mbo*I, and the appropriate fragments were excised from agarose gels and ligated to *Bam*HI-cut M13mp11. The nucleotide sequences of the altered fragments, determined by the dideoxy chain termination method, are presented in Fig. 4. In all the strains, DNA from elsewhere in the mitochondrial genome was fused to the 5'-flanking sequences of the *oxi2* gene upstream of the 5' end of the structural gene. In four of the strains, the point of recombination was within 25 bp of the *oxi2* ATG initiation codon, and in the fifth strain recombination had occurred much further upstream, at −450 relative to the ATG codon. Since all the strains carried an unmodified *oxi2* structural gene, it is likely that they accumulated a wild-type coxIII protein in the absence of PET494. Thus, it is unlikely that the function of PET494 is to stabilize the coxIII protein.

Since the PET494 protein acts at a posttranscriptional step in coxIII synthesis (42), it appeared that these mitochondrial gene rearrangements conferred *PET494* independence by causing an alteration in the structure of the *oxi2* mRNA, making it translatable in the absence of the PET494 protein. The 5' end of the wild-type *oxi2* transcript is at –600 relative to the initiation codon (42). None of the suppressor [*rho*$^-$] genomes could possibly give rise to a wild-type transcript,

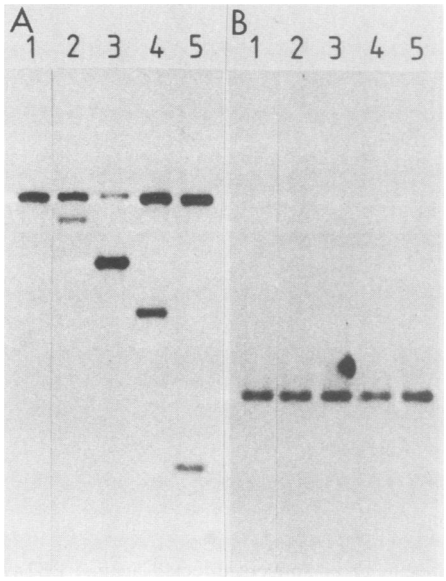

FIG. 3. Mutant *oxi2* genes that suppress a *pet494* deletion are altered at their 5' ends but not at their 3' ends. Mitochondrial DNA was purified from respiring MCC3 revertants grown in glycerol-ethanol medium (YPEG) as previously described (21). The DNA was digested with *Mbo*I and subjected to electrophoresis on two identical 1.5% agarose gels as follows: lanes 1, wild-type mitochondrial DNA (the D273-10B mitochondrial genome from strain DA1); lanes 2, MCC3 revertant strain carrying the mitochondrial *pet494* suppressor *MSU494-6*; lanes 3, MCC3 revertant carrying *MSU494-5*; lanes 4, MCC3 revertant carrying *MSU494-4*; lanes 5, MCC3 revertant carrying *MSU494-2*. The gels were blotted to nitrocellulose, and the filters were hybridized with nick-translated plasmid DNA. One filter was probed with pZHS1 (42) carrying the 5'-end and 5'-flanking sequences of *oxi2* (A), and the other was probed with pZHS3 (42) carrying the 3'-end and 3'-flanking sequences of *oxi2* (B). The 5'-end probe hybridized with a fragment of altered mobility in each revertant strain.

since all were lacking part of this region. To elucidate the structures of the novel *oxi2* mRNAs in the revertant strains, we first identified the sequences joined to the *oxi2* genes by comparing them with published mitochondrial gene sequences, either manually or, for *MSU494-6*, by a computer search of a collection of mitochondrial DNA sequences compiled by L. Grivell. In every case, the region encoding the 5'-untranslated leader of another mitochondrial transcript had been joined to the *oxi2* gene (Fig. 5). The suppressor [*rho*$^-$] genome *MSU494-2*, for example, carried a perfect fusion of the 5'-flanking region of the *aap1* gene, which encodes subunit 8 of ATPase (35, 49), to the *oxi2* structural gene. In three of the strains, recombination occurred between the leader region of the cytochrome *b* gene (*cob*) and the *oxi2* gene, resulting in fusions in which nearly all of the sequence encoding the 940-nucleotide *cob* leader (4) was joined either to a short segment of the *oxi2* leader (*MSU494-3* and *MSU494-4*) or to a longer 450-bp segment (*MSU494-5*). The fifth suppressor examined, *MSU494-6*, carried most of the 5' leader-encoding region of the *oli1* gene, which encodes ATPase subunit 9 (18, 54), fused to a short portion of the *oxi2* leader. The recombination events which created all these suppressor *oxi2* genes had occurred at short regions of homology between the two partners (85 to 100% identity over 10 to 13 bp).

Revertants contain novel *oxi2* mRNAs with 5' leader sequences of other mitochondrial transcripts. The simplest

Wild-type	ATTTATTTATTTAATTATATTATATATATAATATATATATAACAATAAATTT	ATG ACA
MSU494-2	ATTAAATTATTATAATGTTGTTATTAATCTTATTAAAAAATATATATAAAA	ATG ACA
MSU494-3	TTAGTAATAAATAATATTATTAATATTTTATAAATAAATAAT AATAAATTT	ATG ACA
MSU494-6	GAATAATAAAAAGATAATATTAATATATTAAT TATATATAACAATAAATTT	ATG ACA
MSU494-4	TAATATATATAAAATATTAGTAATAA ATAATATATATATAACAATAAATTT	ATG ACA
MSU494-5	ATATTAGTA ATAAATAATAAAAAA/435 bp wild-type sequence/	ATG ACA

FIG. 4. Nucleotide sequences of *oxi2* alterations that suppress a *pet494* deletion. *MSU494-6* arose spontaneously; *MSU494-1*, *MSU494-3*, and *MSU494-4* were induced by $MnCl_2$ mutagenesis; and *MSU494-5* was induced by ethidium bromide mutagenesis (see Materials and Methods). *Mbo*I fragments from the 5′ ends of mutant *oxi2* genes were cloned in bacteriophage M13. An oligonucleotide primer was synthesized which corresponded to nucleotides +49 through +68 of the coding strand of the *oxi2* structural gene, to facilitate sequence determination upstream of the gene (the *Mbo*I site within the *oxi2* gene from which sequencing began with an M13 primer is relatively distant for convenient sequence analysis, at +231). For one suppressor gene, *MSU494-5*, deletions of the cloned fragment were made with exonuclease III and S1 nuclease as described by Henikoff (26). The sequence of the wild-type *oxi2* gene from nucleotides −52 and +6 (51) is shown on the top line; below are shown sequences of the 5′ ends of altered *oxi2* genes from four MCC3 revertant strains, aligned at the *oxi2* ATG initiation codon. New DNA fused to the *oxi2* gene is underlined.

prediction for the structures of the *oxi2* mRNAs arising from these altered genes would be that their 5′ ends correspond to the normal 5′ ends of the transcripts whose leader-encoding sequences were fused to the *oxi2* gene, as diagrammed in Fig. 5. S1 nuclease protection experiments were performed to test this prediction. To prepare DNA probes, we purified replicative-form DNA from the bacteriophage M13 clones carrying the 5′ ends and flanking sequences of the altered *oxi2* genes of *MSU494-2*, *MSU494-5*, and *MSU494-6*. The bacteriophage DNAs were digested with *Sau*3A, the cloned inserts were isolated by preparative agarose gel electrophoresis, and their 5′ ends were radioactively labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Uniquely end-labeled probes were prepared by digestion with a second restriction endonuclease and isolation of the appropriate fragment or, for the *MSU494-2* fragment, by separating the DNA strands (see Materials and Methods). Each end-labeled radioactive probe was hybridized with total RNA from both the corresponding revertant strain and a wild-type strain; the hybrids were treated with S1 nuclease and then subjected to electrophoresis on denaturing acrylamide gels (Fig. 6). *MSU494-2* DNA fragments estimated to be 525 and 220 nucleotides in size were protected from S1 digestion by total RNA from the respiring revertant strain carrying *MSU494-2*. RNA from a wild-type strain protected only the smaller fragment. The size of the smaller fragment protected by both wild-type and revertant RNA corresponds well to the 231-bp distance between the radioactively labeled end of the probe and the point of fusion of *oxi2* and *aap1* sequences, while the size of the larger fragment protected only by revertant RNA is that predicted to be protected by a novel transcript with the same 5′ end as the normal *aap1* transcript. Since mitochondria of the revertant strain carried both [*rho*$^+$] and suppressor [*rho*$^-$] genomes, the revertant RNA protected the same 220-nucleotide fragment protected by wild-type RNA. Thus, wild-type *oxi2* mRNA as well as a novel transcript were synthesized in the revertant strain although only the novel transcript was translatable in the *pet494-41* mutant background.

Similar results were obtained for *MSU494-6* (Fig. 6) and *MSU494-5* (data not shown). In both cases, RNA from the revertant strain protected DNA fragments corresponding to the predicted novel transcript and to the point of divergence between wild-type *oxi2* mRNA and the suppressor *oxi2* gene probe. Therefore, at least for the three cases investigated, replacement of the normal *oxi2* leader with all or part of the untranslated leader region of another mitochondrial transcript made coxIII translation independent of the PET494 protein.

DISCUSSION

The product of yeast nuclear gene *PET494* was previously shown to be required for a posttranscriptional step in the expression or accumulation of the mitochondrial gene product coxIII (7, 16, 17, 42). Experiments reported in this paper show that *PET494* encodes a protein which acts in mitochondria to promote translation of the *oxi2* mRNA. Genetic evidence is presented which suggests that the site of action of the PET494 protein is in the 5′ leader region of the *oxi2* mRNA.

PET494 was shown to be a mitochondrial protein by probing subcellular fractions of yeast cells with PET494-specific antisera. The PET494 protein was specifically associated with mitochondria, fractionating with the organelles during purification by differential centrifugation and sucrose equilibrium density gradient centrifugation. Because the PET494 protein was undetectable on Western blots of proteins from strains carrying a single copy of the *PET494* gene under control of its own promoter, these experiments were performed with subcellular fractions of cells carrying a PET494 expression plasmid, constructed by placing the gene under control of the strong *ADC1* promoter in pAAH5 (1). Apparently PET494 is expressed at very low levels in wild-type cells.

These experiments showed that, like most other nucleus-encoded mitochondrial proteins (25), PET494 is proteolytically processed as it enters mitochondria. The mature PET494 protein, detected on Western blots, has an apparent molecular weight of approximately 44,000, although the

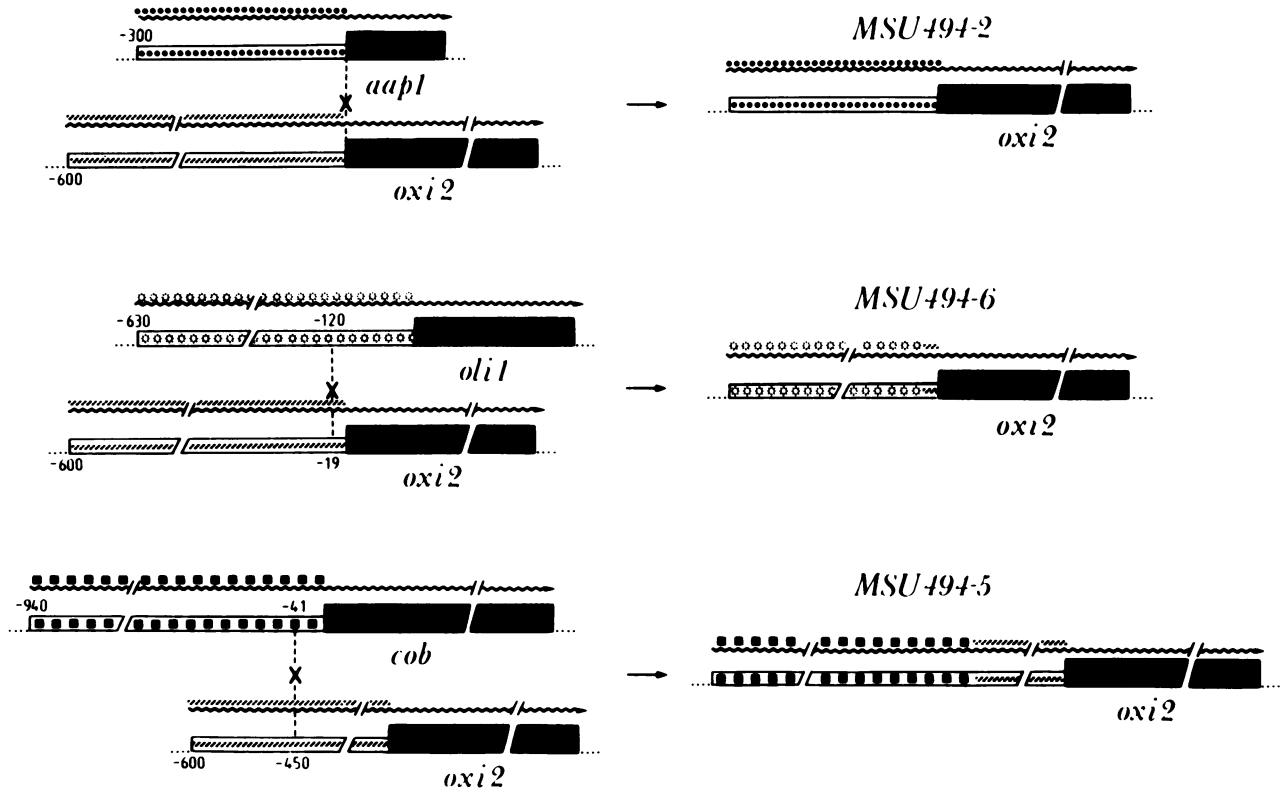

FIG. 5. Rearrangements of the *oxi2* gene that permit coxIII translation in a *pet494* mutant. New sequences fused to *oxi2* 5′-flanking sequences were identified either by manual comparison to published sequences or, for *MSU494-6*, by a computer comparison (performed at the Department of Botany, Edinburgh University, Scotland) to a collection of *S. cerevisiae* mitochondrial DNA sequences compiled by L. Grivell. The recombination events which gave rise to three of the suppressor *oxi2* genes are diagrammed. Large black boxes represent structural genes; patterned boxes represent 5′-untranslated leader-encoding regions; wavy lines represent mRNAs; and patterned wavy lines represent 5′-untranslated mRNA leaders. Dotted lines between pairs of genes show points of recombination, and the suppressor *oxi2* genes resulting from the recombination events are shown to the right. Where recombination occurred at regions of perfect homology between the two partners, points of recombination are arbitrarily designated as the 5′ boundaries of the regions. Top, Recombination occurred between the ATG initiation codons of *aap1* and *oxi2* to create *MSU494-2*, a precise fusion of the *aap1* 5′ leader-encoding region to the *oxi2* structural gene. Middle, Recombination occurred between the leader-encoding regions of *oli1* and *oxi2*, at −120 relative to the *oli1* ATG initiation codon and −19 relative to the *oxi2* initiation codon, to create *MSU494-6*. (A difference from previous *oli1* sequences was seen 6 nucleotides upstream of the point of fusion between *oli1* and *oxi2* sequences, where an A residue rather than a G was found.) Bottom, Recombination occurred between the *cob* gene and *oxi2*, at −41 relative to the *cob* initiation codon and −450 relative to the *oxi2* initiation codon, to form *MSU494-5*. The synthesis of novel transcripts from the suppressor genes as shown was verified by S1 nuclease mapping. Not shown: The suppressor genes *MSU494-3* and *MSU494-5* resulted from recombination between *cob* and *oxi2*, at −6 relative to *cob* and −9 relative to *oxi2* in *MSU494-3* and at −38 relative to *cob* and −25 relative to *oxi2* in *MSU494-4*.

predicted molecular weight of the protein encoded by the *PET494* gene is 56,000. The 56-kDa precursor is synthesized in a rabbit reticulocyte lysate translation system programmed with in vitro-made *PET494* mRNA (unpublished data), confirming the molecular weight prediction from the gene sequences. The disparity between the sizes of the precursor and mature protein indicates that approximately 80 to 100 amino acids are cleaved from the PET494 precursor upon import into mitochondria. These residues must be removed from the amino terminus, since antisera directed against a synthetic peptide corresponding to the predicted carboxy-terminal 11 amino acids of PET494 reacted with the mature protein. Although the signal sequence directing PET494 to mitochondria has not yet been precisely defined, the demonstration that a PET494–β-galactosidase fusion protein carrying the amino-terminal 119 amino acids of PET494 was localized to mitochondria shows that the signal lies within the first 119 residues.

Once in the mitochondrion, the PET494 protein promotes the expression of the mitochondrial *oxi2* gene. Two main classes of model for its mechanism of action were consistent with previous work: (i) that PET494 is required for translation of the *oxi2* mRNA; or (ii) that it is required to stabilize the coxIII protein. To distinguish between these models, we isolated mitochondrial mutations making coxIII expression independent of PET494. All these *pet494* suppressor mutations resulted in changes around the 5′ end of the *oxi2* gene. In five suppressor *oxi2* genes which were examined in detail by nucleotide sequencing, the *oxi2* coding sequence was intact but the region encoding the mRNA 5′-untranslated leader had been altered. This indicates that PET494 is not required to stabilize the coxIII protein during its assembly into the cytochrome *c* oxidase complex, since these revertant strains accumulated wild-type coxIII in the absence of the PET494 protein. Rather, it suggests that PET494 promotes coxIII expression by specifically activating translation of the *oxi2* mRNA.

The fact that the *oxi2* 5′-untranslated leader-encoding

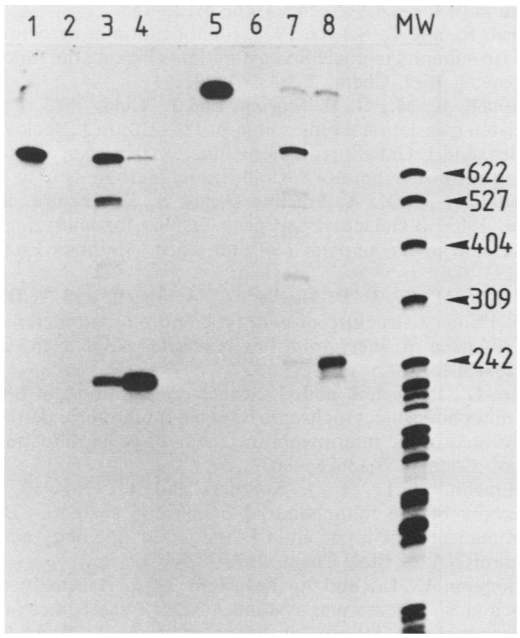

FIG. 6. S1 nuclease mapping of novel *oxi2* transcripts arising from suppressor genes *MSU494-2* and *MSU494-6*. Uniquely end-labeled radioactive DNA probes complementary to *MSU494-2* or *MSU494-6* mRNAs were prepared (see Materials and Methods). The probes were hybridized to *E. coli* tRNA, to total RNA from the MCC3 revertant strain carrying the corresponding suppressor *oxi2* gene, or to total RNA from a wild-type strain, and the hybrids were treated with S1 nuclease. DNA fragments protected from S1 digestion were subjected to electrophoresis on a 5% polyacrylamide–7 M urea gel along with end-labeled *Msp*I fragments of pBR322 as molecular weight standards (MW). The 750-nucleotide *MSU494-2* probe was loaded onto the gel without any treatment (lane 1) or was treated with S1 nuclease after hybridization to various RNAs as follows: lane 2, *E. coli* tRNA; lane 3, total RNA from the MCC3 revertant strain carrying *MSU494-2*; lane 4, total RNA from wild-type strain DA1. *MSU494-2* revertant RNA protected DNA fragments of 525 and 220 nucleotides from S1 digestion, while wild-type RNA protected only the 220-nucleotide fragment (the origin of these protected fragments is described in the text). The 1.8-kb *MSU494-6* probe was subjected to electrophoresis without any treatment (lane 5) or was treated with S1 nuclease after hybridization to RNAs as follows: lane 6, *E. coli* tRNA; lane 7, total RNA from the MCC3 revertant strain carrying *MSU494-6*; lane 8, total RNA from wild-type strain DA1. *MSU494-6* revertant RNA protected DNA fragments of 705 and 238 nucleotides from S1 digestion; wild-type RNA protected only the 238-nucleotide fragment. The 238-nucleotide fragment corresponds to the point of divergence between wild-type *oxi2* mRNA and the *MSU494-6* probe, while the 705-nucleotide fragment was protected by a transcript of the *MSU494-6* gene whose 5′ end corresponds to the normal 5′ end of the *oli1* mRNA.

region was altered in all five of the suppressor genes examined closely may indicate that in wild-type cells, the site of PET494 action is in the leader region of the *oxi2* transcript. Furthermore, the leader-encoding regions of the suppressor *oxi2* genes were all fused to the 5′-flanking sequences of different mitochondrial genes. In three cases analyzed by S1 nuclease mapping, it was shown that the novel transcripts arising from the suppressor genes carried most or all of the normal 5′ leader of another mitochondrial transcript attached to the *oxi2* transcript. This leads to the speculation that perhaps translation of many mitochondrial mRNAs requires specific activation by nuclear-encoded proteins which act on their 5′ leaders. Thus, the mRNAs encoded by the suppressor *oxi2* genes may recruit the translational activator protein or proteins corresponding to the attached leader to allow coxIII translation in the absence of PET494.

Consistent with this hypothesis, candidates for specific translational activators of several mitochondrial mRNAs have been identified genetically. For example, the product of the *PET111* gene is apparently required for a posttranscriptional step in the expression of the mitochondrial gene encoding subunit II of cytochrome *c* oxidase (7, 16, 17; Poutre and Fox, in preparation). The products of two nuclear genes, *CBP6* (13) and *MK2* (45), are required at posttranscriptional steps in the expression of cytochrome *b*, encoded by the mitochondrial *cob* gene. Because an *mk2* mutation can be suppressed by a mitochondrial gene rearrangement similar to the *pet494* suppressors described above, *MK2* appears to be required for translation rather than for protein stability and to act on the 5′ leader of the *cob* transcript (45). Recent evidence suggests that PET494 may also be one of a set of proteins required for coxIII translation. A mutation in a nuclear gene unlinked to *PET494*, termed *PET54*, results in a mutant phenotype identical to that of *pet494* mutations and is suppressible by the mitochondrial mutations described above that suppress a *pet494* deletion (Costanzo et al., submitted). It is not yet known whether the *PET54* gene product acts in concert with the PET494 protein or whether it is required at a different point in the same pathway for activation of coxIII translation.

In considering the possibility that PET494 and other nucleus-encoded proteins mediate positive control by interacting with the leader regions of mitochondrial mRNAs, it is interesting to note that the 5′ leader has been shown to be the target of translational activation of several specific eucaryotic cellular and viral mRNAs. The tripartite leader of adenovirus late mRNAs is necessary for translation late in infection and can confer late translational activation upon a transcript normally translated only early in infection (34). Similarly, the 5′ leader of the *Drosophila melanogaster hsp70* heat shock mRNA is required for translation at high temperature, and attachment of the *hsp70* leader to a non-heat shock mRNA allows the resulting hybrid transcript to be translated at high temperature (32, 38). Certain deletions of the leader region of another *Drosophila* heat shock mRNA, *hsp22*, block its translation at high temperature, showing that translational activation of this mRNA also requires the 5′ leader (28). Another case of specific translational activation has recently been reported to occur in the human immunodeficiency virus (HIV): the product of the viral *tat-III* gene mediates translational activation of viral mRNA at a site in the 5′ leader (47).

The mechanism by which the PET494 protein specifically activates translation of the *oxi2* mRNA is as yet unknown. The molecular mechanism of translational control is best understood for translational repression in several procaryotic systems. In some RNA bacteriophages, in the autoregulation of *E. coli* ribosomal protein synthesis, and in regulation by the products of bacteriophage T4 genes *32* and *regA*, translational repression is mediated by binding of specific proteins to specific sites on mRNAs to block translation, ususally by obscuring the ribosome-binding site or initiation codon of the transcript or both (reviewed in reference 8). Perhaps the PET494 protein works in a similar way, binding to the *oxi2* mRNA leader to activate rather than repress translation. PET494 bound to the *oxi2* mRNA could facilitate ribosome binding by interacting with the ribosome, or by changing the secondary structure of the message into one more favorable for ribosome binding. Alternatively,

perhaps PET494 makes *oxi2* mRNA translatable by catalyzing a specific chemical modification of the transcript. Another possibility is that PET494 promotes coxIII translation by antagonizing a specific translational repressor.

It remains to be seen whether PET494-mediated translational activation is the mechanism by which mitochondrial levels of coxIII are modulated in response to environmental conditions. The increase in coxIII expression during release from glucose repression is known to occur at the translational level (19, 56). Consistent with a role for PET494 in this process, preliminary evidence indicates that PET494 expression may be elevated four- to five-fold in the absence of glucose compared with glucose-repressed levels (D. Marykwas and T. D. Fox, unpublished data). With the ability to manipulate PET494 expression by placing the gene under the control of various promoters and to measure PET494 levels by immunological detection of the protein, the question of whether PET494 modulates coxIII expression can now be addressed in detail.

Translational control of gene expression may in fact be a common regulatory mechanism for both cytoplasmic and organellar mRNAs. Examples of translational control in yeasts (27, 52) and in higher eucaryotes (3, 28, 31, 32, 34, 38; reviewed in reference 29) have recently been reported. Of particular interest is the observation that some chloroplast genes are under translational control. In both the unicellular eucaryote *Euglena gracilis* and the higher plant amaranth, levels of the large subunit of ribulose 1,5-bisphosphate carboxylase, a chloroplast gene product, increase rapidly in response to light although levels of its mRNA remain roughly constant (2, 40). Light regulation of the large subunit gene in *Spirodela* chloroplasts and also of the *psbA* gene encoding the 32-kDa photosystem II polypeptide occurs mainly at the translational level as well (22). Thus PET494 activation of coxIII translation, a process amenable to both genetic and biochemical dissection, may well be an example of a general phenomenon in eucaryotic gene control.

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