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Pet127p, a Membrane-Associated Protein Involved in Stability and Processing of *Saccharomyces cerevisiae* Mitochondrial RNAs

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Nuclear mutations that inactivate the *Saccharomyces cerevisiae* gene *PET127* dramatically increased the levels of mutant *COX3* and *COX2* mitochondrial mRNAs that were destabilized by mutations in their 5′ untranslated leaders. The stabilizing effect of *pet127*Δ mutations occurred both in the presence and in the absence of translation. In addition, *pet127*Δ mutations had pleiotropic effects on the stability and 5′ end processing of some wild-type mRNAs and the 15S rRNA but produced only a leaky nonrespiratory phenotype at 37°C. Overexpression of *PET127* completely blocked respiratory growth and caused cells to lose wild-type mitochondrial DNA, suggesting that too much Pet127p prevents mitochondrial gene expression. Epitope-tagged Pet127p was specifically located in mitochondria and associated with membranes. These findings suggest that Pet127p plays a role in RNA surveillance and/or RNA processing and that these functions may be membrane bound in yeast mitochondria.

The biogenesis of mitochondria is a complex process which requires the concerted action of nuclearly and mitochondrially encoded proteins. The *Saccharomyces cerevisiae* mitochondrial genome codes for seven protein subunits of membrane-bound energy-transducing complexes and includes several genes required for their expression: a mitochondrial ribosomal gene termed *VAR1* and genes for two rRNAs, a full set of tRNAs, and a few low-abundance proteins required for intron splicing (7, 23). All other known proteins required for mitochondrial gene expression are encoded in the nuclear genome and imported into mitochondria after synthesis in the cytosol (52).

Most yeast mitochondrial RNAs are produced by processing of polycistronic precursors that are transcribed from at least 12 distinct promoters (11). The levels of cotranscribed mature RNAs can differ substantially (47), indicating that RNA stability plays an important role in governing transcript abundance. Factors controlling mitochondrial RNA stability have not been extensively studied, except in the case of the nuclear *CBP1* gene, whose product specifically stabilizes the *COB* mRNA (5, 45, 46, 61, 62, 68). A novel nucleoside triphosphate-dependent 3′-to-5′ exoribonuclease, which plays a role in the degradation of excised group I introns in vivo, has been purified from yeast mitochondria (38, 43), and a protein complex that binds mitochondrial mRNA 3′ ends and could protect mRNAs from degradation by the exoribonuclease has been identified (44). However, these components have not been shown to affect mRNA stability in vivo.

In this study we have selected directly for nuclear mutations that increase the level of unstable mutant mitochondrial mRNAs. We had previously generated a series of defined deletion mutations affecting the 5′ untranslated leader (5′-UTL) of the mitochondrially coded *COX3* mRNA (8, 69) to study the target of the membrane-bound mRNA-specific translational activation system controlling expression of *COX3* (17). Some of these deletion mutations dramatically reduced the steady-state level of *COX3* mRNA but did not completely block translation (69). Nuclear mutations that partially suppress the respiratory growth defects of two such mitochondrial *cox3* deletion mutations affect the same nuclear gene, *PET127*, whose activity is necessary to destabilize the mutant mitochondrial mRNAs. The mitochondrially located, membrane-bound Pet127p is also necessary for efficient 5′ processing of several precursor transcripts. Our findings suggest that RNA turnover and processing in yeast mitochondria may involve components that are not soluble in the matrix.

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MATERIALS AND METHODS

Strains, media, and genetic methods. The *S. cerevisiae* strains used in this study are isogenic or congenic to D273-10B. Complete media were YP (1% yeast extract, 2% peptone) containing the following carbon sources: 2% glucose (YPD), 2% galactose (YPGal), 2% raffinose (YPRaf), or 3% ethanol and 3% glycerol (YPEG). Synthetic minimal media (0.67% yeast nitrogen base containing 2% glucose [SD], 2% galactose [SGal], or 3% ethanol and 3% glycerol [SEG]) were supplemented as previously described when appropriate (57). [*rho*^{0}] derivatives were obtained by growing cultures for about 30 generations in YPD containing 50 μg of ethidium bromide per ml. Genetic manipulations were performed by standard procedures as described previously (19, 57). Spontaneous revertants of the *cox3-662* mutant GW139 were selected from independent cultures grown in YPD and spread on YPEG plates. Independent revertants were streaked on YPEG plates and then tested for mitotic stability by restreaking on YPD and then printing the restreaks to YPEG plates; 22 of 24 independent revertants, including strain 62R1, were mitotically stable and were studied further (see Results).

Isolation and alteration of *PET127*. Complementation of the recessive, leaky, heat-sensitive Pet$^-$ phenotype caused by the suppressor of *cox3-662* (see Results) was used to isolate the wild-type allele from a yeast genomic bank in the vector YCp50 (56). Ura$^+$ transformants of strain GW149 were incubated for 24 h at 30°C and then shifted to 37°C for 2 days. Transformants were replica plated at 37°C to prewarmed YPEG plates, incubated at 37°C for 1 day, and replica plated again to prewarmed YPEG plates. After 2 days, Pet$^+$ candidates were checked by streaking on YPEG plates incubated at 37°C. Plasmid DNA was isolated from 32 Pet$^+$ transformants, analyzed by restriction digestion, and reintroduced into strain GW149. The plasmids were also introduced into GW176 to check for complementation of the recessive *cox3-662* suppression phenotype. One of the five distinct plasmids isolated was able to complement both the heat-sensitive Pet$^-$ and recessive suppression phenotypes. The smallest complementing fragment (Fig. 1) was subcloned into the vector YCplac33 (21), creating pGW694. DNA sequence analysis from the *Sal*I site identified the DNA as *PET127*. Frameshift mutations in *PET127* were generated by cleaving pGW694 with *Mlu*I or *Sal*I (Fig. 1), filling in the ends with Klenow polymerase, and religating. Creation of frameshifts at the *Mlu*I or *Sal*I sites was checked by cutting the plasmids with *Bss*HII or *Pvu*I, respectively. The *Cla*I sites in pGW694 were similarly destroyed, and the resulting frameshifts were confirmed by cleavage with *Nru*I. The chromosomal copy of *PET127* was deleted by replacing the 2.2-kb *Sal*I-*Xba*I fragment in pGW694 (Fig. 1) with a 3.8-kb *Sal*I-*Xba*I *hisG-URA3-hisG* fragment (1), creating pGW731. A 6.4-kb *Sph*I-*Kpn*I fragment of pGW731 was used to transform strain PTY22. Ura$^+$ transformants were grown nonselectively

FIG. 1. Restriction map of the 4.8-kb *Sph*I-*Kpn*I insert of pGW694. The open box represents the *PET127* open reading frame. The sequence of this region is available under GenBank accession no. Z74925.

on YPD and then screened for loss of *URA3* to isolate the *pet127*Δ::*hisG* strain, GW202, which was checked by Southern blotting (not shown). To overproduce Pet127p, we constructed a plasmid, pGW797, that contains the entire *PET127* coding sequence, 13 bp of its 5′-UTL, and 353 bp of the 3′ untranslated region under control of the *ADC1* promoter and terminator (2) in the vector YEp352 (31).

To epitope tag Pet127p, a 1.3-kb *Mun*I-*Xba*I fragment (Fig. 1) comprising the 3′ half of *PET127* was cloned into *Eco*RI-*Xba*I-cleaved Ylplac211 (21). The resulting plasmid, pGW735, was then cut with *Xba*I, treated with mung bean nuclease to remove single-stranded ends, and then cleaved with *Sph*I. A 0.4-kb fragment containing three tandem copies of the hemagglutinin (HA) epitope (65) and the actin terminator was isolated from pCS124 (a gift from C. Shamu and J. Nunnari) by cleavage with *Nco*I, treatment with Klenow polymerase to make flush ends, and subsequent cleavage with *Sph*I. This fragment was ligated into pGW735 treated as described above, yielding pGW739. To integrate the epitope-tagged and control genes, pGW739 and pGW735 were linearized within the *PET127* sequence by cutting with *Age*I and used to transform strain PTY22 to Ura^+. This procedure generates a chromosomally integrated complete *PET127* gene under the control of its own promoter, followed by the actin terminator, integrated vector sequences, and a copy of the 3′ half of *PET127*. Integration of the plasmids at *PET127* was verified by Southern blot analysis (not shown).

Isolation, fractionation, and immunological analysis of mitochondria. Strains GW200 and GW201 were grown in YPGal supplemented with adenine (20 mg/liter) to early stationary phase. Mitochondria were isolated as described previously (71), except that spheroplasts were disrupted in a Parr Bomb (Parr Instrument Co. Moline, Ill.) (41). Crude mitochondria were purified on a 20 to 70% sucrose gradient in 50 mM Tris HCl (pH 7.4)–1 mM phenylmethylsulfonyl fluoride and centrifuged for 17 h in an SW41 rotor (Beckman) at 20,000 rpm. Fractionation of mitochondria was performed as described previously (10). Proteins were separated on sodium dodecyl sulfate (SDS)–8% polyacrylamide gels (58), transferred to Immobilon-P membranes (Millipore), and probed in a buffer of 1% dry milk powder–150 mM NaCl–50 mM Tris HCl (pH 7.6)–0.1% Tween 20 with the following antisera: protein A-purified monoclonal anti-HA from 12CA5 cells (35) (BAbCO, Berkeley, Calif.) at a 1:7,500 dilution; monoclonal anti-CoxII from CCO6 cells (a gift from T. L. Mason) (51) at a 1:100 dilution; polyclonal anti-Arg8p (63), treated with acetone powder (28), at a 1:10,000 dilution; and polyclonal anti-cytochrome b_2 (a gift from B. Glick and G. Schatz) (22) at a 1:1,000 dilution. The secondary antibody was either horseradish peroxidase-conjugated goat anti-mouse serum or horseradish peroxidase-conjugated goat anti-rabbit serum (Bethesda Research Laboratories) at a dilution of 1:30,000. The ECL chemiluminescence detection kit (Amersham Corp.) was used to visualize immune complexes.

Hybridization and primer extension analysis of RNAs. Yeast cells were grown in YPGal, YPRaf, or SGal (containing supplements) to an optical density at 600 nm (OD_{600}) of 1. Single-stranded RNA was isolated, subjected to electrophoresis, and blotted to filters as described previously (69). The filters were hybridized at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer as described previously (58). For detection of the mitochondrial mRNAs, the following fragments were used to produce the radiolabeled probes by random priming (Boehringer Mannheim): for *COX3*, a 1.2-kb *Acc*I-*Xba*I fragment containing the *COX3* coding sequence and 3′ flanking region; for *COX2*, a 0.45-kb *Rsa*I fragment within the *COX2* coding region; for *COX1*, a 145-bp fragment from pA1A2 carrying exons 1 and 2 of *COX1*; for *COB*, a 0.6-kb *Eco*RI-*Bam*HI fragment covering parts of the 5′-UTL and the first exon; for *ATP9*, a 1-kb *Bam*HI fragment from plasmid pT1/15 (36); for *ATP6*, a 1.5-kb *Xba*I-*Acc*I fragment from pYGT7 (24); for *VAR1*, a 1.7-kb *Eco*RI-*Hin*dIII fragment from pBV3-1 (15); for 15S rRNA, plasmid pT82 (59); and for 21S rRNA, plasmid p19 (obtained from T. L. Mason). For normalization, the filters were rehybridized with a radiolabeled probe specific for the actin mRNA (20).

The 2.7-kb *PET127* transcript was detected in RNA isolated from PTY22, using GW202 as a negative control. Cells were grown to an OD_{600} of 1 in complete medium containing 10% or 2% dextrose, 3% ethanol plus 3% glycerol, 2% raffinose, or 2% galactose. Fifteen micrograms of each RNA was analyzed by RNA gel blot hybridization with the radiolabeled 2.2-kb *Sal*I-*Xba*I fragment of pGW694 and subsequently rehybridized with the actin probe. Sizes were estimated by using a 0.24- to 9-kb RNA ladder (Bethesda Research Laboratories). mRNA levels were quantitated with a Betascope 603 Blot Analyzer (Betagen Corp.). The relative normalized amounts of *PET127* mRNA were as follows: YPD (10% dextrose), 91%; YPD (2% dextrose), 127%; YPEG, 167%; YPRaf, 174%; and YPGal, 100%.

Primer extension analysis was carried out as previously described (69), except that total cellular RNA was used as a template. The primer for the *COB* transcript was 15 pmol of oligonucleotide cob5B+3 (46). The primer for the 15S rRNA was 110 pmol of oligonucleotide 15S-1 (5′-ATGACTCGTATGCGTCA TGTCC).

RESULTS

Nuclear mutations that suppress specific *cox3* mutations by increasing the levels of mutant *cox3* mRNAs. Our previous deletion analysis of the mitochondrially coded *COX3* 5′-UTL generated several mutations that decreased mRNA stability (69). One such mutation, *cox3-662*, reduced the level of mRNA to 9% of the wild-type level but did not block activator-dependent translation. Cells bearing the *cox3-662* mutation grew weakly on nonfermentable carbon sources at 16 and 30°C but not at 37°C. We found that the *cox3-662* mutant strain GW139 (Table 1) reverted spontaneously, giving rise to colonies that grew substantially better at 30°C on nonfermentable (YPEG) medium. Twenty-two independent pseudorevertants all grew approximately equally well on YPEG at 16 and 30°C but not at all at 37°C. RNA gel blot hybridization analyses of total RNA isolated from pseudorevertant 62R1 (Fig. 2) and two others (not shown) revealed that they contained greatly increased levels of *cox3* mRNA.

Mating of the pseudorevertants to a [rho^0] strain with a wild-type nucleus (DAU1rho^0) yielded diploids whose slow growth on YPEG was comparable to that of the *cox3-662* mutant strain GW139, indicating that pseudoreversion was due to recessive nuclear mutations. A complementation test was carried out by crossing a [rho^0] strain carrying the suppressor mutation derived from pseudorevertant 62R1 (GW149rho^0 [Table 1]) to all of the pseudorevertants. In every case, the diploids had the suppressed growth phenotype, indicating that all suppressor mutations were in one complementation group.

Another mitochondrial DNA (mtDNA) deletion affecting the *COX3* mRNA 5′-UTL, *cox3-438*, had previously been shown to be suppressible by nuclear mutations in at least two genes, one of which is the translational activator *PET122* (69). Pseudorevertants of the *cox3-438* mutant GW16, due to unmapped recessive nuclear mutations, were crossed to GW149rho^0 to test for complementation between the independently isolated suppressors of *cox3-438* and *cox3-662*. The resulting diploids exhibited the suppressed phenotype, indicating failure to complement. Furthermore, tetrad analyses revealed that the *cox3-662* and *cox3-438* nuclear suppressor mutations were linked (not shown).

The nuclear suppressor allele derived from pseudorevertant 62R1 also improved the leaky respiratory growth phenotype caused by two other 5′-UTL deletion mutations, *cox3-512* and *cox3-516*, that caused decreased levels of *COX3* mRNA (69). In the case of two 5′-UTL mutations that completely blocked translation and lowered mRNA levels, *cox3-666* and *cox3-652* (69), the nuclear suppressor failed to restore respiratory growth but did cause substantial increases in mRNA levels (not shown).

Identification of the suppressor gene as *PET127*. To test whether the *cox3-662* suppressor mutations caused respiratory phenotypes by themselves, the pseudorevertants were converted to [rho^0] strains and mated to a wild-type [rho^+] strain (DAU1), and tetrads from the resulting diploids were analyzed. In each case we observed 2:2 segregation for a leaky Pet^- phenotype at 37°C. Like suppression, this heat-sensitive Pet^- phenotype was recessive to the wild-type phenotype. This intrinsic phenotype was used to clone the corresponding wild-

TABLE 1. Yeast strains used in this study

Strain	Genotype (nuclear [mitochondrial])	Source or reference
62R1	*MAT***a** *lys2 pet127-R1* [*rho*$^+$ *cox3-662*]	This study
DAU1	*MAT*α *ade2 ura3-del* [*rho*$^+$]	This study
DAU1rho^0	*MAT*α *ade2 ura3-del* [*rho*^{0}]	This study
DL2	*MAT***a** *lys2* [*rho*$^+$]	69
GW16	*MAT***a** *lys2* [*rho*$^+$ *cox3-438*]	69
GW84	*MAT***a** *ade2 leu2-3,112 ura3-52* [*rho*$^+$ *cox3-438*]	69
GW139	*MAT***a** *lys2* [*rho*$^+$ *cox3-662*]	69
GW146	*MAT***a** *ade2 leu2-3,112 ura3-52* [*rho*$^+$ *cox3-662*]	69
GW149	*MAT*α *ura3-del pet127-R1* [*rho*$^+$]	This study
GW149rho^0	*MAT*α *ura3-del pet127-R1* [*rho*^{0}]	This study
GW176	*MAT*α *ura3-del pet127-R1* [*rho*$^+$ *cox3-662*]	This study
GW200	*MAT***a** *ade2 leu2-3,112 ura3-52 PET127-HA-URA3* [*rho*$^+$]	This study
GW201	*MAT***a** *ade2 leu2-3,112 ura3-52 PET127-URA3* [*rho*$^+$]	This study
GW202	*MAT***a** *ade2 leu2-3,112 ura3-52 pet127*Δ*::hisG* [*rho*$^+$]	This study
GW208	*MAT***a** *ade2 leu2-3,112 ura3-52* [*rho*$^+$ *cox3-5*]	This study
GW209	*MAT***a** *ade2 leu2-3,112 ura3-52 pet127*Δ*::URA3* [*rho*$^+$ *cox3-5*]	This study
GW212	*MAT***a** *ade2 leu2-3,112 ura3-52 pet54-5* [*rho*$^+$]	This study
GW213	*MAT***a** *ade2 leu2-3,112 ura3-52 pet54-5 pet127*Δ*::hisG* [*rho*$^+$]	This study
GW217	*MAT***a** *ade2 leu2-3,112 ura3-52 pet127*Δ*::hisG* [*rho*$^+$]	This study
GW220	*MAT***a** *ade2 leu2-3,112 ura3-52 pet54-5* [*rho*$^+$ *cox3-662*]	This study
GW221	*MAT***a** *ade2 leu2-3,112 ura3-52 pet54-5 pet127*Δ*::hisG* [*rho*$^+$] *cox3-662*]	This study
GW225	*MAT*α *ade2 leu2-3,112 ura3-52 pet122-7* [*rho*$^+$]	This study
GW228	*MAT***a** *ade2 leu2-3,112 ura3-52 pet122-7 pet127*Δ*::hisG* [*rho*$^+$]	This study
GW229	*MAT***a** *ade2 leu2-3,112 ura3-52 pet494-41* [*rho*$^+$]	This study
GW231	*MAT*α *ade2 leu2-3,112 ura3-52 pet54-5 pet122-7 pet494-41* [*rho*$^+$]	This study
GW267	*MAT***a** *ade2 leu2-3,112 ura3-52* [*rho*$^+$ *cox2-107*]	This study
GW268	*MAT***a** *ade2 leu2-3,112 ura3-52 pet127*Δ*::hisG* [*rho*$^+$ *cox2-107*]	This study
PTY22	*MAT***a** *ade2 leu2-3,112 ura3-52* [*rho*$^+$]	P. E. Thorsness

type gene by complementation as described in Materials and Methods. Five different plasmids that complemented the heat-sensitive phenotype of strain GW149 were isolated. However, only one of them also complemented the recessive suppressor phenotype in the *cox3-662* background of strain GW176, suggesting that it corresponded to the suppressor locus itself. The complementing activity was subcloned to a 4.8-kb *Sph*I-*Kpn*I fragment (pGW694) whose partial sequence revealed that it contained the gene *PET127* (Fig. 1), which was previously identified by this lab in a screen for suppressors of a Pet122p carboxy-terminal truncation (25). We confirmed by tetrad analysis that the *cox3-662* suppressor, which was derived from the pseudorevertant 62R1, was indeed linked to a *pet127*Δ::*URA3* marker (not shown). These experiments also revealed

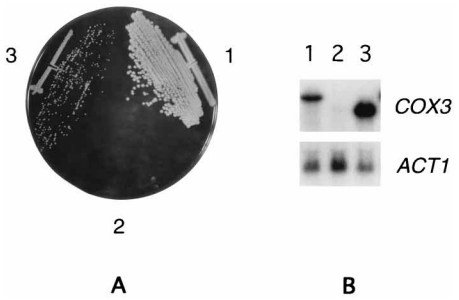

FIG. 2. Suppression of *cox3-662* correlates with greatly increased levels of the mutant *cox3* mRNA. (A) The strains indicated below were streaked on YPEG plates (nonfermentable carbon source) and incubated at 30°C for 6 days. (B) Total RNA was isolated from each of the strains grown in YPGal at 30°C and analyzed by RNA gel blot hybridization (see Materials and Methods). The blots were hybridized with a radiolabeled *COX3* probe and subsequently rehybridized with a probe detecting the actin mRNA (*ACT1*). Strains (see Table 1 for complete genotypes): 1, DL2 (*PET127*[*COX3*]); 2, GW139 (*PET127* [*cox3-662*]); 3, 62R1 (*pet127* [*cox3-662*]).

that chromosomal deletion of *PET127* caused suppression of *cox3-662* (see below). Introduction of a frameshift mutation at the *Mlu*I site (Fig. 1) (see Materials and Methods) prevented complementation of the suppressor phenotype by the plasmid-borne DNA fragment, demonstrating that the suppressor corresponded to the *PET127* open reading frame. As previously reported (25), deletion of the chromosomal copy of *PET127* caused no apparent growth phenotype at 30°C and a leaky respiratory deficiency at 37°C in strains carrying wild-type mtDNA.

A 2.2-kb *Sal*I-*Xba*I fragment (Fig. 1) had previously been identified in this lab as the smallest fragment complementing the heat-sensitive Pet$^-$ *pet127* phenotype in a different strain background (25). However, it is now clear that the gene extends upstream of the *Sal*I site, as suggested by the genomic DNA sequence (GenBank accession no. Z74925), since frameshift mutations introduced at both the *Sal*I and *Cla*I sites (Fig. 1) (see Materials and Methods) abolished complementation. The predicted Pet127p is 93.4 kDa in size, is hydrophilic with a calculated isoelectric point of 10.1, and appears to lack sequences that could form membrane-spanning alpha-helices (14). Pet127p is not strongly homologous to any known protein. The correct amino-terminal sequence of the Pet127p is more consistent with the pattern of mitochondrial targeting signals (29, 55, 67) than that previously reported (25).

Expression and overexpression of *PET127*. Transcriptional regulation of gene expression in response to the carbon source has been observed for several proteins involved in mitochondrial gene expression (66). We examined by gel blot hybridization the relative levels of *PET127* mRNA in total RNA from cells grown in glucose, ethanol plus glycerol, raffinose, and galactose (see Materials and Methods). A 2.7-kb *PET127* transcript was detected in RNA from a wild-type strain but not in that from a *pet127*Δ strain. The levels of this transcript varied

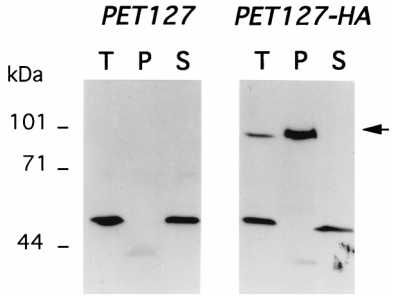

FIG. 3. Pet127p cofractionates with mitochondria. Cells of the wild-type strain GW201 (*PET127*) and a strain expressing HA-tagged Pet127p, GW200 (*PET127-HA*), were grown in YPGal supplemented with adenine (20 mg/liter) at 30°C, disrupted to yield total cell extracts (lanes T), and fractionated into crude mitochondrial pellets (lanes P) and postmitochondrial supernatants (lanes S) (see Materials and Methods). Ten micrograms of each fraction was loaded on an SDS–8% polyacrylamide gel, blotted, and probed with the anti-HA monoclonal serum 12CA5 (see Materials and Methods). The arrow indicates Pet127p-HA.

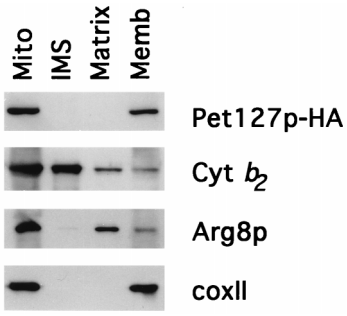

FIG. 4. Pet127p is associated with mitochondrial membranes. Crude mitochondria from the *PET127-HA* strain GW200 grown in YPGal supplemented with adenine (20 mg/liter) at 30°C were purified by sucrose gradient centrifugation and fractionated (see Materials and Methods). Forty micrograms of purified mitochondria, and amounts of the submitochondrial fractions derived from 40 μg, were electrophoresed on an SDS–8% polyacrylamide gel, blotted, and probed with anti-HA monoclonal serum 12CA5 (see Materials and Methods) to detect Pet127p-HA. Ten micrograms of purified mitochondria, and equivalent amounts of the submitochondrial fractions, were analyzed separately on SDS–12% polyacrylamide gels, blotted, and probed with antisera to detect the following proteins (see Materials and Methods): cytochrome b_2 (Cyt b_2), an intermembrane space (IMS) marker (22); Arg8p, a matrix marker (33); and cytochrome oxidase subunit II (coxII), an inner membrane (Memb) marker.

by no more than twofold from the most repressing conditions (10% glucose) to the least (either ethanol plus glycerol or 2% raffinose) (not shown). Thus, expression of *PET127* is not strongly regulated by the carbon source at the level of transcription.

To determine the effects of *PET127* overexpression, the entire *PET127* open reading frame was placed under the control of the strong *ADC1* promoter (2) on a multicopy plasmid, pGW797 (see Materials and Methods). Transformants of a wild-type strain (PTY22) carrying pGW797 or the corresponding empty vector (YEp352) were selected on minimal medium containing glucose (SD) and tested for the ability to grow on either minimal (SEG) or complete (YPEG) medium containing ethanol plus glycerol. The transformants overexpressing *PET127* failed to grow on the nonfermentable media, while the control transformants grew well. Overexpression of *PET127* apparently caused the loss of mtDNA, since the pGW797 transformants failed to yield respiring diploids when mated to a [*rho*^{0}] strain, and subsequent loss of the plasmid did not restore respiratory competence.

Pet127p is a mitochondrial membrane protein. To determine whether Pet127p might act directly on mitochondrial gene expression, we examined its subcellular localization. An allele of *PET127* was constructed that encoded the entire protein followed by three copies of the influenza virus HA epitope (65) fused at the carboxy terminus and chromosomally integrated at the *PET127* locus (see Materials and Methods). The resulting protein appeared to function almost normally, since respiratory growth of a strain expressing Pet127p-HA (GW200) was identical to that of an isogenic control strain (GW201) at 37°C. However, the *PET127-HA* allele weakly suppressed *cox3-662* (not shown).

Immunological examination of total cell protein from a strain containing the *PET127-HA* gene revealed a tagged protein of approximately 100 kDa, the expected size, that was absent from the control (Fig. 3). This protein was enriched in the crude mitochondrial pellet and could not be detected in the postmitochondrial supernatant (Fig. 3). Mitochondria from the *PET127-HA* strain were purified on a 20 to 70% sucrose gradient, and the 100-kDa tagged protein cosedimented with cytochrome oxidase subunit II (not shown). To further localize the protein within the purified mitochondria, we analyzed intermembrane space, matrix, and membrane fractions (Fig. 4). Pet127p-HA cofractionated with cytochrome oxidase subunit II, demonstrating that it is tightly associated with mitochondrial membranes.

Pleiotropic effects of a *pet127*Δ mutation on transcripts of wild-type mtDNA. To further elucidate the possible functions of Pet127p, we compared the sizes and steady-state levels of various mitochondrial transcripts in a *pet127*Δ strain (GW202) with those in the wild type (PTY22) by RNA gel blot hybrid-

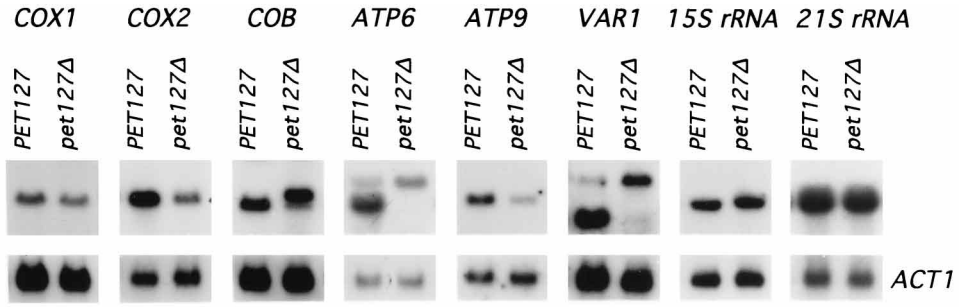

FIG. 5. Deletion of *PET127* affects the levels and processing of various mitochondrial transcripts differently. Strains PTY22 (*PET127*) and GW202 (*pet127*Δ) were grown in YPGal at 30°C to an OD_{600} of 1. Total RNAs were isolated and analyzed by RNA gel blot hybridization (see Materials and Methods). mRNA levels were quantitated with a Betascope 603 Blot Analyzer (Betagen Corp.) and normalized to the levels of *ACT1* mRNA. The amounts of mature transcripts in the *pet127*Δ strain relative to their wild-type levels were as follows: *COX1*, 102%; *COX2*, 32%; *COB*, 69%; *ATP6*, 25%; *ATP9*, 40%; *VAR1*, 18%; 15S rRNA, 107%; and 21S rRNA, 102%.

ization (Fig. 5). Deletion of *PET127* had no detectable effect on either the *COX1* mRNA or the 21S rRNA (Fig. 5), while it increased the level of the *COX3* mRNA slightly (see below). The steady-state levels of two other mRNAs, *COX2* and *ATP9*, were reduced to about 32 and 40% of the wild-type level, respectively (Fig. 5). While the sizes of the *COX2* and *ATP9* mRNAs were not detectably altered, the *COX2* probe detected several larger, unidentified transcripts specific for the *pet127*Δ strain (not shown).

Pet127p is apparently necessary for complete processing of several mitochondrial transcripts. For example, the *pet127*Δ mutant accumulated substantial amounts of a *COB* transcript that was slightly larger than the mature wild-type mRNA (Fig. 5) but accumulated no other larger species (not shown). *COB* is cotranscribed with an upstream $tRNA^{Glu}$ and a spacer of 143 bases between the $tRNA^{Glu}$ and the mature *COB* mRNA 5′ end, after which the transcript is processed in several steps (46). Since the longer *COB* transcript detected in the *pet127*Δ strain appeared to be an unprocessed *COB* mRNA precursor, we performed a primer extension experiment to examine the 5′ ends (Fig. 6). This experiment demonstrated that 5′ processing was indeed defective in the *pet127*Δ strain. There was no band corresponding to the wild-type mature 5′ end, although several minor novel cleavages occurred in this region. Furthermore, the level of a precursor transcript, processed downstream of the $tRNA^{Glu}$ moiety but retaining the 143-base spacer, was dramatically increased.

Both the *ATP6* (24, 60) and *VAR1* (72) mRNAs are known to be processed at their 5′ ends from larger precursors, and the *pet127*Δ mutation appears to block these pathways (Fig. 5). In both cases, the wild-type strain contains a predominant mature mRNA and smaller amounts of longer precursor molecules, while the *pet127*Δ mutant contains predominantly unprocessed precursor and only traces of mature mRNA. In these cases, however, we have not demonstrated that 5′ end processing is defective.

The mature 15S rRNA is produced from a precursor by endonucleolytic cleavage 80 bases downstream of the transcription initiation site (3, 6, 50). Careful examination of several RNA gel blots, including that in Fig. 5, suggested that the 15S rRNA might be slightly longer in the *pet127*Δ mutant than in the corresponding wild-type strains. We tested this hypothesis by primer extension of the 15S rRNA (Fig. 6). RNA from the wild type contained both the mature 15S rRNA, with several 5′ ends as previously reported (6), and the precursor transcript. While the wild-type level of precursor transcript relative to that of mature 15S rRNA appears to be high, it is consistent with a previous nuclease mapping experiment (50). In the *pet127*Δ strain the ratio between precursor and mature 15S RNAs is substantially higher than that in the wild type. Thus, Pet127p is required for normal 5′ end processing of an rRNA, in addition to several mRNAs.

The effect of *pet127*Δ on *COX3* mRNA levels is independent of translational activation. In the absence of mRNA-specific translational activation, the levels of both the *COX2* (53) and *COX3* (see below) mRNAs are decreased relative to wild-type levels, presumably due to decreased stability. This fact raises the possibility that 5′-UTL mutations such as *cox3-662* might destabilize the *COX3* mRNA by interfering with translation and that a *pet127*Δ mutation might allow increased translation of the mutant mRNA and only indirectly increase the mRNA steady-state level.

In testing this hypothesis we have carefully reexamined the effect of translational activation on levels of the wild-type *COX3* mRNA by direct measurement of mRNA levels in Northern blots with a Betascope 603 Blot Analyzer (Fig. 7).

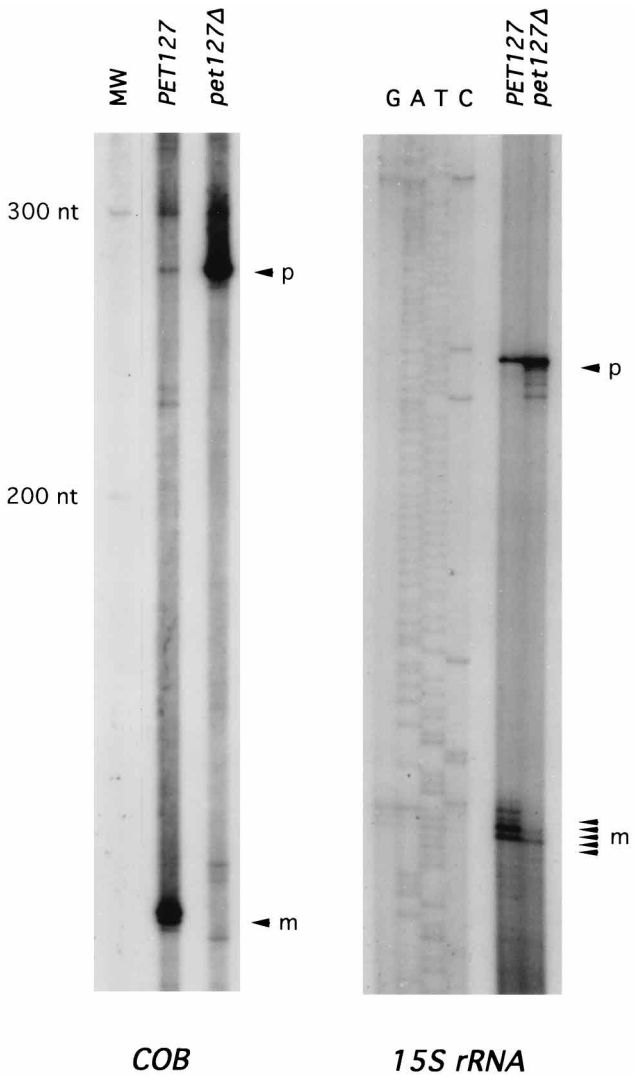

FIG. 6. 5′ end processing of the *COB* mRNA and the 15S rRNA is altered in a *pet127*Δ strain. Primer extension experiments were performed on total RNA isolated from strains PTY22 (*PET127*) and GW202 (*pet127*Δ) with primers specific for either the *COB* mRNA or the 15S rRNA (see Materials and Methods). Cells were grown in YPGal at 30°C. A radioactively labeled 100-bp DNA ladder (Bethesda Research Laboratories) was used as a size standard (MW) in the left panel. Precursors (p) and mature transcripts (m) are indicated. nt, nucleotides.

Using cruder quantitation procedures, we and others had concluded that loss of *COX3*-specific translational activation did not affect the mRNA level (9, 18, 34, 48). However, we now find that steady-state *COX3* mRNA levels in strains lacking any or all of the three activator genes (*PET54*, *PET122*, and *PET494*) are approximately 50% of wild-type levels (Fig. 7B, lanes 1, 7, 11, 13, and 14). Furthermore, simultaneous overexpression of all three activator genes increased the wild-type *COX3* mRNA level by 3.9-fold (Fig. 7A, lanes 1 and 2). Overexpression of the activator genes also increased the level of the *cox3-662* mRNA 6.5-fold (Fig. 7A, lanes 3 and 4) and that of the *cox3-438* mRNA 4.6-fold (Fig. 7A, lanes 5 and 6), consistent with the fact that activator overexpression partially suppressed the respiratory growth phenotypes of these mutations (69).

Despite the fact that translational activators affect mRNA

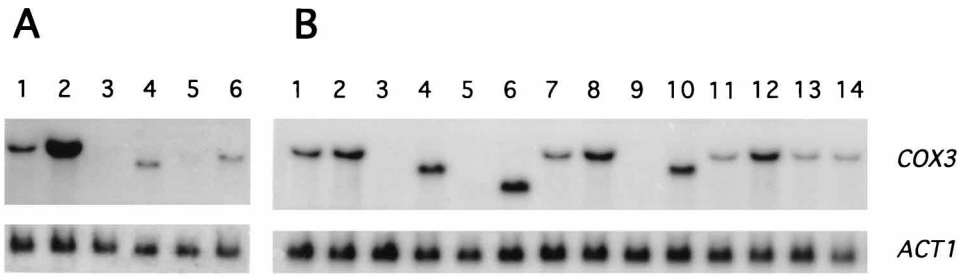

FIG. 7. Overproduction of *COX3* mRNA-specific translational activator subunits elevates *COX3* mRNA levels, but elevation due to deletion of *PET127* is independent of mRNA-specific translational activation. (A) Strains PTY22 [*COX3*], GW146 [*cox3-662*], and GW84 [*cox3-438*] were each transformed with three plasmids overexpressing *PET54*, *PET122*, and *PET494* (69) (indicated below as overproducing). Controls were the same strains transformed with three empty vectors (69) bearing the same selectable markers (indicated below as empty). Transformants were grown at 30°C in minimal medium containing 2% galactose to an OD_{600} of 1, and total RNA was isolated and analyzed by RNA gel blot hybridization with a *COX3* probe and subsequently with an *ACT1* probe (see Materials and Methods). mRNA levels were quantitated with a Betascope 603 Blot Analyzer (Betagen Corp.). The normalized amounts of *COX3* mRNAs relative to empty wild-type amounts were as follows: lane 1, PTY22 ([*COX3*] empty), 100%; lane 2, PTY22 ([*COX3*] overproducing), 388%; lane 3, GW146 ([*cox3-662*] empty), 10.0%; lane 4, GW146 ([*cox3-662*] overproducing), 65.1%; lane 5, GW84 ([*cox3-438*] empty), 12.6%; lane 6, GW84 ([*cox3-438*] overproducing), 58.3%. (B) Strains were grown at 30°C in YPGal to an OD_{600} of 1. Total RNAs were isolated and analyzed by RNA gel blot analysis as described for panel A. The normalized amounts of *COX3* mRNA relative to wild-type amounts were as follows: lane 1, PTY22 (*PET127* [*COX3*]), 100%; lane 2, GW202 (*pet127*Δ [*COX3*]), 167%; lane 3, GW146 (*PET127* [*cox3-662*]), 2.8%; lane 4, GW217 (*pet127*Δ [*cox3-662*]), 138%; lane 5, GW208 (*PET127* [*cox3-5*]), 6.3%; lane 6, GW209 (*pet127*Δ [*cox3-5*]), 143%; lane 7, GW212 (*PET127 pet54*Δ [*COX3*]), 60%; lane 8, GW213 (*pet127*Δ *pet54*Δ [*COX3*]), 161%; lane 9, GW220 (*PET127 pet54*Δ [*cox3-662*]), 4.5%; lane 10, GW221 (*pet127*Δ *pet54*Δ [*cox3-662*]), 104%; lane 11, GW225 (*PET127 pet122*Δ [*COX3*]), 45%; lane 12, GW228 (*pet127*Δ *pet122*Δ [*COX3*]), 173%; lane 13, GW229 (*PET127 pet494*Δ [*COX3*]), 43%; lane 14, GW231 (*PET127 pet54*Δ *pet122*Δ *pet494*Δ [*COX3*]), 53%.

levels, we found that deletion of *PET127* caused increases in levels of wild-type and mutant forms of the *COX3* mRNA whether or not translation could occur. An otherwise wild-type *pet127*Δ strain contained approximately 1.5 times more *COX3* mRNA than an isogenic *PET127* strain (Fig. 7B, lanes 1 and 2). In strains lacking *PET54* or *PET122*, the *pet127*Δ mutation also increased *COX3* mRNA levels to approximately 1.5 times wild-type levels (Fig. 7B, lanes 7, 8, 11, and 12).

The more dramatic effects of the *pet127*Δ mutation on the levels of mutant *COX3* mRNAs were also largely independent of translational activation. The *cox3-662* mRNA level was increased approximately 50-fold by *pet127*Δ in an otherwise wild-type strain and by 23-fold in an isogenic *pet54* mutant (Fig. 7B, lanes 3, 4, 9, 10). The *cox3-5* mutation is a deletion that removes almost the entire *COX3* 5′-UTL and part of the open reading frame (8). It encodes an mRNA that cannot be translated and accumulates at low levels. The *pet127*Δ mutation increased the level of this untranslatable mRNA by approximately 23-fold (Fig. 7B, lanes 5 and 6).

Deletion of *PET127* increases the level of a mutant *COX2* mRNA. In light of the dramatic effect of *pet127*Δ on the levels of apparently unstable *cox3* mutant mRNAs, it seemed possible that other mutant mRNAs might also be stabilized. To test this idea, we combined the *pet127*Δ allele with the *cox2-107* mutation, which was generated by a block sequence substitution that alters 7 bp in the region of mtDNA encoding the *COX2* mRNA 5′-UTL (13). *cox2-107* does not affect the *COX2* promoter, but it substantially reduces the level of mRNA and completely blocks respiratory growth (13). Introduction of the *pet127*Δ mutation into a *cox2-107* strain did not suppress the *cox2-107* nonrespiratory growth phenotype (not shown). However, the *pet127*Δ mutation did increase the level of the *cox2-107* mRNA 4.4-fold compared to that for the isogenic *PET127* [*cox2-107*] strain (Fig. 8).

DISCUSSION

Loss of *PET127* function does not prevent respiratory growth of yeast cells under optimal conditions but has pleiotropic effects on mitochondrial transcripts. One of these effects is to stabilize aberrant mitochondrial mRNAs that are unstable in wild-type cells. In this study, we identified nuclear *pet127* mutations as suppressors of mitochondrial mutations affecting the *COX3* mRNA 5′-UTL that limited respiratory growth by reducing *COX3* mRNA levels. We argue that suppression results from stabilization of the mutant mRNAs, rather than increased transcription, for the following reasons. First, while a *pet127*Δ mutation increased the levels of *cox3* mutant mRNAs by up to 50-fold, it increased the level of wild-type *COX3* mRNA by only 1.7-fold. Similarly, a *pet127*Δ mutation caused a 4.4-fold increase in the level of a destabilized *cox2* mutant mRNA but caused a 3-fold decrease in the level of the corresponding wild-type *COX2* mRNA. Finally, while a *pet127*Δ mutation had no effect on the level of the *COX1* mRNA, it reduced the level of the cotranscribed (11) *ATP6* mRNA by fourfold (in addition to its effect on processing of the *ATP6* mRNA).

The 5′ ends of the 15S rRNA, and the *COB*, *VAR1*, and *ATP6* mRNAs, are generated from precursors by processing

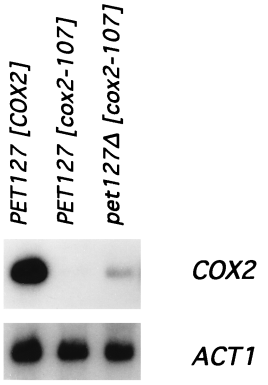

FIG. 8. A *pet127*Δ mutation increases levels of a mutant *COX2* mRNA. Total RNAs were isolated from strains PTY22 (*PET127* [*COX2*]), GW267 (*PET127* [*cox2-107*]), and GW268 (*pet127*Δ [*cox2-107*]) grown in YPGal at 30°C and subjected to RNA gel blot hybridization analysis (see Materials and Methods). The blot was hybridized with a *COX2* probe and subsequently with an *ACT1* probe. mRNA levels were quantitated with a Betascope 603 Blot Analyzer (Betagen Corp.). The relative amounts were as follows: *PET127* [*COX2*], 100%; *PET127* [*cox2-107*], 2.2%; and *pet127*Δ [*cox2-107*], 9.6%.

events (11, 39). We found that a *pet127*Δ mutation caused increased accumulation of unprocessed precursors for each of these transcripts. In the cases of the 15S rRNA and *COB* mRNA, we demonstrated that 5′ end formation was affected. However, the *pet127*Δ mutations did not detectably affect generation of the *COX3* mRNA 5′ end. The *COX3* mRNA differs from the other processed mRNAs in that its 5′ end is generated by the same cleavage event that produces the 3′ end of $tRNA^{Val}$, with which it is cotranscribed (69). The 5′ ends of the other transcripts examined in this study, *COX1*, *COX2*, *ATP9*, and the 21S rRNA, are formed by transcription initiation (11, 39) and were not affected by the *pet127*Δ mutation.

Pet127p is located in mitochondria and is thus likely to be directly involved in organellar RNA metabolism. It remains unclear whether the pleiotropic effects of *pet127* mutations reflect directly the loss of several activities carried out by Pet127p or reflect indirect effects of the loss of a single function. Our data are consistent with the idea that Pet127p has important roles in both mitochondrial RNA turnover and 5′-processing cleavages. Loss of these functions does not have dramatic effects on the respiratory growth of otherwise wild-type yeast cells. However, overproduction of Pet127p causes cells to lose their mtDNA (become [*rho*$^-$]). This phenotype could result from destruction, induced by excess Pet127p, of any RNA necessary for mtDNA replication or global mRNA translation (49). Pet127p could itself be an RNase or could be a protein that modulates the activity of one or more distinct catalytic proteins.

We had previously identified *pet127* mutations as weak recessive suppressors of carboxy-terminal truncations affecting a subunit of the *COX3* mRNA-specific translational activator complex, Pet122p (25). Our present results suggest two possible mechanisms for this suppression. First, carboxy-terminal truncations of Pet122p are known to be suppressed by mutations affecting three mitochondrial ribosomal small-subunit proteins (25–27, 41). Since *pet127* mutations reduce processing of the mitochondrial small-subunit rRNA (15S), it is possible that ribosomes with this unprocessed rRNA can also partially compensate for the translational activator defect. Alternatively, *pet127* mutations cause a modest increase (1.7-fold) in the level of *COX3* mRNA. This increase in mRNA level could promote interaction with the defective translational activator, leading to low levels of Cox3p synthesis.

Surprisingly, we observed that overproduction of the *COX3* mRNA-specific translational activator complex stabilized the *COX3* mRNA roughly fourfold, implying that mRNA unassociated with the complex is degraded. Since loss of Pet127p did not produce an equivalent stabilization, there must be other functions active in wild-type mRNA turnover.

The mitochondrial phenotype of *pet127*Δ mutations resembles, in several respects, the phenotypes of recessive mutations in the nonsense-mediated mRNA decay, or mRNA surveillance, systems that operate on nuclearly encoded mRNAs of *S. cerevisiae* and *Caenorhabditis elegans*. Inactivation of yeast *upf* genes or of *C. elegans smg* genes stabilizes mRNAs containing nonsense codons, as well as some unspliced mRNA precursors (30, 37, 54), revealing the existence of a system for rapid elimination of aberrant mRNAs. Pet127p could play a similar role in mitochondria, for example, eliminating precursor RNAs with unprocessed 5′ ends. The absence of such a surveillance function could lead to the observed lower levels of mature *COB*, *ATP6*, and *VAR1* mRNAs if the accumulated precursors competed with mature mRNAs for stabilizing factors (e.g., Cbp1p [see below]). However, while cytoplasmic mRNA surveillance is dependent on translation (4, 32), our data indicate that both translated and untranslated *COX3* mRNAs, as well as untranslated *COX2* mRNAs, are subject to *PET127*-dependent destabilization in mitochondria.

The protein encoded by the yeast nuclear gene *CBP1* is required specifically to stabilize the *COB* mRNA (5) and may also play a role in promoting the 5′-processing event that generates it (46, 61). Cbp1p is located in mitochondria and appears to be a soluble protein in wild-type cells (68). One mechanism for Cbp1p action would be to bind to the *COB* mRNA 5′ region and protect the mRNA from degradation. A candidate for a general degradative function antagonized by Cbp1p was identified by recessive nuclear mutations in a gene termed *SOC1* that suppress temperature-sensitive *cbp1* mutations (62). The *SOC1* gene has not been isolated by molecular cloning, but it does not appear to correspond to *PET127*. In *soc1* mutant cells the steady-state levels of *COX2*, *ATP6*, and *ATP9* mRNAs are increased compared to wild-type levels (62), while in *pet127* mutant cells these mRNAs are reduced. Furthermore, *soc1* and *pet127* mutations appear to complement each other (70).

Yeast mitochondria contain a nucleoside triphosphate-dependent 3′-to-5′ exoribonuclease activity, termed mtEXO, that copurifies with polypeptides of 75, 90, and 110 kDa (43). The 90-kDa subunit of this enzyme is Suv3p, a putative RNA helicase (38, 64). The 110-kDa subunit may be the product of the *DSS1* gene (12). mtEXO functions in vivo to destroy excised group I intron RNAs, but its role in turnover of other mitochondrial transcripts is unclear (38). The submitochondrial location of mtEXO has not been reported. Yeast mitochondria also contain three soluble proteins of 19, 60, and 70 kDa that bind specifically to the dodecamer sequence at the 3′ ends of mRNAs (44). RNAs bound by these proteins are protected in vitro from degradation by mtEXO, but their role in vivo has not been tested genetically.

The mitochondrial genetic system appears to be specialized for the production of membrane proteins, as reflected in the fact that mRNA-specific translational activators are membrane bound (7, 16, 17, 40, 42). As demonstrated here and in earlier work (53), mRNA-specific translational activators can stabilize wild-type and mutant yeast mitochondrial mRNAs. This study has also revealed that yeast mitochondrial RNA metabolism may involve membrane-bound components, such as Pet127p, whose activity could involve translation-independent RNA surveillance.

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