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The *Saccharomyces cerevisiae ATP22* Gene Codes for the Mitochondrial ATPase Subunit 6-Specific Translation Factor

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ABSTRACT

Mutations in the *Saccharomyces cerevisiae ATP22* gene were previously shown to block assembly of the F_0 component of the mitochondrial proton-translocating ATPase. Further inquiries into the function of Atp22p have revealed that it is essential for translation of subunit 6 of the mitochondrial ATPase. The mutant phenotype can be partially rescued by the presence in the same cell of wild-type mitochondrial DNA and a ρ^- deletion genome in which the 5′-UTR, first exon, and first intron of *COX1* are fused to the fourth codon of *ATP6*. The *COX1/ATP6* gene is transcribed and processed to the mature mRNA by splicing of the *COX1* intron from the precursor. The hybrid protein translated from the novel mRNA is proteolytically cleaved at the normal site between residues 10 and 11 of the subunit 6 precursor, causing the release of the polypeptide encoded by the *COX1* exon. The ability of the ρ^- suppressor genome to express subunit 6 in an *atp22* null mutant constitutes strong evidence that translation of subunit 6 depends on the interaction of Atp22p with the 5′-UTR of the *ATP6* mRNA.

STUDIES of respiratory-deficient mutants of *Saccharomyces cerevisiae* have led to the discovery of at least a dozen nuclear gene products that are necessary for biogenesis of the mitochondrial proton-translocating ATPase (Ackerman and Tzagoloff 2005). Three genes *ATP11* (Ackerman and Tzagoloff 1990), *ATP12* (Ackerman and Tzagoloff 1990), and *FMC1* (Lefebvre-Legendre *et al.* 2001) code for mitochondrial proteins that aid assembly of the α- and β-subunits of F_1 ATPase into a catalytically active oligomer. The other nine genes affect expression of the F_0 unit of the ATPase. Their products target the mitochondrially encoded subunits 6 and 9 of F_0 by promoting processing, stability, and translation of the mRNAs (Payne *et al.* 1991; Camougrand *et al.* 1995; Paul *et al.* 2000; Helfenbein *et al.* 2003; Ellis *et al.* 2004). One such protein, encoded by *ATP22* and located in the inner membrane of mitochondria, was shown to be necessary for expression of F_0 (Helfenbein *et al.* 2003). Mitochondria of *atp22* null and point mutants have a catalytically active F_1 ATPase but are deficient in oligomycin-sensitive ATPase activity (Helfenbein *et al.* 2003). The lack of inhibition of ATPase activity by oligomycin is characteristic of mutants defective in F_0. Like other ATPase-deficient mutants of yeast, *atp22* mutants are difficult to study because of their propensity to undergo deletions in mitochondrial DNA (mtDNA) (Helfenbein *et al.* 2003).

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Although earlier studies pointed to a requirement of Atp22p for F_0 assembly, its specific role in this process remained unclear. The present study was undertaken to gain a better insight into the function of Atp22p. The key to understanding the function of this protein came from studies of partial revertants of an *atp22* null mutant. Such revertants were found to have mitochondrial deletion (ρ^-) genomes, which when present together with wild-type mtDNA, partially restored oligomycin-sensitive ATPase in the *atp22* null mutant. The sequence of one such suppressor genome, combined with Northern analysis of mitochondrial transcripts in the revertant, has led us to conclude that Atp22p is required for translation of subunit 6. Thus, Atp22p is a mitochondrial translation factor (Costanzo and Fox 1990) specific for subunit 6 of the ATPase.

MATERIALS AND METHODS

Yeast strains and growth media: The genotypes and sources of the *S. cerevisiae* strains used in this study are listed in Table 1. The compositions of the media for growth of yeast were: 2% glucose, 1% yeast extract, 2% peptone (YPD); 2% galactose, 1% yeast extract, 2% peptone (YPGal); and 2% ethanol, 3% glycerol, 1% yeast extract, 2% peptone (YEPG).

Preparation of yeast mitochondria and ATPase assays: Mitochondria were prepared by the method of Faye *et al.* (1974) except that Zymolyase 20,000 (MP Biochemicals, Aurora, OH) instead of glusulase was used to convert cells to spheroplasts. ATPase activity was assayed by measuring release of inorganic phosphate (King 1932) from ATP at 37° in the presence and absence of oligomycin.

***In vivo* and *in organello* labeling of mitochondrial gene products:** Cells grown overnight in YPGal were inoculated into

TABLE 1

Genotypes and sources of yeast strains

Strain	Genotype	Source
W303-1A	**a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	[a]
W303-1B	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	[a]
aM10-150-4D	**a** *ade1 cox1*	Tzagoloff *et al.* (1975)
aM9-94-4B	**a** *ade1 cox2*	Tzagoloff *et al.* (1975)
aM9-3-6C	**a** *ade1 cox3*	Tzagoloff *et al.* (1975)
aM7-40-5D	**a** *ade1 cob*	Tzagoloff *et al.* (1975)
aM6-200-2C	**a** *ade1 cob*	Tzagoloff *et al.* (1975)
aJC3/M28-82	**a** *kar1 lys1 ade2 atp6*	This study
C208	α *met6 atp22-1*	Helfenbein *et al.* (2003)
C290	α *met6 atp22*	Helfenbein *et al.* (2003)
C329	α *met6 atp22*	Helfenbein *et al.* (2003)
E44	α *met6 atp22*	Helfenbein *et al.* (2003)
E675	α *met6 atp22*	Tzagoloff and Dieckmann (1990)
N417	α *met6 atp22*	Helfenbein *et al.* (2003)
N608	α *met6 atp22*	Tzagoloff and Dieckmann (1990)
W303ΔATP22	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp22::HIS3*	Helfenbein *et al.* (2003)
aW303ΔATP22	**a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp22::HIS3*	Helfenbein *et al.* (2003)
W303ΔATP22/R1, -R2, -R3	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp22::HIS3* (ρ^+, ρ^{-supR})	This study
W303ΔATP22/ρ^{-supR3}	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp22::HIS3* (ρ^{-sup})	This study
aW303/I°	**a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* (intronless ρ^+ mtDNA)	This study
aMY375/I°	**a** *ura3-52,his4Δ34 kar1* (intronless mtDNA)	[b]
aW303ΔATP22/R3/I°	**a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp22::HIS3* (intronless ρ^+mtDNA, ρ^{-supR3})	aMY375/I° × W303ΔATP22/ρ^{-supR3}

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10 ml of medium containing 0.67% yeast nitrogen base without amino acids, the appropriate auxotrophic requirements, and 2% galactose. Cells equivalent to an A_{600} of 0.5 were harvested at a growth density of 1–2 A_{600}. After centrifugation and washing with 40 mm potassium phosphate pH 6 containing 2% galactose, the cells were suspended in 500 μl of the same buffer and 10 μl of a freshly prepared aqueous solution of cycloheximide (7.5 mg/ml) were added. The cells were incubated at 24° for 5 min prior to addition of 4 μl of [^{35}S]methionine (10 Ci/ml). The reaction was terminated after 30 min with 500 μl of 20 mm methionine and 75 μl of 1.8 m NaOH, 1 m β-mercaptoethanol, and 0.01 m phenylmethylsulfonyl fluoride. An equal volume of 50% TCA was added and the mixture was centrifuged. The precipitated proteins were washed once with 0.5 m Tris (free base) and two times with water and were suspended in 50 μl of sample buffer (Laemmli 1970).

Mitochondria prepared by the method of Herrmann *et al.* (1994) were labeled at 24° with [^{35}S]methionine for 30 min (Hell *et al.* 2000). The radiolabeled proteins were separated on a 12.5% polyacrylamide gel containing 4 m urea and 25% glycerol, transferred to nitrocellulose, and exposed to Kodak X-OMAT film.

Extraction and Northern blot analysis of mitochondrial RNAs: Mitochondria were solubilized in 1% sodium dodecyl sulfate (SDS), 10 mm Tris–Cl, pH 7.5, 0.1 m NaCl at a concentration of ~10 mg/ml and immediately extracted with an equal volume of water-saturated phenol. After separation of the two phases by centrifugation at 2000 × *g* for 10 min, the upper phase was collected and nucleic acids were precipitated with 3 vol of ethanol in the presence of 0.25 m NaCl. The precipitate was rinsed twice with 80% ethanol and dried under vacuum.

Equivalent amounts of RNA were separated on a 1% agarose gel, stained with ethidium bromide, and blotted to a nylon membrane (Nytran, SuPerCharge; Schleicher & Schuell, Keene, NH). Following crosslinking with UV light, the blot was prehybridized at 43° for 4 hr with 125 μg of salmon sperm DNA in 5× SSC, 5× Denhardt's, 0.5% SDS. The blot was hybridized overnight at 43° with probes from the first exon of *COX1*, first intron of *COX1*, and *ATP6*. The *COX1* intron and *ATP6* probes were labeled with [α-^{32}P]dATP by random priming (Feinberg and Vogelstein 1983). The COX1 exon probe was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Mapping of mitochondrial suppressors: Revertant cells were converted to ρ^- mutants (respiratory-deficient mutants with a partially deleted mitochondrial genome) with ethidium bromide (Slonimski and Tzagoloff 1976). Purified mutants were crossed to the *atp22* mutant and to testers with mutations in the mitochondrial *COX1*, *COX2*, *COX3*, *COB*, or *ATP6* genes. After prototrophic selection on minimal glucose, the diploid cells were replicated on rich glycerol/ethanol medium (YEPG) and scored for growth after incubation for 2–3 days at 30°.

Miscellaneous procedures: Standard methods were used for the preparation and ligation of DNA fragments and for transformation and recovery of plasmid DNA from *Escherichia coli* (Maniatis *et al.* 1989). Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). Western blots were treated with a rabbit polyclonal antibody against yeast subunit 6 followed by a second reaction with peroxidase-coupled anti-rabbit IgG. The antibody complexes

were visualized with the Super Signal chemiluminescent substrate kit (Pierce Chemical, Rockford, IL). Protein concentrations were determined by the method of Lowry *et al.* (1951).

RESULTS

***In vivo* and *in organello* labeling of mitochondrial translation products:** Complementation group G99 consists of nine independent *atp22* point mutants (Tzagoloff and Dieckmann 1990). Western analysis of ATPase subunits indicated that *atp22* point mutants are grossly deficient in subunit 6 (Helfenbein *et al.* 2003). A subunit 6 deficit is not necessarily related to translation of the protein or absence of the mRNA (Ellis *et al.* 2004) but can also be caused by its turnover in mutants defective in F_0 assembly (Paul *et al.* 1989, 2000; Arselin *et al.* 1996). In our earlier studies of the *atp22* mutant, N417, some incorporation of ^{35}S-methionine into subunit 6 was observed when cells were labeled *in vivo* in the presence of cycloheximide to arrest cytoplasmic protein synthesis (Helfenbein *et al.* 2003). In subsequent studies we found that with the exception of N417, other *atp22* mutants tested failed to synthesize subunit 6 when assayed either *in vivo* with whole cells (Figure 1A) or *in organello* with isolated mitochondria (Figure 1C). The *atp22* mutants are able to translate subunits 8 and 9, the other two mitochondrially encoded constituents of F_0 (Figure 1B). The slow growth of N417 on rich glycerol/ethanol medium suggests that the genetic lesion does not abolish expression of F_0 totally, which would explain why this strain is able to synthesize subunit 6. Like other ATPase mutants that display a pleiotropic deficiency of cytochrome oxidase (Marzuki *et al.* 1989; Paul *et al.* 1989), most of the *atp22* mutants showed a substantial decrease in translation of subunit 1 of this respiratory complex.

Revertants of the atp22 null mutant: Revertants of the *atp22* null mutant with extragenic suppressors were isolated by spreading W303ΔATP22 at high density on rich glycerol/ethanol medium. Revertant colonies became discernible after 3–4 days and continued to appear over a period of several weeks. Three revertants were purified and their growth on glycerol/ethanol was compared to the mutant and the wild type parental strain. Unlike the null mutant, which has a very clear growth defect on glycerol and ethanol, the three revertants grew on the nonfermentable carbon sources but at a rate considerably slower than that of the parental wild type (Figure 2A).

Several criteria were used to ascertain that the revertants assemble a functional F_0. Sensitivity of mitochondrial ATPase to oligomycin is a simple and reliable test for the presence of a functional F_1–F_0 complex. The partial restoration of the F_1–F_0 complex in the revertants is supported by the 10–15% inhibition of the mitochondrial ATPase activity by oligomycin (Table 2). The presence of F_0 is also supported by the detection of

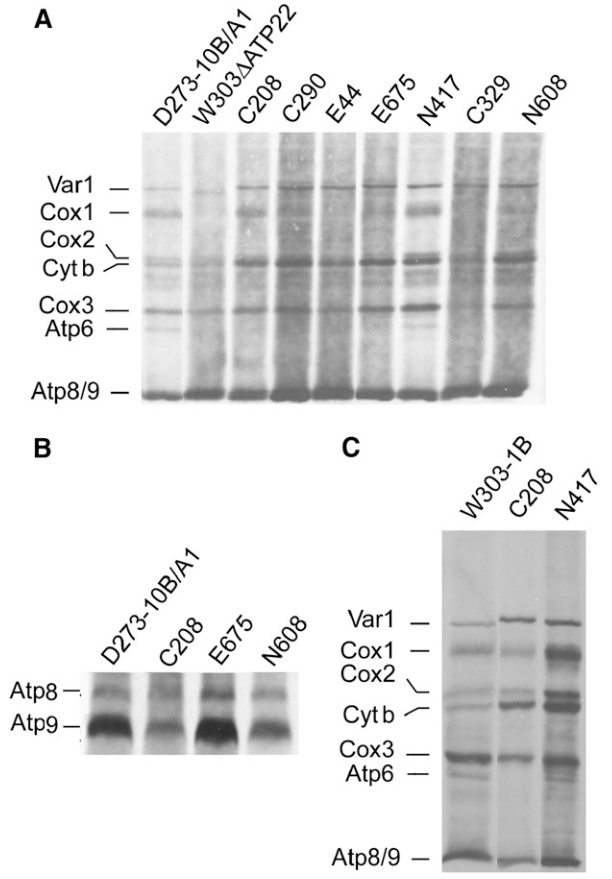

Figure 1.—*In vivo* and *in organello* labeling of mitochondrial gene products. (A) The wild-type strain D273-10B/A1, the *atp22* null mutant W303ΔATP22, and seven independent *atp22* point mutants were labeled with ^{35}S-methionine in the presence of cycloheximide as detailed in materials and methods. Total cellular proteins were separated on a 12.5% polyacrylamide gel containing 4 M urea and 25% glycerol. The proteins were transferred to nitrocellulose paper and exposed to X-ray film. The radioactively labeled products of mitochondrial translation are identified in the margin. (B) The wild type and *atp22* mutants were labeled as in A but separated on a 17.5% polyacrylamide gel (Laemmli 1970) without urea or glycerol to increase the resolution between subunits 8 and 9 of the ATPase. (C) Mitochondria of the wild-type strain W303-1B and two different *atp22* point mutants were labeled with ^{35}S-methionine as described in materials and methods. The proteins were separated and visualized as in A.

subunit 6 in mitochondria of the revertant (Figure 2B), although in much lower concentrations than in wild type.

Suppression depends on the coexistence in the atp22 null mutant of wild type and a ρ^- mitochondrial genome: The suppressors in the three *atp22* revertants behaved as dominant mutations in mtDNA. Diploid cells issued from crosses of the revertants to the mutants displayed the growth phenotype of the haploid revertants. When the crosses to the *atp22* mutant were repeated with revertants that had been converted to ρ^o mutants (respiratory-deficient mutants lacking mitochondrial

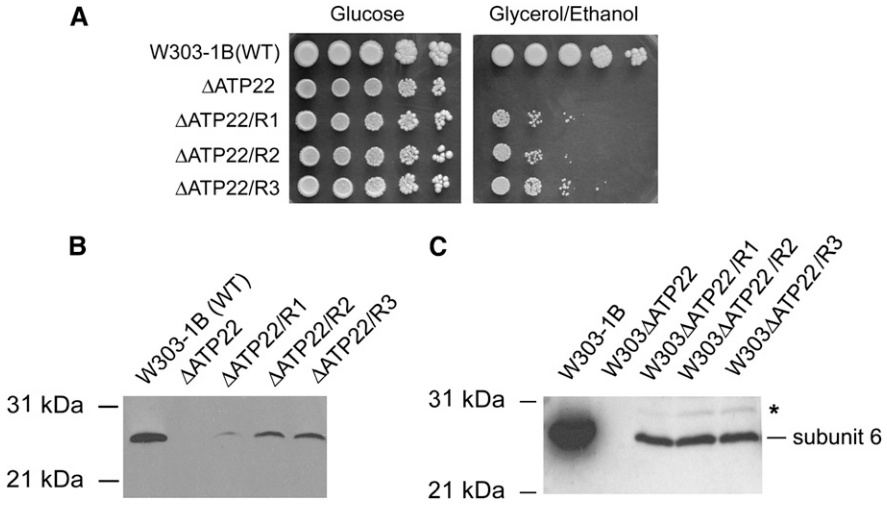

FIGURE 2.—Growth properties and expression of subunit 6 in *atp22* revertants. (A) Serial dilutions of the wild-type parental strain W303-1B, of the *atp22* null mutant W303ΔATP22 (ΔATP22), and of three different revertants W303ΔATP22/R1, -R2, and -R3 (ΔATP22/R1, -R2, and -R3) were spotted on rich glucose and glycerol plus ethanol medium. The plates were incubated at 30° for 3 days. (B) Mitochondria were prepared from the wild type, the *atp22* null mutant, and the three revertants used for the growth tests shown in A. Total mitochondrial proteins (40 μg) were separated on a 12% polyacrylamide gel (LAEMMLI 1970), transferred to nitrocellulose, and reacted with a rabbit polyclonal antibody against subunit 6 of the yeast mitochondrial ATPase. The blot was further reacted with anti-rabbit IgG and developed with the Super Signal chemiluminescent kit (Pierce Chemical, Rockford, IL). (C) Mitochondria (40 μg) prepared from the same strains as in A were separated by SDS–PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose paper, and probed as in B with twice the concentration of the rabbit polyclonal antibody against subunit 6. The bands corresponding to subunit 6 and the novel protein (asterisk) are marked.

DNA), none of the diploid progeny grew on glycerol/ethanol, indicating that the suppressors were dominant mutations in mtDNA.

Unlike normal mtDNA mutations, which are transmitted to vegetative progeny, the *atp22* suppressors were very unstable and rapidly lost when revertants were grown under nonselective conditions (Table 3). Following overnight growth of the revertant W303ΔATP22/R3 in rich glucose medium, 94% of the cells had lost the ability to grow on nonfermentable substrates. When grown in selective medium (glycerol/ethanol), the percentage of respiratory-competent cells was increased to 25%. The rapid loss of respiratory function, even under selective conditions, suggested that the revertants might be heteroplasmic cells containing both the wild-type and suppressor ρ⁻ genomes. This type of mitochondrial suppressor has been reported for a number of mitochondrial protein-coding genes that fail to be expressed because of mutations in nuclear gene products that promote translation of the mRNAs by interacting with their 5′-UTRs (DIECKMANN *et al.* 1984; MULLER *et al.* 1984; RODEL and FOX 1987; MANTHEY and MCEWEN 1995). The presence in W303ΔATP22/R3 of a suppressor ρ⁻ genome was confirmed by the mitochondrial genotypes of 104 respiratory-deficient segregants (Table 3). Approximately 72% of the cells analyzed were not complemented by either the ρ° tester or the *atp22* null mutant. These correspond to revertants that lost the suppressor and because of the instability of wild-type mtDNA in the *atp22* null background had degraded to ρ° and ρ⁻ mutants. A second genotype, represented by 24% of the segregants corresponded to cells that had lost the suppressor but retained wild-type mtDNA. These cells were complemented by the ρ° tester but did not confer respiratory growth when they were backcrossed to the null mutant. The least frequent segregants represented by only five of the colonies tested were not complemented by the ρ° tester but were able to restore respiratory growth to the mutant. These segregants had lost the wild-type genome but had retained the ρ⁻ suppressor genome.

TABLE 2

Mitochondrial ATPase activities in wild type and *atp22* mutants

Strain	ATPase activity (μmol/mg/min) − Oligomycin	ATPase activity (μmol/mg/min) + Oligomycin	Inhibition (%)
W303-1A (WT)	4.80	1.20	75
W303ΔATP22	3.15	3.09	1.9
W303ΔATP22/R1	3.38	3.03	10.4
W303ATP22/R2	3.84	3.37	12.2
W303ΔATP22/R3	3.89	3.33	14.4

W303ΔATP22/R1, -R2, and -R3 are three independent revertants of W303ΔATP22. ATPase activity was measured at 37° in the absence and presence of 10 μg/ml oligomycin.

A more complete genotype of the ρ⁻ suppressor mtDNA was obtained from crosses of one of the segregants that complemented the *apt22* mutant but was not complemented by the ρ⁻ testers, to different mit⁻ mutants (mit⁻ mutants have loss-of-function point mutations or deletions in mitochondrial genes). Crosses of the mutant with the ρ⁻ suppressor to mit⁻ testers indicated the presence in the suppressor genome of the *OLI2/ATP6* and *COB* but not the *COX1*, *COX2*, and *COX3* markers. The *cox1* tester (aM10-150-4D) used in these crosses contained a large deletion in *COX1*.

Mapping and sequence analysis of the ρ⁻ suppressor: The locus of mtDNA responsible for suppressing the *atp22* null mutation was assessed by ρ⁻ deletion mapping.

TABLE 3

Segregation of suppressor in W303ΔATP22/R3

Strain	Total no. of respiration-deficient segregants analyzed	No. of segregants	Growth on YPEG: Haploid cells	Growth on YPEG: Diploid cells × ΔATP22	Growth on YPEG: Diploid cells × CB11ρ°	Genotype
ΔATP22/R3	104	75	−	−	−	ρ^- or ρ^o
		24	−	−	+	ρ^+
		5	−	+	−	ρ^{-supR3}

CB11ρ° is an "**a**" mating-type strain with an *ade1* mutation and lacking mtDNA.

A library of ρ⁻ clones was obtained by mutagenesis of the revertant W303ΔATP22/R3 with ethidium bromide. Crosses of the purified clones to the *atp22* null mutant and to several mit⁻ testers representing different regions of mtDNA indicated that the suppressor activity was coretained with the *ATP6* but not the *COB* marker (data not shown). This result pointed to *ATP6* as the target of Atp22p.

The ρ⁻ genome of one of the clones, containing only the *ATP6* marker, was partially sequenced. The sequence indicated that the suppressor genome had the wild-type *ATP6* sequence except for the absence of the normal 5′-UTR and the first three codons of the gene. In their place the second nucleotide of the fourth codon of *ATP6* was fused to the 3′ end of the first intron of *COX1* (Figure 3A). Although the ρ⁻ mtDNA was not sequenced in its entirety, the continuous sequence obtained indicated that the entire *COX1* 5′-UTR together with its first exon and the entire first intron was fused to the *ATP6* coding sequence (Figure 3B). The same gene fusion was found in the mtDNA isolated from the revertants W303ΔATP22/R1 and W303ΔATP22/R2. The sequence of the ρ⁻ mtDNA also indicated the presence of *ENS2* (Nakagawa *et al.* 1991), a gene that lies downstream of *ATP6* in the mitochondrial genome of W303.

Analysis of mitochondrial transcripts in the atp22 revertant: The presence of subunit 6 in the *atp22* revertants indicates that this mitochondrial gene product is translated from a transcript emanating from the *ATP6/COX1* fusion gene in the ρ⁻ suppressor. In wild type, *COX1* is cotranscribed with *ATP6*, *ATP8*, and *ENS2* (Pelissier *et al.* 1995). This primary polycistronic transcript is cleaved after *ENS2* and at two sites 600 bp apart located between *COX1* and *ATP8* (Pelissier *et al.*

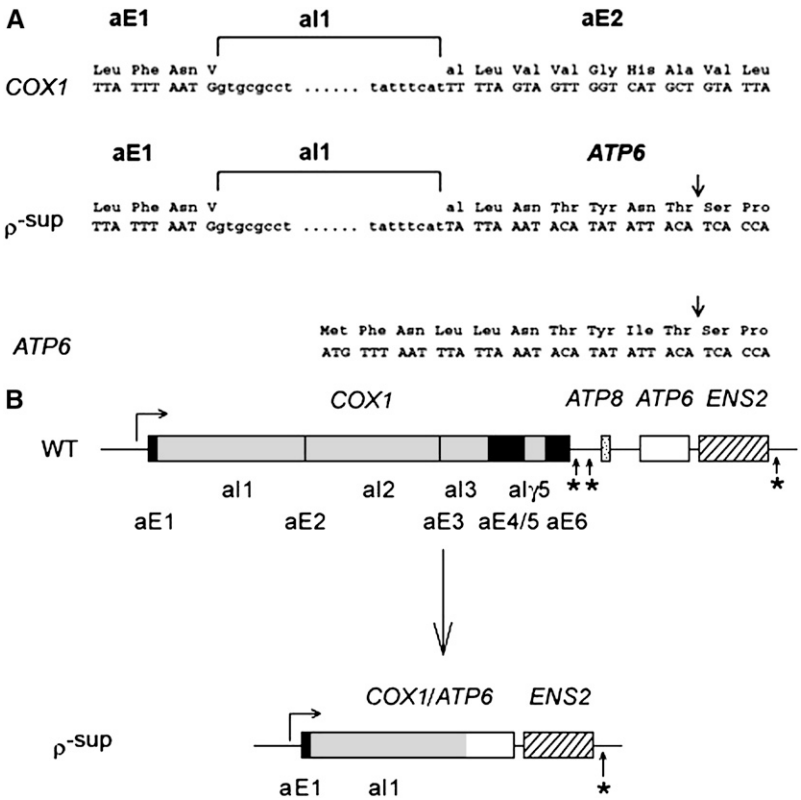

FIGURE 3.—Sequence and structure of the *ATP6* suppressor. (A) Sequence at the junction of the 3′ end of *COX1* intron aI1 and *ATP6*. The sequence of wild-type *ATP6* near the N-terminal coding region of the gene is shown at the bottom. The cleavage site at which the N-terminal 10 residues of the subunit 6 precursor are removed is shown by the arrow. The top sequence shows the aE1/aI1 and aE2/aI1 junctions of *COX1*. The ρ⁻ suppressor with the aE1–aI1 sequence of *COX1* fused to the last two nucleotides of the fourth codon of *ATP6* is shown in the middle. The exonic sequences of *COX1* and the coding sequence of *ATP6* are in uppercase boldface type and the aI1 intron sequence is in lowercase type. The *COX1* and *ATP6* coding sequences are translated above the sequence. (B) The top diagram shows the *COX1* gene of the W303 strain with exons aE1–aE6 indicated by the solid boxes and lines and introns aI1–aIγ5 by the shaded boxes. The downstream *ATP8*, *ATP6*, and *ENS2* genes are represented by the stippled, open, and hatched boxes, respectively. The start of transcription of this region is indicated by the arrow and the processing sites of the primary transcript by the asterisks. The region of the ρ⁻ suppressor with the hybrid *ATP6* and downstream *ENS2* genes is shown in the bottom diagram.

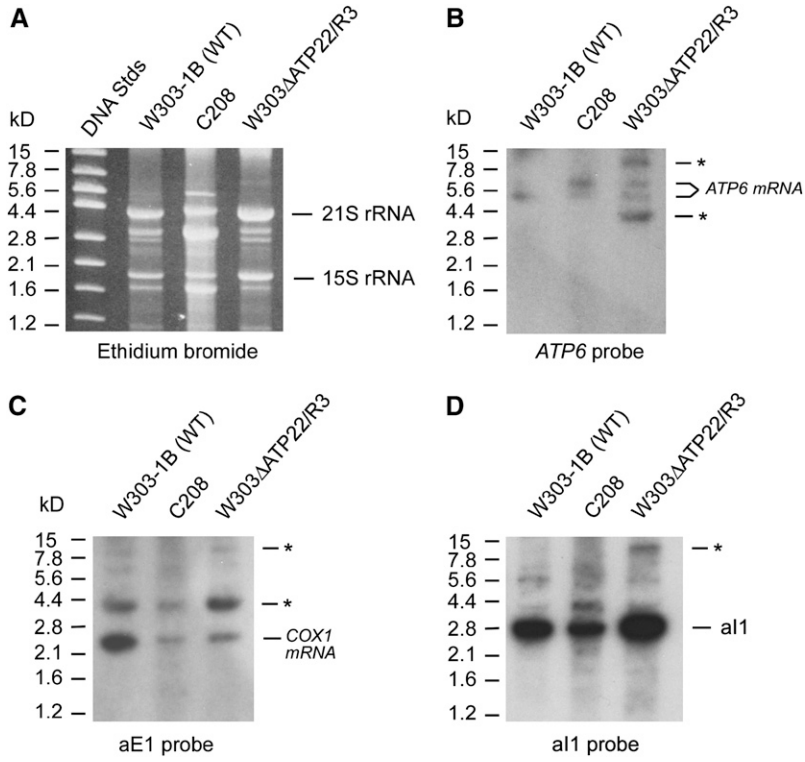

FIGURE 4.—Northern analysis of *COX1* and *ATP6* transcripts. RNAs were extracted as described in MATERIALS AND METHODS from mitochondria of the wild-type strains W303-1B, the *atp22* point mutant C208, and the revertant W303ΔATP22/R3. The RNAs were separated on a 1% agarose gel, stained with ethidium bromide, and photographed (A). The RNAs were transferred to a Nytron membrane and the blot was hybridized to *ATP6* (B), *COX1* exon aE1 (C), and *COX1* intron aI1 (D) ^{32}P-labeled probes. The migration of double-stranded DNA standards is indicated next to each part. The 21S and 15S rRNAs are identified in A. The 5.2- and 4.6-kb *ATP6* mRNAs are marked in B. Processing of the larger 5.2-kb transcript to the 4.6-kb transcript is less efficient in the mutant and the revertant than in wild type. The novel transcripts detected by the *ATP6*, aE1, and aI1 probes are indicated by asterisks.

1995). These endonucleolytic cleavages produce two distinct *ATP6* mRNAs of 4.6 and 5.2 kb, the latter having a 5′ terminus coincident with the 3′ end of *COX1* mRNA. On the basis of the structure of *COX1/ATP6* in the suppressor and assuming transcription of the gene is initiated from the normal *COX1* promoter, the primary transcript should be 6.6 kb. This transcript should be reduced by 2.5 kb after splicing of the *COX1* intron. Mitochondrial RNAs were prepared from wild type, the *atp22* point mutant C208, and the revertant W303ΔATP22/R3. The point mutant was used instead of the null mutant because of the extreme instability of wild-type mtDNA in the latter. To maximize the number of heteroplasmic cells with both wild-type and suppressor genomes, the revertant was grown in rich glycerol/ethanol medium. Even under these selective growth conditions, only 24% of the cells were ascertained to be heteroplasmic following overnight growth. Northern blots of the different RNAs from the three strains were hybridized to an *ATP6* probe, a *COX1* exonic probe, and a probe from the first intron of *COX1*. The *ATP6* probe hybridized to the expected 4.6- and 5.2-kb transcripts in the wild type and the point mutant. (Since the agarose gel was run under nondenaturing conditions the true sizes of all the RNAs should be less than those estimated from their positions relative to the DNA size standards.) The two *ATP6* transcripts were less abundant in the mutant because 75% of the cells used to prepare mitochondria consisted of ρ^- and ρ^o mutants. The 4.6- and 5.2-kb transcripts were present in the revertant but in addition two novel transcripts of ~4 and 7 kb were also detected (Figure 4B). Hybridization of the Northern blot with a *COX1* exon aE1 probe revealed the expected *COX1* mRNA of 2.2 kb and a less abundant 4-kb transcript corresponding to a partially spliced intermediate in wild type and the mutant (Figure 4C). The *COX1* exonic probe detected the same transcripts in the revertant but the larger 4-kb transcript was more abundant relative to the 2.2-kb mRNA, implying that more than one transcript contribute to the 4-kb signal in this strain. The similar migration of the 4-kb transcripts detected by the *COX1* aE1 and the *ATP6* probes in the revertant suggests that the *ATP6* transcript is responsible for the stronger signal. This was confirmed by hybridization of the aE1 probe to mitochondrial RNA from a revertant stain (aW303ΔATP22/R3/I^o) in which the ρ^+ genome of W303 was substituted by the ρ^+ genome of aMY375/I^o, a strain devoid of all mitochondrial introns. In this background there was no contribution of the partially spliced *COX1* transcript in the 4-kb region (not shown). The different RNAs were also hybridized to a *COX1* aI1 intron probe. This probe detected the aI1 circular group II intron in all three strains (Figure 4D). The probe also revealed a 6.6-kb transcript in the revertant. This transcript was similar to the 6.6-kb RNA seen in the Northern of the revertant hybridized to the *ATP6* and *COX1* exon aE1 probes (Figure 4, B and C).

On the basis of the results of these hybridizations we conclude that the 6.6-kb *ATP6* transcript in the revertant contains a hybrid gene in which the aE1 exon and aI1 intron of *COX1* are joined to the fourth codon of *ATP6* as shown in Figure 3. The novel 4-kb transcript is a

spliced product of the 6.6-kb transcript from which the intron has been excised, presumably at the same splice sites as in the native *COX1* gene, resulting in a transcript (*COX/ATP6*) with an in-frame fusion of the *COX1* aE1 exonic sequence to the *ATP6* sequence.

Unprocessed subunit 6 in the atp22 revertant: The primary translation product expressed from the spliced novel *COX1/ATP6* mRNA of the revertant should be a hybrid protein starting with the entire sequence encoded by the first exon of *COX1* followed by the subunit 6 sequence beginning with the fourth codon of the ATPase sequence and differing only at that position where the leucine is substituted by a valine (Figure 3A). The valine codon is formed as a result of splicing of the aI1 intron. This protein has a predicted mass of 35 kDa or ~6 kDa larger than that of the normal mature subunit 6. *In vivo* labeling of mitochondrial gene products disclosed the presence in the revertants but not in wild type of a novel protein that migrated to a position midway between the 31- and 21-kDa markers (not shown). A protein with the same migration was also detected by Western analysis of mitochondria with an antibody to subunit 6 (Figure 2C), confirming the identity of this protein as the primary translation product from the fusion gene.

Since the primary translation product of the *COX/ATP6* fusion gene retains the proteolytic cleavage site of the subunit 6 precursor (Figure 3A), processing would be expected to remove the sequence encoded by the first exon of COX1 together with the remaining seven residues of the subunit 6 presequence. The protein resulting from this cleavage has a sequence identical to that of wild-type mature subunit 6. This is supported by the identical electrophoretic behavior of subunit 6 in wild type and the revertants (Figure 2, B and C).

DISCUSSION

The proton-translocating ATPase is composed of the two functionally distinct sectors, F_1 and F_0. The yeast nuclear *ATP22* gene was previously shown to code for a mitochondrial protein essential for assembly of F_0 (Helfenbein *et al.* 2003). The earlier studies, however, fell short of defining the function of Atp22p. Here we present evidence that Atp22p is required for translation of ATPase subunit 6 encoded by the mitochondrial *ATP6* gene.

Both *in vivo* and *in organello* labeling of mitochondrial gene products indicate that mutations in *ATP22* elicit a deficit of subunit 6. The failure of *atp22* mutants to synthesize subunit 6 can stem from instability/impaired processing of the *ATP6* transcript or inability of the mutants to translate the protein. Subunit 6 is translated from two transcripts of 4.6 and 5.2 kb, both of which contain *ATP6*, *ATP8*, and *ENS2*. The latter gene is not present in all yeast strains but is a feature of the mitochondrial genome of W303. *ATP8* and *ENS2* code for subunit 8 of the ATPase (MacReadie *et al.* 1983) and an endonuclease (Nakagawa *et al.* 1991), respectively. Three nuclear genes have been implicated in processing and translation of the transcript. Mutations in *NCA2* and *NCA3* when present in the same strain produce an increased ratio of the 4.6-kb relative to the 5.2-kb transcript and decreased translation of subunits 6 and 8 (Camougrand *et al.* 1995; Pelissier *et al.* 1995). Mutations in *AEP3* render the *ATP6/ATP8/ENS2* transcripts unstable (Ellis *et al.* 2004). Northern analysis of *ATP6* transcripts in an *atp22* point mutant indicates the presence of both the 4.6- and the 5.2-kb transcripts, although processing of the larger 5.2- to the 4.6-kb transcript is less efficient than in wild type. The presence of the two mRNAs suggests that the absence of subunit 6 is unlikely to be caused by instability of the *ATP6* mRNAs or a defect in processing of the primary transcript, which in addition to the aforementioned *ATP6*, *ATP8*, and *ENS2* also contains *COX1* (Pelissier *et al.* 1992). The Northern results instead imply that the subunit 6 deficit is caused by a translation defect.

Translation of subunit 6, oligomycin sensitivity, and growth of the *atp22* null mutant on nonfermentable carbon sources are partially restored by mutations in mtDNA. The mitochondrial suppressors in the three revertants studied have been mapped to *ATP6* and shown to consist of ρ^- genomes with rearrangements such that the 5′-UTR and first exon and intron of *COX1* are fused to *ATP6* starting with the fourth codon of the gene. The partial rescue of the *atp22* mutant by the ρ^- suppressor depends on its coexistence with a ρ^+ genome in the same cell. This heteroplasmic condition is very unstable and under nonselective conditions the revertant phenotype is rapidly lost as a result of rapid segregation of the two mtDNAs. The requirement of both genomes for respiratory growth suggested that the subunit 6 detected in the revertant is derived from the ρ^- suppressor while all the other gene products are expressed from the ρ^+ genome. Translation of subunit 6 in the revertant, therefore, is a function of the substitution of the *COX1* sequence for the normal 5′-UTR of *ATP6*.

Mitochondrial ρ^- genomes with heterologous 5′ upstream sequences have been shown to suppress mutations in translation factors for *COX1* (Manthey and McEwen 1995), *COX2* (Poutre and Fox 1987), *COX3* (Muller *et al.* 1984), and *COB* (Rodel and Fox 1987). These transcript-specific translation factors interact with the 5′-UTRs of their cognate mRNAs. In each case the presence of the suppressor obviates the requirement for the missing or mutated translation factor by recruitment of an alternate factor specific for the new 5′-UTR (Costanzo and Fox 1988, 1990). In this study translation of subunit 6 in the *atp22* revertants is probably mediated by interaction of *COX1* translation factors with the *COX1* 5′-UTR in the novel *ATP6* transcript. Our results are, therefore, consistent with Atp22p being a translation factor specific for the *ATP6* mRNA. The

specificity of Atp22p for *ATP6* is also supported by the ability of *atp22* mutants to translate subunits 8 and 9. This indicates that even though *ATP6* and *ATP8* are located on the same transcript, their translation is controlled by different translation factors.

The ρ^- suppressor in the revertant is of some interest in itself. Unlike other previously described suppressors with novel 5′-UTR fusions, the *COX1/ATP6* gene contains not only the 5′-UTR but also the first exon and intron of *COX1*. The primary transcript resulting from the *COX1/ATP6* gene must be spliced to remove the intronic sequence and to achieve an in-frame fusion of the *COX1* aE1 exon to the *ATP6* coding sequence. This is supported by the presence in the *atp22* revertant of two new transcripts, only the larger of which hybridizes to the aI1 intron probe. Splicing of the first *COX1* intron depends on a splicing factor (maturase) encoded by the first intron of *COB* (De La Salle *et al.* 1982). The presence of *COB* in the ρ^- genome of W303ΔATP22/R3 accounts for its ability to function as a suppressor even in the background of intronless mtDNA.

The subunit 6 synthesized in the revertant has an electrophoretic migration identical to that of the wild-type protein, indicating that the protease responsible for processing the amino-terminal 10 residues of the primary translation product (Michon *et al.* 1988) can also process the new hybrid protein, most likely at its normal site despite the presence of the N-terminal sequence of cytochrome oxidase subunit 1. That the primary translation product of the *COX1/ATP6* fusion gene contains the sequence encoded by aE1 is supported by the presence in the revertant of a novel translation product of a size commensurate with the extra residues contributed by the aE1 sequence. This protein is detected both by pulse labeling of mitochondrial translation products in the revertant cells and by Western blot analysis of mitochondria with an antibody against subunit 6.

This study identifies a new translational factor specific for subunit 6 of the ATPase. Currently *ATP8* and *VAR1* are the only genes of the yeast mitochondrial genome for which translational factors are not known.

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LITERATURE CITED

Ackerman, S. H., and A. Tzagoloff, 1990 Identification of two nuclear genes (*ATP11, ATP12*) required for assembly of the yeast F_1-ATPase. Proc. Natl. Acad. Sci. USA **87:** 4986–4990.

Ackerman, S. H., and A. Tzagoloff, 2005 Function, structure, and biogenesis of mitochondrial ATP synthase. Prog. Nucleic Acid Res. Mol. Biol. **80:** 95–133.

Arselin, G., J. Vaillier, P. V. Graves and J. Velours, 1996 ATP synthase of yeast mitochondria. Isolation of the subunit h and disruption of the *ATP14* gene. J. Biol. Chem. **271:** 20284–20290.

Camougrand, N., P. Pelissier, J. Velours and M. Guerin, 1995 *NCA2*, a second nuclear gene required for the control of mitochondrial synthesis of subunits 6 and 8 of ATP synthase in *Saccharomyces cerevisiae*. J. Mol. Biol. **247:** 588–596.

Costanzo, M. C., and T. D. Fox, 1988 Specific translational activation by nuclear gene products occurs in the 5′ untranslated leader of a yeast mitochondrial mRNA. Proc. Natl. Acad. Sci. USA **85:** 2677–2681.

Costanzo, M. C., and T. D. Fox, 1990 Control of mitochondrial gene expression in *Saccharomyces cerevisiae*. Annu. Rev. Genet. **24:** 91–113.

De La Salle, H., C. Jacq and P. P. Slonimski, 1982 Critical sequences within mitochondrial introns: pleiotropic mRNA maturase and cis-dominant signals of the box intron controlling reductase and oxidase. Cell **28:** 721–732.

Dieckmann, C. L., T. J. Koerner and A. Tzagoloff, 1984 Assembly of the mitochondrial membrane system. *CBP1*, a yeast nuclear gene involved in 5′ end processing of cytochrome b pre-mRNA. J. Biol. Chem. **259:** 4722–4731.

Ellis, T. P., K. G. Helfenbein, A. Tzagoloff and C. L. Dieckmann, 2004 Aep3p stabilizes the mitochondrial bicistronic mRNA encoding subunits 6 and 8 of the H^+-translocating ATP synthase of *Saccharomyces cerevisiae*. J. Biol. Chem. **279:** 15728–15733.

Faye, G., C. Kujawa and H. Fukuhara, 1974 Physical and genetic organization of petite and grande yeast mitochondrial DNA. IV. *In vivo* transcription products of mitochondrial DNA and localization of 23 S ribosomal RNA in *petite* mutants of *Saccharomyces cerevisiae*. J. Mol. Biol. **88:** 185–203.

Feinberg, A. P., and B. Vogelstein, 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13.

Helfenbein, K., T. Ellis, C. L. Dieckmann and A. Tzagoloff, 2003 *ATP22*, a nuclear gene required for expression of the F_0 sector of mitochondrial ATPase in *Saccharomyces cerevisiae*. J. Biol. Chem. **278:** 19751–19756.

Hell, K., A. Tzagoloff, W. Neupert and R. A. Stuart, 2000 Identification of Cox20p, a novel protein involved in the maturation and assembly of cytochrome oxidase subunit 2. J. Biol. Chem. **275:** 4571–4578.

Herrmann, J. M., H. Foelsch, W. Neupert and R. A. Stuart, 1994 Isolation of yeast mitochondria and study of mitochondrial protein translation, pp. 538–544 in *Cell Biology: A Laboratory Handbook*, Vol. I, edited by J. E. Celis. Academic Press, San Diego.

King, E. J., 1932 The colorimetric determination of phosphorus. Biochem. J. **26:** 292–297.

Laemmli, U. K., 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.

Lefebvre-Legendre, L., J. Vaillier, H. Benabdelhak, J. Velours, P. P. Slonimski *et al.*, 2001 Identification of a nuclear gene (*FMC1*) required for the assembly/stability of yeast mitochondrial F(1)-ATPase in heat stress conditions. J. Biol. Chem. **276:** 6789–6796.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:** 265–275.

Macreadie, I. G., C. E. Novitski, R. J. Maxwell, U. John, B. G. Ooi *et al.*, 1983 Biogenesis of mitochondria: the mitochondrial gene (*ATP8*) coding for mitochondrial ATPase subunit 8 in *Saccharomyces cerevisiae*. Nucleic Acids Res. **11:** 4435–4451.

Maniatis, T., E. F. Fritsch and J. Sambrook, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Manthey, G. M., and J. E. McEwen, 1995 The product of the nuclear gene *PET309* is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial *COX1* locus of *Saccharomyces cerevisiae*. EMBO J. **14:** 4031–4043.

Marzuki, S., L. C. Watkins and W. N. Choo, 1989 Mitochondrial H^+-ATPase in mutants of *Saccharomyces cerevisiae* with defective subunit 8 of the enzyme complex. Biochim. Biophys. Acta **975:** 222–230.

Michon, T., M. Galante and J. Velours, 1988 NH2-terminal sequence of the isolated yeast ATP synthase subunit 6 reveals post-translational cleavage. Eur. J. Biochem. **172:** 621–625.

Muller, P. P., M. K. Reif, S. Zonghou, C. Sengstag, T. L. Mason *et al.*, 1984 A nuclear mutation that post-transcriptionally blocks

accumulation of a yeast mitochondrial gene product can be suppressed by a mitochondrial gene rearrangement. J. Mol. Biol. **175:** 431–452.

Nakagawa, K., N. Morishima and T. Shibata, 1991 A maturase-like subunit of the sequence-specific endonuclease endo.SceI from yeast mitochondria. J. Biol. Chem. **266:** 1977–1984.

Paul, M. F., J. Velours, G. Arselin De Chateaubodeaup, M. Aigle and B. Guerin, 1989 The role of subunit 4, a nuclear-encoded protein of the F_0 sector of yeast mitochondrial ATP synthase, in the assembly of the whole complex. Eur. J. Biochem. **185:** 163–171.

Paul, M. F., A. Barrientos and A. Tzagoloff, 2000 A single amino acid change in subunit 6 of the yeast mitochondrial ATPase suppresses a null mutation in *ATP10*. J. Biol. Chem. **275:** 29238–29243.

Payne, M. J., E. Schweizer and H. B. Lukins, 1991 Properties of two nuclear *pet* mutants affecting expression of the mitochondrial *oli1* gene of *Saccharomyces cerevisiae*. Curr. Genet. **19:** 343–351.

Pelissier, P., N. Camougrand, G. Velours and M. Guerin, 1995 *NCA3*, a nuclear gene involved in the mitochondrial expression of subunits 6 and 8 of the F_o-F_1 ATP synthase of *S. cerevisiae*. Curr. Genet. **27:** 409–416.

Pelissier, P. P., N. M. Camougrand, S. T. Manon, G. M. Velours and M. G. Guerin, 1992 Regulation by nuclear genes of the mitochondrial synthesis of subunits 6 and 8 of the ATP synthase of *Saccharomyces cerevisiae*. J. Biol. Chem. **267:** 2467–2473.

Poutre, C. L., and T. D. Fox, 1987 *PET111*, a *Saccharomyces cerevisiae* nuclear gene required for translation of the mitochondrial mRNA encoding cytochrome *c* oxidase subunit II. Genetics **115:** 637–647.

Rodel, G., and T. D. Fox, 1987 The yeast nuclear gene *CBS1* is required for translation of mitochondrial mRNAs bearing the *cob* 5′ untranslated leader. Mol. Gen. Genet. **206:** 45–50.

Slonimski, P. P., and A. Tzagoloff, 1976 Localization in yeast mitochondrial DNA of mutations expressed in a deficiency of cytochrome oxidase and/or coenzyme QH2-cytochrome *c* reductase. Eur. J. Biochem. **61:** 27–41.

Tzagoloff, A., and C. L. Dieckmann, 1990 *PET* genes of *Saccharomyces cerevisiae*. Microbiol. Rev. **54:** 211–225.

Tzagoloff, A., A. Akai, R. B. Needleman and G. Zulch, 1975 Assembly of the mitochondrial membrane system. Cytoplasmic mutants of *Saccharomyces cerevisiae* with lesions in enzymes of the respiratory chain and in the mitochondrial ATPase. J. Biol. Chem. **250:** 8236–8242.

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