ATP25, a New Nuclear Gene of *Saccharomyces cerevisiae* Required for Expression and Assembly of the Atp9p Subunit of Mitochondrial ATPase

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We report a new nuclear gene, designated *ATP25* (reading frame YMR098C on chromosome XIII), required for expression of Atp9p (subunit 9) of the *Saccharomyces cerevisiae* mitochondrial proton translocating ATPase. Mutations in *ATP25* elicit a deficit of *ATP9* mRNA and of its translation product, thereby preventing assembly of functional F_0 . Unlike Atp9p, the other mitochondrial gene products, including ATPase subunits Atp6p and Atp8p, are synthesized normally in *atp25* mutants. Northern analysis of mitochondrial RNAs in an *atp25* temperature-sensitive mutant confirmed that Atp25p is required for stability of the *ATP9* mRNA. Atp25p is a mitochondrial inner membrane protein with a predicted mass of 70 kDa. The primary translation product of *ATP25* is cleaved in vivo after residue 292 to yield a 35-kDa C-terminal polypeptide. The C-terminal half of Atp25p is sufficient to stabilize the *ATP9* mRNA and restore synthesis of Atp9p. Growth on respiratory substrates, however, depends on both halves of Atp25p, indicating that the N-terminal half has another function, which we propose to be oligomerization of Atp9p into a proper size ring structure.

INTRODUCTION

Expression of the mitochondrial gene products of *Saccharomyces cerevisiae* depends on messenger-specific stabilization and translation factors, some of which have been shown to interact with sequences in the 5' untranslated leaders of their respective RNAs. Such factors have been described for the three mitochondrially encoded subunits of cytochrome oxidase, cytochrome *b* and subunits of the proton translocating ATPase or F_1 - F_0 complex (reviewed in Costanzo and Fox, 1990; Dieckmann and Staples, 1994). Two nuclear genes are known to be required for expression of Atp9p (subunit 9 or subunit c) of F_0 , the proton translocating sector of the ATPase complex. *AEP1/NCA1* has been shown to facilitate translation of Atp9p (Payne *et al.*, 1991; Payne *et al.*, 1993), whereas *AEP2/ATP13* acts at an earlier step by promoting stability and processing of the *ATP9* mRNA (Ackerman *et al.*, 1991; Ellis *et al.*, 1999). Similar factors have been described for Atp6p, another subunit of the F_0 sector (Zeng *et al.*, 2007a). Nuclear genes have also been described for proteins that promote posttranslational steps in the F_0 assembly

pathway. These include Atp10p and Oxa1p both of which have been shown to chaperone the interaction of Atp6p with the Atp9p ring (Tzagoloff *et al.*, 2004; Jia *et al.*, 2007) and Atp23p that cleaves the Atp6p precursor but in addition is required for some still unknown step of F_0 assembly (Zeng *et al.*, 2007b; Osman *et al.*, 2007).

Most of the currently known mRNA-specific translational and stabilization factors of yeast mitochondria have been identified through biochemical characterizations of respiratory-deficient mutants (*pet*) (Ebner *et al.*, 1973; Michaelis *et al.*, 1982; Tzagoloff and Dieckmann, 1990). As part of continuing efforts to identify and functionally characterize nuclear genes involved in mitochondrial biogenesis, mutants representative of different complementation groups in our collection of *pet* strains have been screened for defective ATPase. Here, we present evidence that the ATPase lesion of mutants in complementation group G29 stems from a deficit of Atp9p. The gene responsible for this phenotype, designated *ATP25*, corresponds to reading frame YMR098C on yeast chromosome XIII. Like Aep1p and Aep2p/Atp13p (Payne *et al.*, 1991; Payne *et al.*, 1993), the *ATP25* product seems to target solely Atp9p. We present evidence that the Atp9p deficit in *atp25* mutants is a consequence of increased turnover of *ATP9* mRNA, and we conclude that Atp25p is a new stabilization factor specific for the *ATP9* mRNA. In addition to stabilizing the *ATP9* mRNA, Atp25p acts posttranslationally by promoting assembly of Atp9p into the oligomeric ring structure. Our results indicate that RNA stability and oligomerization of Atp9p are catalyzed by two separate domains corresponding to the C- and N-terminal halves of Atp25p, respectively.

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Abbreviations used: BN, blue native; mtDNA, mitochondrial DNA; ρ^0 mutant, respiratory-deficient mutant lacking mitochondrial DNA; ρ^- mutant, respiratory-deficient mutant with a partially deleted mitochondrial genome; PAGE, polyacrylamide gel electrophoresis; *pet* mutant, respiratory-deficient mutant of yeast with a mutation in a nuclear gene; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

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 solid and liquid growth media have been described previously (Myers *et al.*, 1985).

Table 1. Genotype and sources of yeast strains

Strain	Nuclear genotype	mtDNA	Source
W303-1A	<i>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i>	ρ^+	Rodney Rothstein (Department of Human Genetics, Columbia University, New York, NY)
W303-1B	<i>MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i>	ρ^+	Rodney Rothstein
CB11 ρ^0	<i>MATa ade1</i>	ρ^0	ten Berge <i>et al.</i> (1974)
C279	<i>MATα met6 atp25-1</i>	ρ^+	Tzagoloff and Dieckmann (1990)
N210	<i>MATα met6 atp25</i>	ρ^+	Tzagoloff and Dieckmann (1990)
N124	<i>MATα met6 atp25</i>	ρ^+	Tzagoloff and Dieckmann (1990)
C279/U1	<i>MATα ura3-1 atp25-1</i>	ρ^+	C279 $\times$ W303-1A
aW303 Δ ATP25	<i>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp25::LEU2</i>	ρ^+	This study
W303 Δ ATP25	<i>MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp25::LEU2</i>	ρ^+	This study
aW303 Δ ATP25(H)	<i>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp25::HIS3</i>	ρ^+	This study
W303 Δ ATP25(H)	<i>MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp25::HIS3</i>	ρ^+	This study
W303 Δ ATP25/ST23	<i>MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp25::HIS3 + pG29/ST23</i>	ρ^+	This study
W303 Δ ATP25/ST40	<i>MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp25::HIS3 + pG29/ST40</i>	ρ^+	This study
aW303 Δ ATP25/ST32	<i>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp25::HIS3 + pG29/ST32</i>	ρ^+	This study
a/ α W303 Δ ATP25/ST23, ST32	<i>MATa/α ade2-1/ade2-1 his3-1,15/his3-1,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 + pG29/ST23 + pG29/ST32</i>	ρ^+	W303 Δ ATP25/ST23 $\times$ aW303 Δ ATP25/ST32
W303/ATP25ts-1	<i>MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp25::HIS3 atp25ts-1</i>	ρ^+	This study
W303 Δ ATP10	<i>MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp10::LEU2</i>	ρ^+	Ackerman and Tzagoloff (1990)
RKY48-1	<i>MATa ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG</i>	ρ^+ <i>atp6Δ2-10a</i>	Zeng <i>et al.</i> (2007c)

Preparation of Mitochondria

Depending on their use, mitochondria were prepared by one of several methods. For enzyme assays and most other purposes, mitochondria were obtained by the method of Faye *et al.* (1974) except that Zymolyase 20T was substituted for glucuronase during the conversion of cells to spheroplasts. Mitochondria with intact outer membrane, used to localize Atp25p and to label mitochondrial gene products in organelle, were isolated by the methods of Glick and Pon (1995) and Herrmann *et al.* (1994), respectively.

Cloning of ATP25

ATP25 was cloned by transformation of C279/U1 with a yeast genomic plasmid library consisting of partial Sau3A fragments of yeast nuclear DNA averaging 12 kb in YEp24 (Botstein and Davis, 1982). This library was kindly provided by Marian Carlson (Department of Human Genetics, Columbia University, NY). Transformation of $\sim 10^8$ cells with 10 μ g of library DNA yielded three uracil-independent and respiratory-competent clones, of which one clone (C279/U1/T2) was used to subclone the gene.

Construction of Clones Expressing the Entire and Partial Atp25p Tagged with hemagglutinin (HA)

A short sequence coding for the HA tag was introduced immediately after the termination codon of ATP25 by polymerase chain reaction (PCR) amplification of the region of ATP25 starting from the unique BstX1 site with primers 5'-cattttccaaacatttgagaga and 5'-ggcaagctttcaagcgtagctggagcgtc-gtatgggtattgattgtttgtccacgactag. The PCR fragment was digested with BstX1 and HindIII, and then it was substituted for the native sequence in pG29/ST8 (see Figure 3). The resultant plasmid pG29/ST17 contained ATP25 with an in-frame 27-nucleotide-long sequence coding for nine extra C-terminal amino acids constituting the HA tag. The fusion gene was also transferred as a SacI-HindIII fragment from pG29/ST17 to the CEN plasmid pRS316 (Sikorski and Hieter, 1989). This construct was designated as pG29/ST18.

The sequence coding for the N-terminal half of Atp25p was PCR amplified with primers 5'-ggcggatccgaactatcgtactttag and 5'-ggcctgcagtcgaagcgtatgctggagcgtcgtatggg-taataccttctcttggc. The fragment containing 300 base pairs of

5' untranslated sequence followed by the ATP25 sequence coding for the first 292 residues and ending with a short sequence coding for the HA tag was digested with BamHI and PstI, and cloned into YEp351 (Hill *et al.*, 1986). The resultant plasmid pG29/ST32 was used to transform aW303 Δ ATP25(H) to obtain aW303 Δ ATP25/ST32. Similarly, the sequence coding for the C-terminal half of Atp25p was amplified with primers 5'-ggcggatccatgtcaactatcaacccaaacggg and 5'-ggcaagctttcaagcgtagctggagcgtcgtatgggtattgattgtttgtccacggactag. This PCR product consisting of the C-terminal 332 codons of ATP25 preceded by a methionine codon and terminating with the sequence coding for the HA tag was digested with BamHI and HindIII, and cloned into YEp352 and YEp351 (Hill *et al.*, 1986), yielding pG29/ST23 and pG29/ST40. Both plasmids were introduced into W303 Δ ATP25(H).

Expression of trpE-ATP25 Fusion Proteins in Escherichia coli

A *trpE*/ATP25 fusion protein was obtained by PCR amplification of the sequence from the 313th codon to the end of the gene with primers 5'-caaaaggaagagctcaaaggagcc and 5'-tggtgtgaagatccacgttggga. This fragment was digested with SacI and BamHI and cloned in pATH20 (Koerner *et al.*, 1991). *E. coli* transformed with this construct expressed a protein of ~ 70 kDa, corresponding to the N-terminal half of anthranilate synthase component 1 fused to the C-terminal sequence of ATP25 starting from residue 313. The insoluble fusion protein was isolated from *E. coli*, dissociated in 1% SDS, and size fractionated on a Biogel A-5 column. The highly enriched protein was used to immunize rabbits (Koerner *et al.*, 1991).

Construction of a Temperature-sensitive (ts) Allele of atp25 in W303

ATP25 was mutagenized by PCR amplification of the gene under conditions causing misincorporation of deoxynucleotides (Barros *et al.*, 2002). The gene was amplified from pG29/T2 with primers 5'-ggcggatccgaactatcgtactttag and 5'-ggcggatccgaagattaagatataatatacagg in four separate reactions containing 0.03 mM MnCl₂, 1.25 mM MgCl₂, and 0.2 mM dNTPs but having a threefold lower concentration of one of the four deoxynucleotides. The prod-

ucts of the four reactions were pooled digested with BamHI and cloned into YCplac22, a centromeric plasmid containing the *TRP1* marker (Gietz and Sugino, 1988). The resultant library was used to transform the *atp25* null mutant W303ΔATP25(H). Tryptophan prototrophic transformants were obtained at 30°C and checked for growth at 37°C. The ts mutants W303/ATP25ts-1 was selected based on its clear ts growth phenotype on YEPG (rich glycerol/ethanol).

Miscellaneous Procedure

Standard methods were used for the preparation and ligation of DNA fragments and for transformation and recovery of plasmid DNA from *E. coli* (Sambrook *et al.*, 1989). The conditions for Northern hybridizations have been described previously (Barros *et al.*, 2006). The *ATP25* coding sequence plus 290 nucleotides of 5' and 500 nucleotides of 3' untranslated regions were sequenced by the method of Maxam and Gilbert (1977). The sequence obtained was identical to a region of chromosome XIII containing reading frame YMR098C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Where indicated the composition of the polyacrylamide gel were modified to permit better resolution of ATPase subunits. Native proteins were extracted with 2% final concentration of digitonin and separated by native blue (BN)-PAGE on a 4–13% polyacrylamide gel (Wittig *et al.*, 2006). Western blots were treated with a rabbit polyclonal antibody against yeast Atp25p followed by a second reaction with anti-rabbit immunoglobulin G (IgG) coupled to peroxidase. The antibody complexes were visualized with the SuperSignal chemiluminescent substrate kit (Pierce Chemical, Rockford, IL). Protein concentrations were determined by the method of Lowry *et al.* (1951).

RESULTS

Properties of Mutants in Complementation Groups G29

This complementation group consists of seven independent respiratory-deficient *pet* mutants with recessive point mutations in nuclear DNA. The mutations confer a secondary instability in mitochondrial DNA, which depending on the mutant strain causes the production of 30–90% ρ^{-}/ρ clones during vegetative growth. The high lability of mitochondrial DNA (mtDNA) is a hallmark of several different classes of nuclear mutants (Tzagoloff and Dieckmann, 1990). Most commonly, it occurs in strains with mutations in F_1 - F_0 ATPase (Paul *et al.*, 1989; Helfenbein *et al.*, 2003) or in components of the mitochondrial translation system (Myers *et al.*, 1985). Mitochondrial translation mutants display multiple deficiencies in cytochromes *a*, *a*₃, and *b*. Because such cytoplasmic petite mutants are able to synthesize F_1 but not F_0 , their ATPase is oligomycin insensitive. ATPase mutants have a similar pleiotropic phenotype with deficiencies in the bc1 and cytochrome oxidase complexes, both of which contain subunits translated on mitochondrial ribosomes. ATPase mutants either lack ATPase activity altogether when F_1 biosynthesis is impaired or the ATPase is oligomycin insensitive if the mutations prevent F_0 assembly (reviewed in Ackerman and Tzagoloff, 2005). The latter phenotype, however, is also encountered among mitochondrial translation mutants.

Assays of mitochondrial cytochromes in mutants from complementation group G29 revealed severe reductions in all but *c*-type cytochromes (Figure 1A). Although the mutant mitochondria had ATPase activity, the enzyme was not inhibited by oligomycin, indicative of a lesion in F_0 (data not shown). Because the absence of oligomycin sensitivity was observed even in strains that underwent <50% conversion to ρ^{-}/ρ mutants, this suggested that the F_0 deficiency was not secondary to a translation defect but rather resulted from a mutation in an F_0 subunit or in a factor necessary for F_0 assembly. This was confirmed by analysis of mitochondrial translation products labeled in vivo in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Because of the high percentage of ρ^{-}/ρ in the cultures of the mutants used for labeling, they incorporated less [³⁵S]methionine than the wild type (Figure 1B). With the exception

Figure 1. Phenotype of G29 mutants. (A) Mitochondria of the wild-type W303-1A and of the G29 mutant aC279/U1 were extracted with 1% potassium deoxycholate at a final protein concentration of 5 mg/ml (Tzagoloff *et al.*, 1975). Half of the extract was reduced with sodium dithionite, and the other half was oxidized with potassium ferricyanide. The difference spectrum was recorded at room temperature. The absorption maxima of the α -bands of cytochromes *a*, *a*₃, *b* and *c*, *c*₁ are indicated. (B) In vivo labeling of mitochondria gene products. The parental wild-type strain D273-10B/A1 and C279, N124, and N210, three independent mutants from complementation group G29, were labeled with [³⁵S]methionine (1,000 Ci/mmol; GE Healthcare, Piscataway, NJ) in the presence of cycloheximide as described previously (Zeng *et al.*, 2007a). Total cellular proteins were separated by SDS-PAGE on three differently prepared polyacrylamide gels. The 17.5% polyacrylamide gel on the extreme left was prepared from a 30:0.8 acrylamide:bis-acrylamide stock, and it was run in the normal buffer system of Laemmli (1970). The middle gel was prepared from a stock solution of 30:0.2 acrylamide:bis-acrylamide, and it was run with a buffer adjusted to pH 8.3. The 12% polyacrylamide gel on the right was prepared from a 30:0.8 acrylamide:bis-acrylamide stock, and it contained 4 M urea and 25% glycerol. The relative migration of some proteins differs depending on the gel system used. Cox1p, Cox2p, and Cox3p are subunit of cytochrome oxidase; cytochrome *b* (Cyt. b) is a subunit of the bc1 complex; and Atp6p, Atp8p, and Atp9p are subunits of F_0 . (C) The wild-type parent D273-10B/A1 and the G29 mutant N210 were labeled in vivo for the indicated times with [³⁵S]methionine in the presence of cycloheximide, and the labeled products were separated by SDS-PAGE on a 17.5% polyacrylamide gel with a 30:0.8 ratio of acrylamide:bis-acrylamide as in B.

of Atp9p, the three mutants analyzed synthesized all the mitochondrial gene products including Atp6p and Atp8p, the other two mitochondrially derived subunits of F_0 (Figure 1B). The absence of Atp9p in the mutants was unlikely to be due to turnover of the protein, because there was no evidence of labeling even at very short pulse times with [35 S]methionine (Figure 1C). The normal synthesis of the other mitochondrial gene products suggested that the pleiotropic phenotype of the mutants stemmed from an ATPase deficiency and that the mutation was in a nuclear gene essential for proper expression of Atp9p.

ATP9 is cotranscribed with *tRNA^{ser}* and *VAR1* (Zassenhaus *et al.*, 1984). The primary transcript undergoes several endonucleolytic cleavages to yield the tRNA and *ATP9* and *VAR1* mRNAs (Figure 2E; Zassenhaus *et al.*, 1984). To ascertain whether the absence of Atp9p in G29 mutants stemmed from a deficiency of its mRNA, Northern blots of total mitochondrial RNAs from C279, N210, and the parental wild-type D273-10B/A1 were hybridized to an *ATP9* probe. The Northern analysis indicated a severe reduction in both mutants of the transcript corresponding to the *ATP9* mRNA

but not of the 1.5-kb intermediate (Figure 2B). C279 had another abundant transcript of 2.2 kb. The size of this transcript and its hybridization to the seryl-tRNA probe (Figure 2C) indicated it to be the product of endonucleolytic processing at 3' but not 5' of the tRNA. The high concentration of the 2.2-kb intermediate in C279 and its absence in N210 suggested that C279 may be enriched in a subpopulation of ρ^- clones with an amplified genome(s) containing *ATP9* and the tRNA region of mitochondrial DNA. Processing of the transcript at the 5' end of the tRNA in such ρ^- clones would not occur because of the requirement of the mitochondrially encoded RNA cofactor of RNase P (Hollingsworth and Martin, 1986). Northern analysis also confirmed the presence in C279 and N210 of two transcripts corresponding to the *ATP6* mRNAs (Figure 2D).

Cloning of *ATP25*

The gene responsible for the Atp9p deficiency was cloned by transformation of C279/U1 with a yeast genomic plasmid library based in the episomal plasmid YEp24 (Botstein and

Figure 2. Northern analysis of *ATP9*, *tRNA^{ser}*, and *ATP6* transcripts in wild-type and G29 mutants. Mitochondria were isolated from the parental respiratory competent strains D273-10B/A1 and from the G29 mutants C279 and N210. The C279 and the N210 cultures consisted of 2 and 20% ρ^+ cells, respectively. Total RNAs were extracted from mitochondria representing 1 mg of protein as described previously (Barros *et al.*, 2006). After precipitation with alcohol, the RNA extracts were dried and dissolved in 20 μ l of sterile water. The extracts (3 μ l) were separated on a 1% agarose gel under nondenaturing conditions, and were stained with ethidium bromide (A). After transfer to a Nytran membrane, the blot was hybridized as described in Barros *et al.* (2006) with *ATP9* (B), *tRNA^{ser}* (C), and *ATP6* (D) probes that had been labeled with [α - 32 P]dCTP by random priming (Feinberg and Vogelstein, 1983). The blots were exposed to x-ray film for 30 min. The sizes of some of the DNA standards and the migration of the mitochondrial 15S and 21S rRNA are indicated in the margins of the stained gel. The mature seryl-tRNA; the 1.5-, 2.2-, and 0.95-kb *ATP9* transcripts; and the two *ATP6* mRNAs are identified by the arrows. Because the gel was run under nondenaturing conditions the migration of the RNAs is slightly retarded. (E) Diagram of the *ATP9/tRNA^{ser}/VAR1* region of mtDNA showing the *ATP9* transcripts detected in the Northern hybridizations. The transcripts shown are based on Zassenhaus *et al.* (1984).

Davis, 1982). Two respiratory-competent clones obtained from the transformations were used to isolate plasmids capable of restoring growth of the mutant on nonfermentable carbon sources. One of the plasmids (pG29/T2) was used to subclone the gene. The smallest region (pG29/ST8) capable of restoring respiratory growth in C279/U1 revealed a single reading frame of 1839 nucleotides. This gene is identical to reading frame YMR098C on chromosome XIII, and it has been designated *ATP25* in keeping with the convention used to name other genes involved in biogenesis of mitochondrial ATPase. It is of interest that another subclone (pG29/ST1) lacking the N-terminal 68 codons of the gene is also competent in conferring respiratory growth on the mutant.

To verify that C279/U1 had a mutation in *ATP25*, nuclear DNA obtained from the mutant was digested with a combination of PstI and SphI and fragments of ~2.1 kb were used to construct a library in the integrative vector YIp352 (Hill *et al.*, 1986). The library was screened by colony hybridization with a probe consisting of the BglII fragment internal to *ATP25*. The sequence of *atp25* in a clone isolated from the library disclosed the presence of a C-to-T transition at nucleotide 1231 of the gene creating a TAA stop at codon 410. The mutation confirmed that restoration of respiratory growth in the mutant by pG29/T2 (Figure 3A) was the result of complementation rather than suppression.

A null allele of *ATP25* was obtained by replacing the 800-base pairs coding region between the two BglII sites with a 3-kb BglII fragment containing *LEU2* or a 1-kb BamHI fragment with *HIS3*. Transformation of W303 haploid strains with a linear fragment containing the partially deleted gene yielded respiratory-deficient and leucine (or histidine)-independent transformants that were confirmed by Southern hybridization of their nuclear DNA to harbor the null mutations. Crosses of the *HIS3* and *LEU2* deleted/disrupted *atp25* mutants to the G29 point mutants failed to produce respiratory competent diploid cells. The null mutants were unstable, producing >95% secondary ρ^{-}/ρ mutants, and they were not complemented by integration of the C279 allele at the *URA3* locus of nuclear DNA. The ATPase activity of mitochondria of the null mutants like those of the point mutants was insensitive to oligomycin (data not shown).

Isolation of Temperature-sensitive Alleles of *ATP25*

The absence of *ATP9* mRNA in *atp25* mutants could be consequent to a block in transcription, RNA processing, or

instability of the mRNA. Studies aimed at distinguishing among these possible functions of the *ATP25* product were hindered by the mtDNA instability of the null and most *atp25* point mutants. To circumvent this problem, temperature-sensitive alleles of the gene were obtained by low-stringency PCR amplification of the gene. Several such mutants were isolated and one mutant, W303/ATP25ts-1, was used to probe the biochemical lesion causing the Atp9p deficiency.

Growth of the ts mutant on ethanol/glycerol at 24°C was ~2 times slower than that of wild type, and it was almost completely inhibited at 37°C (Figure 3A). Spectra of mitochondrial cytochromes showed decreases in cytochrome oxidase and to a lesser extent of cytochrome *b* in the mutant grown at 37°C (Figure 3B). Although the wild-type strain also sustained some loss of "a" and "b" type cytochromes when grown at 37°C, the decrease was less pronounced than in the mutant. The presence of cytochromes *a*, *a₃*, and *b* in the ts mutant grown at the nonpermissive temperature made it highly unlikely that Atp25p is involved in transcription, because translation of these respiratory carriers depends on both the seryl-tRNA and the ribosomal protein Var1p.

The ts mutant corroborated the results obtained with the point mutants, indicating that the product of *ATP25* is required for expression of Atp9p and oligomycin-sensitive ATPase. The ATPase activity of mitochondria was inhibited by >60% in the presence of oligomycin when the ts mutant was grown at 24°C, but it was essentially unaffected by oligomycin when the cells were grown at 37°C, the nonpermissive temperature (Table 2). In vivo labeling of mitochondrial products in cells grown at 24°C and switched to 37°C for different times showed an almost complete arrest of Atp9p synthesis after incubation at 37°C for 4 h (Figure 4A). A temperature shift experiment was also done to assess the effect of the ts mutation on *ATP9* transcripts. Mitochondria were prepared from cells that were first grown to early stationary phase at 24°C and subsequently incubated at 37°C for up to 7 h. Northern analysis of mitochondrial RNAs revealed a time-dependent loss of the Atp9p mRNA (Figure 4, B and C). The decrease of Atp9p mRNA and of in vivo synthesis of the protein both occurred after 4-h incubation at the restrictive temperature. Interestingly, the 1.5-kb *ATP9* precursor normally seen in wild type was not affected at 37°C.

Figure 3. Phenotype of the *atp25* temperature-sensitive mutant W303/ATP25ts-1. (A) Dilutions of the wild-type W303-1B and of W303/ATP25ts-1, grown to early stationary phase in liquid YPD, were spotted on two YPD and YEPG plates and incubated at 24 and 37°C for 2–3 d. (B) Spectra of mitochondrial cytochromes in W303-1B and W303/ATP25ts-1 grown at 24 and 37°C in YPGal to early stationary phase. Mitochondria were prepared and extracted, and spectra were recorded as described in Figure 1A.

Table 2. ATPase activity of mitochondria from wild type and the *atp25* ts mutant

Strain	Growth temp (°C)	% ρ^+	ATPase ($\mu\text{mol}/\text{min}/\text{mg}$)		% inhibition
			–Oligomycin	+Oligomycin	
W303	24	>99	5.23	0.6	88.5
W303	37	>99	4.55	1.05	76.9
W303/ <i>ATP25ts-1</i>	24	61	4.50	1.47	67.3
W303/ <i>ATP25ts-1</i>	37	50	1.87	1.83	2.1

Mitochondria were prepared from wild-type W303-1B and an *atp25* ts mutant. Samples of the cultures were used to determine the percentage of ρ^-/ρ^0 cells. The mitochondrial ATPase activities were measured with or without 10 $\mu\text{g}/\text{ml}$ oligomycin at 37°C.

Detection and Characterization of a 35-kDa Proteolytic Product of *ATP25* in Mitochondria

ATP25 was modified to express a C-terminally HA-tagged protein. HA-tagged Atp25p from a multicopy or low copy plasmid restored wild type growth in the C279/U1 and in the null mutant W303 Δ ATP25, indicating that the presence of the tag did not impair its function. Western analysis of mitochondria from C279/U1 or the null mutant expressing Atp25p-HA from a low copy CEN plasmid (C279/U1/ST18 and W303 Δ ATP25/ST18 in 5A) disclosed the presence of a 35-kDa protein. This protein was much more abundant in mitochondria of the mutants transformed with the gene in a high copy plasmid (C279/U1/ST17 and W303 Δ ATP25/ST17 in Figure 5A). In the latter strains, however, the antibody also detected a minor protein of ~60 kDa, a mass 10 kDa less than that of the primary translation product predicted from the gene sequence (Figure 5A). This protein is not detected in mitochondria of wild type or of mutants expressing the modified gene from a low copy plasmid, suggesting that it corresponds to the full-length mature protein. Addition of phenylmethylsulfonyl fluoride (PMSF) at every step of the mitochondrial purification procedure or additional purification of mitochondria on a Nycodenz gradient did not increase the ratio of the 60-kDa to the 35-kDa protein in the transformant with the gene on a multicopy plasmid or lead to its appearance in the strain harboring the gene on a low copy CEN plasmid.

Western blots of mitochondria were also probed with a polyclonal antibody raised against the C-terminal half of the protein starting from Lys313. The antibody detected the 35-kDa HA-tagged protein in the null mutant with the gene in low copy plasmid or on a multicopy plasmid (W303 Δ ATP25/ST18 and ST17, respectively; Figure 5B). The polyclonal antibody also detected a protein in wild-type mitochondria that migrated slightly faster than Atp25p-HA, presumably because of the absence of the HA tag (Figure 5B). Neither mitochondria of the *atp25* null mutant or the postmitochondrial supernatant fraction corresponding to the cytosolic proteins of wild-type cells contained the 35-kDa protein (data not shown). Because the polyclonal antibody cross-reacted with a number of proteins in the 60- to 70-kDa region it could not be used to study the 60-kDa product in wild-type yeast.

To confirm that the 35-kDa protein is a product of Atp25p and to identify the proteolytic cleavage site, six consecutive histidines codons were added to the 3' end of the of *ATP25*. When expressed from a multicopy plasmid, the hybrid gene expressed a protein with an electrophoretic migration similar to that of the 35-kDa HA-tagged protein. The polyhistidine-tagged protein was affinity purified on a nickel-NTA

acid column and was further purified by SDS-PAGE electrophoresis. The N-terminal five residues of the purified protein had a sequence identical to that of Atp25p starting at serine 293.

Localization of the 35-kDa C-Terminal Fragment of Atp25p

The 35-kDa product of Atp25p cofractionated with submitochondrial membranes after disruption of mitochondria by ultrasound (Figure 5C). Extraction of mitochondria at alkaline pH with sodium carbonate released ~30% of the HA-tagged Atp25p, indicating that it is probably a peripherally associated membrane protein. No Atp6p, an integral membrane protein was present in the carbonate extract, indicating that the Atp25p in this fraction was not due to contamination by membrane fragments. Some F_1 , detected by the β -subunit antibody, was sheared from the membrane after sonic disruption of mitochondria. An additional 60% of the β -subunit in the submitochondrial membrane particles was extracted by carbonate (Figure 5C).

The carboxy-terminal 35-kDa protein is a constituent of the inner membrane facing the matrix side based on its resistance to digestion by externally added proteinase K in mitochondria and mitoplasts (Figure 5D). This was true both of wild-type mitochondria probed with the polyclonal antibody against Atp25p and of mitochondria from the transformant expressing the Atp25p-HA from a low copy plasmid probed with a monoclonal antibody against the HA tag. The Western blots of the mitochondria were also reacted with antibodies against cytochrome b_2 (a soluble intermembrane protein), against Sco1p (an inner membrane protein facing the intermembrane space), and against α -ketoglutarate dehydrogenase (a subunit of the matrix localized α -ketoglutarate dehydrogenase complex). The depletion of cytochrome b_2 in the mitoplasts and the sensitivity of Sco1p but not the α -ketoglutarate dehydrogenase to proteinase K confirmed that most of the mitochondria had been converted to mitoplasts by the hypotonic treatment (Figure 5D). Treatment of mitochondria with proteinase K in the presence of 0.05 or 0.1% Triton X-100 resulted in complete digestion of HA-tagged Atp25p (Figure 5E).

Both the N-Terminal and C-Terminal Halves of Atp25p Are Required for Restoration of Respiratory Growth

The Atp25p antibody was obtained against the sequence starting from residue 313. This antibody does not recognize the N-terminal sequence of Atp25p preceding the cleavage site. Complementation tests, however, indicated that both halves of the protein are necessary for respiratory growth.

Figure 4. Northern analysis of *ATP9* mRNA and of its translation product in the *atp25* ts mutant after shift to the nonpermissive temperature. (A) The wild-type haploid parent W303-1B and the ts mutant W303/ATP25ts-1 were grown at 24°C overnight in YPGal, transferred to minimal galactose medium, and incubated at 24°C for 7 h or at 37°C for the indicated times. Samples of the cultures were labeled with [³⁵S]methionine in the presence of cycloheximide to inhibit cytoplasmic protein synthesis (Zeng *et al.*, 2007a). Total cellular proteins were separated by SDS-PAGE on a 17.5% polyacrylamide gel prepared with a 30:0.8 solution of acrylamide:bis-acrylamide. After transfer to nitrocellulose, the blot was exposed to x-ray film. (B) The wild-type and ts mutant were grown in YPGal to early stationary phase at 24°C. The culture was transferred to 37°C, and growth was continued. Equal samples of the culture was removed at the indicated times and used to prepare mitochondria. Only a 20% increase in mass occurred after 7 h at 37°C. Total mitochondrial RNAs were extracted as described in Figure 2, and then they were separated on a 1% nondenaturing gel. The amount of RNA loaded per lane was adjusted based on the ethidium bromide stain associated with the 21S and 15S rRNAs (marked in the margin). More of the mutant RNAs was loaded to compensate for the presence of 40% $\rho^{-/o}$ in the culture. (C) The gel shown in B was blotted to Nytran and hybridized to a radioactively labeled *ATP9* probe as described in Figure 2. The *ATP9* precursor and mRNA are identified in the margin.

Transformants harboring only the N- or C-terminal half of the gene were either not complemented for their respiratory defect (Δ ATP25/ST32 in Figure 6A), or they showed only a very marginal restoration of growth on ethanol/glycerol (Δ ATP25/ST23 in Figure 6A). Growth of the null mutant on glycerol/ethanol, however, was restored when plasmids coding for the N- or C-terminal half of Atp25p were present jointly in the same cell (Δ ATP25/ST23, ST32).

The requirement of both halves of Atp25p for restoration of respiration was also evident from the spectra of mitochondrial cytochromes in the different strains used to determine the growth phenotype. Only the transformant that contained plasmids coding for both halves of the protein had a normal complement of cytochromes (Figure 6B). The transformant with only the N-terminal half of Atp25p displayed a pleiotropic deficiency of “a” and “b” type cytochromes similar to the null mutant. This was also true of the transformant with the C-terminal half, even though this strain was able to grow very slowly on the respiratory substrates (Figure 6, A and B). The slow growth and the increase in the percentage of ρ^{+} cells in the mutant containing only the C-terminal half suggested that this domain was able to partially restore expression of Atp9p. This was supported by the results of labeling of mitochondrial gene products in whole cells (Figure 6C). The null mutant containing the N-terminal half of the Atp25p (Δ ATP25/ST32) failed to show any significant incorporation of [³⁵S]methionine into any of the mitochondrial translation products. This transformant had highly unstable mtDNA and consisted of <1% ρ^{+} cells. In contrast, both Δ ATP25/ST23, ST32 and Δ ATP25/ST23 harboring both halves or only the C-terminal half of Atp25p, respectively, synthesized all the endogenous gene products, including Atp9p (Figure 6C). The ability of the C-terminal half of Atp25p, by itself, to restore Atp9p expression indicates that this domain is sufficient to stabilize the *ATP9* mRNA.

Detection of the N and C Half of Atp25p Expressed from Constructs Containing the Partial Genes

The N- and C-terminal halves of Atp25p tagged at their C termini with HA were cloned in multicopy plasmids. Expression of the two halves of Atp25p in transformants harboring either or both partial genes was confirmed by Western blot analysis of mitochondria (Figure 7A).

The submitochondrial localization of the two halves of Atp25p was analyzed in the double transformant by proteinase treatment of intact and hypotonically lysed mitochondria (Figure 7B). The intactness of mitochondria and their conversion to mitoplasts with sealed inner membranes was verified by the detection of the intermembrane marker cytochrome *b*₂ in mitochondria treated with proteinase K and of the matrix marker α -ketoglutarate dehydrogenase in the mitoplasts. Interestingly, approximately the same amount of the C-terminal half of Atp25p was lost in the proteinase K-treated mitochondria and mitoplasts. This proteinase-sensitive fraction of Atp25p probably represents protein that is either attached to the outer membrane or only partially translocated into the interior compartment. Because the C-terminal half of Atp25p expressed from the pG29/ST23 construct lacks a mitochondrial targeting sequence it is not surprising that its transport into mitochondria is inefficient. Approximately the same amount of the C-terminal Atp25p fragment in mitochondria and mitoplasts was resistant to proteinase K, indicating that this fraction was translocated to the matrix where the natural C-terminal fragment of wild type was also detected (Figure 5).

In contrast, all of the N-terminal polypeptide of Atp25p was resistant to proteinase K in mitochondria, indicating that the presence of the mitochondrial targeting sequence facilitated transport of all of the protein into mitochondria. The N-terminal fragment was partially sensitive to proteinase K in mitoplasts as was the intermembrane Sco1p maker, suggesting that like the latter fragment, this fragment is associated with the inner membrane facing the intermembrane compartment.

Figure 5. Detection of and localization of Atp25p. (A) Mitochondria were prepared from the wild-type strain W303-1B and from W303ΔATP25/ST18 and C279/U1/ST18, the *atp25* null and point mutant, respectively, with *ATP25-HA* in a CEN plasmid. W303ΔATP25/ST17 and C279/U1/ST17 contain *ATP25-HA* on a multicopy plasmid. Mitochondrial proteins (40 μg) were separated by SDS-PAGE on a 12% polyacrylamide gel, and were transferred to nitrocellulose. The protein blot was probed with a monoclonal antibody against the HA tag followed by a peroxidase-coupled rabbit antibody against mouse IgG. The antibody-antigen complexes were visualized with the SuperSignal kit (Pierce Chemical). (B) Mitochondria were prepared from the wild-type strain W303-1B and from W303ΔATP25/ST18 and W303ΔATP25/ST17 (see above). Mitochondrial proteins (40 μg) were separated and transferred to nitrocellulose as described in A. The blot on the left was processed as described in A. The blot on the right was first reacted with a rabbit polyclonal antibody against the C-terminal half of Atp25p followed by a peroxidase-coupled goat antibody against rabbit IgG. Antibody-antigen complexes were visualized as described in A. The slower migrating bands above the Atp25p and Atp25p-HA are cross-reacting protein of unidentified nature. (C) Mitochondria from W303ΔATP25/ST18 were suspended in 0.6 M sorbitol, 20 mM HEPES, pH 7.5, at a protein concentration of 10 mg/ml. The mitochondria (0.4 ml) were disrupted with a Branson microprobe by irradiation for 5 s at half-maximal output. The suspension was centrifuged at $90,000 \times g_{av}$ for 20 min. The supernatant was collected and the pellet consisting of submitochondrial membrane vesicles was resuspended in the starting volume of 0.6 M sorbitol, 20 mM HEPES, pH 7.5. The mitochondria were also extracted with an equal volume of 0.2 M sodium carbonate. After incubation on ice for 20 min, the mixture was centrifuged at $90,000 \times g_{av}$. The supernatant was collected and the extracted mitochondria were suspended in the starting volume of 0.6 M sorbitol, 20 mM HEPES, pH 7.5. A 40-μg sample of mitochondria (Mito) and equivalent volumes of the submitochondrial vesicles (SMP), sonic extract (supernatant), carbonate extracted mitochondria (Carb.-P), and carbonate extract (Carb.-E) were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose, and then they were probed with a mAb against the hemagglutinin tag and polyclonal antibodies against Atp6p and the β subunit of F_1 . (D) Mitochondria of the wild-type W303-1B and W303ΔATP25/ST18 at a protein concentration of 10 mg/ml were diluted with 6 volumes of either 0.6 M sorbitol, 20 mM HEPES, pH 7.5, or 20 mM HEPES, pH 7.5, in the presence and absence of 100 μg/ml proteinase K. After incubation at 4°C for 30 min the mitochondria were treated with PMSF at a final concentration of 0.5 mM. The mitochondria and mitoplasts formed under these hypotonic conditions were centrifuged, suspended in 0.6 M sorbitol, 20 mM HEPES, pH 7.5, and treated with 0.1 volumes of 50% trichloroacetic acid. The denatured proteins were centrifuged at 14,000 rpm for 5 min in a microfuge, rinsed with water, and dissolved in Laemmli sample buffer (Laemmli, 1970). Proteins were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose, and reacted with the rabbit polyclonal antibodies against the C-terminal half of Atp25p and with a monoclonal antibody against the HA tag as described in A. (E) W303ΔATP25/ST18 mitochondria were incubated as described in D in the absence or presence of proteinase K and the indicated concentrations of Triton X-100. Proteins were precipitated with 5% final concentration of trichloroacetic acid, rinsed in water before depolymerization in Laemmli sample buffer, separated by SDS-PAGE on a 12% polyacrylamide, and probed with a monoclonal antibody against the HA tag as described in A.

Function of the N-Terminal Half of Atp25p

The requirement of both halves of Atp25p for restoration of ATPase suggested that the N-terminal half of Atp25p catalyzes a function distinct from stabilization of the *ATP9* mRNA. A reasonable hypothesis was that this function might be related to some aspect of Atp9p biogenesis. To test this possibility, the status of the ATPase in cells expressing only the C-terminal domain of Atp25p was probed immunologically with antibodies against different subunits of F_1 or F_0 and by labeling mitochondrial translation products in vivo.

The ATPase complex of wild type and different mutants was extracted from mitochondria with digitonin and separated by BN-PAGE. Antibodies against the β -subunit of F_1 revealed that most of the ATPase extracted from wild-type mitochondria migrated as the monomer F_1 - F_0 complex (Figure 8A). This was also true of RKY48-1, a mutant expressing Atp6p without its N-terminal presequence (Zeng *et al.*, 2007c). No ATPase complex was detected in W303ΔATP25/ST40, a transformant expressing the C-terminal domain of *ATP25* or in the *atp10* mutant, which synthesizes the Atp9p

ring but is blocked in its interaction with Atp6p (Tzagoloff *et al.*, 2004).

An antibody against Atp9p recognized the ATPase monomer in wild type and RKY48-1 but not in the *atp10* null mutant or in W303ΔATP25/ST40 (Figure 8A). Consistent with previous data, the Atp9p antibody also detected the Atp9p ring in RKY48-1 (Zeng *et al.*, 2007c) and in the *atp10* mutant (Tzagoloff *et al.*, 2004). Significantly, the ring was absent in W303ΔATP25/ST40, even though this strain synthesizes Atp9p (Figure 8B).

The absence of the Atp9p ring in mitochondria of W303ΔATP25/ST40 could be the consequence of turnover, resulting in a steady-state concentration of Atp9p too low for immunological detection. Although turnover of Atp9p cannot be excluded, it is unlikely in view of experiments showing that there is no difference in the stability of newly translated Atp9p in wild type and in the mutant harboring only the C-terminal half of Atp25p (data not shown). Alternatively, assembly of Atp9p into the ring may be blocked in the absence of the N-terminal domain. This explanation is consistent with in vivo pulse-labeling results. In the exper-

Figure 6. Growth and biochemical phenotype of *atp25* null mutants transformed with the N- and C-terminal halves of *ATP25* singly and in combination. (A) The wild-type W303-1A, the *atp25* null mutant W303Δ*ATP25* (Δ*ATP25*), and the mutant transformed with plasmids coding either the N-terminal (ST32), C-terminal (ST23), or both halves of the protein were grown overnight in liquid YPD. Serial dilution was spotted on YPD and YEPG plates and incubated at 30°C for 2–3 d. (B) The null mutant expressing either the N-terminal half (Δ*ATP25*/ST32), C-terminal half (Δ*ATP25*/ST23), or both halves of Atp25p were grown in YPGal to early stationary phase. Mitochondria were prepared and their spectra recorded as in Figure 1A. (C) The point mutant C279 and the strains used for the spectral analysis in B were labeled *in vivo* in the presence of cycloheximide, as described in Figure 1B. Total cellular proteins were separated by SDS-PAGE on a 17.5% polyacrylamide gel. The radioactively labeled Atp8p and Atp9p are identified in the margin.

iment of Figure 8B, newly translated mitochondrial gene products were extracted with digitonin and displayed by BN-PAGE. The pulse-labeling conditions used in this experiment preempted the newly translated Atp9p from assembling into the ATPase complex, and as a result, it was present as a ring in both wild type and the *atp10* mutant (Figure 8B). No newly translated Atp9p, however, was incorporated into the ring structure in the transformant expressing only the C-terminal half of Atp25p (Figure 8B).

DISCUSSION

Atp9p is a low-molecular-weight hydrophobic subunit of mitochondrial and bacterial proton-translocating ATPases. It exists as a 10- to 11-membered ring structure (Arechaga *et al.*, 2002), which together with Atp6p defines the channel

through which protons are translocated during ATP formation or hydrolysis (Fillingame and Dmitriev, 2002). In *S. cerevisiae*, expression of the mitochondrial gene coding for Atp9p has been shown to depend on several nuclear gene products (Payne *et al.*, 1991, 1993). Although our understanding of the functions of these factors is still fragmentary, there is good evidence that they are required for processing/stability of the *ATP9* transcript and for translation of the mRNA (Ackerman *et al.*, 1991; Payne *et al.*, 1993; Ellis *et al.*, 1999). The product of the nuclear *ATP25* gene reported here is a new member of this class of proteins.

The *atp25* mutants have a phenotype similar to *aep1* and *aep2/atp13* mutants reported previously (Ackerman *et al.*, 1991; Ellis *et al.*, 1999). They contain catalytically active F_1 ATPase, but they are unable to incorporate it into the larger proton-translocating complex because of a block in assembly of F_0 , the membrane sector of this inner membrane complex. In the current study, the absence of functional F_0 in *atp25* mutants has been correlated with a severe deficit of the Atp9p subunit. *In vivo* labeling revealed that *atp25* mutants are capable of translating all the mitochondrial gene products except Atp9p. This failure to synthesize Atp9p was unlikely to be due to a defect in transcription of the gene or processing of the primary transcript for the following reason. *ATP9* is part of a polycistronic transcript that includes *tRNA^{ser}* and *VAR1* (Zassenhaus *et al.*, 1984). The ability of *atp25* mutants to express all the normal mitochondrial gene products except Atp9p attests to the presence of both functional ribosomes and the seryl-tRNA. This in turn suggested that the defect was likely to be related to translation of Atp9p or stability of its mRNA. To further probe the biochemical basis of the Atp9p deficit, two of the more stable *atp25* mutants were used to analyze *ATP9* transcripts in mitochondria. Northern analysis of mitochondrial RNA confirmed the presence in the mutants of seryl-tRNA (the *VAR1* transcript was not analyzed) and of the low abundance 1.5-kb *ATP9* precursor transcript. The mutants, however, were grossly deficient in the 0.95-kb *ATP9* mRNA.

The Northern results are most consistent with an aberrantly high rate of *ATP9* mRNA turnover in the mutants. This was corroborated by Northern analysis of *ATP9* mRNA stability in an *atp25* temperature-sensitive mutant. The level of *ATP9* mRNA in mitochondria of the mutant grown at the

Figure 7. Detection and localization of HA-tagged N- and C-terminal halves of Atp25p expressed from the respective partial genes. (A) Mitochondria prepared from the wild-type W303-1B, the *atp25* null mutant (Δ*ATP25*), and the *atp25* null mutant expressing either C-terminal half (Δ*ATP25*/ST23) or N-terminal (Δ*ATP25*/ST32) of Atp25p from multicopy plasmids were separated by SDS-PAGE on a 12% polyacrylamide gel. The proteins were transferred to nitrocellulose, and then they were probed with a monoclonal antibody against the HA tag as described in Figure 5A. (B) Mitochondria of the *atp25* null mutant transformed with both halves of Atp25p (Δ*ATP25*/ST23, ST32) were diluted with 6 volumes of either 0.6 M sorbitol, 20 mM HEPES, pH 7.5, or 20 mM HEPES, pH 7.5, and incubated at 4°C for 30 min in the absence or presence of 100 μg/ml proteinase K and further processed as described in Figure 5D. The HA antibody does not always detect the N-terminal fragment either in the single or double transformant, probably because of its lower concentration relative to the C-terminal fragment.

Figure 8. Assembly of Atp9p in wild-type and mutant mitochondria. (A) Mitochondria from wild-type W303-1B, the *atp25* null mutant expressing only C-terminal half of *ATP25* (Δ ATP25/ST40), the *atp6* leaderless mutant (RKY48-1), and the *atp10* null mutant (Δ ATP10) were solubilized with 2 mg digitonin/mg mitochondrial protein, and were analyzed by BN-PAGE on a 4–13% polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with polyclonal antibodies against the β subunit of F_1 (left) and Atp9p (right). (B) The strains used in A (except RKY48-1) were labeled in vivo with [35 S]methionine in the presence of cycloheximide as in Figure 1B. The labeled cells were converted to spheroplasts by incubation at 30°C for 10 min in the presence of 14 mg/ml Zymolyase 20,000. Mitochondria were prepared, mixed with unlabeled wild-type mitochondria, and extracted with digitonin as described in A. The soluble fraction obtained after centrifugation at $70,000 \times g_{av}$ for 15 min was separated either by SDS-PAGE on a 17.5% polyacrylamide gel (left) or by BN-PAGE (right), transferred to a PVDF membrane, and exposed to x-ray film. The band identified as the Atp9p ring was confirmed to contain Atp9p by SDS-PAGE in a second dimension (data not shown).

permissive temperature was estimated to be comparable with the wild type when corrected for the percentage of ρ^- and ρ^0 cells in the culture, but there was an almost total depletion of this transcript between 1.5 and 4 h after shift to the nonpermissive temperature. The *ATP9* mRNA of wild-type cells grown and incubated under similar conditions was completely stable. As expected, the *ts* mutant showed a similar time-dependent loss of Atp9p translation after shift to the restrictive temperature. Based on these results, we

propose Atp25p to be a novel RNA stabilization factor specific for the 0.95-kb *ATP9* mRNA. Because the abundance of the 1.5-kb precursor, normally also seen in wild type, does not seem to be affected either in *atp25* point mutants or the *ts* mutant grown under nonpermissive conditions, the function of Atp25p must be restricted to the fully processed mRNA. Several other mitochondrial RNAs are known to require protein factors for their stability. Cbp1p has been shown to affect the stability of the cytochrome *b* mRNA (Mittelmeier and Dieckmann, 1990), but in addition it also functions during translation of apocytochrome *b* (Islas-Osuna *et al.*, 2002). Aep3p is another recently described factor that stabilizes the *ATP8-ATP6* bicistronic mRNA (Ellis *et al.*, 2004). Although the evidence that Atp25p is involved in stability of the 0.95-kb *ATP9* mRNA is compelling, we cannot exclude the possibility that this factor, not unlike Cbp1p, may also play a role in translation of Atp9p.

Based on the sequence of *ATP25*, the primary translation product is predicted to have a mass of 70 kDa. Unexpectedly, an antibody directed against the C-terminal half of Atp25p detected a 35-kDa protein. Because the antibody cross reacted with proteins in the 60- to 70-kDa region it was not possible to decide whether the full size protein was also present. This question was resolved with an *atp25* null mutant expressing Atp25p C-terminally tagged with an HA epitope. Mitochondria of strains expressing the HA-tagged protein from a low copy plasmid contained only the 35-kDa protein. The full-size product was not detected even when Western blots were overexposed. A 60 kDa HA-tagged protein was present in mitochondria of the *atp25* null mutant transformed with the gene on a high copy plasmid. The 60-kDa band, however, represented only a small fraction of the signal associated with the 35-kDa protein. No increase in the 60-kDa product was observed by inclusion of PMSF at all stages of the mitochondrial isolation procedure or by purification of mitochondria on a gradient to remove possible lysosomal contamination. The 35-kDa Atp25p product resulting from cleavage after Tyr292 is, therefore, unlikely to be an artifact of the mitochondrial isolation procedure, but rather it seems to be a physiological and functional unit of the protein. The C-terminal half of Atp25p is associated with the inner membrane and it is protected from external protease, indicating that it faces the matrix side of the membrane, a location consistent with its proposed role.

The evidence that the N-terminal half of Atp25p is also required for respiratory competence is unambiguous. Transformation of the null mutant with constructs coding for either the N- or C-terminal half of Atp25p is not sufficient to produce respiration. Nearly wild-type growth on nonfermentable carbon sources is elicited, however, when both constructs are present in the mutant simultaneously. Transformants containing the gene coding for the C-terminal half of Atp25p are only very partially complemented for their growth defect, they display the same pleiotropic phenotype as the mutant, and they are deficient in oligomycin-sensitive ATPase. Nonetheless, this domain of Atp25p is sufficient to promote expression of Atp9p. This observation strongly implies that Atp25p is a bifunctional protein with the C-terminal half affecting the stability of the *ATP9* mRNA and the N-terminal half serving some other function.

The function of the N-terminal half of Atp25p was studied by assessing the status of Atp9p in the *atp25* null mutant expressing only the C-terminal half of Atp25p (W303 Δ ATP25/ST40). Atp9p was present as the oligomeric ring in an *atp10* mutant completely blocked in assembly of F_0 (Ackerman and Tzagoloff, 1990) or in an *atp6* leaderless mutant, which assembles only 50% of the nor-

mal amount of F_0 (Zeng *et al.*, 2007c). No ring, however, was detected in the *atp25* mutant expressing only the C-terminal half of Atp25p. This was true both when Atp9p was analyzed under steady-state condition with an antibody or when it was assayed radiochemically after *in vivo* pulse labeling. Under the latter conditions, Atp9p is synthesized in all three strains, but it is assembled into the ring only in wild type and the *atp10* mutant. These results indicate that the inability of the C-terminal domain to restore growth of the *atp25* mutant on respiratory substrates stems from its failure to assemble Atp9p ring. The absence of the ring in the *atp25* mutant lacking N-terminal half of Atp25p implies that this domain either promotes the interaction of the Atp9p monomer or it prevents the monomer from forming polymers of diverse size. It is of interest to note that the N-terminal half of Atp25p has homologues in fungi and contains the DUF143 domain found in a group of bacterial proteins of unknown function. In contrast the homology of the C-terminal half is confined to proteins found only in some budding and filamentous yeast such as *Ashbya* that are closely related to *S. cerevisiae*.

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REFERENCES

- Ackerman, S. H., Gatti, D. L., Gellefors, P., Douglas, M. G., and Tzagoloff, A. (1991). Characterization of *ATP12*, a yeast nuclear gene required for the assembly of the mitochondrial F_1 -ATPase. *J. Biol. Chem.* 266, 7517–7523.
- Ackerman, S. H., and Tzagoloff, A. (1990). *ATP10*, a yeast nuclear gene required for the assembly of the mitochondrial F_1 - F_0 complex. *J. Biol. Chem.* 265, 9952–9959.
- Ackerman, S. H., and Tzagoloff, A. (2005). Function, structure, and biogenesis of mitochondrial ATP synthase. *Prog. Nucleic Acid Res. Mol. Biol.* 80, 95–133.
- Arechaga, I., Butler, P. J., and Walker, J. E. (2002). Self-assembly of ATP synthase subunit c rings. *FEBS Lett.* 515, 189–193.
- Barros, M. H., Myers, A. M., Van Driesche, S., and Tzagoloff, A. (2006). *COX24* codes for a mitochondrial protein required for processing of the *COX1* transcript. *J. Biol. Chem.* 281, 3743–3751.
- Barros, M. H., Nobrega, F. G., and Tzagoloff, A. (2002). Mitochondrial ferredoxin is required for heme A synthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 277, 9997–10002.
- Botstein, D., and Davis, R. W. (1982). Principles and practice of recombinant DNA research with yeast. In: *The Molecular Biology of the Yeast Saccharomyces cerevisiae: Metabolism and Gene Expression*, ed. J. N. Strathern, E. W. Jones, and J. R. Broach, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 607–636.
- Costanzo, M. C., and Fox, T. D. (1990). Control of mitochondrial gene expression in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* 24, 91–113.
- Dieckmann, C. L., and Staples, R. R. (1994). Regulation of mitochondrial gene expression in *Saccharomyces cerevisiae*. *Int. Rev. Cytol.* 152, 145–181.
- Ebner, E., Mason, T. L., and Schatz, G. (1973). Mitochondrial assembly in respiration-deficient mutants of *Saccharomyces cerevisiae*. II. Effect of nuclear and extrachromosomal mutations on the formation of cytochrome c oxidase. *J. Biol. Chem.* 248, 5369–5378.
- Ellis, T. P., Helfenbein, K. G., Tzagoloff, A., and Dieckmann, C. L. (2004). Atp3p 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.
- Ellis, T. P., Lukins, H. B., Nagley, P., and Corner, B. E. (1999). Suppression of a nuclear *aep2* mutation in *Saccharomyces cerevisiae* by a base substitution in the 5'-untranslated region of the mitochondrial *oli1* gene encoding subunit 9 of ATP synthase. *Genetics* 151, 1353–1363.
- Faye, G., Kujawa, C., and Fukuhara, H. (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 Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132, 6–13.
- Fillingame, R. H., and Dmitriev, O. Y. (2002). Structural model of the transmembrane F_0 rotary sector of H^+ -transporting ATP synthase derived by solution NMR and intersubunit cross-linking. *Biochim. Biophys. Acta* 1565, 232–245.
- Gietz, R. D., and Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* 74, 527–534.
- Glick, B. S., and Pon, L. A. (1995). Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods Enzymol.* 260, 213–223.
- Helfenbein, K., Ellis, T., Dieckmann, C., and Tzagoloff, A. (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.
- Herrmann, J. M., Foelsch, H., Neupert, W., and Stuart, R. A. (1994). Isolation of yeast mitochondria and study of mitochondrial protein translation. In: *Cell Biology: A Laboratory Handbook*. Vol. I, ed. J. E. Celis, San Diego, CA: Academic Press, 538–544.
- Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986). Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* 2, 163–167.
- Hollingsworth, M. J., and Martin, N. C. (1986). RNase P activity in the mitochondria of *Saccharomyces cerevisiae* depends on both mitochondrial and nucleus-encoded components. *Mol. Cell Biol.* 6, 1058–1064.
- Islas-Osuna, M. A., Ellis, T. P., Marnell, L. L., Mittelmeier, T. M., and Dieckmann, C. L. (2002). Cbp1 is required for translation of the mitochondrial cytochrome b mRNA of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 277, 37987–37990.
- Jia, L., Dienhart, M. K., and Stuart, R. A. (2007). Oxa1 directly interacts with Atp9 and mediates its assembly into the mitochondrial F_1F_0 -ATP synthase complex. *Mol. Biol. Cell* 18, 1897–1908.
- Koerner, T. J., Hill, J. E., Myers, A. M., and Tzagoloff, A. (1991). High-expression vectors with multiple cloning sites for construction of *trpE* fusion genes: pATH vectors. *Methods Enzymol.* 194, 477–490.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Maxam, A. M., and Gilbert, W. (1977). A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA* 74, 560–564.
- Michaelis, G., Mannhaupt, G., Pratje, E., Fischer, E., Naggert, J., and Schweizer, E. (1982). Mitochondrial translation products in nuclear respiratory-deficient pet mutants of *Saccharomyces cerevisiae*. In: *Mitochondrial Genes*, ed. P. Slonimski, P. Borst, and G. Attardi, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 311–321.
- Mittelmeier, T. M., and Dieckmann, C. L. (1990). CBP1 function is required for stability of a hybrid *cob-oli1* transcript in yeast mitochondria. *Curr. Genet.* 18, 421–428.
- Myers, A. M., Pape, K. L., and Tzagoloff, A. (1985). Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae*. *EMBO J.* 4, 2087–2092.
- Osman, C., Wilmes, C., Tatsuta, T., and Langer, T. (2007). Prohibitins interact genetically with Atp23, a novel processing peptidase and chaperone for the F_1F_0 -ATP synthase. *Mol. Biol. Cell* 18, 627–635.
- Paul, M. F., Velours, J., Arselin de Chateaubodeau, G., Aigle, M., and Guerin, B. (1989). The yeast ATP synthase subunit 4, structure and function. *Eur. J. Biochem.* 185, 163–171.
- Payne, M. J., Finnegan, P. M., Smooker, P. M., and Lukins, H. B. (1993). Characterization of a second nuclear gene, *AEP1*, required for expression of the mitochondrial *OLI1* gene in *Saccharomyces cerevisiae*. *Curr. Genet.* 24, 126–135.
- Payne, M. J., Schweizer, E., and Lukins, H. B. (1991). Properties of two nuclear pet mutants affecting expression of the mitochondrial *oli1* gene of *Saccharomyces cerevisiae*. *Curr. Genet.* 19, 343–351.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

- Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19–27.
- ten Berge, A. M., Zoutewelle, G., and Needleman, R. B. (1974). Regulation of maltose fermentation in *Saccharomyces carlsbergensis*. 3. Constitutive mutations at the MAL6-locus and suppressors changing a constitutive phenotype into a maltose negative phenotype. *Mol. Gen. Genet.* 131, 113–121.
- Tzagoloff, A., Akai, A., and Needleman, R. B. (1975). Assembly of the mitochondrial membrane system. Characterization of nuclear mutants of *S. cerevisiae* with defects in respiratory enzymes and ATPase. *J. Biol. Chem.* 250, 8228–8235.
- Tzagoloff, A., Barrientos, A., Neupert, W., and Herrmann, H. (2004). Atp10p assists assembly of Atp6p into the F₀ unit of the yeast mitochondrial ATPase. *J. Biol. Chem.* 279, 19775–19780.
- Tzagoloff, A., and Dieckmann, C. L. (1990). *PET* genes of *Saccharomyces cerevisiae*. *Microbiol. Rev.* 54, 211–225.
- Wittig, I., Braun, H. P., and Schagger, H. (2006). Blue native PAGE. *Nat. Protoc.* 1, 418–428.
- Zassenhaus, H. P., Martin, N. C., and Butow, R. A. (1984). Origins of transcripts of the yeast mitochondrial *var1* gene. *J. Biol. Chem.* 259, 6019–6027.
- Zeng, X., Hourset, A., and Tzagoloff, A. (2007a). The *Saccharomyces cerevisiae* *ATP22* gene codes for the mitochondrial ATPase subunit 6-specific translation factor. *Genetics* 175, 55–63.
- Zeng, X., Kucharczyk, R., di Rago, J. P., and Tzagoloff, A. (2007c). The leader peptide of yeast Atp6p is required for efficient interaction with the Atp9p ring of the mitochondrial ATPase. *J. Biol. Chem.* 282, 36167–36176.
- Zeng, X., Neupert, W., and Tzagoloff, A. (2007b). The metalloprotease encoded by *ATP23* has a dual function in processing and assembly of subunit 6 of mitochondrial ATPase. *Mol. Biol. Cell* 18, 617–626.