**ATP22, a Nuclear Gene Required for Expression of the F₀ Sector of Mitochondrial ATPase in Saccharomyces cerevisiae**

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Expression of the mitochondrial proton-translocating ATPase of *Saccharomyces cerevisiae* has been shown to depend on chaperones that target the F₁ and F₀ sectors of this inner membrane complex. Here we report a new gene, designated *ATP22* (reading frame YDR350C on chromosome IV), that provides an essential function in the assembly of F₀. *ATP22* was cloned by transformation of C208/L2, a strain previously assigned to complementation group G99 of a collection of respiration-defective nuclear *pet* mutants. C208/L2 and the other *atp22* mutants have oligomycin-insensitive F₁-ATPase, suggesting that the lesion is confined to F₀. This is supported by the sedimentation properties of the mutant ATPase and results of immunochemical analysis of F₀ subunit polypeptides. Northern analysis of ATPase transcripts and in vivo pulse labeling of the mitochondrial translation products in the mutant indicate normal expression of subunits 6, 8, and 9, the three mitochondrial gene products of F₀. Atp22p therefore functions at a post-translational stage in assembly of F₀. Localization studies indicate Atp22p to be a component of the mitochondrial inner membrane. Protease protection experiments further indicate that Atp22p faces the matrix side of the membrane where most of the ATPase proteins are located and assembled.

The proton-translocating ATPase of mitochondria consists of two functionally distinct parts, F₁ and F₀. The F₁-ATPase catalyzes the reversible synthesis of ATP from ADP and inorganic phosphate. It consists of 5 distinct polypeptides with a stoichiometry of 3α3βγ-δεσ (1, 2). This extrinsic protein of the mitochondrial inner membrane is attached by means of a double stalk to F₂, the proton-transferring hydrophobic component of the inner membrane (3, 4). In most bacteria, F₀ consists of 3 subunits. The exceptions are photosynthetic bacteria that have an F₀ with 4 subunits. The F₀ of *Saccharomyces cerevisiae* is composed of 9 different polypeptides (5, 6). Three other ATPase-associated subunits have been shown recently (7) to be required for dimerization of the yeast enzyme. The most abundant constituent of F₀ is a low molecular weight proteolipid (subunit 9 or subunit C) that forms the proton channel of the complex (8, 9).

Studies of respiration-deficient yeast mutants have disclosed the existence of at least two nuclear gene products necessary for the expression of the F₁-ATPase. *ATP11* and *ATP12* code for chaperones that interact with the β and α subunits of F₁, respectively, thereby minimizing their aggregation through nonspecific hydrophobic interactions (10, 11). A number of nuclear genes have also been implicated in assembly of yeast F₀. At least 4 genes have been shown to be required for expression of the mitochondrial encoded subunits 6, 8, and 9 (12–15). Another nuclear gene, *ATP10*, codes for a subunit 6-specific chaperone of the inner membrane protein (16).

Most ATPase-deficient mutants have an unstable mitochondrial genome causing them to accumulate ρ− and ρ0 mutants.¹ ATPase mutants also incur secondary losses of the b₉, and cytochrome oxidase complexes. Even though this phenotype is fairly widespread among *pet* mutants (e.g. leaky mitochondrial protein synthesis mutants), it nonetheless is a good starting point for identifying strains with lesions in the ATPase. In continuing efforts to catalogue nuclear gene products that contribute to the maintenance of respiration-competent mitochondria, we have screened the class of pleiotropic pet mutants (17) for defects in ATPase. In this communication we report a new gene *ATP22*, which is required for the biogenesis of the ATPase. The encoded product, Atp22p, is a mitochondrial protein that provides an essential function in assembly of the F₀ sector.

**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 I. The compositions of the media for growth of yeast have been described elsewhere (20).

**Preparation of Yeast Mitochondria and ATPase Assays**—Mitochondria were prepared by the method of Faye et al. (21) except that Zymolyase 20,000 instead of Glusulase was used to convert cells to spheroplasts. ATPase activity was assayed by measuring release of inorganic phosphate from ATP at 37 °C in the presence and absence of oligomycin (22). For localization of Atp22p, mitochondria were prepared by the method of Glick (23).

**Cloning and Sequencing of ATP22**—*ATP22* was cloned by transformation of the respiration-deficient mutant C208/L2 (MATα leu2-3,112 pet22-1) with a yeast genomic library consisting of partial *Sac3A* fragments of nuclear DNA from strain D273-10B/A1 cloned in YEp13 (24). Transformation of 10⁶ cells with 10 µg of library DNA yielded a single leucine-independent and respiratory-competent clone (C208/L2/T1). The plasmid (pG99/T1) conferring respiratory competence to the mutant was amplified in *Escherichia coli*. BR1 and used to subclone the gene. The insert of subclone pG99/ST4 was sequenced by the method of Maxam and Gilbert (25) following single strand separation of 5′ end-labeled restriction fragments. All the restriction sites used for labeling were crossed from neighboring sites. The sequence of *ATP22* is identical to reading frame YDR350C on chromosome IV.

**Disruption of ATP22**—The following strategies were used to delete

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¹ The abbreviations used are: ρ− mutant, respiration-deficient mutant with a partially deleted mitochondrial genome; ρ₀ mutant, respiration-deficient mutant lacking mitochondrial DNA; pet mutant, respiration-deficient mutant of yeast with a mutation in a nuclear gene.
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Table I

| Strain          | Genotype          | Source |
|-----------------|-------------------|--------|
| D273-10B/A1     | MATa met6         | 18     |
| CB11            | ade1              | 19     |
| W303            | ade2-1 his3-1,15 leu2-3,112 trp1-1::URA3-1 |    |
| W303-1A         | ade2-1 his3-1,15 leu2-3,112 trp1-1::URA3-1 |    |
| W303-1B         | ade2-1 his3-1,15 leu2-3,112 trp1-1::URA3-1 |    |
| C208            | met6 atp22-1      | 17     |
| B208            | ade1 atp22-1      |        |
| C208/L2         | leu2-3,112 atp22-1| C208 × CB11 |
| C290            | met6 atp22       |        |
| N417/L2         | leu2-3,112 atp22  | N417 × W303-1A |
| C326            | met6 atp22       |        |
| N9-168          | met6 atp13       | 13     |
| E44             | met6 atp22       |        |
| E232            | met6 atp22       |        |
| W3033ATP22      | ade2-1 his3-1,15 leu2-3,112 trp1-1::URA3-1 atp22::HIS3 | This study |
| aW3033ATP22     | ade2-1 his3-1,15 leu2-3,112 trp1-1::URA3-1 atp22::HIS3 | This study |
| LL203ATP22      | his3-1,1 leu2-3,112 atp22::HIS3 | This study |

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results

Phenotype of atp22 Mutants—The atp22 mutants reported here are part of a collection of respiration-deficient pet mutants of S. cerevisiae (17). Complementation group G99 of this collection consists of 9 independent isolates all of which were complemented by a ρ0 tester strain indicating that the respiratory deficiency stems from recessive mutations in a nuclear gene. The mutants tend to degrade to secondary ρ0 or ρ− mutants as a result of deletion in their mtDNA. The frequency of ρ0/ρ− mutants with deleted mitochondrial genomes ranged from 5 to 75% in the five strains tested (Table II). Increased instability of mitochondrial DNA is one of the characteristics of ATPase mutants (35, 36). The most stable mutant, N417/L2, grew slowly on glycerol/ethanol, probably because the mutation in this strain causes only a partial loss of function.

Asays of ATPase in isolated mitochondria indicated that the mutant enzyme is oligomycin-insensitive (Table II). This phenotype is associated with mutations that prevent the F1-ATPase from interacting with F0, and can be elicited by lesions in subunits of F0 proteins required for expression of one of the three mitochondrial encoded subunits of F0 (12, 13), and accessory factors that function in F0 assembly (36). Additionally, because of the dependence of F0 assembly on the endogenously expressed subunits, loss of oligomycin sensitivity can also be a consequence of mutations that impair mitochondrial translation (17). Cytoplasmic petite mutants deficient in mitochondrial protein synthesis assemble catalytically active and oligomycin-insensitive F1-ATPase, which exists as a soluble matrix protein (38).

The ATPase defect of atp22 mutants is not due to the accumulation of large numbers of ρ0/ρ− clones. For example, the ATPase activity measured in C290 and N417/L2 was insensitive to oligomycin, even though the percentage of ρ0/ρ− cells in the cultures used to prepare mitochondria was only 30 and 5%, respectively (Table II). Like other ATPase mutants, atp22 mutants are partially deficient in cytochromes a, a3, and b and have reduced NADH and succinate oxidase activities (Fig. I, Table II (13, 14). A requirement of Atp22p for expression of the mitochondrial ATPase genes ATP6 (OLI2), ATP8 (AAP1) (44), and ATP9 (OLI1) was also excluded. Northern analysis of mitochondrial transcripts confirmed the presence of the fully processed ATP9 and the bistronic ATP6/ATP8 mRNAs (Fig. 1B). The two ATP6/ATP8 transcripts seen in yeast mitochondria result from processing at two different 5′ sites (43). The mitochondrial ATPase-specific mRNAs were also studied in an atp22 null mutant constructed in strain LL20 which produces ~50% ρ0/ρ− mutants. In this background the ATP9 transcript normalized to the 21 S rRNA was 98% of wild type. The normalized longer ATP6/ATP8 transcript was 90% and the shorter transcript was
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Respiratory and ATPase activities of mitochondria from wild type and atp22 mutants

The atp22 mutants C290, C326, E44, E232, and N417 are isogenic with the parental strain D273-10B/A1. Purified ρ+ cells were grown in YPGal to early stationary phase, and mitochondria were prepared. Samples of the cultures were used to test for the percentage of ρ+ and ρ− cells. NADH and succinate oxidase were measured at 24 °C (37) and ATPase at 37 °C. The values reported are averages of duplicate assays with the ranges indicated.

| Strain              | ρ+ % | NADH oxidase | Succinate oxidase | ATPase |
|---------------------|------|--------------|-------------------|--------|
|                     |      | μmol/min/mg  | μmol/min/mg       | μmol/min/mg |
| D273-10B/A1         | >99  | 0.422 ± 0.015 | 0.209 ± 0.012     | 4.35   |
| N417/L2             | 95   | 0.146 ± 0.004 | 0.030 ± 0.002     | 2.26   |
| C290                | 70   |              |                   | 2.94   |
| E44                 | 22   |              |                   | 1.56   |
| E232                | 20   |              |                   | 1.50   |
| C326                | 23   |              |                   | 1.90   |

51% of wild type. The lack of requirement of Atp22p for expression of the mitochondrial ATPase genes is also supported by in vivo translation assays indicating that atp22 mutants synthesize subunits 6, 8, and 9 of F0 (Fig. 1C). Finally, the sequence of ATP22 does not correspond to any known subunit of F1 or F0 (see below). Based on these results, Atp22p is likely to play a role at a post-translational stage of F0 assembly.

Cloning and Disruption of ATP22—The ATP22 gene was cloned by transformation of C208/L2 (MATa leu2-3,112 atp22−) with a yeast genomic plasmid library. One of 4,000 leucine-independent transformants obtained was rescued for the growth defect on non-fermentable carbon sources. The plasmid pG99/T1, responsible for restoration of

FIG. 1. Phenotype of atp22 mutants. A, spectra of mitochondrial cytochromes in wild type and an atp22 mutant. Mitochondria of the wild type strain D273-10B/A1 (D273) and the atp22 mutant N417/L2 were extracted with potassium deoxycholate at a protein concentration of 5 mg/ml (39), and difference spectra of the extracts oxidized with potassium ferricyanide and reduced with sodium dithionite were recorded at 580 nm (39), and difference spectra of the extracts oxidized with potassium ferricyanide and reduced with sodium dithionite were recorded at 580 nm (39), and difference spectra of the extracts oxidized with potassium ferricyanide and reduced with sodium dithionite were recorded at 580 nm (39), and difference spectra of the extracts oxidized with potassium ferricyanide and reduced with sodium dithionite were recorded at 580 nm (39).
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respiration in the mutant, was amplified in E. coli and used to subclone the gene. Various regions of the nuclear DNA insert in pG99/T1 were transferred to YEp351 (29), and the resultant constructs were tested for their ability to confer respiratory competence to C208/L2. Based on the ability of the subclones to rescue the mutant phenotype, the gene was localized between a HindIII and a Bgl II site of pG99/ST1 (Fig. 2A). The sequence of this region revealed a single reading frame corresponding to open reading frame YDR350c/TCM10 on chromosome IV. Because TCM10, the previous three letter designation for this gene, is not based on any known function of the protein, we propose ATP22 as a more appropriate alternative designation based on earlier names of mitochondrial ATPase-related genes.

A mutant allele of ATP22 was constructed by replacing most of the coding sequence with the yeast HIS3 gene (see “Materials and Methods”). A linear fragment of DNA containing the null allele was used to transform the respiration-competent diploid strain W303. Two independent His+ transformants were sporulated, tetrad dissected, and the meiotic products scored for their growth phenotype. Seven complete tetrads obtained from the two transformants (α/w303ΔATP22) showed a 2:2 segregation of growth on non-fermentable substrates (glycerol/ethanol). In each instance the loss of respiratory competence co-segregated with the histidine prototrophy. The respiratory defect of the haploid segregants was complemented by a ρ− mutant but not by ATP22 testers, indicating close linkage of the point mutation to the ATP22/HIS3 null allele. Two haploid ATP22 segregants, W303ΔATP22 and w303ΔATP22, were also checked for stability of their mtDNA. In both cases less than 1% of freshly grown cultures of either strain had wild type mtDNA (ρ−). A similar deletion in strain LL20, however, had a less severe effect on mtDNA. In this genetic background only 50% of vegetatively grown cells were ρ−. Nonetheless, because of their high genome instability, ATP22 null mutants were of limited usefulness for biochemical studies.

Analysis of ATPase—The absence of oligomycin-sensitive ATPase activity in ATP22 mutants was most consistent with a lesion in the F0 sector of the complex. This is supported by the sedimentation properties of the ATPase in the ATP22 mutant. In agreement with previous studies (45), the ATPase extracted with Triton X-100 from wild type mitochondria sediments as the F1-F0 complex with an estimated mass of 500 kDa (Fig. 3A). The distribution of ATPase activity following centrifugation of a similar extract from the mutant mitochondria indicated a mass of ~310 kDa, a value within experimental error of the mass of the yeast F1-ATPase determined by this method as reported previously (45).

A lesion in the F0 sector was also supported by the steady-state concentrations of ATPase subunits. Some components of F0, particularly subunit 6, are unstable in mutants that fail to assemble the F1-F0 complex (16). Western analysis indicated that mitochondria of ATP22 mutants had normal amounts of the α subunit of F1, but were almost totally depleted of subunit 6 of F0 (Fig. 3B). The αtp22 mutant N9-168, included as a control, also displays the absence of subunit 6, even though this
gene targets subunit 9 (12, 13). The low level of subunit 6 detected in N417/L2 is consistent with the slow growth phenotype of this mutant. Because synthesis of subunit 6 is not affected in the mutants (Fig. 1C), its low steady-state level is most likely the result of increased protein turnover as a result of impaired F_{0} assembly.

Localization of Atp22p—Atp22p is a 71-kDa basic protein with an overall hydrophilic character. There are, however, several hydrophobic stretches in the protein of sufficient length to qualify for transmembrane domains (not shown). No homologues of Atp22p, except for other species of Saccharomyces, were detected in the most recent protein data banks.

Atp22p was localized in mitochondria immunochemically and by expressing it as a fusion protein containing a carboxy-terminal 7-kDa polypeptide with a biotinylation signal (49). The antibody recognized a protein of ~70 kDa in the mitochondrial fraction of a wild type strain but not the atp22 null mutant (Fig. 4A). The greatly increased signal seen in a mutant (Fig. 4A) indicates the presence of the transformant (data not shown). A biotinylated protein of 12 mg/ml in 0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA (STE) was separated on a 12% polyacrylamide gel and processed as in Fig. 4A. B, separation of Atp22p on isopycnic gradients. Mitochondria (Mito) of the wild type strain W303-1A were converted to submitochondrial particles with potassium deoxycholate (DOC). Mitochondria from the wild type strain W303-1A were converted to submitochondrial particles as in Fig. 5A. The membranes were suspended in 0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA (STE) at a final concentration of 12 mg of protein/ml. The suspension was adjusted to 1 M KCl and centrifuged at 100,000 × g, for 20 min, the supernatants were collected, and the pellets were resuspended in the starting volume of STE. Equivalent volumes of the pellets and supernatants were separated by SDS-PAGE on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose, the blot was probed with antibody against Atp22p and subunit 5 of cytochrome oxidase (Cox5p) as in Fig. 4A. B, separation of Atp22p on isopycnic gradients. Mitochondria (Mito) of the wild type strain W303-1A were converted to submitochondrial (SMP) particles by sonic irradiation as in Fig. 5A. The mitochondrial suspensions were also diluted with 8 volumes of 0.6 M sorbitol, 20 mM Hepes, pH 7.5, as a control.

Proteinase K (Prot. K) was added to one-half of each sample after dilution to a final concentration of 100 μg/ml. After incubation for 60 min on ice, phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM, and the mitochondria and mitoplasts were recovered by centrifugation at 100,000 × g, for 10 min. The pellets were suspended in 0.6 M sorbitol, 20 mM Hepes, pH 7.5, and precipitated by addition of 0.1 volume of 50% trichloroacetic acid and heated for 10 min at 65 °C. Mitochondrial (Mt) and mitoplast (Mp) proteins (40 μg) were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with antibody against Atp22p and cytochrome b_{2} (Cyt b_{2}). Antibody-antigen complexes were visualized by a second reaction with 125I-protein A and exposure to x-ray film.

Localization of Atp22p—Atp22p is a 71-kDa basic protein with an overall hydrophilic character. There are, however, several hydrophobic stretches in the protein of sufficient length to qualify for transmembrane domains (not shown). No homologues of Atp22p, except for other species of Saccharomyces, were detected in the most recent protein data banks.

Atp22p was localized in mitochondria immunochemically and by expressing it as a fusion protein containing a carboxy-terminal 7-kDa polypeptide with a biotinylation signal (49). The antibody recognized a protein of ~70 kDa in the mitochondrial fraction of a wild type strain but not the atp22 null mutant (Fig. 4A). The greatly increased signal seen in a mutant transformed with ATP22 on a multicopy plasmid further confirmed this band to be Atp22p. Atp22p was not detected in the post-mitochondrial supernatant fraction of wild type yeast or of the transformant (data not shown). A biotinylated protein of the expected size (~7 kDa larger than the native Atp22p) was also detected in mitochondria of the atp22 mutant C208/L2 transformed with the ATP22-BIO fusion gene on a high copy plasmid or integrated in single copy at the LEU2 locus of chromosomal DNA (Fig. 4B). Both transformants were complemented for the respiratory defect indicating the presence of the carboxyl-terminal biotinylated peptide did not interfere with the activity of the protein.

The presence of several hydrophobic domains in Atp22p suggested it might be a membrane protein. This was supported by...
its resistance to extraction with sodium carbonate and co-
fractination with sub mitochondrial particles following sonic
disruption of mitochondria (Fig. 5A). In contrast to the inter-
membrane marker cytochrome b_6, most of which was released
after conversion of mitochondria to mitoplasts, all the Atp22p
remained associated with the mitoplasts (Fig. 5B). Treatment
of mitochondria and mitoplasts with proteinase K under con-
ditions that cause loss of proteins that face the cytoplasmic
side of the inner membrane had no effect on Atp22p. The presence
of Atp22p in a protease-protected compartment of mitoplasts
(together with its failure to be released from sonically disrupted
mitochondria and mitoplasts with proteinase K under con-
ditions that cause loss of proteins that face the cytoplasmic
side of the inner membrane).

Unlike most mitochondrial membrane proteins, Atp22p is
not solubilized with deoxycholate. A titration indicated that
even at a final concentration of 1% deoxycholate, none of the
Atp22p was extracted (Fig. 6A). Under the same conditions,
increasing concentrations of the detergent caused a progressive
removal of the inner membrane protein Sco1p (40) from the
membranes. The ineffectiveness of deoxycholate to solubilize
Atp22p was also evident in its banding behavior on isopycnic
gradients. Centrifugation of mitochondria or sub mitochondrial
particles through a step sucrose gradient showed that Atp22p
banded at densities similar to that of Cox5p, a subunit of the
inner membrane marker cytochrome oxidase. When mitochon-
dria were treated with 1% deoxycholate, Atp22p was concen-
trated at a denser region of the gradient, whereas most of the
cytochrome oxidase was shifted to a region of lesser density.
The increased density is probably due to extraction of the bulk
phospholipids but not of Atp22p from the membrane by
dehydrate.

DISCUSSION
Nuclear genes of S. cerevisiae reported previously (12–15) to
affect F_0 assembly are involved in processing/translation of the
mitochondrial ATPase-specific transcripts for subunits 6, 8, and
9. The exception is Atp10p, a mitochondrial inner mem-
brane protein that functions post-translationally as a chaper-
one of subunit 6 (16, 36). The ATP22 product reported here is the
second example of a mitochondrial protein with a post-
translational role essential for F_0 assembly. This is supported by
the presence in atp22 mutants of the fully processed ATP6,-8,
and -9 mRNAs and their normal translation when cyclohex-
imide-inhibited cells are pulse-labeled with a radioactive
precurser. Furthermore, the presence in the mutants of cataly-
ically active F_1-F_0-ATPase excludes a role of Atp22p in assembly
of this oligomeric protein. The nearly complete absence under
steady-state conditions of subunit 6 in the mutants further
argues for the importance of Atp22p for biogenesis of the F_1-F_0
complex. This evidence, however, does not necessarily mean
that Atp22p is involved in biogenesis of subunit 6 because
mutations that prevent formation of F_0, independent of their
specific functions, produce a similar phenotype. For example,
the atp13 (aep2) mutant N9-168 is also grossly deficient in
subunit 6 despite the fact that the product of this gene is
involved in expression of subunit 9 (12, 13).

Although the precise function of Atp22p is not clear at pres-
ent, its localization and topology in the inner membrane of
mitochondria is consistent with a role in assembly of the F_0
sector. Unlike ATP10 for which homologues exist in plants,
some fungi, but not animals, ATP22 appears to be present only
in the genus Saccharomyces. Searches of current protein data
banks have failed to reveal even distant relationships to pro-
teins of other organisms. This suggests that the function of
Atp22p is related to some unique feature of F_0 in this yeast.
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