

Formation of an Energized Inner Membrane in Mitochondria with a γ -Deficient F_1 -ATPase

Christopher P. Smith and Peter E. Thorsness*

Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071

Received 4 August 2005/Accepted 27 September 2005

Eukaryotic cells require mitochondrial compartments for viability. However, the budding yeast *Saccharomyces cerevisiae* is able to survive when mitochondrial DNA suffers substantial deletions or is completely absent, so long as a sufficient mitochondrial inner membrane potential is generated. In the absence of functional mitochondrial DNA, and consequently a functional electron transport chain and F_1F_o -ATPase, the essential electrical potential is maintained by the electrogenic exchange of ATP^{4-} for ADP^{3-} through the adenine nucleotide translocator. An essential aspect of this electrogenic process is the conversion of ATP^{4-} to ADP^{3-} in the mitochondrial matrix, and the nuclear-encoded subunits of F_1 -ATPase are hypothesized to be required for this process in vivo. Deletion of *ATP3*, the structural gene for the γ subunit of the F_1 -ATPase, causes yeast to quantitatively lose mitochondrial DNA and grow extremely slowly, presumably by interfering with the generation of an energized inner membrane. A spontaneous suppressor of this slow-growth phenotype was found to convert a conserved glycine to serine in the β subunit of F_1 -ATPase (*atp2-227*). This mutation allowed substantial ATP hydrolysis by the F_1 -ATPase even in the absence of the γ subunit, enabling yeast to generate a twofold greater inner membrane potential in response to ATP compared to mitochondria isolated from yeast lacking the γ subunit and containing wild-type β subunits. Analysis of the suppressing mutation by blue native polyacrylamide gel electrophoresis also revealed that the $\alpha_3\beta_3$ heterohexamers can form in the absence of the γ subunit.

The mitochondrial compartment is the site of oxidative phosphorylation and numerous other metabolic processes and is required for cell viability, even when the compartment is compromised by the loss of the mitochondrial genome (mtDNA) (1). An essential feature of mitochondria is an energized inner mitochondrial membrane, which allows for insertion of key proteins and, depending on environmental and genetic conditions, the synthesis of ATP^{4-} from ADP^{3-} and P_i (5, 10, 13, 29). Cells that contain an intact mitochondrial genome, referred to as ρ^+ , can generate an energized inner membrane by creating a membrane potential ($\Delta\Psi_M$) in three ways: (i) by pumping protons into the intermembrane space as electrons are passed to O_2 via the electron transport chain, (ii) by coupling the pumping of protons into the intermembrane space to ATP hydrolysis by the F_1F_o -ATPase, and (iii) by exchanging ATP^{4-} for ADP^{3-} through the adenine nucleotide translocator (ANT). Because four subunits of the electron transport chain and three subunits of the F_o portion of the F_1F_o -ATPase are encoded by mtDNA, viable cells lacking a mitochondrial genome (ρ^o cells) must maintain $\Delta\Psi_M$ by the exchange of ATP^{4-} for ADP^{3-} through ANT (14). ADP^{3-} is provided by the hydrolysis of ATP^{4-} , catalyzed by the remaining F_1 portion of the ATPase (F_1 -ATPase).

Numerous observations illustrate the dependence of ρ^o cells upon ATP hydrolysis and ANT activity in different organisms. For example, *Saccharomyces cerevisiae* (budding yeast) tolerates loss of mtDNA (petite-positive phenotype), but loss-of-function mutations in the major isoform of ANT (e.g., the

point mutation encoded by *op1*) render yeast petite-negative (19). Yeast cells bearing mutations that inactivate F_1 -ATPase or the mitochondrial AAA protease (*yme1* Δ) also exhibit a petite-negative phenotype (7, 14, 18, 35, 37). The *yme1* Δ petite-negative phenotype can be suppressed by point mutations that create changes of certain residues in the α and γ subunits of F_1 -ATPase, leading to increased ATP hydrolysis activity and an elevated $\Delta\Psi_M$ (17, 37). Some of the changes occur in the same residues that have also been shown to allow the petite-negative yeast *Kluyveromyces lactis* to grow without mtDNA (8). Finally, as in yeast, ANT and F_1 -ATPase activity are essential in ρ^o human cells (3, 6).

F_1 -ATPase activity in *S. cerevisiae* is severely compromised by deletion of *ATP3*, which encodes the γ subunit of F_1 -ATPase. *atp3* Δ yeast cells also quantitatively lose their mtDNA and grow extremely slowly (20). Presumably, there is sufficient ATP hydrolysis in the mitochondrial matrix to support at least the minimal $\Delta\Psi_M$ required for viability. The loss of mtDNA in *atp3* Δ yeast is likely the result of the dissipation of the mitochondrial inner membrane electrical potential that is created by an assembled proton-leaking, γ -deficient F_1F_o -ATPase (23). The slow-growth phenotype of *atp3* Δ yeast is suppressed by loss of the gene encoding either the α subunit (*atp1* Δ) or the β subunit (*atp2* Δ) (20). In *atp1* Δ *atp3* Δ and *atp2* Δ *atp3* Δ yeast cells, the F_1F_o -ATPase never assembles, so a proton leak is not formed. Therefore, mtDNA is stable, and the membrane potential is maintained by the electron transport chain (23).

Based upon the model for generation of $\Delta\Psi_M$ in ρ^o yeast, we hypothesized that the slow-growth phenotype of *atp3* Δ yeast can also be overcome by improved ATP hydrolysis activity in the mitochondrial matrix as opposed to mutations that stabilize mtDNA. Utilizing an unbiased genetic approach, we designed a

* Corresponding author. Mailing address: Department of Molecular Biology, University of Wyoming, Laramie, WY 82071. Phone: (307) 766-2038. Fax: (307) 766-5098. E-mail: thorsnes@uwyo.edu.

TABLE 1. Yeast strains

Strain	Genotype ^a	Source or reference
PTY44	<i>MATα ura3-52 lys2 leu2-3,112 trp1-Δ1</i> [ρ^+ , <i>TRP1</i>]	34
JTY3	<i>MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 atp3-Δ1::kanMX4</i> [ρ^0]	This study
JTY6	<i>MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 atp2-227 atp3-Δ1::kanMX4</i> [ρ^0]	This study
TCY55	<i>MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 atp2-227</i> [ρ^+ , <i>TRP1</i>]	This study
TCY60	<i>MATα ura3-52 ade2 leu2-3,112 trp1-Δ1 atp2-Δ1::kanMX4 atp3-Δ1::kanMX4</i> [ρ^+ , <i>TRP1</i>]	This study
TCY46	<i>MATα ura3-52 ade2 leu2-3,112 trp1-Δ1 atp1-Δ1::kanMX4</i> [ρ^+ , <i>TRP1</i>]	This study
TCY64	<i>MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 atp1-Δ1::kanMX4 atp3-Δ1::kanMX4</i> [ρ^+ , <i>TRP1</i>]	This study
TCY102	<i>MATα ura3-52 ade2 leu2-3,112 trp1-Δ1 atp1-Δ1::kanMX4 atp2-227 atp3-Δ1::kanMX4</i> [ρ^+ , <i>TRP1</i>]	This study
TCY103	<i>MATα ura3-52 ade2 leu2-3,112 trp1-Δ1 atp1-Δ1::kanMX4 atp2-227</i> [ρ^+ , <i>TRP1</i>]	This study
TCY38	<i>MATα ura3-52 ade2 leu2-3,112 trp1-Δ1 his3-Δ1::hisG opl</i> [ρ^+ , <i>TRP1</i>]	This study

^a The mitochondrial genotype is bracketed.

suppressor selection strategy to isolate a mutation(s) that allows vigorous growth of *atp3 Δ ρ^0* yeast. The selection strategy yielded one spontaneous suppressing mutation that changed a conserved residue near the active site of F₁-ATPase, leading to a more active, γ -deficient F₁-ATPase. The observed increase in ATP hydrolysis activity and consequent increase in $\Delta\Psi_M$ are consistent with the model that a threshold of ATP hydrolysis is required in the mitochondrial matrix for formation of the essential membrane potential in ρ^0 yeast. Analysis of the suppressing mutation by blue native polyacrylamide gel electrophoresis (BN-PAGE) also revealed that the $\alpha_3\beta_3$ heterohexamers of the F₁-ATPase forms in the absence of the γ subunit, providing additional insight into the assembly of the F₁F₀-ATPase.

MATERIALS AND METHODS

Strains and media. All *S. cerevisiae* strains were derived from D273-10B, and the genotypes of the strains used in this study are summarized in Table 1 (34). Standard genetic techniques were used to construct and analyze various yeast strains (30). Yeast strains were grown in complete glucose (YPD) medium containing 2% glucose, 2% Bacto peptone, 1% yeast extract, 40 mg/liter adenine, 40 mg/liter tryptophan; complete ethanol-glycerol (YPEG) medium containing 3% glycerol, 3% ethanol, 2% Bacto peptone, 1% yeast extract, 40 mg/liter adenine, 40 mg/liter tryptophan; complete raffinose (YPRaf) medium containing 2% raffinose, 2% Bacto peptone, 1% yeast extract, 40 mg/liter adenine, 40 mg/liter tryptophan; or synthetic dextrose (SD) medium containing 2% glucose and 6.7 g/liter yeast nitrogen base without amino acids (Difco), supplemented with the appropriate nutrients (34). Where indicated, ethidium bromide (EtBr) was added at a concentration of 25 μ g/ml (37), or Geneticin was added at a concentration of 300 μ g/ml.

Nucleic acid techniques. All manipulations of DNA were performed using standard techniques (26). Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). Plasmid DNA was prepared from *Escherichia coli* by boiling lysis (26).

Null mutations of *ATP1*, *ATP2*, and *ATP3* were created by homologous gene replacement using DNA fragments generated by PCR. Genomic DNA prepared from a yeast deletion library (Research Genetics) containing the appropriate null allele (replaced with the KanMx4 cassette) was used as a template. Oligonucleotides used in the PCR to generate DNA for the disruptions were the following: 5'-CCATCTTCCCATTGACGTT-3' (forward primer) and 5'-CTTGCAGGC GATATTTTCCTT-3' (reverse primer) for *ATP1*, 5'-AAGTGCTCCTCCTCA AGTCAA-3' (forward primer) and 5'-AACAAGAAGGAAGCAGGGAA-3' (reverse primer) for *ATP2*, and 5'-ACGTGATGGAGAACCAATGA-3' (forward primer) and 5'-GCGAGAAGGACTTTTCAAAAAA-3' (reverse primer) for *ATP3*. PCR-generated DNAs were used to transform the yeast strain PTY44. Transformants resistant to Geneticin (Sigma Chemical Corp.) were putative null alleles of *ATP1*, *ATP2*, or *ATP3* and were verified by PCR.

ATP2 (pATP2) and *atp2-227* (pATP2-227) were amplified by PCR and cloned into the BamHI and SacI sites of pRS316 (*CEN6*, *ARS*, and *URA3*). Genomic DNA from appropriate yeast strains was used for PCRs using the following oligonucleotides: 5'-CGGATTGGTACCTTTGGATCCGGACAGGCCAAT TGATAAA-3' (forward primer) and 5'-GTGGGATCCTTTGAGCTCTTA

TCGCAGGTTGTGTGCTT-3' (reverse primer). The primers were located 192 bp upstream and 319 bp downstream of the *ATP2* open reading frame.

Isolation of mitochondria. Mitochondrial isolation was performed essentially as described (9, 38). Yeast were grown in YPRaf medium, treated with zymolyase (Seikagaku America) to create spheroplasts and broken with a Dounce homog-

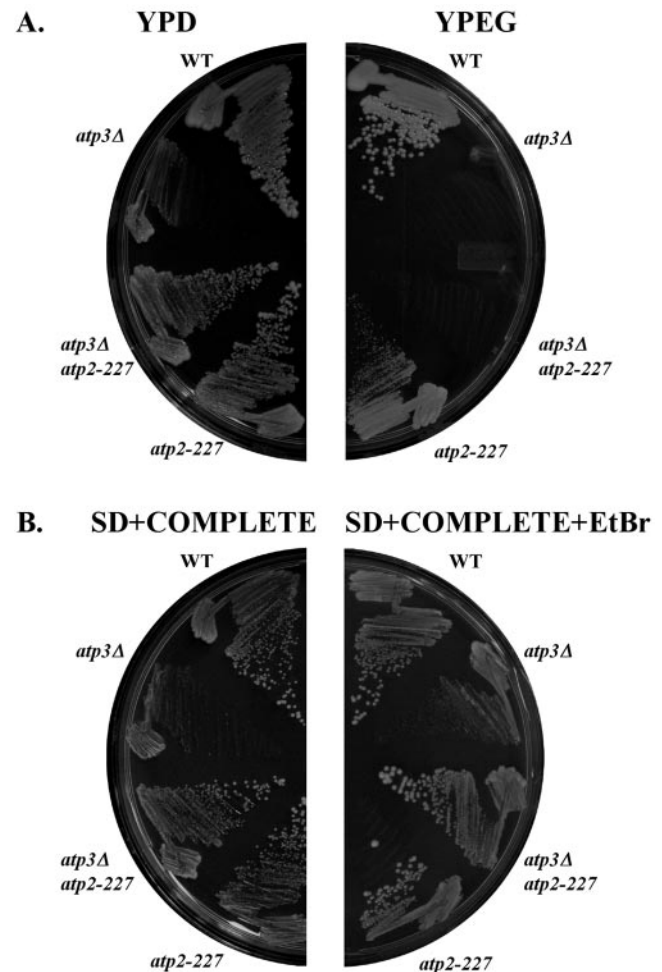


FIG. 1. Suppression of the *atp3 Δ* slow-growth phenotype by *atp2-227*. The indicated strains were streaked on YPD and YPEG media (A) and on synthetic dextrose SD medium lacking or containing 25 μ g/ml EtBr (B) and incubated for 5 days at 30°C. Growth of yeast in the presence of EtBr induces the quantitative loss of mtDNA (12, 31). Strains: wild type (WT), PTY44; *atp3 Δ* , JTY3; *atp2-227 atp3 Δ* , JTY6; *atp2-227*, TCY55.

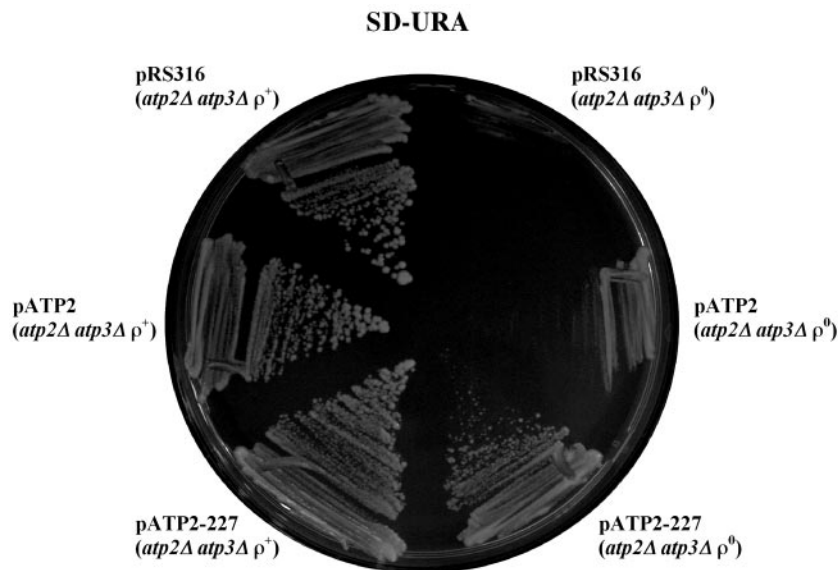


FIG. 2. Rescue of *atp3Δ* slow-growth phenotype by *atp2-227*. *atp2Δ atp3Δ* yeast cells were transformed with pRS316 (vector control), pATP2 (wild-type *ATP2*), and pATP2-227 (*atp2-227*). Transformed strains were grown on plates of SD medium lacking uracil (URA) and lacking (ρ^+) or containing (ρ^0) 25 $\mu\text{g/ml}$ EtBr and then streaked to SD medium lacking uracil and incubated for 5 days at 30°C to compare growth.

enizer, and mitochondria were collected by differential centrifugation. Mitochondrial yield was determined by spectrophotometric analysis (A_{280}).

BN gel electrophoresis. BN-PAGE was performed as previously described (25, 27, 28). Isolated mitochondrial pellets (200 to 300 μg of protein) were lysed in 35 μl of ice-cold digitonin buffer (1% [wt/vol] digitonin, 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10% [vol/vol] glycerol). After a clarifying spin, 5 μl of sample buffer (5% [wt/vol] Serva Blue [Invitrogen], 100 mM Bis-Tris, pH 7.0, 500 mM ϵ -aminocaproic acid, 25% glycerol) was added to the supernatants. The samples (100 μg of protein for ρ^+ yeast and 150 μg of protein for ρ^0 yeast) were separated on a 4 to 10% polyacrylamide gradient gel at 4°C. Atp2p and Atp1p were detected using antisera that were a gift from Trevor Lithgow. Signals were detected using the ECL detection method (Amersham).

Determination of mitochondrial F_1F_0 -ATPase activity and mitochondrial membrane potential. ATPase activities were determined using isolated mitochondria essentially as previously described (36). Studies were performed in parallel, with and without 10 μg of oligomycin (Sigma). Each reaction was performed in triplicate from the same isolated mitochondria preparation. The reaction mixtures contained 120 μg of mitochondria and were incubated at 37°C for 12 min.

The effect of the addition of ATP upon the inner mitochondrial membrane potential was assayed by monitoring the potential-dependent quenching of the fluorescent dye rhodamine-123 (Molecular Probes, Eugene, OR) (11, 14). Fluorescence was monitored using a FluoroMax-2 spectrofluorometer operating in steady-state mode. Samples were excited at 502 nm, and emission was measured at 525 nm. Each reaction was performed using 250 μg of mitochondria in a final volume of 2.5 ml. At the indicated time, ATP was added to a final concentration of 1 mM. A total of 50 ng of valinomycin in ethanol (50 μl) was subsequently added at the indicated times. All experiments were performed in triplicate.

RESULTS

***atp2-227* suppresses the slow-growth phenotype of *atp3Δ* yeast.** *atp3Δ* yeast cells grow slowly, even on rich glucose medium. Suppressing mutations of this phenotype were selected in complete SD medium containing EtBr, which induces the quantitative loss of mtDNA (31). Fifty independent cultures were grown to saturation, and approximately 1×10^7 cells/culture were plated to separate solid growth medium plates containing glucose as a carbon source. A single faster-growing

colony was isolated (*atp3Δ atp2-227*), indicating that such suppressing mutations are rare. Yeast containing *atp2-227* grew at a rate comparable to wild-type ρ^+ cells on fermentable carbon sources (YPD medium and SD plus complete medium), regardless of the presence or absence of the γ subunit (Fig. 1, *ATP3* versus *atp3Δ*). Similarly, *atp2-227 atp3Δ* ρ^0 and *atp2-227 ATP3* ρ^0 yeast cells grow at a rate similar to wild-type ρ^0 yeast (Fig. 1B, SD+COMPLETE+EtBr).

atp3Δ and *atp3Δ atp2-227* yeast cells failed to grow on the nonfermentable carbon source (YPEG medium) because cells lacking the γ subunit of F_1F_0 -ATPase are incapable of respiratory growth (24, 37) (Fig. 1A). *atp3Δ* yeast cells containing the suppressing *atp2-227* mutation were quantitatively ρ^0 , like unsuppressed *atp3Δ* yeast, after reintroduction of mtDNA upon backcrossing (data not shown). *atp2-227 ATP3* yeast showed a dramatic reduction in growth on YPEG medium compared to wild-type yeast (Fig. 1A). This intrinsic slow-growth phenotype of *atp2-227* ρ^+ yeast on YPEG medium was not the result of increased ρ^0 formation, since cultures of both wild-type ρ^+ and *atp2-227* yeast contained approximately 98% ρ^+ cells.

***atp2-227* encodes a mutant form of the β subunit of F_1F_0 -ATPase.** The respiratory-deficient phenotype of *atp2-227* ρ^+ yeast was used to clone the wild-type gene corresponding to the suppressing mutation. Plasmids that complemented the *atp2-227* respiratory defect were identified and sequenced. The complementing clones encoded *ATP2*, the structural gene for the β subunit of the F_1F_0 -ATPase. The wild-type and suppressing *ATP2* alleles were cloned by PCR into a replicating plasmid and expressed in yeast. *atp2Δ atp3Δ* yeast cells bearing a plasmid expressing the *atp2-227* suppressing allele grew more vigorously in the absence of mtDNA than did the same yeast strain containing a plasmid expressing the wild-type *ATP2* allele or a plasmid control (Fig. 2).

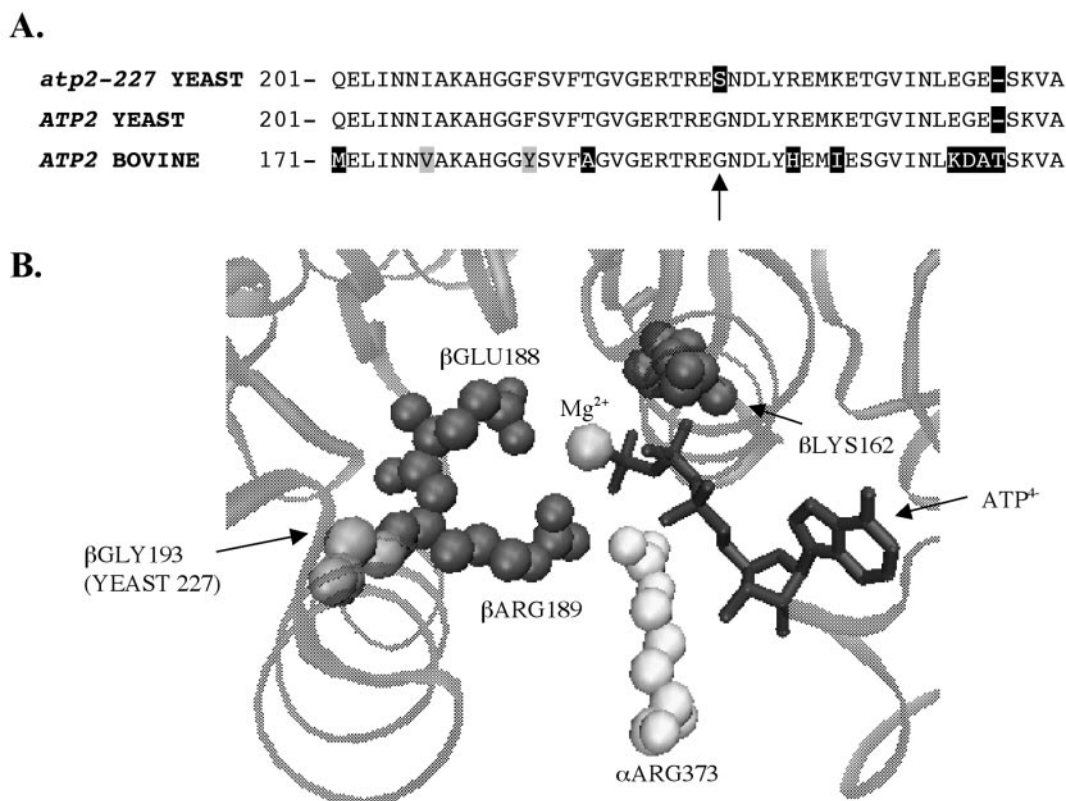


FIG. 3. *atp2-227* encodes a change from glycine to serine at residue 227 of the β -subunit of the F_1 -ATPase. (A) Predicted amino acid sequence of residues 201 to 249 of the yeast wild-type *ATP2* and *atp2-227* alleles aligned with the comparable region of bovine *ATP2* using CLUSTALW (33). Black boxes indicate nonconserved amino acid substitutions, and gray boxes indicate conservative substitutions. The arrow marks position 227. (B) A three-dimensional rendering of the bovine F_1 -ATPase active site with the equivalent glycine residue (yeast residue 227 is equivalent to bovine β GLY193) was created using the program VMD, version 1.8.2 (15).

Analysis of the sequence for *atp2-227* reveals a point mutation in codon 227 (GGT \rightarrow AGT) that causes a change from glycine to serine. This codon is located in a highly conserved region of the β subunit (33) (Fig. 3A). Probing databases with the BLASTP algorithm identified only two orthologs out of 500 hits that do not contain a glycine at this position (2). The ortholog found in the purple sea urchin *Strongylocentrotus purpuratus* contains a serine in this position, like the *atp2-227* allele, while one of several reported sequences for *Anopheles gambiae* contains a valine at the equivalent residue. Utilizing the high-resolution X-ray crystal structure data for the bovine ATP synthase, the equivalent glycine (193) residue was found

to be near the ATP hydrolysis/synthesis active site (1, 15) (Fig. 3B). The location of this residue led us to hypothesize that the mutation suppressed the slow-growth phenotype of *atp3 Δ* yeast by increasing ATP hydrolysis activity.

***atp2-227* increases ATP hydrolysis activity in *atp3 Δ* yeast.** The ATP hydrolysis activity measured in isolated mitochondria is summarized in Table 2. The ATP hydrolysis assays were performed in the presence and absence of the F_1F_0 -ATPase proton-translocation inhibitor, oligomycin (36). Wild-type ρ^+ cells showed an approximate fourfold reduction in ATP hydrolysis in the presence of oligomycin, indicating that much of the ATP hydrolysis was a result of an assembled F_1F_0 -ATPase.

TABLE 2. ATPase activity of isolated mitochondria

Strain	Relevant genotype	ATP hydrolysis activity (μ mol of P_i /min/mg)		Total ATP hydrolysis activity (% of control level) ^a
		With oligomycin	Without oligomycin	
PTY44	<i>ATP3 ATP2</i> ρ^+	1.33 \pm 0.09	5.89 \pm 0.03	100
TCY55	<i>ATP3 atp2-227</i> ρ^+	1.02 \pm 0.09	4.09 \pm 0.38	69.4
JTY6	<i>atp3Δ atp2-227</i> ρ^0	1.06 \pm 0.12	1.12 \pm 0.07 ^b	19.0
PTY44 (ρ^0)	<i>ATP3 ATP2</i> ρ^0	0.84 \pm 0.05	1.00 \pm 0.13	16.9
JTY3	<i>atp3Δ ATP2</i> ρ^0	0.40 \pm 0.11	0.33 \pm 0.01 ^b	5.6
TCY60	<i>atp3Δ atp2Δ</i> ρ^+	0.0130 \pm 0.0030	0.0122 \pm 0.0028	0.2

^a ATPase activity was normalized to wild-type (PTY44) in the absence of oligomycin.

^b The ATPase activity levels of the two strains are statistically different as determined by a *t* test of independent samples, with $P < 0.0001$.

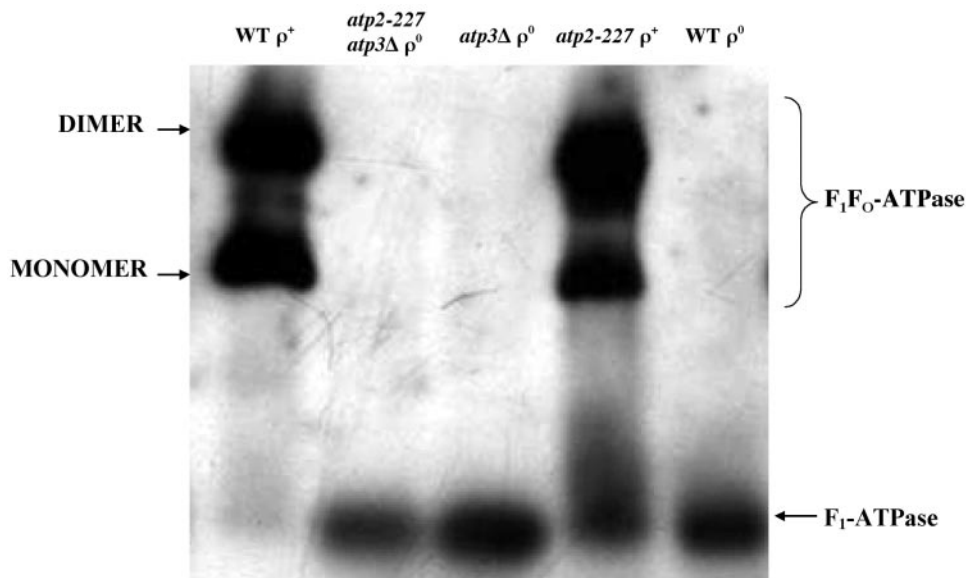


FIG. 4. F_1F_o -ATPase assembly in *atp2-227*, *atp3Δ*, and *atp3Δ atp2-227* yeast. A total of 100 μ g (ρ^+ yeast) or 150 μ g (ρ^0 yeast) of mitochondria isolated from the indicated strains in 1% digitonin buffer was resolved by BN-PAGE on a 4 to 10% polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with antibodies against Atp2p. The band corresponding to the monomer of the F_1F_o -ATPase migrated with a molecular weight slightly smaller than thyroglobulin (669 kDa; data not shown). Strains: wild type (WT) ρ^+ , PTY44 ρ^+ ; *atp2-227 atp3Δ* ρ^0 , JTY6; *atp3Δ* ρ^0 , JTY3; *atp2-227* ρ^+ , TCY55; WT ρ^0 , PTY44 ρ^0 .

atp2-227 ρ^+ yeast cells also showed a fourfold reduction in ATP hydrolysis upon the addition of oligomycin, indicating that the mutation does not impact assembly of an intact F_1F_o -ATPase. However, *atp2-227* ρ^+ yeast showed an overall reduction of F_1F_o -ATPase activity relative to wild-type ρ^+ yeast, indicating that the assembled F_1F_o -ATPase has impaired catalytic ability, which is consistent with the reduced growth of *atp2-227* ρ^+ yeast on nonfermentable carbon sources (Fig. 1A). Mitochondria prepared from yeast that lack mtDNA show an overall reduction in ATP hydrolysis compared to mitochondria prepared from ρ^+ yeast, and the ATPase activity is insensitive to oligomycin. Mitochondria isolated from *atp3Δ* ρ^0 yeast have very low ATPase activity, reduced to approximately one-third the level found in mitochondria of wild-type ρ^0 yeast. In contrast, mitochondria from *atp2-227 atp3Δ* ρ^0 yeast exhibit a comparable rate of ATP hydrolysis with that found in mitochondria prepared from wild-type ρ^0 yeast and show a statistically significant threefold increase of activity relative to that measured in *atp3Δ* ρ^0 yeast. Finally, *atp2Δ atp3Δ* ρ^+ yeast showed virtually no ATP hydrolysis activity, consistent with the absence of a functional F_1 -ATPase. We conclude that the improved hydrolysis of ATP by F_1 -ATPase containing β subunits encoded by *atp2-227* in yeast lacking the γ subunit is the likely mode of suppression of the slow-growth phenotype of *atp3Δ* ρ^0 yeast.

F_1F_o -ATPase assembly in *atp2-227 atp3Δ*, and *atp3Δ atp2-227* yeast. Previous efforts found that a mutation in yeast that changed glycine-227 to aspartate impaired F_1F_o -ATPase assembly, leading to a dramatic reduction in ATP hydrolysis activity in the presence of the γ subunit (22). In contrast, we found that the substitution of serine for glycine at position 227 resulted in only 25% less activity than wild-type F_1F_o -ATPase and similar sensitivity to inhibition by oligomycin. To directly

address the physical state of F_1F_o -ATPase, BN-PAGE was used to assess assembly of the multisubunit complex (Fig. 4). Isolated mitochondria were subjected to BN-PAGE, blotted to nitrocellulose membranes, and probed with antibody for the β subunit of F_1F_o -ATPase (anti-Atp2p) (Fig. 4). All samples showed bands at the gel front, presumably indicating that free β subunits were present in each of the yeast strains (data not shown). The wild-type ρ^+ and *atp2-227* ρ^+ yeast showed bands that correspond to the monomer and dimer forms of the F_1F_o -ATPase (4, 32). Therefore, the change of glycine to serine at residue 227 in the yeast F_1F_o -ATPase does not significantly impact enzyme-complex assembly.

None of the mitochondria isolated from ρ^0 yeast cells contained assembled F_1F_o -ATPase, consistent with the absence of the F_o -portion, which is largely encoded by mtDNA (Fig. 4). However, all three ρ^0 mitochondrial preparations exhibited a band that has previously been attributed to an F_1 -ATPase (3, 4, 32). The band is of similar size, regardless of the presence (wild-type ρ^0) or absence (*atp3Δ* ρ^0 and *atp3Δ atp2-227* ρ^0) of the γ subunit. When probed with antisera directed against the α subunit of F_1F_o -ATPase (anti-Atp1p), all of the ρ^0 yeast cells showed a similar band corresponding to a free F_1 -ATPase (data not shown). Based upon BN-PAGE, the $\alpha_3\beta_3$ heterohexamers of the F_1 -ATPase can assemble without the γ subunit when the β subunit is encoded by either *ATP2* or *atp2-227*.

***atp2-227* requires the α subunit (*ATP1*) of F_1F_o -ATPase and a fully functional ANT for suppression of the slow-growth phenotype of *atp3Δ* yeast.** Based upon the proximity of the altered residue to the α subunit and the ability of the $\alpha_3\beta_3$ ring to assemble in *atp3Δ* yeast, we hypothesized that the substitution of serine for glycine at position 227 in the β subunit alters the conformation of the F_1 -ATPase active site by interacting with residues of both the α and β subunits. Consequently,

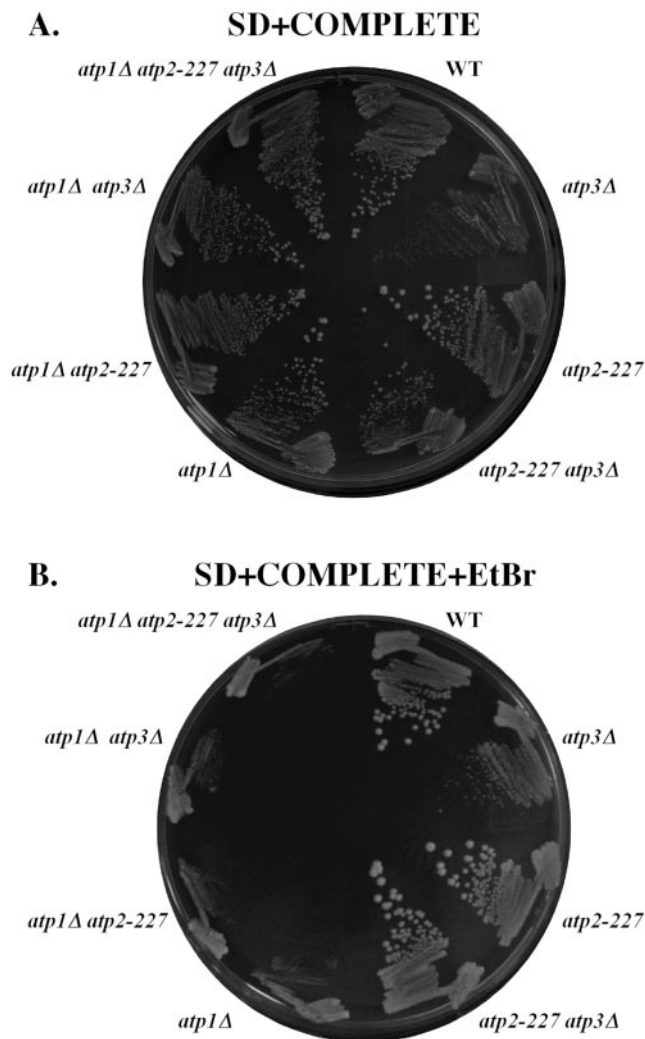


FIG. 5. *atp2-227 atp3Δ* ρ° and *atp2-227* ρ° yeast require the α subunit (*ATP1*) of the F_1F_0 -ATPase for vigorous growth. The indicated strains were streaked to SD medium lacking (A) and containing (B) 25 μ g/ml EtBr and incubated for 5 days at 30°C. Strains: wild type (WT), PTY44; *atp3Δ*, JTY3; *atp2-227*, TCY55; *atp2-227 atp3Δ*, JTY6; *atp1Δ*, TCY46; *atp1Δ atp2-227*, TCY103; *atp1Δ atp3Δ*, TCY64; *atp1Δ atp2-227 atp3Δ*, TCY102.

suppression of the *atp3Δ* slow-growth phenotype by *atp2-227* would require the α subunit. Yeast cells lacking both the α (*atp1Δ*) and γ subunits (*atp3Δ*) of the F_1F_0 -ATPase remain ρ^+ and grow faster than *atp3Δ* ρ° yeast (21) (Fig. 5A). However, *atp1Δ atp3Δ* yeast cells grow extremely slowly without mtDNA (Fig. 5B). The suppressing mutation *atp2-227* was introduced into yeast lacking both the α and γ subunits of F_1 -ATPase to assess its ability to suppress the extreme-slow-growth phenotype of these yeast cells when they are ρ° . As previously observed, *atp2-227 atp3Δ* yeast grew better than *atp3Δ* yeast on both media (both are quantitatively ρ°) (Fig. 5A). However, the suppressing mutation did not affect the growth of *atp1Δ* ρ^+ or *atp1Δ atp3Δ* ρ^+ yeast (Fig. 5A). In addition, the presence of the altered β subunit encoded by *atp2-227* did not improve growth of *atp1Δ* ρ° or *atp1Δ atp3Δ* ρ° yeast (Fig. 5B). Consequently, *atp2-227* does not disrupt the interaction between α

and β subunits but in fact requires this interaction to suppress the *atp3Δ* growth defect.

Tetrad analysis of genetic crosses of haploid yeast containing *atp3Δ*, *op1* (a loss-of-function point mutation of ANT), and *atp2Δ* or *atp2-227* was performed to determine if ANT activity was required for suppression of the *atp3Δ* slow-growth phenotype by *atp2-227*. First, 100% of yeast spores bearing both *atp3Δ* and *op1* were nonviable ($n = 19$). Similarly, 100% of *atp3Δ op1 atp2-227* spores were nonviable ($n = 5$). These results are consistent with our hypothesis that *atp3Δ* ρ° and *atp3Δ atp2-227* ρ° yeast require ANT to generate a sufficient $\Delta\Psi_M$ for viability.

***atp2-227* increases $\Delta\Psi_M$ in yeast lacking the γ subunit (*atp3Δ*) of F_1F_0 -ATPase.** Mitochondria isolated from *atp2-227 atp3Δ* ρ° yeast have increased ATPase activity compared to mitochondria prepared from *atp3Δ* ρ° yeast. Theoretically, this improved ATP hydrolysis provides an increased amount of ADP^{3-} to exchange for ATP^{4-} through ANT, increasing the inner membrane potential and consequently improving growth. To test this hypothesis, changes of $\Delta\Psi_M$ in isolated mitochondria were monitored in response to added ATP by measuring the uptake of the fluorescent dye, rhodamine 123 (11). These changes were recorded after the addition of mitochondria, ATP, and the ionophore valinomycin, which dissipated the $\Delta\Psi_M$ generated by hydrolysis of ATP. Rhodamine 123 fluorescence was quenched in response to ATP^{4-} in the absence of mitochondria but showed little response to valinomycin after an initial increase in intensity (<1%) (data not shown). Therefore, the response to valinomycin can be used to compare the $\Delta\Psi_M$ of mitochondria prepared from different strains of yeast.

Mitochondria prepared from wild-type ρ^+ yeast had a 5% increase in relative fluorescence upon the addition of valinomycin, compared to a 2% increase in fluorescence under similar conditions with mitochondria prepared from wild-type ρ° cells (Fig. 6A). Mitochondria isolated from cells that lack the γ subunit (*atp3Δ*) exhibited a 2% increase in relative fluorescence upon valinomycin addition, similar to mitochondria prepared from wild-type ρ° yeast (Fig. 6B). Mitochondria isolated from *atp2-227 atp3Δ* ρ° yeast had a 5% increase in fluorescence upon valinomycin treatment, equivalent to that found in mitochondria prepared from wild-type ρ^+ yeast (Fig. 6B). Mitochondria prepared from yeast lacking β and γ subunits (*atp2Δ atp3Δ*) showed essentially no response to valinomycin (Fig. 6C), indicating that very little ATP hydrolysis occurred, consistent with the inability to grow without mtDNA (Fig. 2). Finally, mitochondria isolated from *atp2-227* ρ^+ yeast also showed a 5% increase in fluorescence in response to valinomycin (Fig. 6C). The greater response of mitochondria prepared from *atp3Δ atp2-227* ρ° yeast to valinomycin indicates that *atp3Δ atp2-227* ρ° yeast cells have a greater $\Delta\Psi_M$ than *atp3Δ* ρ° yeast.

DISCUSSION

For yeast that lack mtDNA, and consequently a functional electron transport chain and ATP synthase, the major role of the F_1 -ATPase is to generate ADP^{3-} in support of the electrogenic exchange of ADP^{3-} for ATP^{4-} through ANT in ρ° yeast. Yeast cells lacking the γ subunit of F_1 -ATPase quantitatively lose mtDNA and grow extremely slowly, likely because

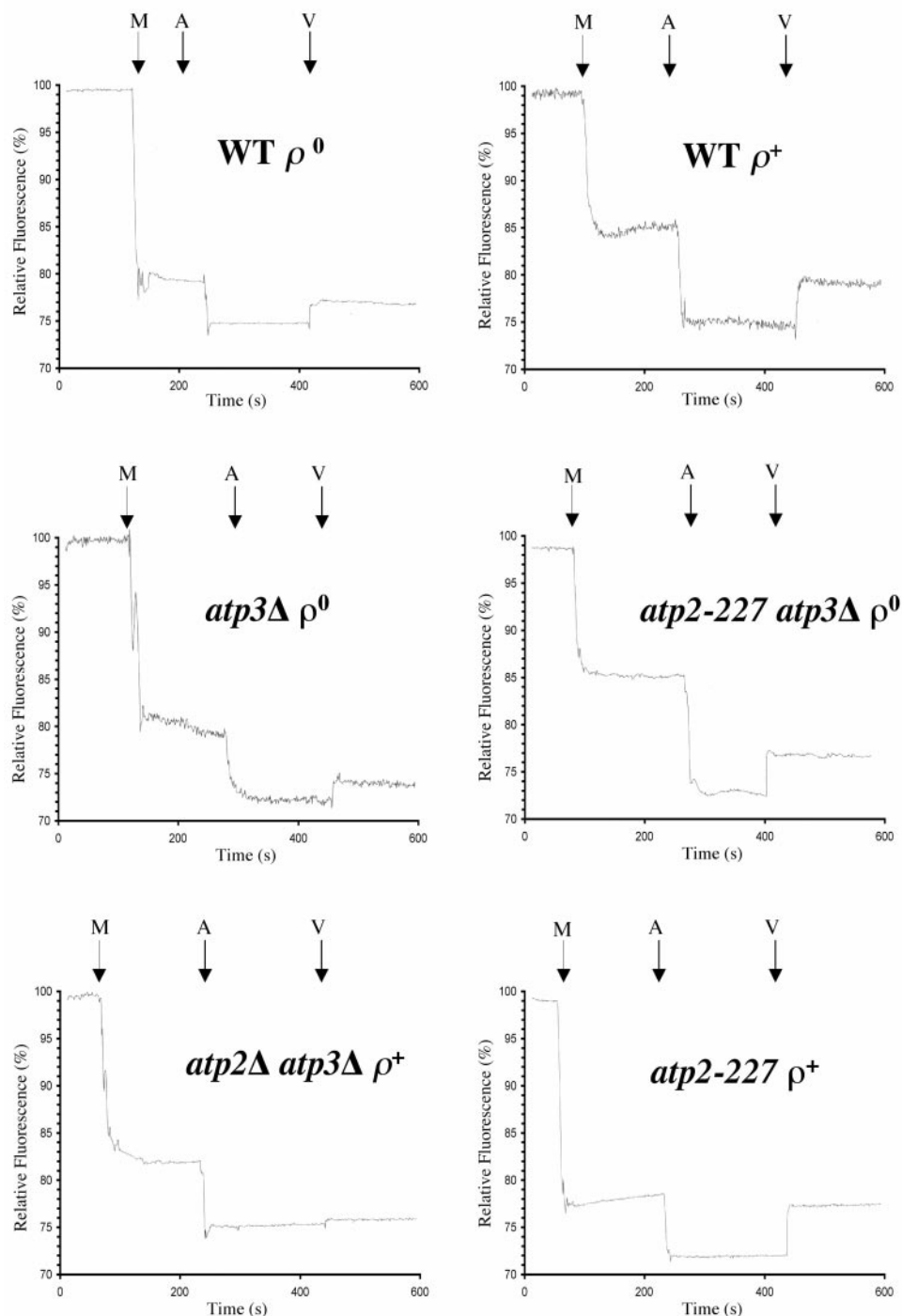


FIG. 6. *atp2-227 atp3Δ ρ⁰* yeast generates a greater inner mitochondrial membrane potential in response to ATP compared to *atp3Δ ρ⁰* yeast. At the indicated times, 250 μ g of mitochondria isolated from the indicated strains (M), ATP to a final concentration of 1 mM (A), and 50 ng of valinomycin in 95% ethanol (V) were added to a final volume of 2.5 ml of reaction buffer containing 1 μ M rhodamine 123 (Molecular Probes) (11, 14). The relative fluorescence was monitored using a FluoroMax-2 spectrofluorometer operating in the steady-state mode. The samples were excited at 502 nm, and emission was measured at 525 nm. Strains: wild type (WT) ρ^+ , PTY44 ρ^+ ; WT ρ^0 , PTY44 ρ^0 ; *atp3Δ ρ⁰*, JTY3; *atp2-227 atp3Δ ρ⁰*, JTY6; *atp2Δ atp3Δ ρ⁺*, TCY60; *atp2-227 ρ⁺*, TCY55.

ATP hydrolysis activity in the mitochondrial matrix is severely compromised. By employing an unbiased genetic approach, we identified a suppressing mutation in the β subunit (*atp2-227*) that improves growth of *atp3Δ ρ⁰* yeast (Fig. 1) by improving

ATP hydrolysis activity (Table 2) and consequently increasing $\Delta\Psi_M$ (Fig. 6). In addition, we have demonstrated that *atp3Δ ρ⁰* and *atp2-227 atp3Δ ρ⁰* yeast cells assemble a functional F_1 -ATPase (Fig. 4) and require both an interaction with the α

subunit and the presence of a fully function ANT for growth (Fig. 5) (see Results).

The arginine residue (α Arg373) of the bovine α subunit, which is part of the conserved arginine-finger domain of the active site, is proposed to sense the presence or absence of the γ -phosphate of the bound ATP (16). Because of the relative proximity of the β subunit residue changed by *atp2-227* to the active site of the enzyme (corresponding to G193S in the bovine enzyme) (Fig. 3B), we hypothesize that the introduction of a serine at position 227 changes the active site either through alteration of the hydrogen bonding network or steric effects, potentially affecting either the position of α Arg373 and/or the β Arg189. Such an alteration of the active site may lead to improved ATP hydrolysis in the absence of the γ subunit. This postulated repositioning of key active-site residues in the *atp2-227* allele would be the structural basis for suppression of the slow-growth phenotype of *atp3 Δ* yeast. The corresponding alteration of the interaction between the active site formed by the α and β subunits and the rotating γ subunit may also explain the reduced ability of strains bearing the *atp2-227* allele to grow on carbon sources that require oxidative phosphorylation.

Finally, the isolation of the spontaneous suppressing mutation and its subsequent characterization also provide further insight into the assembly of the F_1F_0 -ATPase. Previously, deletions in the α and β subunits were shown to suppress the slow-growth phenotype of *atp3 Δ* yeast (23). The current model of suppression suggests that the F_0 -portion of the ATPase "senses" the $\alpha_3\beta_3$ heterohexamer during assembly, and the important proton-channel subunit encoded by *ATP6* is added to the complex. Once formed, the uncoupled, γ -less enzyme freely passes protons down an electrochemical gradient, dissipating $\Delta\Psi_M$ and ultimately destabilizing mtDNA. In the work presented here, we provide BN-PAGE evidence suggesting that the $\alpha_3\beta_3$ heterohexamer of the F_1 -ATPase is assembled in the presence or absence of the γ subunit, supporting this model. This suggests that an interaction between the α and β subunits and the F_0 -portion of the ATPase, independent of the γ subunit, is a key step during assembly of the ATP synthase enzyme-complex.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health (GM068066).

We thank Sharon Ackerman for assistance with the protocol for the ATP hydrolysis assay, Trevor Lithgow for generously providing antisera, and Michael T. Ryan for the BN-PAGE protocol. We are also grateful to Mary Brownson and Jeremy Tiller for technical assistance and to Brian Francis, Mary Thorsness, and Karen White for critical review of the manuscript.

REFERENCES

1. Abrahams, J. P., A. G. W. Leslie, R. Lutter, and J. Walker. 1994. Structure at 2.8 Å resolution of F_1 -ATPase from bovine heart mitochondria. *Nature (London)* **370**:621–628.
2. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
3. Appleby, R. D., W. K. Porteous, G. Hughes, A. M. James, D. Shannon, Y. H. Wei, and M. P. Murphy. 1999. Quantitation and origin of the mitochondrial membrane potential in human cells lacking mitochondrial DNA. *Eur. J. Biochem.* **262**:108–116.
4. Arnold, I., K. Pfeiffer, W. Neupert, R. A. Stuart, and H. Schagger. 1998. Yeast mitochondrial F_1F_0 -ATP synthase exists as a dimer: identification of three dimer-specific subunits. *EMBO J.* **17**:7170–7178.
5. Baker, K. P., and G. Schatz. 1991. Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. *Nature (London)* **349**:205–208.
6. Buchet, K., and C. Godinot. 1998. Functional F_1 -ATPase essential in maintaining growth and membrane potential of human mitochondrial DNA-depleted ρ^0 cells. *J. Biol. Chem.* **273**:22983–22989.
7. Chen, X. J., and G. D. Clark-Walker. 1999. Alpha and beta subunits of F_1 -ATPase are required for survival of petite mutants in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **262**:898–908.
8. Clark-Walker, G. D., P. M. Hansbro, F. Gibson, and X. J. Chen. 2000. Mutant residues suppressing ρ^0 -lethality in *Kluyveromyces lactis* occur at contact sites between subunits of $F(1)$ -ATPase. *Biochim. Biophys. Acta* **1478**:125–137.
9. Daum, G., P. C. Böhni, and G. Schatz. 1982. Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. *J. Biol. Chem.* **257**:13028–13035.
10. Dunn, C. D., and R. E. Jensen. 2003. Suppression of a defect in mitochondrial protein import identifies cytosolic proteins required for viability of yeast cells lacking mitochondrial DNA. *Genetics* **165**:35–45.
11. Emaus, R. K., R. Grunwald, and J. J. Lemasters. 1986. Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim. Biophys. Acta* **850**:436–448.
12. Fox, T. D., L. S. Folley, J. J. Mulero, T. W. McMullin, P. E. Thorsness, L. O. Hedin, and M. C. Costanzo. 1991. Analysis and manipulation of yeast mitochondrial genes. *Methods Enzymol.* **194**:149–165.
13. Gasser, S. M., G. Daum, and G. Schatz. 1982. Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. *J. Biol. Chem.* **257**:13034–13041.
14. Giraud, M. F., and J. Velours. 1997. The absence of the mitochondrial ATP synthase delta subunit promotes a slow growth phenotype of ρ^- yeast cells by a lack of assembly of the catalytic sector F_1 . *Eur. J. Biochem.* **245**:813–818.
15. Humphrey, W., A. Dalke, and K. Schulten. 1996. VMD: visual molecular dynamics. *J. Mol. Graph.* **14**:33–38, 27–28.
16. Kagawa, R., M. G. Montgomery, K. Braig, A. G. Leslie, and J. E. Walker. 2004. The structure of bovine $F(1)$ -ATPase inhibited by ADP and beryllium fluoride. *EMBO J.* **23**:2734–2744.
17. Kominsky, D. J., M. P. Brownson, D. Updike, and P. E. Thorsness. 2002. Genetic and biochemical basis for viability of yeast lacking mitochondrial genomes. *Genetics Society of America, Bethesda, M.D.*
18. Kominsky, D. J., and P. E. Thorsness. 2000. Expression of the *Saccharomyces cerevisiae* gene *YME1* in the petite-negative yeast *Schizosaccharomyces pombe* converts it to a petite-positive yeast. *Genetics* **154**:147–154.
19. Kovacova, V., J. Irmelrova, and L. Kovac. 1968. Oxidative phosphorylation in yeast, IV: combination of a nuclear mutation affecting oxidative phosphorylation with cytoplasmic mutation to respiratory deficiency. *Biochim. Biophys. Acta* **162**:157–163.
20. Lai-Zhang, J., and D. M. Mueller. 2000. Complementation of deletion mutants in the genes encoding the F_1 -ATPase by expression of the corresponding bovine subunits in yeast *S. cerevisiae*. *Eur. J. Biochem.* **267**:2409–2418.
21. Lai-Zhang, J., Y. Xiao, and D. M. Mueller. 1999. Epistatic interactions of deletion mutants in the genes encoding the F_1 -ATPase in yeast *Saccharomyces cerevisiae*. *EMBO J.* **18**:58–64.
22. Liang, Y., and S. H. Ackerman. 1996. Characterization of mutations in the beta subunit of the mitochondrial F_1 -ATPase that produce defects in enzyme catalysis and assembly. *J. Biol. Chem.* **271**:26522–26528.
23. Mueller, D. M. 2000. Partial assembly of the yeast mitochondrial ATP synthase. *J. Bioenerg. Biomembr.* **32**:391–400.
24. Paul, M.-F., S. Ackerman, J. Yue, G. Arselin, J. Velours, and A. Tzagoloff. 1994. Cloning of the yeast *ATP3* gene coding for the γ -subunit of F_1 and characterization of *atp3* mutants. *J. Biol. Chem.* **269**:26158–26164.
25. Ryan, M. T., H. Muller, and N. Pfanner. 1999. Functional staging of ADP/ATP carrier translocation across the outer mitochondrial membrane. *J. Biol. Chem.* **274**:20619–20627.
26. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning, a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. Schagger, H., W. A. Cramer, and G. von Jagow. 1994. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **217**:220–230.
28. Schagger, H., and G. von Jagow. 1991. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* **199**:223–231.
29. Schleyer, M., B. Schmidt, and W. Neupert. 1982. Requirement of a membrane potential for the posttranslational transfer of proteins into mitochondria. *Eur. J. Biochem.* **125**:109–116.

30. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. *Methods in yeast genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Slonimski, P. P., G. Perrodin, and J. H. Croft.** 1968. Ethidium bromide induced mutation of yeast mitochondria: complete transformation of cells into respiratory deficient non-chromosomal "petites." *Biochem. Biophys. Res. Commun.* **30**:232–239.
32. **Soubannier, V., J. Vaillier, P. Paumard, B. Coulary, J. Schaeffer, and J. Velours.** 2002. In the absence of the first membrane-spanning segment of subunit 4(b), the yeast ATP synthase is functional but does not dimerize or oligomerize. *J. Biol. Chem.* **277**:10739–10745.
33. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
34. **Thorsness, P. E., and T. D. Fox.** 1993. Nuclear mutations in *Saccharomyces cerevisiae* that affect the escape of DNA from mitochondria to the nucleus. *Genetics* **134**:21–28.
35. **Thorsness, P. E., K. H. White, and T. D. Fox.** 1993. Inactivation of *YME1*, a member of the *ftsH-SEC18-PAS1-CDC48* family of putative ATPase-encoding genes, causes increased escape of DNA from mitochondria in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**:5418–5426.
36. **Tzagoloff, A.** 1979. Oligomycin-sensitive ATPase of *Saccharomyces cerevisiae*. *Methods Enzymol.* **55**:351–358.
37. **Weber, E. R., R. S. Rooks, K. S. Shafer, J. W. Chase, and P. E. Thorsness.** 1995. Mutations in the mitochondrial ATP synthase gamma subunit suppress a slow-growth phenotype of *yme1* yeast lacking mitochondrial DNA. *Genetics* **140**:435–442.
38. **Yaffe, M. P.** 1991. Analysis of mitochondrial function and assembly. *Methods Enzymol.* **194**:627–643.