Yeast Cells Lacking the Mitochondrial Gene Encoding the ATP Synthase Subunit 6 Exhibit a Selective Loss of Complex IV and Unusual Mitochondrial Morphology

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Atp6p is an essential subunit of the ATP synthase proton translocating domain, which is encoded by the mitochondrial DNA (mtDNA) in yeast. We have replaced the coding sequence of Atp6p gene with the non-respiratory genetic marker ARG8m. Due to the presence of ARG8m, accumulation of p−p° petites issued from large deletions in mtDNA could be restricted to 20–30% by growing the atp6 mutant in media lacking arginine. This moderate mtDNA instability created favorable conditions to investigate the consequences of a specific lack in Atp6p. Interestingly, in addition to the expected loss of ATP synthase activity, the cytochrome c oxidase respiratory enzyme steady-state level was found to be extremely low (<5%) in the atp6 mutant. We show that the cytochrome c oxidase-poor accumulation was caused by a failure in the synthesis of one of its mtDNA-encoded subunits, Cox1p, indicating that, in yeast mitochondrial proteins, Cox1p synthesis is a key target for cytochrome mtDNA-encoded subunits, Cox1p, indicating that, in yeast mitochondria, Cox1p synthesis is a key target for cytochrome c oxidase abundance regulation in relation to the ATP synthase activity. We provide direct evidence showing that in the absence of Atp6p the remaining subunits of the ATP synthase can still assemble. Mitochondrial cristae were detected in the atp6 mutant, showing that neither Atp6p nor the ATP synthase activity is critical for their formation. However, the atp6 mutant exhibited unusual mitochondrial structure and distribution anomalies, presumably caused by a strong delay in inner membrane fusion.

In the mitochondrial inner membrane, the F1F0-type ATP synthase produces ATP from ADP and inorganic phosphate by using the energy of the transmembrane electrochemical proton gradient generated by the respiratory chain in the course of electron transfer to oxygen. The ATP synthase harbors two major structural domains, a transmembrane component (F0) containing a proton-permeable pore and a peripheral, matrix-localized, catalytic component (F1), where the ATP is synthesized (1–4). In the F0, the core of the proton channel consists of a ring of c subunits (ten in yeast (4)) and one a subunit (Atp6p). Proton movement through this channel coincides with rotation of the subunit c ring (5–9), which results in conformational changes favoring ATP synthesis in the F1 (1).

Due to its good fermenting capacity the yeast Saccharomyces cerevisiae has been extensively used as a genetic system for the study of the mitochondrial ATP synthase (for reviews see Refs. 10 and 11). As in most eukaryotes, the yeast ATP synthase has a dual genetic origin, nuclear and mitochondrial. The yeast mitochondrial ATP synthase genes (ATP6, ATP9, and ATP8) encode the proton channel subunits a and c (usually referred to in yeast as Atp6p and Atp9p), respectively, and a third F0 subunit (Atp8p) of unknown function. Dozens of mutations in the nuclear ATP synthase genes have provided much information on their protein products (10, 11). In contrast, only a very few mutants of the mitochondrial ATP synthase genes have been reported. Random generation of respiratory growth-deficient yeast strains issued from point lesions in the mtDNA (mit−), by several groups in the 70s (12, 13), systematically produced one-hundred times more mutations in respiratory chain subunits (e.g. cytochrome b and Cox1p) than in the mtDNA-encoded ATP synthase subunits. The few isolated mitochondrial ATP synthase mutants were often genetically unstable in the form of p−p° petites, i.e. cells bearing large deletions in the mtDNA (p−) or totally lacking mtDNA (p°) (14–16). Those showing moderate mtDNA instability were often leaky mutants exhibiting slow growth on respiratory substrates. It has thus been assumed that the scarcity of mitochondrial ATP synthase gene mutants was due to their inherent genetic instability, and full inactivation of the mitochondrial ATP synthase genes was believed to result in nearly 100% petites (17). Although frameshift and nonsense mutations in these genes were found, it has been suggested that they are not automatically stringent mutations because of the error-prone character of the mitochondrial translation apparatus (see Ref. 18 for an example of a leaky frameshift mutation in the Atp6p gene). These early reports on

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4 The abbreviations used are: mtDNA, mitochondrial DNA; Atp6p, deletion of the S. cerevisiae ATP6 gene, coding for ATP synthase Atp6p subunit; GFP, green fluorescent protein; MOPS, 4-morpholinepropanesulfonic acid; BN, blue native; CN, clear native.
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TABLE 1

Genotypes and sources of yeast strains

| Strain | Nuclear genotype | mtDNA | Source |
|--------|------------------|-------|--------|
| DFS160 | Mata leu2Δ ura3-52 ade2-101 arg8::URA3 kar1-1 | † | (19) |
| NB40-3C | Mata lys2 lys3-11 ura3-52 his3ΔHindIII arg8::HisG | † cos2-62 | N. Bonnefoy |
| NB104 | Mata ade2-101 ura3-52 leu2Δ arg8::URA3 kar1-1 | † cos2-60 | This study |
| MR1 | Mata leu2Δ ura3-52 ade2-101 arg8::URA3 kar1-1 | † atp6::ARG8m | This study |
| MR6 | Mata ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::HIS3 | † WT | This study |
| MR10 | Mata ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::HIS3 | † atp6::ARG8m | This study |
| SDC30 | Mata leu2Δ ura3-52 ade2-101 arg8::URA3 kar1-1 | † ATP6 | This study |

yeast mitochondrial ATP synthase mutants, and later on the observation that mutations in nuclear-encoded subunits can also dramatically increase petite production (up to 100%), might have discouraged those who would have been interested in studying the mitochondrial ATP synthase proton channel subunits through the isolation of mutants, which may explain the absence of further mutational studies of these proteins over the last 20 years.

Here, we aimed to determine whether yeast cells where ATP6 is completely deleted (i.e. where informational suppression is not possible) can be obtained and maintained in a \( p^+ \) state. To this aim, we have used an approach, developed by Fox and co-workers (19), based on the insertion of non-respiratory genes, e.g. \( \text{ARG8}^m \), into the mtDNA. \( \text{ARG8}^m \) is a mitochondrial version of the nuclear \( \text{ARG8} \) gene encoding a mitochondrial protein involved in arginine biosynthesis. When \( \text{ARG8}^m \) is present in \( p^+ \) mtDNA, in a nuclear \( \Delta \text{arg8} \) context and in the absence of external arginine, \( p^-/p^0 \) cells cannot divide. They should therefore accumulate less rapidly in cultures of mutants with an increased propensity to produce petites, if the systems in charge of the mtDNA are still functional and if retention of \( p^+ \) mtDNA is not detrimental to the cell. In media lacking arginine, a yeast strain in which the entire ATP6 gene coding sequence had been replaced with \( \text{ARG8}^m \) open reading frame, produced only 20–30% \( p^-/p^0 \) cells. This moderate mtDNA instability created favorable conditions for investigating the consequences of a specific lack of ATP6.

MATERIALS AND METHODS

Yeast Strains and Media—The \( S. \text{cerevisiae} \) strains and their genotypes are listed in Table 1. The media used for growth of yeast were: YPGA (1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose, and 40 mg/liter adenine); N3 (1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glycerol, and 50 mM potassium phosphate buffer, pH 6.2); YPGALA (1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) galactose, and 40 mg/liter adenine); WO (0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose, and other supplements depending on the strain auxotrophic markers); CSMD (0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose, and 0.8% (w/v) of a CSM mixture of amino acids and bases (from BIO-101); CSMG (CSMG containing 2% (w/v) galactose instead of glucose); CSMD-R (CSMD medium with a CSM mixture lacking arginine); CSMG-R (CSMG medium with a CSM mixture lacking arginine); CSMD-U (CSMD medium with a CSM mixture lacking uracil); and CSMD-R-U (CSMD medium with a CSM mixture lacking both uracil and arginine). Solid media contained 2% (w/v) agar.

Deletion and Replacement of the ATP6 Gene with \( \text{ARG8}^m \)—The plasmid pDS24 containing \( \text{ARG8}^m \) (19) was used as a template for PCR amplification of an \( \text{atp6::ARG8}^m \) DNA cassette with primers 5'-GCcttagaTAATAAGAGTATTGATTATT-TATTATAAAGTTATGTTTATATATTTATAATTATATTAT-TGACACATTAGAAAGAG and 5'-GCCggatcCTTTAT-TTATATTTAATACCCATATGAAATATTTTTTATATTTTTGAACGATACAGCTTCG.'
AGGAG and 5'-CGggatccGAGGGGAAAGAGTAGGAT. Owing to the presence of a BamHI site at the 5’-end of these primers (lowercase letters), the PCR product was cloned as a BamHI-BamHI fragment into pJM2. The resulting plasmid, pSDC21, was introduced by biotic transformation into DFS160 mitochondria as described above (DFS160 transformation with pMR1), to give the p~ synthetic strain SDC30.

**In Vivo Labeling of Mitochondrial Translation Products**—The wild-type strain MR6 and the atp6::ARG8 deletion strain MR10 were grown to early exponential phase (10^7 cells/ml) in 10 ml of liquid CSMD and CSMD-R media, respectively. The cells were harvested by centrifugation and washed twice with a low sulfate medium (23) containing 2% glucose, supplemented with histidine, tryptophan, leucine, uracil, adenine, and arginine (50 mg/liter each). Cells were resuspended in 1 ml of the same medium, and 1 mm cycloheximide was added. After a 10-min incubation at 28 °C, 0.5 mCi of [35S]methionine plus [35S]cysteine (Amersham Biosciences) was added. The cell suspension was further incubated for 10 or 60 min at 28 °C. Mitochondrial membranes were then extracted using the procedure described in a previous study (23) and resuspended in 20 μl of Laemmli sample buffer (24).

Fluorescence Microscopy Analyses of Yeast Cells—For visualization of mitochondria by fluorescence microscopy, the wild-type strain MR6 and the atp6::ARG8 deletion strain MR10 were transformed with the URA3 plasmid pGALClbGFP bearing a galactose-inducible GFP fused to the pre-sequence of mitochondrial isocitrate synthase (25). Transformed MR6 and MR10 cells were grown to early exponential phase (10^7 cells/ml) in liquid CSMD-U and CSMD-R media, respectively. The cells were observed in a fully automated Zeiss 200M inverted microscope (Carl Zeiss, Thornwood, NY) equipped with an MS-2000 stage (Applied Scientific Instrumentation, Eugene, OR), a Lambda LS 175-watt xenon light source (Sutter, Novato, CA), a 100 × 1.4 numerical aperture plan-apochromat objective, and a five-position filter turret. Filter cubes were for Alexa-phalloidin 568: Cy3 (excitation: HQ535/50; emission: HQ500lp), and for fixed cells GFP Endow GFP long pass (excitation: HQ470/40, emission: HQ495lp, and beam splitter: Q495lp), and for fixed cells GFP co-localization Narrowband HQ fluorescein isothiocyanate (excitation: HQ487/25, emission: HQ535/40, and beam splitter: Q505lp, Chroma Technology Corp., Rockingham, VT). Images were acquired using a CoolSnap HQ camera (Roper Scientific, Tucson, AZ). The microscope, camera, and shutters (Uniblitz, Rochester, NY) were controlled by SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO). For live cell microscopy, 2 μl of the culture was spotted directly onto a glass slide and immediately imaged at room temperature (~22 °C). For fixed cells imaging, formaldehyde was added (3.7% final) directly in the culture.

Phalloidin staining was performed as previously described (26) with Alexa-phalloidin-568 (Molecular Probes, Invitrogen). All the pictures are maximum projection of z-stacks acquired with a Z step of 0.3 mm.

**Freezing and Freeze Substitution for Ultrastructural Analyses of Yeast Cells**—The wild-type strain MR6 and atp6::ARG8 deletion strain MR10 were grown to early exponential phase (10^7 cells/ml) in liquid CSMD and CSMD-R media, respectively. The yeast pellets were placed on the surface of a copper electron microscopy grid (400 mesh) coated with Formvar. Each loop was very quickly submerged in pre-cooled liquid propane and held at −180 °C with liquid nitrogen. The loops were then transferred to a pre-cooled solution of 4% osmium tetroxide in dry acetone in a 1.8-ml polypropylene vial and kept at −82 °C for 48 h (substitution fixation), warmed gradually to room temperature, and washed three times in dry acetone. Specimens were stained for 1 h with 1% uranyl acetate in acetone at 4 °C in a dark room. Following another rinse in dry acetone, the loops were progressively infiltrated with araldite (epoxy resin, Fluka). Ultrathin sections were stained with lead citrate.

**Miscellaneous Procedures**—Mitochondrial enzyme assays and membrane potential analyses were performed on isolated mitochondria prepared by the enzymatic method (27), from cells grown in CSMD (MR6 strain) or CSMD-R (MR10). Protein amounts were determined by the procedure of (28) in the presence of 5% SDS. Oxygen consumption rates were measured with a Clark electrode in the respiration buffer (0.65 M mannitol, 0.36 mM EGTA, 5 mM Tris phosphate, 10 mM Tris maleate, pH 6.8) as described previously (29). For ATP synthesis rate measurements, mitochondria (0.3 mg. ml⁻¹) were placed in a 2-ml thermostatically controlled chamber at 28 °C in respiration buffer. The reaction was started by the addition of 4 mM NADH and 1 mM ADP and stopped by 3.5% perchloric acid, 12.5 mM EDTA. Samples were then neutralized to pH 6.5 by addition of KOH, 0.3 M MOPS. ATP was quantified by luciferin/ luciferase assay (ThermoLabsystems) on an LKB bioluminometer. Participation of the F1F0-ATP synthase to ATP production was assessed by oligomycin addition (20 μg/mg of protein). The specific ATPase activity was measured at pH 8.4 using a described procedure (30). Variations in transmembrane potential (ΔΨ) were evaluated in the respiration buffer by measure-
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Influence of the ATP6 Deletion on mtDNA Stability—The influence of the ATP6 deletion on mtDNA stability was evaluated by scoring the number of \( \rho^-/\rho^0 \) petites in MR10 cultures. Two fermentable carbon sources were tested: galactose, which does not elicit catabolite repression, and glucose. In the presence of arginine, where \( \rho^- \) mtDNA is not required for MR10 growth, the glucose and galactose cultures contained \( \sim 40 \) and \( 80\% \) \( \rho^-/\rho^0 \) petites, respectively (after 20 generations, see Table 2). In the absence of arginine, two times less petites was found, 20\% with glucose and 40\% with galactose, respectively. This is because once they form MR10 \( \rho^-/\rho^0 \) cells cannot divide with-

**RESULTS**

**Construction of a Yeast Strain Where ATP6 Is Replaced with ARG8**—Acylornithinaminotransferase (Arg8p) is a nuclear encoded mitochondrial protein involved in ornithine and arginine biosynthesis (36, 37). Although it is normally synthesized in the cytosol and then imported into mitochondria, Fox and co-workers (19) have shown that Arg8p can be directly synthesized inside the mitochondrion from a recoded gene, \( \text{ARG8}^m \), inserted into the mtDNA. In this study we have used \( \text{ARG8}^m \) to delete the \( \text{ATP6} \) gene. This gene is co-transcribed on a polycistronic RNA with \( \text{COX1} \) and \( \text{ATP8} \) and, in some strains, \( \text{ENS2} \), which encodes an optional DNA endonuclease, in the order \( \text{COX1} \rightarrow \text{ATP8} \rightarrow \text{ATP6} \rightarrow \text{ENS2} \) (38–40). Processing of the primary transcript yields two mRNAs containing both \( \text{ATP8} \) and \( \text{ATP6} \) (and \( \text{ENS2} \) if present) and \( \text{COX1} \) mRNA (41).

An \( \text{atp6}:\text{ARG8}^m \) DNA cassette was constructed to precisely delete and replace the \( \text{ATP6} \) coding sequence with the \( \text{ARG8}^m \) open reading frame, in strain MR6 bearing a null allele in nuclear \( \text{ARG8} \) gene (see “Materials and Methods”). The \( \text{atp6}:\text{ARG8}^m \) isolates (called MR10) were selected by virtue of their capacity to grow on media lacking arginine. As expected, these clones were respiratory growth-deficient (Fig. 1).

Several verifications proved that \( \text{ARG8}^m \) was correctly integrated at the \( \text{ATP6} \) locus in MR10. Southern blot analyses (Fig. 2A) and PCR reactions (Fig. 2B) on MR10 mtDNA revealed the expected DNA fragments (Fig. 2D). Moreover, MR10 recovered respiratory competence when crossed with SDC30, a \( \rho^- \) strain containing the wild-type \( \text{ATP6} \) gene (Fig. 2C).

**Mitochondrial RNA were extracted by the procedure of a previous study (34) from MR6 and MR10 strains grown to exponential phase in CSMD and CSMD-R, respectively. They were separated (10 \( \mu \)g) on a horizontal 1% (v/v) agarose-6% (v/v) formaldehyde gel in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA). The RNAs were then transferred to a Nytran membrane (Schleicher & Schuell) and hybridized with the following DNA probes: p14 (cytochrome b (34)), pYGT21 (Cox1p (35)); pYIL25 (containing the entire 15 S rRNA gene cloned into pUC13, provided by J. Lazowska); a11 (HindII-MboI fragment of a11 cloned into pUC13; provided by J. Lazowska); for \( \text{ATP9} \), we used as a probe a PCR product amplified from strain FY1679 mtDNA with primers 5’-GGAATATTATTAATAAGTCC and 5’-GAATGTTATGATTTTCAAAGGAG; for \( \text{ATP6} \), we used as a probe a fragment of the coding sequence (from position 340 to 780) that was PCR-amplified with primers 5’-GTATGGATCC-CAATCTATTG and 5’-TATGCATTTTTAAATATGAT-GCTG; for \( \text{ARG8}^m \), we used as probe a fragment (from position 1 to 692 of \( \text{ARG8}^m \) open reading frame) that was PCR-amplified with primers 5’-ATGACACATTTAGAAAGAGTTAGA and 5’-GTTGAAGTTGTTATTTCC. Hybridizations were carried out in 50% (v/v) formamide, 5\% SSPE, 0.5% SDS, 7% polyethylene glycol 5000, 5\% Denhardt’s solution, 100 \( \mu \)g/ml carrier DNA at 42 °C.

Mitochondrial DNA for Southern blot analysis was prepared according to a previous study (33) from MR6 and MR10 strains grown to exponential phase in CSMD and CSMD-R, respectively. The mtDNA (5 \( \mu \)g) was digested with SwaI or AccI, electrophoresed on a horizontal 1% agarose gel in 1\% TAE buffer (Tris acetate 20 mM, 0.5 mM EDTA), and then transferred to a Nytran membrane (Schleicher & Schuell). Hybridizations with the \( \text{ATP6} \) and \( \text{ARG8}^m \) probes were carried out in 6 \( \times \) SSC, 1\% Denhardt’s solution, 0.1% SDS, 100 \( \mu \)g/ml carrier DNA at 60 °C. The DNA probes were labeled with [\( \alpha^-\text{32P} \)]dCTP using the Rediprime™ II kit and purified on MicroSpin™ G-25 columns from Amersham Biosciences.
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TABLE 2
Accumulation of ρ−/ρ0 petites in MR10 cultures

| Medium                     | t0  | t12 | t20  |
|----------------------------|-----|-----|------|
| Glucose minus Arg          | 23 ± 3 | 21 ± 3 | 21 ± 2 |
| Glucose plus Arg           | 23 ± 3 | 39 ± 1 | 48 ± 1 |
| Galactose minus Arg        | 23 ± 3 | 43 ± 5 | 52 ± 8 |
| Galactose plus Arg         | 23 ± 3 | 58 ± 8 | 87 ± 3 |

Without an external source of arginine. We have no explanation for the increase in petite production with galactose.

Expression of the mtDNA Genes in the Atp6 Deletion Strain—The limited mtDNA instability in MR10 (see above) allowed us to properly investigate the consequences of a lack in Atp6p on mitochondrial structure and function. In all the experiments described below, using either glucose or galactose to grow MR10, there were never more than 20–30% petites (in galactose media, MR10 was grown for only five to six generations to limit the number of petites). We first analyzed the expression of the mitochondrial genes. As expected, Atp6p could not be detected in MR10 by pulse labeling of mtDNA-encoded proteins (Fig. 3). Interestingly, the Cox1p subunit of cytochrome c oxidase (complex IV or aa3) was very poorly synthesized in MR10, whereas the other mtDNA-encoded proteins showed a rather good synthesis. A radioactive signal detected only in MR10 near the position of Cox1p possibly corresponded to the ARG8m protein product whose size is similar to that of Cox1p. We did not identify the two faint bands seen below Cox3p in the MR10 lanes. A possibility is that these bands are degradation products of Arg8p, which may become more susceptible to mitochondrial proteases when synthesized inside the organelle.

Northern blot analyses of mitochondrial RNAs with COX1 probes, one purely exonic, and one intron-specific, aI1, showed that COX1 transcripts were synthesized and correctly processed in MR10 (Fig. 4). The COX1 mRNA was in only a slightly reduced amount (70% compared with the wild type), indicating that the failure in Cox1p expression mainly resulted from a post-transcriptional defect. Consistent with the successful synthesis of cytochrome b and Atp9p in MR10, the mRNAs of these proteins and the 15 S rRNA accumulated normally. As expected, with a probe internal to the ATP6 coding sequence transcripts were detected only in MR6, whereas ARG8m responding transcripts were seen only in MR10 (not shown). Thus, deletion of ATP6 gene had no significant effect on the expression of the other mtDNA-encoded proteins except Cox1p, which was very poorly synthesized.

Influence of Lack in Atp6p on the ATP Synthase Activity and Oxygen Consumption—Consistent with the failure in Cox1p expression, MR10 mitochondria exhibited very low respiration rates, <5% compared with the wild type, with either NADH or

FIGURE 3. In vivo labeling of mitochondrial translation products. The wild-type strain MR6 and the atp6::ARG8m deletion strain MR10 were labeled with [35S]methionine plus cysteine for 10 or 60 min, as indicated, in the presence of cycloheximide to inhibit cytosolic protein synthesis. Mitochondrial membranes were extracted from the cells, and the labeled proteins were separated by SDS-PAGE on a 16.5% polyacrylamide gel (left). For a better resolution of Cox3p and Atp6p, a 12% polyacrylamide gel containing 6 M urea was used (right). 200,000 DPM were loaded onto each lane. The gels were dried and analyzed with a PhosphorImager.

FIGURE 4. Northern blot analysis of mitochondrial transcripts in the atp6::ARG8m deletion strain MR10. Approximately 15 μg of mitochondrial RNA of wild-type strain MR6 and atp6::ARG8m deletion strain MR10 was separated on a 1.3% agarose/formaldehyde gel. The RNAs were blotted onto Nytran and hybridized separately to probes specific for individual mitochondrial transcripts. For each strain, the different RNAs were normalized to 15 S rRNA. Shown are the relevant hybridization signals obtained with probes specific for Atp9, COX1, and cytochrome b (CYTB) mRNAs, intron aI1 of COX1 gene, and 15 S rRNA. The values in percentage are the normalizations for the mutant expressed relative to those estimated for the wild type.
an electron donor (ascorbate/N,N,N,N-tetramethyl-p-phenylenediamine) that directly reduces the cytochrome c oxidase (Table 3). In the presence of the membrane potential uncoupler carbonyl cyanide m-chlorophenylhydrazone, where respiration is maximal, the respiratory activity was still very low in MR10 indicating a strong decrease in respiratory enzyme content. As a consequence of this low respiratory activity, only a very weak electrical potential difference across the inner membrane could be maintained by the respiratory chain in MR10 (not shown).

The mitochondrial ATPase activity in MR10 was decreased by ~30% only compared with wild-type, indicating that the F$_{1}$, which is the major mitochondrial ATPase, was still well expressed and functional in the absence of Atp6p. However, as usually observed in strains with lesions in the ATP synthase F$_{0}$ component, the mitochondrial ATPase activity in MR10 was very poorly inhibited by oligomycin (Table 3), a drug known to specifically block the F$_{1}$F$_{0}$ complex proton channel. Not surprisingly, no oligomycin-sensitive ATP synthesis activity and no ATP-driven proton translocation via the ATP synthase were detected in MR10 mitochondria (data not shown).

A Lack in Atp6p Results in a Selective Decrease in Cytochrome c Oxidase Accumulation—The spectral cytochrome c oxidase (aa$_{3}$) absorption signal was barely detectable in MR10 mitochondria, <5% compared with the wild type (Fig. 5). The other mitochondrial cytochromes (c + c$_{1}$ and b) were clearly visible. Calculations from the shown spectra gave a cytochrome b hemes content in MR10 diminished by ~20% compared with the wild-type (378 versus 466 pmol/mg of mitochondrial proteins). The c + c$_{1}$ content was significantly higher in MR10 than in the wild-type (826 versus 673 pmol/mg), presumably because of a stronger accumulation in cytochrome c. Indeed, this soluble cytochrome of the mitochondrial intermembrane space has been found to accumulate more in many yeast mitochondrially deficient mutants, including mutants of the ATP synthase (see Ref. 46 for an example). The absorption band near 575 nm in MR10, not seen in wild-type mitochondria (Fig. 5), possibly corresponded to unassembled hemes, similarly to what has been observed in several cytochrome c oxidase or bc$_{1}$ complex assembly defective mutants (42).

Western blots of mitochondrial proteins resolved by SDS-PAGE revealed a cytochrome b subunit content in MR10 ~70% that of the wild-type, whereas the cytochrome c oxidase Cox1p and Cox2p subunits were in trace amounts in MR10 (Fig. 6A). These findings were corroborated by BN-PAGE analyses showing only a modest decrease in the cytochrome bc$_{1}$ complex content and almost complete disappearance of cytochrome aa$_{3}$ complex in MR10, recorded at room temperature. The mitochondria were oxidized with potassium ferricyanide and reduced with sodium dithionite. The positions of the $\alpha$-absorption bands of cytochromes a, a$_{3}$, c, and c$_{1}$ are indicated.

These findings were corroborated by BN-PAGE analyses showing only a modest decrease in the cytochrome bc$_{1}$ complex content and almost complete disappearance of cytochrome aa$_{3}$ complex in MR10 (Fig. 6B). In the BN-PAGE conditions used, the cytochrome bc$_{1}$ complex is normally found in “supercomplexes” containing the cytochrome c oxidase (43). This explains the faster electrophoretic migration rate of the MR10 cytochrome bc$_{1}$ complex compared with the one isolated from the wild-type (Fig. 6B). Altogether, the results of these analyses showed that in the absence of Atp6p the cytochrome c oxidase accumulated very poorly (<5% compared with wild type), whereas the cytochrome bc$_{1}$ complex was much less affected (70–80% compared with wild type).

ATP Synthase Assembly in the Absence of Atp6p—The assembly of the ATP synthase in mitochondria is believed to be an ordered sequence in which Atp6p would be the last subunit to be added to the inner mitochondrial membrane. Western blots of mitochondrial proteins resolved by SDS-PAGE showed a decrease in the F$_{1}$, the major mitochondrial ATPase, which is in trace amounts in MR10 (Fig. 6B). The figure shows difference spectra of isolated mitochondria (2.5 mg/ml) from wild-type strain MR6 and atp6::ARG8$^{+}$ deletion strain MR10, recorded at room temperature. The mitochondria were oxidized with potassium ferricyanide and reduced with sodium dithionite. The positions of the $\alpha$-absorption bands of cytochromes a, a$_{3}$, c, and c$_{1}$ are indicated.

The assemblage of the ATP synthase in mitochondria is believed to be an ordered sequence in which Atp6p would be the last subunit to

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**TABLE 3**

| Strain | Respiration rates | ATPase specific activity |
|--------|------------------|-------------------------|
|        | State 4          | State 3                 | Ascorbate/TPD +CCCP | +CCCP | - oligomycin | + oligomycin | inhibition |
| MR6    | 144 ± 16         | 358 ± 4                 | 657 ± 38            | 1235 ± 50 | 1535 ± 36 | 163 ± 78  | 90 |
| MR10   | <20              | <20                     | 30 ± 6              | 38 ± 11    | 1023 ± 89 | 964 ± 55  | 6  |

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*J.-P. di Rago, unpublished results.*
be inserted (Ref. 44 and references therein). Direct evidence for the ability of the ATP synthase subunits to assemble in bacteria in the absence of subunit a (Atp6p homolog) has been provided before (21). Our ATP6 deletion strain MR10 offered a good opportunity to test whether ATP synthase assemblies lacking only Atp6p can form in yeast cells. We first determined the accumulation levels of a number of ATP synthase subunits in MR10 mitochondria, by Western blots of SDS-PAGE gels (Fig. 6A). As expected Atp6p was not detected, whereas the other tested ATP synthase subunits were all present but in somewhat reduced amounts. We next analyzed the ATP synthase assembly state in MR10 by BN-PAGE (Fig. 6B). In the conditions used, intact ATP synthases are resolved mainly as dimers and monomers, which can be revealed by labeling the gels for ATPase activity (45). The only ATPase signal detected in the MR10 lane corresponded to free F1-ATPase. Western blotting of the BN-PAGE gels with antibodies against the ATP synthase γ subunit did not reveal other assemblies than free F1γ, and this particle was in significantly higher amounts in MR10 compared with wild-type (not shown). With antibodies against Atp9p, assemblies much smaller than F1γ, which may correspond to free Atp9p oligomeric rings were detected in MR10 (not shown). With antibodies against Atp4p, only small size Atp4p-containing complexes were found in the mutant (not shown). Although these data do not preclude that in the absence of Atp6p the remaining ATP synthase subunits can still assemble, they indicated that if it exists the putative Atp6p-less ATP synthase assembly is rather fragile.

The ATP synthase assembly state in MR10 was further analyzed by CN-PAGE, which is reputed to preserve, better than BN-PAGE, interactions within fragile protein assemblies (69). Once again, the ATPase signal on gel corresponding to free F1γ was stronger in MR10 compared with wild type (Fig. 6C). Interestingly, two additional ATPase signals (noted “1” and “2” in Fig. 6C) appeared in the MR10 lane. With a size not very different from that of intact ATP synthase monomers, the ATPase signal 1 could correspond to a complex containing most if not all of the ATP synthase subunits but Atp6p. The ATPase signal 2, which migrated slightly faster, could, in addition to Atp6p, miss a few other subunits. Whether these ATPase signals actually corresponded to partial F1Fo complexes was checked by Western blotting. To this end, the bands 1 and 2 were cut out from the CN-PAGE gel, and their proteins were resolved by SDS-PAGE followed by Western blot with antibodies against several subunits representative of the different ATP synthase domains (F1γ (subunit γ), peripheral stalk (Atp4p and Atp7p) and proton translocating domain (Atp9p)). These four subunits were all detected in band 1, whereas band 2 lacked Atp7p only (Fig. 6C). These data provide the first direct evidence that, in the absence of Atp6p, the other subunits of the mitochondrial ATP synthase complex can still assemble.

**Mitochondrial Structure Is Altered in the ATP6 Deletion Strain**—Mitochondrial structure was found to be altered in some yeast ATP synthase mutants (45–47) giving support to the idea that the ATP synthase would be involved in mitochondrial cristae formation (47) (see “Discussion”). To determine whether deletion of ATP6 had some influence on mitochondrial morphology, Δatp6 cells were embedded in araldite resin and examined for ultrastructure by transmission electron microscopy. A striking feature in most MR10 cell electron micrographs was the presence of tightly associated and reciprocally deformed mitochondria (Fig. 7, A–C). In comparison to a recent study (48), these structures suggest that mitochondrial inner membrane fusion was delayed in MR10 (see “Discussion”).

Cristae-like structures were perfectly discernable in MR10 mitochondria (Fig. 7, C and D), although they appeared quite larger and longer than those seen in the wild-type control (Fig. 7, E–G). Quite frequently, electron dense bodies, never observed in the wild-type control, accumulated in the matrix of MR10 mitochondria (Fig. 7C). The nature of these inclusion bodies is not known.

We also analyzed mitochondrial structure in Δatp6 cells by fluorescence microscopy using a matrix-targeted GFP. In wild-type cells, the mitochondria were organized as a classic network of extended tubular structures (Fig. 8, A and C). In the mutant,
the mitochondrial compartment appeared much more condensed. Actin cables and patches were clearly detectable and normally polarized in MR10 (Fig. 8B) indicating that the aberrant mitochondrial distribution was not caused by defects in actin cytoskeleton organization.

DISCUSSION

In this study we report on the construction and analysis of yeast cells in which the coding sequence of mitochondrial ATP6 gene has been entirely deleted and replaced with ARG8m. As expected from previous reports (see the introduction) the atp6 deletion strain MR10 (Deltaatp6) showed an increased propensity to produce rho-/rho- petites. However, with the ARG8m gene inserted at the ATP6 locus, petite accumulation could be significantly restricted, to only 20–30%, by growing Deltaatp6 yeast in glucose media lacking arginine. These results argue against a direct role for Atp6p in mtDNA inheritance as previously suggested (17).

With such a moderate mtDNA instability, the consequences of a lack in Atp6p on mitochondrial structure and function could be investigated in good conditions. An interesting finding was the very poor accumulation (<5%) of cytochrome c oxidase in the Deltaatp6 strain, whereas that of cytochrome bc1 complex was much less affected (70–80% of wild type). The low content in cytochrome c oxidase was attributed to a failure in the synthesis of the mitochondrially encoded Cox1p subunit of this enzyme. One could argue that the loss of Cox1p expression is not related to the loss of ATP6 function. Indeed, the COXI and ATP6 genes belong (together with ATP8) to the same polycistronic unit. Replacing the whole ATP6 coding sequence by a totally different nucleotide sequence (ARG8m) could prevent the normal processing of that unit. Several lines of evidence prove that the failure in Cox1p expression in the Deltaatp6 yeast was not such an artifact but rather indeed the consequence of ATP synthase function inactivation. First, the Cox1p mRNA was correctly processed and accumulated rather well (70% compared with wild-type) in Deltaatp6 yeast, showing that Cox1p synthesis impairment resulted from a block in translation. Secondly, as it will be described elsewhere, we observed similar defects in Cox1p expression with point ATP6 mutations that compromise ATP synthase functioning (ARG8m is not present in the mtDNA of these mutants).6 Thus, what clearly controls the level of Cox1p expression here is the ATP synthase activity.

As a consequence of the poor accumulation of cytochrome c oxidase, isolated mitochondria from the Deltaatp6 strain exhibited a dramatically low respiration rate, <5% in comparison to the wild type. A decreased respiratory activity is a feature shared by many yeast ATP synthase-defective mutants. Similarly to what occurs in Deltaatp6 yeast, the cytochrome c oxidase was found to be more affected than the other respiratory enzymes in strains with mutations in ATP4 (49), ATP7 (50), ATP8 (14), or ATP9 (16). However, because they produce many petites (>70%), it was not easy to assess to which degree and how the cytochrome c oxidase was specifically affected in these mutants. In the Deltaatp4 mutant, as in Deltaatp6 yeast, the poor accumulation of cytochrome c oxidase was suggested to be the result of a decrease in Cox1p synthesis. In contrast, in atp9 and atp8

6 M. Rak, E. Tetaud, S. Duvezin-Caubet, N. Ezkurdia, J. Rytka, and J.-P. di Rago, manuscript in preparation.
mutants, the various cytochrome c oxidase subunits were reported to be normally synthesized and still able to associate to each other. The cytochrome c oxidase deficiency in these mutants was suggested to be caused by a block in heme insertion or synthesis. The present study where the petite issue could be minimized provides clear evidence that defects in the ATP synthase may have dramatic and selective consequences on the cytochrome c oxidase by lowering Cox1p synthesis.

Cox1p synthesis has been shown in previous studies (51, 52) to be a key regulatory target for cytochrome c oxidase expression. Indeed, with a few exceptions, Cox1p translation was found to be selectively slowed down in cox mutants where newly synthesized Cox1p polypeptides cannot assemble (51). The down-regulation of Cox1p involves Mss51p, which is required to activate Cox1p translation, and Cox14p. Both proteins were shown to transiently interact with newly translated Cox1p polypeptides (51). It was proposed that the entry of Cox1p into the COX assembly pathway would release Mss51p from the Cox1p-Mss51p-Cox14p ternary complex allowing Mss51p to initiate a new round of translation. Such interactions would couple translation of Cox1p to COX assembly, a regulatory scheme similar to the “control by epistasy of synthesis” mechanism that has been proposed to regulate biogenesis of the photosystem apparatus in chloroplasts of *Chlamydomonas reinhardtii* (74). It will be especially interesting to determine whether proteins like Mss51p and Cox14p are involved in the control of Cox1p expression by the ATP synthase activity revealed in this study.

In tightly coupled mitochondria the oxygen consumption rate and ATP synthase activity depend on each other, which makes it not very surprising to see the respiratory activity decreased in many yeast ATP synthase defective mutants. Short regulatory responses involving the cytochrome c oxidase have been described both in yeast and mammals (for review see Ref. 53). For instance, it is well established that mitochondrial respiration rate is modulated by the binding of adenine nucleotides on the cytochrome c oxidase, indicating that the ADP/ATP ratio is an important effector in the co-regulation of cytochrome c oxidase and ATP synthase. The present study points to the existence in yeast of long term regulations in which it is the cytochrome c oxidase synthesis rate that allows the respiratory activity to be modulated in response to the ATP synthase activity.

The import of glycolytic ATP into mitochondria is of vital importance in yeast mutants unable to perform oxidative phosphorylation. ATP can be imported into mitochondria by reversal of the ADP/ATP translocase, which counter-exchanges ATP with ADP across the inner membrane (54). This is an electrogenic reaction exchanging four against three negative

![Fluorescence microscopy analyses of the atp6::ARG8m deletion strain MR10](image-url)
charges. The maintenance of a high ΔΨ across the inner membrane would certainly hamper ATP to enter mitochondria (55). As a consequence, we would like to speculate that the strong decrease of mitochondrial respiration in ATP synthase-defective mutants might be required not only to adjust the electron flow to ATP synthesis rate but also to decrease the membrane potential when ATP needs to be imported into the organelle. Thus we believed that the very poor synthesis of cytochrome c oxidase is a vital requirement allowing Δatp6 yeast to import glycolytic ATP into mitochondria. Consistent with this hypothesis, no decrease in cytochrome c oxidase synthesis was observed in uncoupled ATP synthase mutants where maintenance of a mitochondrial potential is severely compromised by massive proton leaks through the F₁ (56–58).

It has been suggested that the ATP synthase in some way regulates cristae formation (45, 59–62). Allen showed that F₁ complexes are arranged as a double row of particles along the full length of the helically shaped tubular cristae in Paramecium multimicronucleatum (62). Based on these observations, he proposed (47) that the double-cone shape of ATP synthase dimers offers the potential to form a rigid arc, which leads to an inner membrane protrusion that is then amplified to form tubules upon association of additional complexes during mitochondrial biogenesis. In favor of this hypothesis, it was found that loss of ATP synthase subunits e or g, both of which appear to be specifically involved in the enzyme dimerization in yeast (63), results in abnormal mitochondrial morphologies characterized by numerous digitations and onion-like structures (45). In addition, the Devenish laboratory showed that the correct arrangement of F₁F₀-ATP synthase complexes within the inner membrane is crucial for the genesis and/or maintenance of mitochondrial cristae and morphology (60). Also consistent with Allen’s model, we have shown that yeast mutants unable to assemble the αβ₃ subcomplex of F₁ are devoid of cristae (46). In these mutants, not only the F₁ but also many F₀ subunits are missing because of an increased susceptibility to proteolytic degradation when unassembled. In these mutants both the cytochrome bc₁ and aa₃ complexes accumulate poorly (64, 65), presumably because of their lack in cristae. Indeed, these allow much greater amounts of membrane-bound energy-transducing enzymes to be packed in the interior of the organelle (66, 67).

Interestingly, cristae-like structures were clearly discernible in the Δatp6 mutant (Fig. 7, C and D) indicating that neither Atp6p nor the ATP synthase activity is crucial for cristae generation. A corollary, according to Allen’s model, would be that absence of Atp6p should not prevent the formation of the double-cone shape of ATP synthase dimers presumed to be responsible for cristae formation. In favor of this, using the CN-PAGE technique, we provide the first direct evidence that Atp6p is not required for the assembly of the remaining ATP synthase subunits. Not very surprisingly, the Atp6-less assembly proved to be fragmented especially in BN-PAGE where it fully dissociated into several subcomplexes. Other techniques will be required to determine whether the Atp6-less F₁F₀ complex actually exists in the inner membrane in a dimeric form.

Although cristae can form, mitochondrial morphology exhibited profound changes in Δatp6 yeast. A striking feature in electron micrographs of Δatp6 cells was the presence of numerous tightly associated and reciprocally deformed mitochondria (Fig. 8, A and B), anomalies never observed in the other ATP synthase mutants that have been analyzed by electron microscopy. Very similar structures have been observed in in vitro assays of yeast mitochondria fusion when the mitochondrial electrical potential was collapsed with valinomycin (48). In that case, deformed mitochondrial pairs were shown to be the products of outer membrane fusion in the absence of inner membrane fusion. Thus, in comparison to these results, a reasonable view is that inner membrane fusion is strongly delayed in Δatp6 cells. Fluorescence microscopy analyses showed that the mitochondrial compartments are condensed in Δatp6 yeast instead of being distributed as a network of extended tubular structures. We propose that this condensation might be a consequence of the delay in inner membrane fusion by sticking together organelles engaged in fusion events. A possibility is that these mitochondrial anomalies are caused by the poor energization of the inner membrane in the Δatp6 yeast.

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