A Yeast Model of the Neurogenic Ataxia Retinitis Pigmentosa (NARP) T8993G Mutation in the Mitochondrial ATP Synthase-6 Gene*

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NARP (neuropathy, ataxia, and retinitis pigmentosa) and MILS (maternally inherited Leigh syndrome) are mitochondrial disorders associated with point mutations of the mitochondrial DNA (mtDNA) in the gene encoding the Atp6p subunit of the ATP synthase. The most common and studied of these mutations is T8993G converting the highly conserved leucine 156 into arginine. We have introduced this mutation at the corresponding position (183) of yeast Saccharomyces cerevisiae mitochondrially encoded Atp6p. The “yeast NARP mutant” grew very slowly on respiratory substrates, possibly because mitochondrial ATP synthesis was only 10% of the wild type level. The mutated ATP synthase was found to be correctly assembled and present at nearly normal levels (80% of the wild type). Contrary to what has been reported for human NARP cells, the reverse functioning of the ATP synthase, i.e. ATP hydrolysis in the F1 coupled to F0-mediated proton translocation out of the mitochondrial matrix, was significantly compromised in the yeast NARP mutant. Interestingly, the oxygen consumption rate in the yeast NARP mutant was decreased by about 80% compared with the wild type, due to a selective lowering in cytochrome c oxidase (complex IV) content. This finding suggests a possible regulatory mechanism between ATP synthase activity and complex IV expression in yeast mitochondria. The availability of a yeast NARP model could ease the search for mechanisms against this mitochondrial disease.

Most of the cellular ATP requirements in human are produced by the mitochondrial F1F0-ATP synthase. This enzyme synthesizes ATP from ADP and inorganic phosphate by using the electrochemical proton gradient formed across the inner mitochondrial membrane in the course of electron transfer to oxygen by the respiratory chain (complexes I–IV). 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 in a ring of 10–12 Atp9p subunits and one Atp6p subunit (referred to as subunits c and a in Escherichia coli, respectively). Proton movements through this channel coincide with rotation of the Atp9p-ring (5–9), which results in conformational changes favoring ATP synthesis in the F1 (1). The ATP synthase can function reversibly by hydrolyzing ATP coupled to proton extrusion out of the mitochondrial matrix.

NARP (neuropathy, ataxia, and retinitis pigmentosa) and MILS (maternally inherited Leigh syndrome) are mitochondrial disorders associated with point mutations of the mitochondrial DNA (mtDNA) in the Atp6p gene encoding the ATP6 gene. The most common and studied NARP/MILS mutation is T8993G converting a highly conserved leucine residue into arginine, at Atp6p amino acid position 156 (11). Wild type and mutated mtDNAs always co-exist in cells and tissues of the patients. Typically between 70 and 90% of mutated mtDNA result in the NARP syndrome, whereas the far more severe MILS syndrome usually occurs when the mutation load exceeds 90–95% (12).

Numerous studies with cells and transmitochondrial cell hybrids (cybrids) containing high levels of T8993G all showed important decreases in mitochondrial ATP synthesis rate, i.e. 50 to 90% (13–20). However, the precise impact of the leucine to arginine pathogenic change on the ATP synthase is still unknown. Some authors proposed that the underlying mechanism for the impaired ATP production would be a defect in the assembly/stability of the ATP synthase (13, 21, 44), whereas others concluded that the T8993G mutation essentially affects in some unknown way the functioning of the enzyme proton channel (10, 18–20, 45).

The use of model organisms easily tractable to genetic manipulations may help to better understand ATP synthase alterations involved in human diseases. E. coli ATP synthase was found to be very sensitive to the leucine to arginine patho-

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5 The abbreviations used are: NARP, neuropathy, ataxia, and retinitis pigmentosa; MILS, maternally inherited Leigh syndrome; mtDNA, mitochondrial DNA; CCCP, carbonyl cyanide p-chlorophenylhydrazone; TMPD, N,N,N,N-tetramethyl-p-phenylenediamine; MOPS, 4-morpholinepropanesulfonic acid; BN, blue Native.
genic change induced by T8993G (at position 207 of the homologous subunit α) as evidenced by a complete loss of both ATP synthesis and ATP-driven proton pumping activities (22). However, whereas the assembly of L156R-Atp6p in human cells containing high levels, up to 100%, of T8993G was found to be unaffected (19, 45), the mutated L207R-subunit α failed to insert into the bacterial ATP synthase complex (23). The more sophisticated structure of mitochondrial ATP synthase, with at least 10 subunits not present in the bacterial enzyme (24), together with important differences in subunit α and Atp6p structures (10), might be responsible for the different sensitivities of the bacterial and human ATP synthases to the leucine to arginine pathogenic change.

The yeast *Sacharomyces cerevisiae*, a facultative aerobic eukaryote, obviously is a much better model than bacteria for the study of human mitochondrial disorders (46), especially those involving mutations of the ATP synthase, an enzyme that is highly similar in yeast and human (24). As in human, the yeast Atp6p gene lies within the mtDNA, and *S. cerevisiae* is one of the rare organisms tractable to site-directed mutagenesis of the mtDNA (25). Due to its normal incapacity to stably propagate mitochondrial structures in the heteroplasmic state, one can easily obtain yeast populations where all the mtDNA molecules bear a given mutation, thus allowing one to study the consequences of specific mtDNA alterations on mitochondrial structure and function.

**MATERIALS AND METHODS**

**Yeast Strains and Media**—The *S. 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⁻¹ 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⁻¹ adenine); WO (0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose), and other supplements depending on the strain auxotrophic markers. Solid media contained 2% (w/v) agar.

**Construction of ATp6-L183R Mutant Strain MR14**—The ATp6 locus of wild type yeast mtDNA (from strain FY1679 entirely sequenced (26)), from nucleotide position −316 upstream of the ATp6 initiator codon to nucleotide position +275 downstream of the ATp6 stop codon, was PCR amplified as a BamHI-BamHI fragment (described in Ref. 27). Its unique EcoRI site (internal to the ATp6 coding sequence) was cut and the two resulting BamHI-EcoRI fragments cloned separately into pUC19, to give pSDC8 and pSDC9. The pSDC8 plasmid contains the 5′ end of the ATp6 locus where the leucine TTA codon 183 (equivalent to the leucine 156 codon of human ATP6 gene) is located. Using the QuikChange XL Site-directed Mutagenesis Kit of Stratagene, the TTA codon 183 of the yeast ATp6 gene in pSDC8 was changed into the arginine AGA codon with primers 5′-CGCTAGGCTATTTCAAGGT-TTAAGATTAGTCTATATCTTAGCTGG and 5′-CCA-GCTAGATATTAGAACCTAATCTTAAACCTTTGAC- ATAGCTCTAGCG (the mutagenic bases are in bold), to give plasmid pSDC15. The SapI-EcoRI ATp6 fragment of pSDC15 was isolated and ligated with pJM2 cut with the same enzymes, to give pSDC17. The pJM2 plasmid contains the yeast mitochondrial COX2 gene as a marker for mitochondrial transformation (28). The 3′ part of the wild type ATp6 locus in pSDC9 was liberated by a SapI + EcoRI digestion and cloned into the same sites of pSDC17, to give pSDC22, thus reconstructing a whole ATp6 gene containing the L183R mutation. The pSDC22 plasmid was introduced by co-transformation with the nuclear selectable LEU2 plasmid pFL46 into the pSDC16 by microprojectile bombardment using a biolistic PDS-1000/He particle delivery system (Bio-Rad) as described (25). Mitochondrial transformants were identified among the Leu+ transformants by their ability to produce respiring clones when mated to the nonrespiring NB40-3C strain bearing a deletion in the mitochondrial COX2 gene (25). One mitochondrial transformant (synthetic pSDC31) was crossed to the atp6::ARG8 mtDNA deletion strain MR10 (27). The crosses SDC31 x MR10 produced cytoductants (called MR14) harboring the MR10 nucleus and where the ARG8 gene open reading frame had been replaced with the ATp6-L183R gene. The MR14 clones were identified by virtue of their inability to grow in the absence of an external source of arginine and complementation tests with a ρ− strain (SDC30 (27)) containing the wild type yeast ATp6 gene alone. Sequencing of the mutated atp6 locus in MR14 revealed no other changes than L183R.

**Miscellaneous Procedures**—Cells that were ρ− in MR14 cultures were identified by virtue of their ability to form pink colonies in the presence of limiting amounts of adenine, whereas ρ+/ρ− cells gave white colonies. The pink color is due to an intermediate of the adenine biosynthetic pathway that accumulates in strains with an auxotrophic mutation in the ADE2 gene (49, 50). When respiration is totally abolished, this pigment is not oxidized and remains white. For mitochondrial enzyme assays and membrane potential analyses, mitochondria were prepared by the enzymatic method (29), from MR6 and MR14 strains grown to middle exponential phase (3−4 × 10⁷ cells ml⁻¹) in YPGALA medium. Protein amounts were determined by the procedure of Ref. 30 in the presence of 5% SDS. Oxygen consumption rates were measured with a Clark electrode in the

| Yeast Model of NARP Syndrome |
|-----------------------------|
| **TABLE 1** Genotypes and sources of yeast strains |
| **Strain** | **Nuclear genotype** | **mtDNA** | **Source** |
| DFS160 | MATa leu2Δ ura3Δ-S2 ade2-101 arg8::URA3 kar1-1 | ρ⁺ | Ref. 28 |
| NB40-3C | MATa lys2 leu2Δ-S112 ura3Δ-S2 his3ΔHindIII arg8::HIS3 | ρ⁺ cox2-62 | Ref. 28 |
| MR6 | MATa ade2-1 his3Δ-S112 trp1Δ-S1ura2Δ-S1 CAN1 arg8::HIS3 | ρ⁺ WT | Ref. 27 |
| MR10 | MATa ade2-1 his3Δ-S112 trp1Δ-S1ura2Δ-S1 CAN1 arg8::HIS3 | ρ⁺ atp6::ARG8 | Ref. 27 |
| SDC30 | MATa leu2Δ ura3Δ-S2 ade2-101 arg8::URA3 kar1-1 | ρ− ATp6 | Ref. 27 |
| SDC31 | MATa leu2Δ ura3Δ-S2 ade2-101 arg8::URA3 kar1-1 | ρ− atp6-L183R | This study |
| MR14 | MATa ade2-1 his3Δ-S112 trp1Δ-S1ura2Δ-S1 CAN1 arg8::HIS3 | ρ− atp6-L183R | This study |
respiration buffer (0.65 mM mannitol, 0.36 mM EGTA, 5 mM Tris phosphate, 10 mM Tris maleate, pH 6.8) as described previously (31). 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 2 N KOH, MOPS. 0.3 mM ATP was quantified by luciferin/luciferase assay (ThermoLabsystems) on an LKB bioluminometer. Participation of the \(F_2/F_0\)-ATP synthase in ATP production was assessed by oligomycin addition (20 \(\mu\)g mg\(^{-1}\) of protein). The specific ATPase activity was measured at pH 8.4 using a previously described procedure (32). Variations in transmembrane potential (\(\Delta\psi\)) were evaluated in the respiration buffer by measurement of rhodamine 123 fluorescence quenching with a SAFAS Monaco fluorescence spectrophotometer (33). Cytochrome spectral analysis was performed as in Ref. 47. SDS-PAGE was according to Laemmli (34). Western blot analyses were performed as described previously (35). Polyclonal antibodies raised against Atp6p, Atp7p, and OSCP (a gift from J. Velours) were used after dilution to 1:10,000. Polyclonal antibodies against yeast complex III cytochrome \(b\) subunit (a gift from T. Langer) were used after dilution to 1:2000. Monoclonal antibodies against Cox2p subunit of complex IV (from Molecular Probes) were used after dilution to 1:5000. Nitrocellulose membranes were incubated with peroxidase-labeled antibodies at a 1:10,000 dilution and with peroxidase-labeled antibodies at a 1:10,000 dilution and revealed with the ECL reagent of Amersham Biosciences. Pulse labeling of mtDNA-encoded proteins and Northern blot analyses were performed as described in Ref. 27.

**FIGURE 1.** The atp6-L183R mutation very severely compromises the respiratory growth of yeast. Freshly grown cells of wild type (WT) yeast (MR6), a \(\Delta\text{atp6}\) deletion strain (MR10), and the atp6-L183R mutant MR14 (referred to as NARP), were spotted onto glucose (YPGA) and glycerol (N3) media. The plates were incubated at 28 °C and photographed after the indicated number of days.

**TABLE 2**

| Strain | Respiration rates | ATPase activity | ATP synthesis rate |
|--------|------------------|----------------|------------------|
|        | NADH             | NADH + ADP     | NADH + CCCP      | Asc/TMPD + CCCP | \(\text{nmol O}_2\text{ min}^{-1}\text{mg}^{-1}\) | \(\text{nmol Pi min}^{-1}\text{mg}^{-1}\) | \(\text{nmol P}_i\text{ min}^{-1}\text{mg}^{-1}\) | \(\text{nmol Pi min}^{-1}\text{mg}^{-1}\) |
|        | \(\mu\text{mol}\) | \(\mu\text{mol}\) | \(\mu\text{mol}\) | \(\mu\text{mol}\) | +Oligo | +Oligo | +Oligo |
| MR6    | 364 ± 35         | 647 ± 30       | 1231 ± 145       | 1911 ± 246      | 2439 ± 247 | 375 ± 48 | 911 ± 106 | 174 ± 45 |
| MR14   | 132 ± 15         | 139 ± 13       | 281 ± 24         | 430 ± 18        | 2047 ± 92 | 482 ± 91 | 59 ± 6   | <5      |

**RESULTS**

Converting Atp6p Leucine Residue 183 into Arginine Severely Compromises the Yeast Respiratory Growth—Leucine residue 156 of human Atp6p changed into arginine by the T8993G mutation corresponds to leucine residue 183 of yeast Atp6p. The TTA triplet encoding this residue was converted into the arginine AGA codon on a plasmid bearing the yeast wild type ATP6 gene. The resulting mutated plasmid (pSDC22) was introduced by biolistic transformation into the mitochondria of a yeast strain lacking mtDNA (\(\rho^0\)). Crossings were then performed to fix the atp6-L183R mutation by mtDNA recombination into a complete (\(\rho^+]\)) mitochondrial genome, and give strain MR14.

MR14 strain exhibited a very slow growth on media containing a non-fermentable carbon source (e.g. glycerol), both at 28 °C, the optimal temperature for growing yeast (Fig. 1), and at 36 °C (not shown), whereas the growth on glucose was normal (Fig. 1). The MR14 respiratory growth deficiency was rescued by crossing with a synthetic \(\rho^–\) strain (SDC30) containing in its mitochondria the wild type ATP6 gene only (not shown), which proved that no other genetic alteration than atp6-L183R, in nuclear or mitochondrial DNA, was involved in the expression of MR14 respiratory growth-deficiency phenotype.

The atp6-L183R Mutation Has a Minor Influence on Yeast mtDNA Stability—Quite often, in *S. cerevisiae*, mutations of the ATP synthase destabilize the mtDNA in the form of \(\rho^–/\rho^+\) petites issued from large deletions in the mtDNA (36). It was thus important to determine whether the atp6-L183R mutation compromised the stability of the mtDNA. To this end, we scored the number of \(\rho^–/\rho^+\) cells produced by MR14 grown by fermentation with, as carbon source, either galactose, which does not elicit catabolite repression, or glucose. When grown on galactose, MR14 cultures contained only 5–10% of petites versus 2% for the corresponding wild type strain MR6, whereas <5 and <1% of petites accumulated in MR14 and MR6, respectively, when glucose was the carbon source. The poor growth of MR14 on non-fermentable substrates was thus not due to a defect in mtDNA maintenance.

**Influence of the atp6-L183R Mutation on the Yeast Mitochondrial Energy Transducing System**—In the following sections we describe a number of experiments aiming to determine how the atp6-L183R mutation impacts the ATP synthase and respiration. All were performed using MR14 cells grown on galactose, *i.e.* in non-repressing conditions for a good mitochondrial expression, and at 28 °C, the optimal temperature for oxidative phosphorylation in yeast.

The atp6-L183R Mutation Results in a Strong Lowering in Oxygen Consumption Due to a Poor Accumulation of Complex
Mitochondrial cytochrome contents

The mitochondrial cytochrome contents were estimated in mitochondria (2.5 mg/mL) from wild type strain MR6 and atp6-L183R mutant MR14 grown in galactose media (YPGALA). The mitochondria were oxidized with potassium ferricyanide and reduced with sodium dithionite. The spectra were recorded at room temperature and quantified according to the procedure described in Ref. 46. The cytochrome contents are expressed in nanomoles per mg of mitochondrial proteins. The values reported are averages of triplicate assays ± S.D.

| Strain | c + c₁ | b | aa₃ |
|--------|--------|---|-----|
| MR6    | 572 ± 47 | 349 ± 20 | 135 ± 14 |
| MR14   | 722 ± 34 | 329 ± 10 | 39 ± 16 |

IV—Mitochondria isolated from MR14 exhibited a low respiratory activity. With NADH as an electron donor, the basal oxygen consumption rate (state 4) was 3 times lower compared with wild type (Table 2). In the presence of an excess of ADP (state 3), conditions in which the respiration rate normally increases to compensate for the use of the mitochondrial potential (ΔΨ) by the ATP synthase, to phosphorylate the added ADP, the oxygen consumption rate in MR14 was only very weakly stimulated, i.e. 1.1-fold versus 1.8 for the wild type. In the presence of the membrane potential uncoupler CCCP, where it is maximal, the oxygen consumption rate was still very low in MR14, indicating a strong decrease in respiratory enzyme content. However, the uncoupled respiration in MR14 was higher compared with state 3 by a factor of about 2, as in wild type mitochondria, which indicated that the atp6-L183R mutation did not increase the inner mitochondrial membrane passive permeability to protons.

Using ascorbate/TMPD, an electron donor that directly reduces the terminal respiratory complex IV (cytochrome c oxidase), and in the presence of CCCP, MR14 still exhibited a strong respiratory deficit of 78% (Table 2). The drop in complex IV activity correlated with a decrease in the content of spectrally detectable cytochromes aa₃, whereas cytochromes c₁ and b were in normal amounts in MR14 (Table 3), which indicated that the respiratory defect was due to a low content in complex IV. This was confirmed by SDS- and BN-PAGE analyses of mitochondrial proteins. In SDS-PAGE, the complex III cytochrome b and c₁ subunits were in almost normal amounts in MR14, whereas the cytochrome c oxidase Cox2p subunit was much less abundant, only 33% compared with the wild type (Fig. 4C). BN-PAGE was performed in conditions where respiratory complexes III and IV are normally resolved as two distinct “supercomplexes” (III₂IV₂ and III₂IV₁) and free complex III homodimers (III₃) (43). These different complexes were revealed after transfer on membranes blotted with antibodies against Cox2p or cytochrome b. The shown blots (Fig. 4B) support the existence of a selective and substantial decrease in complex IV content in the mutant strain MR14. Of the two supercomplexes, only III₂IV₂ was significantly reduced. Consistent with a specific lack in complex IV, a larger amount of complex III homodimers was detected in the mutant than in the wild type (Fig. 4B). Although a precise quantification of the protein complexes in BN-PAGE is rather difficult, the shown data are perfectly consistent with a specific lack in complex IV in MR14 of about 70% as estimated from the respiration assays, spectral, and SDS-PAGE analyses.

Noteworthy, the relative intensities of the immunological signals corresponding to the III₂IV₂ and III₂IV₁ complexes were very different depending on the antibodies used for their detection. In the wild type MR6 lane, with antibodies against Cox2p, the III₂IV₂ complex gave a much stronger signal than the III₂IV₁ complex, whereas the latter was much better detected than the former with antibodies against cytochrome b. This is certainly due to a better accessibility for the anti-cytochrome b antibodies when the complex III dimer is associated to only one complex IV.

Prior work has shown that the entire deletion of the ATP6 gene also results in an important and selective decrease in complex IV content (of about 95%) (27). The low level of complex IV in Δatp6 yeast was attributed to a specific decrease (>90%) in the synthesis rate of the mitochondrially encoded Cox1p subunit of this complex (27, Fig. 2A). As the Cox1p mRNA was detected in only a partially reduced amount in the Δatp6 yeast (70%), the slower rate in Cox1p synthesis is likely to be due in part to a slowing in translation (27). The rate of Cox1p synthesis in MR14 was also reduced, but somewhat to a lesser extent, about two times compared with the wild type, whereas the other mtDNA-encoded proteins, including the mutated

![Image](https://example.com/image.png)
mitochondrial membranes (not osmotically protected mitochondria buffered at pH 8.4). The ATPase activity in MR14 was reduced by 20% only, compared with the wild type, and showed a slightly diminished sensitivity to oligomycin, i.e. 77 versus 85% in MR6 (Table 2). Thus the F1-ATPase was well expressed and functional in MR14, with apparently only a minor loss in coupling to Fo, but see below.

Energization of the Inner Mitochondrial Membrane—To determine whether F1-catalyzed ATP hydrolysis in MR14 was or not coupled to proton translocation through the Fo, we performed rhodamine 123 fluorescence quenching experiments on intact mitochondria (buffered at pH 6.8). In wild type mitochondria energized with ethanol, as expected, an ADP addition transiently decreased fluorescence quenching reflecting the use of the transmembrane potential (ΔΨ) by the ATP synthase to phosphorylate the added ADP (Fig. 3A). In response to subsequent KCN addition, the ΔΨ collapsed, first rapidly and then more slowly (Fig. 3A). The slow phase decrease in potential reflects an Fo-mediated proton-pumping coupled to F1-catalyzed hydrolysis of the ATP accumulated in the matrix following phosphorylation of the added ADP. Indeed, in the presence of oligomycin, the KCN addition resulted in a single phase of rapid fluorescence increase (not shown). In MR14, despite a reduced respiratory capacity (see above), ethanol induced a ΔΨ of comparable amplitude to the one it induced in wild type mitochondria (Fig. 3B). However, the decrease in ΔΨ induced by a further ADP addition was of much smaller amplitude in MR14 than in the wild type and a much longer time was required to recover the initial ΔΨ established with ethanol (Fig. 3B). This observation is consistent with the low ATP synthesis rate in MR14 (see above). A subsequent KCN addition resulted in a ΔΨ collapse that was much more rapid than in wild type mitochondria. This finding suggested that the reverse functioning of the ATP synthase was compromised in MR14. To better appreciate this feature we followed changes in ΔΨ mediated by externally added ATP. The mitochondria were first energized with ethanol to remove the natural inhibitory peptide (IF1) of the F0-ATPase. The respiratory activity was then blocked with KCN, and less than 1 min later (thus well before rebinding of IF1, see Ref. 47) ATP was added. In wild type mitochondria, the ATP addition promoted a large and stable fluorescence quenching of the dye that was oligomycin-sensitive (Fig. 3C). In MR14, the ΔΨ induced by ATP was unstable and of smaller

Atp6p-L183R, showed good synthesis (Fig. 2B). Northern blot analyses revealed that the Cox1p mRNA was in MR14, as in Δatp6 yeast, in only a partially reduced amount, i.e. 50–70% compared with the wild type (not shown). By contrast, ATP6 transcripts, which are synthesized together with COX1 and ATP8 from a polycistronic unit, in the order COX1-ATP8-ATP6 (see Ref. 27) were in normal amounts compared with the wild type. Thus, lowering in Cox1p synthesis in MR14 was apparently not due to a decreased transcription but rather to a slower translational rate and/or reduced stability of the Cox1p mRNA.

Influence of atp6-L183R Mutation on Mitochondrial ATP Synthesis/Hydrolysis—The impact of atp6-L183R mutation on oxidative phosphorylation was further analyzed by measuring the rate of mitochondrial ATP synthesis (in state 3, with NADH as a respiratory substrate). The ATP synthesis rate in MR14 was reduced by about 90%, compared with the wild type (Table 2). The residual ATP synthesis activity was efficiently inhibited by oligomycin, a specific inhibitor of the ATP synthase. Thus, the atp6-L183R mutation compromises severely but not completely the oxidative phosphorylations, an observation consistent with the slow growth of MR14 on respiratory substrates. We next measured the rate of mitochondrial ATP hydrolysis and the sensitivity of this activity to oligomycin on disrupted mitochondrial membranes (not osmotically protected mitochondria buffered at pH 8.4). The ATPase activity in MR14 was reduced by 20% only, compared with the wild type, and showed a slightly diminished sensitivity to oligomycin, i.e. 77 versus 85% in MR6 (Table 2). Thus the F1-ATPase was well expressed and functional in MR14, with apparently only a minor loss in coupling to Fo, but see below.

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FIGURE 3. Energization of mitochondria. Energization of the mitochondrial inner membrane was monitored by rhodamine 123 fluorescence quenching with intact mitochondria from wild type yeast MR6 (A and C) and atp6-L183R mutant MR14 (B and D). The additions were 0.5 μg/ml rhodamine 123, 0.15 mg/ml mitochondrial proteins (Mito), 10 μl of ethanol (EtOH), 6 μg/ml oligomycin (oligo), 0.2 mm potassium cyanide (KCN), 50 μM ADP, 1 mm ATP, and 3 μM CCCP. Superposed in panel D are two experiments where oligomycin was added at different times. Data are representative of at least 3 experiments.
amplitude (Fig. 3D). These ΔΨ changes in MR14 were sensitive to oligomycin, showing that they were actually mediated by the ATP synthase. Therefore, the yeast *atp6*-L183R mutation led to a rather severe perturbation in the reverse functioning of the yeast ATP synthase, a conclusion that contrasts with what had been reported for human NARP cells (Refs. 10 and 18 and see “Discussion”).

The *atp6*-L183R Mutation Has Little Incidence on the ATP Synthase Assembly/Stability and Supramolecular Organization—We next examined the influence of the *atp6*-L183R mutation on ATP synthase assembly/stability and supramolecular structure. The ATP synthase exists in the inner membrane as a homodimer (37), and evidence indicates that ATP synthase dimers associate with each other into long polymers, a structure presumed to be important for the proper organization/folding of the inner membrane into cristae (38–40). Oligomeric forms of the ATP synthase can be resolved by BN-PAGE analysis of mitochondrial proteins solubilized with 0.75 and 1.0 g of digitonin/g of protein; at higher detergent concentrations, i.e. 1.5 and 2.0 g of digitonin per g of protein, the ATP synthase is isolated mainly as dimers and monomers (40). The different ATP synthase assemblies were revealed by gels labeling for ATPase activity (Fig. 4A, right panel) followed by Coomassie Blue staining to better appreciate their relative amounts (Fig. 4A, left panel). These analyses failed to detect any major difference between MR14 and the wild type, except a modest decrease (20%) in ATP synthase content and a very small increase in free F1-ATPase in the mutant. Western blottings of
BN-PAGE strips with antibodies against different ATP synthase subunits (Atp6p, OSCP, and Atp3p) failed to detect any partial enzyme assemblies in the mutant other than the very small increase amount in F1. Furthermore, using the same antibodies we performed Western blottings of whole BN-PAGE strips resolved by second-dimension SDS-PAGE. These analyses further supported that the ATP synthase was present in the mutant in only slightly diminished amounts, about 80% compared with the wild type, an estimation consistent with the results of SDS-PAGE analyses of total mitochondrial proteins (Fig. 4C). A quantification of the blots revealed that for each ATP synthase assembly, the amount of Atp6p relative to OSCP and Atp7p were similar in MR14 and the wild type (not shown). Thus, the atp6-L183R mutation had apparently no significant incidence on the assembly/stability and supramolecular organization of the yeast ATP synthase. The 20% decrease in ATP synthase content in MR14 is likely due to the profound change in mitochondrial physiology caused by the >90% loss in oxidative phosphorylation rather than to a specific lowering in the kinetics of assembly or to a reduced stability of the complex.

**DISCUSSION**

We report here on the construction and properties of a yeast strain bearing in its mtDNA an equivalent of the NARP/MILS syndrome-causing T8993G mutation. This mutation converts a highly conserved leucine residue into arginine within the Atp6p subunit of the ATP synthase, at amino acid position 156 in human, 183 in yeast. Although the structure of Atp6p is still unknown, there is a lot of information indicating that the leucine residue modified by T8993G would be embedded in the inner mitochondrial membrane within a region of Atp6p critical for proton conduction across the membrane (10). In close proximity to this leucine residue, amino acids (Arg-186 in yeast Atp6p, Glu-59 in yeast Atp9p) are known to be directly involved in proton translocation and/or rotation of the Atp9p-ring within the F0. It is therefore not surprising that replacing in the membrane, close to essential F0 catalytic residues, a highly hydrophobic side chain by a positively charged one is detrimental to the ATP synthase.

In human NARP cells the assembly of the ATP synthase was reported in several studies to be unaffected by T8993G indicating that the impaired ATP production in these cells would be caused by a local disruption of the enzyme proton channel (19, 45). However, others concluded that T8993G destabilizes the ATP synthase or slows down its assembly as indicated by an increased accumulation of ATP synthase subcomplexes in the mutant cells/tissues analyzed (13, 21, 44).

In view of these discrepancies, it was interesting to determine how the leucine to arginine pathogenic change affects the ATP synthase in yeast, an organism tractable to site-directed mutagenesis of the mtDNA and currently used for biochemical studies of the ATP synthase (24, 25). Quite often in *S. cerevisiae*, deleterious mutations of the ATP synthase destabilize the mtDNA giving rise to a stronger accumulation, up 100%, of $\rho^-/\rho^-$ cells issued from large deletions in the mtDNA (36). Fortunately, the atp6-L183R had a very minor incidence on mtDNA maintenance that allowed us to investigate in good conditions the impact of this mutation on mitochondrial structure and function.

The yeast NARP mutant exhibited a very slow growth on respiratory substrates, which was attributed to a major lowering in the mitochondrial ATP synthesis rate, by about 90% compared with wild type. Interestingly, the oxygen consumption rate in the NARP mutant was also compromised, by about 80%, due to a decreased complex IV content. A very strong (95%) and selective decrease in the accumulation of complex IV was found also in a yeast strain lacking the entire ATP6 gene (27). In both the NARP and Δatp6 yeast strains, the drop in complex IV content results from a decrease in the synthesis of the mitochondrially encoded Cox1p subunit of this complex. The modulation of Cox1p synthesis by the activity of the F1F0 complex can be regarded as a possible regulatory mechanism allowing the respiratory flux to adjust to the rate of ATP production in yeast mitochondria, as previously suggested (27). In this respect, it must be stressed that different mitochondrial mechanisms regulating the activity/biogenesis of the respiratory complexes may exist between high and low eukaryotes, the latter having to cope with frequent and important fluctuations in energy and nutrient availability in their environment. Thus, the yeast NARP mutant may not be the best model to determine the influence of T8993G on respiration in human cells, an issue that is still far from being understood. Indeed, there is conflicting evidence in the literature on the existence of a mitochondrial respiratory defect in human NARP cells. Although some studies reported an oxygen consumption defect in isolated mitochondria from cells with high levels of T8993G mutation (16, 41), other studies in intact cybrids failed to confirm these findings (21).

Whether the T8993G mutation affects or not the assembly of the ATP synthase has long been controversial. A recent study of human (homoplasmic) NARP cells where the assembly of the ATP synthase has been thoroughly investigated led to the conclusion that this mutation has no significant effect on the assembly of the ATP synthase (45). In addition, the authors of this study provide evidence that the ATP synthase supramolecular structure is unaffected in NARP cells. This structure, which consists in long polymers of ATP synthase dimers, is presumed to be important for mitochondrial cristae formation (37–40). We failed to detect any significant defects in ATP synthase assembly and supramolecular structure in the yeast NARP mutant as well. Consistent with these observations, the yeast NARP mutant displayed normal mitochondrial morphol- ogy (not shown).

There is general agreement in the literature that T8993G does not affect the reverse functioning of the human ATP synthase, *i.e.* ATP hydrolysis in the F1, coupled to proton translocation through the F0 out of the mitochondrial matrix (10, 18). Consistent with this assertion, numerous studies with human NARP cells all showed a little decrease and efficient inhibition by oligomycin of the mitochondrial ATPase activity, similarly to what we observed here in the yeast NARP mutant. However, it is important to note that the mitochondrial ATPase assays are performed with disrupted mitochondrial membranes (not osmotically protected mitochondria usually buffered at pH 8.4), thus in conditions where an electrical potential difference
across the inner membrane cannot form. The use of intact mitochondria (buffered at a more physiological pH 6.8) is needed to evaluate the proton pumping activity of the F1F0-ATPase complex. Nevertheless, our rhodamine 123 fluorescence quenching experiments clearly show that in intact yeast mitochondria the leucine to arginine pathogenic change induced by T8993G severely compromises the reverse functioning of the ATP synthase. Indeed, addition of ATP to the mutated mitochondria induced a variation in electrical potential ($\Delta \Psi$) that was much smaller and rather unstable compared with the wild type (Fig. 3).

Different explanations can be evoked to account for these observations. First, the $atp6$-L183R mutation could affect the efficiency of the coupling of the ATP9p-ring ATP-driven translocation to proton translocation. In that case, the sensitivity to oligomycin of the F1F0-ATPase activity (in the ATPase assays) could result from a block in rotation of the ATP9p-ring upon addition of the inhibitor. Such an uncoupling of the ATP synthase has been characterized in E. coli subunit a (Atp6p homolog) and subunit c (Atp9p homolog) mutants (48).

Another view is that the ATP driven rotation of the ATP9p-ring is still tightly coupled to proton translocation in NARP mitochondria but protons move slowly through the $F_0$. In apparent contradiction with this is the rather good rate of ATP hydrolysis in the NARP mitochondria. However, in the ATPase assays where a $\Delta \Psi$ cannot build up across the inner membrane, movements of protons through the mutated $F_0$ could be facilitated by the absence of $\Delta \Psi$. In the proton-pumping assays, there is no $\Delta \Psi$ when the ATP is added. However, after the ATP addition, a $\Delta \Psi$ builds up with the consequence that protons then have to progress through the $F_0$ against an electrical potential difference. With the L183R change within a region of Atp6p crucial for proton conduction, the flow of protons during ATP hydrolysis could be dramatically compromised when the outer face of the inner membrane becomes positively charged. Thus, the progressive disappearance of the ATP-induced variation in $\Delta \Psi$ could be due to an $F_0$ activity too low to compensate for the passive permeability to protons of the inner membrane.

To our knowledge there is only one study (18) where the influence of T8993G on ATP-driven proton translocation was investigated in human NARP cells. In this study ATP-driven proton pumping was monitored using sub mitochondrial particles derived from the platelets of patients that were 15–90% heteroplasmic for T8993G. It was concluded that ATP-driven proton translocation was unaffected. From the data reported in the present study, we believe that the influence of T8993G on the reverse functioning of human ATP synthase should be revisited, using experimental conditions more similar to the ones we used, i.e. with mitochondria that are intact and homoplasmic for T8993G.

Although both ATP synthesis and ATP-driven proton translocation were significantly reduced in the yeast NARP mitochondria, at first it seemed that the former was more affected (by 90%) than the latter (apparently by 50%). The strong down-regulation of cytochrome c oxidase in the yeast NARP mutant makes it difficult to interpret the ATP synthesis rate, because energization of the mitochondrial membrane is likely to be inferior in the mutant. The actual impairment in ATP synthesis, on a molecular basis, might therefore be less important than the determined 90%. However, the analysis of other (more than 15) point $atp6$ mutants with various phenotypes shows a linear correlation between mitochondrial ATP synthesis rate and cytochrome c oxidase activity, indicating that the cytochrome c oxidase deficit directly correlates with the extent to which the intrinsic ATP synthesis capacity is reduced. The mitochondrial potential is well known to vary in a non-linear fashion with the proton pumping activity. This is clearly obvious when ethanol is used to energize the mitochondrial membrane in the yeast NARP mutant. Indeed, despite an 80% reduction in respiration, the $\Delta \Psi$ established with ethanol is almost the same in the mutant and the wild type. Thus, the less efficient (and unstable) energization of the inner membrane with ATP in the yeast NARP mutant indicates that the ATP-driven proton translocation activity is certainly much more affected than it seems from the rhodamine 123 fluorescence quenching assays.

More direct methods (e.g. visualization of the rotation of the mobile ATP synthase subunits) will be needed to better understand how the leucine to arginine pathogenic change compromises the functioning of the F1F0 complex. In this respect, the yeast NARP mutant will be very valuable because it will allow production and purification of large quantities of a mitochondrial ATP synthase bearing this mutation, which is extremely difficult to do with human cells homoplasmic for T8993G (19).

When we started this study we had in mind that a yeast NARP model could be of special interest for the search of potential rescuing mechanisms, by seeking genetic suppressors or molecules, that when present in the medium, can restore respiration-dependent growth of the yeast NARP mutant. Such approaches can easily be set up in yeast (42, 43). Although yeast has been extensively used for the study of mitochondrial diseases, and tens of mitochondrial diseases yeast models are available (42), somewhat curiously, the pharmacological approach has been neglected. Our laboratory in collaboration with that of M. Blondel (Roscoff, France) is actively engaged in the search of molecules active against ATP synthase deficiencies in yeast cells. The first promising molecules7 and several genetic suppressors8 active against T8993G in yeast cells have been found.

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