A “Petite Obligate” Mutant of Saccharomyces cerevisiae

FUNCTIONAL mtDNA IS LETHAL IN CELLS LACKING THE $\delta$ SUBUNIT OF MITOCHONDRIAL F$_1$-ATPASE

Received for publication, December 27, 2005, and in revised form, March 24, 2006. Published, JBC Papers in Press, April 11, 2006, DOI 10.1074/jbc.M513805200

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With the mitochondrial F$_1$F$_0$-ATP synthase, the nucleus-encoded $\delta$-F$_1$ subunit plays a critical role in coupling the enzyme proton translocating and ATP synthesis activities. In Saccharomyces cerevisiae, deletion of the $\delta$ subunit ($\Delta\delta$) was shown to result in a massive destabilization of the mitochondrial genome (mitochondrial DNA; mtDNA) in the form of 100% $p^{-}/p^+$ petites (i.e. cells missing a large portion (>50%) of the mtDNA ($p^+$) or totally devoid of mtDNA ($p^{-}$)). Previous work has suggested that the absence of complete mtDNA ($p^+$) in $\Delta\delta$ yeast is a consequence of an uncoupling of the ATP synthase in the form of a passive proton transport through the enzyme (i.e. not coupled to ATP synthesis). However, it was unclear why or how this ATP synthase defect destabilized the mtDNA. We investigated this question using a nonrespiratory gene (ARG8$^{50}$) inserted into the mtDNA. We first show that retention of functional mtDNA is lethal to $\Delta\delta$ yeast. We further show that combined with a nuclear mutation ($\Delta$atp4) preventing the ATP synthase proton channel assembly, a lack of $\delta$ subunit fails to destabilize the mtDNA, and $p^{+}$ $\Delta\delta$ cells become viable. We conclude that $\Delta\delta$ yeast cannot survive when it has the ability to synthesize the ATP synthase. Accordingly, the $p^{-}/p^+$ mutation can be viewed as a rescuing event, because this mutation prevents the synthesis of the two mtDNA-encoded subunits (Atp6p and Atp9p) forming the core of this channel. This is the first report of what we have called a “petite obligate” mutant of S. cerevisiae.

The mitochondrial inner membrane contains the ATP synthase, which utilizes a transmembrane proton gradient to catalyze ATP synthesis from inorganic phosphate and ADP. The ATP synthase has two major structural domains, an F$_0$ component, which forms a proton-permeable pore across the membrane, and a peripheral, matrix-localized, F$_1$ component, where the ATP is synthesized (1).

The F$_1$ domain comprises five different subunits, all nucleus-encoded, with $\alpha_3\beta_2\gamma\delta\epsilon_1$ stoichiometry. The three $\alpha$ subunits and the three $\beta$ subunits alternate in position within a hexamer that contains the adenine nucleotide processing sites (2–4). The $\gamma$, $\delta$, and $\epsilon$ subunits form a subcomplex of F$_1$, named the central stalk, linking the $\alpha_3\beta_2$ subcomplex to the ATP synthase proton channel (5). During catalysis, the central stalk rotates together with a transmembrane ring of 10–12 c subunits in the F$_0$ (6–12). In the course of this rotation, the $\gamma$ subunit sequentially interacts with the three $\alpha\beta$ pairs in a way that favors ATP synthesis in the catalytic sites, as required by the binding change mechanism (1).

In the yeast Saccharomyces cerevisiae, null mutations of the $\delta$ or $\gamma$ subunit massively destabilize the mitochondrial genome in the form of 100% cytoplasmic petites, which are cells with a large (>50%) deletion in mitochondrial DNA (mtDNA)$^{\delta}$ ($p^+$) or completely lacking mtDNA ($p^-$) (13, 14). This is an intriguing observation, since many other mutations impairing mitochondrial oxidative phosphorylation, including a null mutation of the catalytic $\beta$ subunit of the ATP synthase, have no major effect on the stability of the mitochondrial genome (14).

Defects in the synthesis of the $\delta$ or $\gamma$ subunit were shown to result in isolated mitochondria in a major uncoupling of mitochondrial respiration mediated by partial F$_0$F$_1$ assemblies in which proton translocation is not coupled to ATP synthetase in the F$_1$ (15, 16). In media containing oligomycin, a specific inhibitor of the ATP synthase assumed to block the enzyme proton channel, mtDNA maintenance could be observed in growing $\Delta\delta$ or $\Delta\gamma$ yeast, providing in vivo evidence that an ATP synthase uncoupling may be the cause of the loss of mtDNA in cells unable to properly express the $\delta$ or $\gamma$ subunit (16, 17).

However, how the ATP synthase uncoupling caused by the absence of the $\delta$ or $\gamma$ subunit destabilizes the mtDNA is still unknown. It has been hypothesized that the loss of the mtDNA allows $\Delta\delta$ or $\Delta\gamma$ yeast to survive by eliminating the ATP synthase F$_0$ component, which is in part encoded by the mtDNA (16, 17). The idea is that the F$_0$-mediated mitochondrial uncoupling in $\Delta\delta$ or $\Delta\gamma$ yeast would be lethal by preventing essential mitochondrial reactions from occurring. As a consequence, $\Delta\delta$ or $\Delta\gamma$ yeast would need to inactivate the ATP synthase proton channel in order to survive.

However, in apparent contradiction with this idea, although a lack in the third central stalk subunit ($\epsilon$) also results in a major F$_0$-mediated mitochondrial uncoupling, only partial effects were observed on the mtDNA in $\Delta\epsilon$ mutant (18). Indeed, cultures of this mutant usually contain 30% $p^-$ cells, showing that retention of functional mtDNA in $\Delta\epsilon$ yeast is not lethal. Furthermore, it has been reported that a lack in the $\delta$ subunit is viable in Kluyveromyces lactis, a “petite negative” yeast that cannot survive the $p^{-}/p^+$ mutation (19). These observations raise the
arginine biosynthesis. In this study, we have used the nuclear (23). Using standard cloning procedures and two-step PCR strategies

**MATERIALS AND METHODS**

**TABLE 1**

| Strain       | Nuclear genotype, plasmid | MtDNA          | Origin   |
|--------------|----------------------------|----------------|----------|
| KL14-4A/60   | Mata his1 trp2            | p°            | Slonimski |
| DFS160       | Mata leu2Δ ura3-52 ade2-101 arg8ΔIURA3 kar1-1 | p°            | Ref. 20 |
| NB40-3C      | Mata lys2 leu2-3,112 ura3-52 his3ΔHindIII arg8:hisG | p° cox2-62 | Ref. 20 |
| NB151-1B     | Mata pet111:LEU2 lys2 ura3-52 his3ΔHindIII arg8:hisG leu2 | p°            | J. Velours |
| PVY10/60     | Mata atp4ΔIURA3 his3 met6 ura3 | p° ARG8°     | This study |
| SDC12        | Mata/ MATA a lys2+/+ leu2Δ/leu2-3,112 ura3-52/ura3-52 ade2-101/+ | his3ΔHindIII/+ arg8ΔIURA3 arg8:hisG kar1-1/+ | This study |
| SDC13        | Mata/ Mata atp16ΔKAN7R/+ lys2+/+ leu2Δ/leu2-3,112 ura3-52/ura3-52 ade2-101/+ his3ΔHindIII/+ arg8ΔIURA3 arg8:hisG kar1-1/+ | p° ARG8°     | This study |
| SDC15        | Mata/ Mata lys2+/+ leu2Δ/leu2-3,112 ura3-52/ura3-52 ade2-101/+ his3ΔHindIII/+ arg8ΔIURA3 arg8:hisG kar1-1/+ | p°            | This study |
| SDC22        | MATa ade2-1 his3-13,15 trp1-1 leu2-3,112 ura3-1 can1-100 arg8:His3 | p° ARG8°     | Ref. 16 |
| SDC3         | Mata/ Mata pet111:LEU2/+ lys2/lyz2 leu2/? ura3-52/ura3-52 ade2-101/+ his3ΔHindIII/ his3ΔHindIII arg8ΔIURA3 arg8:hisG | p° ARG8°     | This study |
| SDC12-4B     | Mata lys2 leu2 ura3-52 his3ΔHindIII ade2-101 arg8ΔIURA3 | p° ARG8°     | This study |
| SDC12-21A    | Mata lys2 leu2 ura3-52 his3ΔHindIII arg8:hisG | p° ARG8°     | This study |
| SDC13-14B/60 | Mata atp16:KAN7R lys2 leu2 ura3-52 his3ΔHindIII arg8:hisG | p° ARG8°     | This study |
| SDC16-7C     | Mata atp4ΔIURA3 ura3 his3 met6 arg8:hisG | p° ARG8°     | This study |
| SDC17-31B    | Mata atp4ΔIURA3 atp4ΔKAN7R lys2 ura3 his3 met6 arg8:hisG | p° ARG8°     | This study |
| SDC29-10C    | MATa arg8:His3 atp16::KanMX4 atp4::TRP1 ade2-1 his3-13,15 leu2-3,112 trp1 can1-100, pSDC13 | p° ARG8°     | This study |
| SDC3         | arg8:His3 atp16::KanMX4 atp4::TRP1, pSDC13, pSDC20 | p° ARG8°     | This study |
| YSE15a/60    | MATa ade2-1 his3-13,15 trp1-1 leu2-3,112 ura3-1 can1-100, pSDC13 | p° ARG8°     | Ref. 16 |
| SDC6         | MATa ade2-1 his3-13,15 trp1-1 leu2-3,112 ura3-1 arg8:His3 atp16::KAN7R can1-100, pSDC13 | p° ARG8°     | This study |

Fox and co-workers (20) have developed elegant approaches for the study of mitochondria based on the insertion into the mtDNA of non-

respiratory genes like ARG8°. This gene is a mitochondrial version of the nuclear ARG8 gene encoding a mitochondrial protein involved in arginine biosynthesis. In this study, we have used ARG8° to better understand why and how a lack in the δ subunit destabilizes the mtDNA. To this end, we have inserted ARG8° into a noncoding region of the mitochondrial genome (i.e. keeping intact all of the genes that normally reside in yeast mitochondria). Thus, in a Δarg8 nucleus and in the absence of external arginine, this mtDNA is required not only for respiration but also for arginine biosynthesis. With this system, we clearly show that the ATP synthase proton translocating activity is lethal to yeast cells missing the δ subunit and demonstrate that the ρ° / ρ+ mutation is a suppressor allowing survival of Δδ yeast. This is the first report of what we have called a “petite obligate” mutant. We discuss the results in relation with potential mechanisms regulating the assembly of the ATP synthase.

**RESULTS AND DISCUSSION**

**Strains, Media, and Genetic Techniques**—The *S. cerevisiae* strains used are listed in Table 1. *Escherichia coli* XLI-1 Blue strain (Stratagene) was used for the cloning and propagation of plasmids. Complete glucose (YPGA), galactose (YPGALA), or glyceral (N3) and minimal media for growing yeast were prepared as described in Ref. 22. The yeast sporulation medium and the procedure for converting yeast into ρ° strains by ethidium bromide treatment have been described (27). Yeast transformation (28), crosses, and tetrad dissection (29) were performed as described previously.

**Construction of a COX2-ARG8° Transcriptional Unit and Its Insertion Upstream of COX2 in the mtDNA**—To create an ARG8° construct that could direct integration into intergenic regions of the mtDNA, we took advantage of the EcoRI site engineered on plasmid pPT24, 285 bases upstream of the COX2 start codon (i.e. upstream of the promoter) (23). Using standard cloning procedures and two-step PCR strategies (24) with pPT24 (containing COX2) and pDS24 (containing ARG8°) (20) as templates, we constructed an EcoRI cassette containing the ARG8° open reading frame precisely flanked on the 5′ side by 143 bp of the COX2 promoter region and on the 3′ side by 119 bp of the COX2 terminator. This ARG8° cassette was inserted into the EcoRI site of pPT24 in the forward orientation to yield plasmid pSDC10. The initial COX2 locus of pPT24 (from 485 bases upstream to 2015 bases downstream of the start codon) is therefore separated in two parts in pSDC10, the region −485 to −285 before the first EcoRI site and the region −285 to +2015 after the ARG8° cassette and the second EcoRI site (see Fig. 1A). The plasmid pSDC10 was introduced by co-transformation with the nuclear selectable LEU2 plasmid pFL64 into the ρ° strain DFS160 by microprojectile bombardment using a biostatic PDS-1000/He particle delivery system (Bio-Rad) as described (25). Mitochondrial transformants were identified among the Leu+ nuclear transformants by their ability to produce both respiring and arginine-prototrophic diploid clones when mated to the nonrespiring and arginine auxothrophic NB40-3C strain, bearing deletions in both the mitochondrial COX2 gene (cox2-62) and in the nuclear ARG8 gene. The DNA recombination events leading to the simultaneous integration of ARG8° and COX2 in NB40-3C mtDNA are illustrated in Fig. 1A. One respiratory growth-competent and arginine-prototrophic clone (called SDC12) was retained for further analyses, and the ARG8° integration was verified molecularly by Southern analysis and sequencing.

A control diploid strain, SDC15, isogenic to SDC12 but carrying the wild-type mtDNA was constructed by crossing DFS160 with NB80, the COX2 equivalent of NB40-3C (26).

**Construction of ARG8° Strains Carrying atp16 (δ) and/or atp4 Disruptions**—We first created a diploid strain (SDC13) heterozygous for a null mutation of the ATP synthase δ subunit gene (Δδ), homozygous for a null mutation of ARG8 (Δarg8) and containing the ARG8° mtDNA (Δδ+ Δarg8Δarg8 [p° ARG8°]), by deleting one of the two subunit gene copies in SDC12 with the atp16::KanMX4 cassette described previously (16). A Δδ Δarg8 [p° ARG8°] spore derived from SDC13 was converted to ρ° by ethidium bromide treatment (SDC13-14B/60) and crossed with strain SDC16-7C (Δatp4 Δarg8 [p° ARG8°]), a spore derived from the cross of FYV10/60 (Δatp4 ARG8 ρ°) with a spore of SDC12 (SDC12–21A: Δarg8 [p° ARG8°]). The resulting dip-
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A

ARG8m

E

COX2

pSDC10

B

Arg8p

MARG8 

MARG8

MARG8

C

SDC13-G

SDC13-I

Glucose

+Eth. Br.

-Arg

-Arg

-Arg

Glycerol

SDDC29-10c

FIGURE 1. Integration of ARG8m upstream of COX2 into mtDNA allows wild-type accumulation of Arg8p and arginine prototrophy while retaining respiratory competence. A, a plasmid (pSDC10) containing ARG8m flanked by COX2 expression sequences and inserted into the engineered EcoRI site upstream of the wild-type COX2 gene (hatched box, upstream promoter; box with vertical stripes, downstream terminator) was constructed and introduced by biolistic transformation into a strain retaining respiratory competence (Fig. 1A). The resultant synthetic mtDNA was inserted upstream of COX2 in the wild-type strain NB40-3C, which bears a deletion in ARG8 (-) strain NB40-3C, which bears a deletion in ARG8, strain SDC12 lacking ARG8 and containing wild-type mtDNA (Δarg8), and strain SDC12 (Δarg8 ARG8m). The mitochondrial proteins (20 μg) were electrophoresed in SDS-polyacrylamide gel, transferred onto nitrocellulose and probed with antibodies against Arg8p. C, cells from SDC12 (Δarg8 ARG8m) and SDC15 (Δarg8) were spotted onto the indicated media and incubated 5 days at 28°C.

RESULTS

Conversion of S. cerevisiae into a Conditional Petite Negative Yeast with an Intact Respiratory Capacity by Inserting ARG8m into a Noncoding Region of the mtDNA

Acetylornithine aminotransferase (Arg8p) is a nuclear encoded mitochondrial protein involved in ornithine and arginine biosynthesis (34, 35). Although it is normally synthesized in the cytosol and then imported into mitochondria, Fox and co-workers (20, 36–38) have shown that Arg8p can be synthesized as well directly inside the mitochondrion from a recoded gene, ARG8m, inserted into the mtDNA. In the present study, we have inserted ARG8m in the intergenic region upstream of the COX2 gene, using a protocol described by Mireau et al. (39). The ARG8m open reading frame was flanked with the 5’- and 3’-untranslated regions of COX2 and then integrated into the mtDNA by homologous recombination (see “Materials and Methods” and Fig. 1A).

We have isolated in this way a diploid strain (SDC12) with the expected integration of ARG8m in mtDNA and homozygous for a null mutation in ARG8 (Δarg8/Δarg8 [p+ ARG8m]). SDC12 accumulated normal levels of Arg8p (Fig. 1B), grew well on media devoid of arginine, and was respiratory competent (Fig. 1C). As expected, arginine was required for SDC12 to grow in the presence of ethidium bromide, an intercalating agent inducing the loss of mtDNA (Fig. 1C).

The mtDNA in SDC12 showed a good stability. Indeed, after about 15 generations in complete 10% glucose (i.e. conditions where arginine biosynthesis and oxidative phosphorylation are not required for growth), over 98% of the cells were still respiratory competent and arginine-prototrophic. A good mtDNA stability was also observed in SDC12 meiotic segregants.

In SDC12, the ARG8m gene is under control of the COX2 5’-untranslated region. Therefore, ARG8m expression should depend on Pet111p, a nucleus-encoded Cox2p translational activator (40, 41). To make sure
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TABLE 2
Tetrad analyses of heterozygous Δδ+/ yeast

| Strain   | Gly+ | G418° | 2 Gly− G418° | 2 Gly+ G418° | Gly+ | G418° | Number of analyzed tetrads |
|----------|------|-------|--------------|--------------|------|-------|---------------------------|
| SDC12    | 10   |       | 2            |              |      |       |                           |
| SDC13-I  | 3    | 4     |              |              | 2    | 77    | 33                        |
| SDC13-G  | 2    | 2     |              |              | 9    | 91    | 50                        |

that this was the case, an SDC12 meiotic segregant (SDC12-4B) was crossed with a ρ−Δpet111 strain (NB151-3C Δarg8 pet111::LEU2), to give SDC3 (Δarg8/Δarg8 Δpet111+ [ρ− ARG8m]). All the spores from SDC3 are necessarily Δarg8 and should therefore not grow in media lacking arginine if they cannot express ARG8m. As expected, the asci usually contained two spores, both respiratory growth-deficient and leucine-prototrophic, as a result of the pet111 deletion (not shown). These spores were also arginine-axotrophic. Following their transformation with a plasmid-borne wild type PET111 gene, respiratory competence and arginine prototrophy were recovered, demonstrating the requirement for Pet111p to express ARG8m.

Loss of Regulation of the ATP Synthase Caused by Lack of the δ Subunit Is Lethal

Elimination of the δ subunit gene in S. cerevisiae always results in 100% ρ−/ρ− populations, and evidence suggests that this conversion into petites is caused by an uncoupling of the ATP synthase (13–17). Below, we describe experiments we performed with the ARG8m system to determine how this ATP synthase defect impairs mtDNA maintenance.

Δδ+/ Yeast Is Genetically Unstable—We first aimed to analyze the segregation of the δ subunit deletion (atp16::KanMX4) and ARG8m markers upon sporulation of SDC13, a diploid heterozygous for the δ deletion, homozygous for Δarg8, and containing the ARG8m mtDNA. It has been reported that diploid strains with a heterozygous mutation in the δ subunit gene (i) have an increased propensity to produce ρ−/ρ− petites (30–40%), (ii) grow slowly on respiratory substrates, and (iii) exhibit partially uncoupled mitochondria, indicating that the reduced δ subunit gene dosage from 2 to 1 has semidominant negative effects (15).

To see whether such defects occurred also in our Δδ+/ SDC13 strain, SDC13 cells were plated for single colonies and incubated for 6 days on glucose plates (Fig. 2). Very small size colonies, probably corresponding to ρ−/ρ− cells were observed, but in a rather limited proportion (less than 10%). Two other distinct types of colonies were observed. One (named SDC13-G), representing about 20% of the population, consisted of grande colonies like those formed by the corresponding wild type (SDC12). The other type (SDC13-I), which was the most frequent (70%), consisted of colonies with an intermediate size, suggesting a reduced but not complete loss of respiratory capacity. In addition, these colonies had a scalloped shape. This trait is typical of strains with an increased propensity to produce ρ−/ρ− petites (47), but it could also reflect a reduced cell viability. When SDC13-I subclones were plated again for single colonies, a similar colonial heterogeneity was observed, whereas SDC13-G subclones gave essentially grande colonies. Similar observations were made with other, genetically independent, SDC13 isolates (all checked by Southern blot).

Sporulation and Tetrad Analysis of Δδ+/ ARG8m Yeast—One SDC13-I subclone and one SDC13-G subclone were sporulated, and tetrads were dissected on rich glucose plates. In the case of SDC13-I, a rather large proportion of the tetrads (42%, n = 40; see Table 2) were incomplete with only two or three viable spores. A lower proportion of incomplete tetrads (20%, n = 20, Table 2) was obtained from SDC13-G. A number of complete tetrads showing a correct segregation of the mating types was analyzed, 11 for SDC13-G and nine for SDC13-I. Geneticin resistance segregated 2:2 in all SDC13-G tetrads. However, only two tetrads showed the expected co-segregation of Geneticin resistance with respiratory growth deficiency. In the nine other SDC13-G tetrads, all spores were respiratory competent. In the case of SDC13-I, four tetrads showed the expected 2:2 co-segregation of Geneticin resistance and respiratory deficiency. In three other tetrads, all spores grew on glycerol and were Geneticin-sensitive. In the remaining two tetrads, Geneticin resistance segregated 2:2, but all spores were respiratory competent.

All spores from SDC13 are necessarily Δarg8 and depend therefore on their capacity to remain ρ− and to express ARG8m in order to proliferate in media lacking arginine. With no exception, all of the spores that were at the same time respiratory deficient and Geneticin-resistant, thus presumably deleted for the δ subunit, were arginine-axotrophic. Test crosses with a ρ− tester revealed that the early progenies of these spores were entirely composed of ρ−/ρ− cells. Not surprisingly, all of the spores that were respiratory competent were able to grow in media lacking arginine.

Tetrads from SDC13-I were also dissected on a glucose medium lacking arginine. Many were incomplete, but this was seen as well, although to a lesser extent, with ascii from the wild type SDC12 strain. It might be that germination on minimal medium is less efficient. However, significantly, none of the SDC13-I spores that germinated in the absence of external arginine was Δδ.

Altogether, the results of this first set of experiments indicated that Δδ cells were strictly unable to propagate/express the mtDNA or that they were not viable when they contained functional mtDNA. The
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In the Absence of ATP4, a Lack of δ Subunit Fails to Destabilize the Mitochondrial Genome—The primary effect of a lack in δ subunit is an uncoupling of the ATP synthase (15, 16). The absence of ATP synthesis in the F1 is probably not the cause of the instability of the mtDNA in Δδ yeast, since many other mutations impairing oxidative phosphorylation do not have major effects on the mtDNA. More reasonably, the uncoupled F0 is considered responsible for the loss of the mtDNA. We explored this possibility by assaying whether a nuclear mutation that inactivates the ATP synthase proton channel can suppress the deleterious effects on the mtDNA caused by elimination of the δ subunit gene. Although the two subunits (Atp6p and Atp9p) forming the core of the ATP synthase proton channel are encoded by the mtDNA, a number of nuclear mutations are known to inactivate this channel. This is the case with a null mutation of the nuclear ATP4 gene, which encodes a subunit of the ATP synthase peripheral stalk (42). This element connects, via its periphery, the αβ3 subcomplex of F1 to the membrane domain of the ATP synthase (Fig. 4A). The peripheral stalk is believed to function like a stator, preventing the αβ3 particle from rotating together with the central stalk during catalysis. In the absence of ATP4, Atp6p can still be synthesized but fails to accumulate, whereas the ATP synthase F1 component is unaffected (42) (see also Fig. 3). Presumably, ATP4 is required for the insertion of Atp6p in the ATP synthase, and unassembled Atp6p proteins are rapidly degraded by mitochondrial proteases.

In order to determine whether a lack of ATP4 enables Δδ yeast to remain ρ+ and viable, we constructed a diploid strain (SDC17) heterozygous for Δδ and Δatp4, homozygous for Arg8, and containing the ARG8mtDNA (Δδ + Δatp4 + Δarg8/Δarg8 [ρ+ ARG8mtDNA]). All spores from SDC17 are necessarily Δarg8 and therefore depend on their capacity to express ARG8mtDNA to grow on a glucose medium lacking arginine. The spores carrying both the Δatp4 and Δδ mutations were found to be arginine-prototrophic. On the contrary, as expected, the Δδ spores that were ATP4+/ρ+ could grow only in the presence of arginine because of the inability of Δδ yeast to grow in a ρ− state (see above).

To ascertain that inactivation of ATP4 was actually responsible for the ability of the Δatp4 Δδ meiotic segregants to remain ρ+ and viable, we performed a complementation test with a plasmid-borne wild type ATP4 gene. A Δδ Δatp4 clone was transformed with this plasmid or with the corresponding empty vector (pRS313) and then plated for single colonies on plasmid-selective (histidine minus) glucose media containing or not containing arginine. The arginine supplement was not required to grow Δatp4 Δδ cells transformed with the empty vector. On the contrary, Δatp4 Δδ cells transformed with ATP4 could produce colonies only in the presence of arginine (Fig. 4B), and these proved to be exclusively composed of ρ+ cells as revealed by crossings with a wild type ρ+ strain (Fig. 4C). These data demonstrated that elimination of ATP4 suffices to allow Δδ yeast to remain ρ+ and viable.

A Lack in ATP4 Rescues Δδ Yeast Viability by Preventing an F1- mediated Collapsing of the Mitochondrial ΔΨ—A lack in ATP4 is assumed to allow Δδ yeast to remain ρ+ and viable by preventing the assembly of a plasmid-borne ATP4 gene or the corresponding empty plasmid (pRS313). The transformant mixtures were plated equally onto glucose medium containing (+Arg) or not containing (−Arg) arginine and onto a glucose medium devoid of arginine but supplemented with oligomycin (−Arg + Oligo) and incubated 5 days at 28 °C. All of the media were devoid of histidine (−His) for plasmid selection. SDC17-31B cells transformed either with the ATP4-containing or the corresponding empty plasmid were plated for single colonies onto a glucose medium containing arginine (+Arg) or a glucose medium lacking arginine but supplemented with oligomycin (−Arg + Oligo), as in B. The colonies were replica-mated with a ρ+ tester strain (KL14-4A/60) and then replicated onto rich glycerol (N3) plates. Photographs were taken after a 4-day incubation at 28 °C.
the ATP synthase proton channel (as discussed above). Direct evidence for this assumption was sought with a regulatable strain (SDC33) in which δ-H9254 subunit and Atp4p are expressed, from a doxycycline-repressible (tetO) and a galactose-inducible (GAL10) promoter, respectively (Fig. 5A).

We first analyzed SDC33 by fluorescence microscopy with DASPMI, a mitochondrial δ-H9004/H9023-sensitive probe (Fig. 5D). As expected, the mitochondria were normally stained as a continuous reticulate network in SDC33 cells grown in a galactose minus doxycycline medium (i.e., conditions in which both δ-H9254 subunit and Atp4p, and hence the whole ATP synthase, can be synthesized). The mitochondria were also stained when both subunits were repressed (glucose + doxycycline). In these conditions, however, the mitochondria appeared fragmented presumably because of the change in carbon source and/or a weaker energization of the inner membrane due to the loss of mitochondrial oxidative phosphorylation. The SDC33 cells that were grown in glucose plus doxycycline were transferred and incubated for 3 h in galactose + doxycycline, conditions in which Atp4p can be synthesized while the δ-H9254 subunit gene remains repressed. In these conditions, the respiration became over the time gradually less sensitive to CCCP to
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JUNE 16, 2006•VOLUME 281•NUMBER 24
JOURNAL OF BIOLOGICAL CHEMISTRY 16311

reach a nearly CCCP-insensitive state 3 h after the galactose switch. Immunoblotting showed that this progressive uncoupling of mitochondrial respiration was paralleled by a gradual accumulation of Atp4p in the cells (Fig. 5C). These experiments showed that a lack in Atp4p prevents an F$_0$-mediated collapsing of the mitochondrial $\Delta\Psi$ when $\delta$ subunit is missing. Consistent with this, $\Delta\delta$ cells, reconstituted by transforming $\Delta\delta$ Atp4p (p-ARG8$^{\mu}$) cells with ATP4, remained p$^+$ and viable in the absence of external arginine when the growth medium was supplemented with oligomycin (Fig. 4B).

**DISCUSSION**

Previous work has shown that yeast lacking the ATP synthase $\delta$ subunit gene ($\Delta\delta$) produced populations containing p$^-$/p$^+$ cells exclusively (13, 14). Some evidence already suggested that $\Delta\delta$ yeast is unable to maintain intact p$^+$ mtDNA because of the loss of regulation of the ATP synthase in the form of a passive proton transport through the F$_0$ (i.e. not coupled to ATP synthesis in the F$_1$) (15–17). However, it was unclear why and how this ATP synthase defect compromised mtDNA maintenance.

To further investigate this question, we have introduced into the yeast mtDNA a mitochondrial version (ARG8$^{\mu}$) of the nuclear ARG8 gene encoding a mitochondrial protein involved in arginine biosynthesis (20). As a consequence, the mtDNA became required not only for respiration but also for arginine prototrophy (in a $\Delta\delta$ strain context). We used this nuclear-to-mitochondria gene relocation to gain a better understanding of why a lack in $\delta$ subunit destabilizes the mtDNA, according to the following rationale.

The mitochondrial genome is rather unstable in *S. cerevisiae*, even in wild type strains, which always produce a few percent p$^-$/p$^+$ cells. This may account for the relative ease with which many different types of mutations can increase the production of p$^+$ mtDNA because of the loss of regulation of the ATP synthase (21). Indeed, when oxidative phosphorylation is impossible, whether there is or is not functional mtDNA, as in $\Delta\delta$ yeast, p$^+$ and p$^-$/p$^+$ cells might have a similar multiplication rate by using glycolytic ATP. Thus, because of the irreversibility of the p$^-$/p$^+$ mutation, a modest increase in the frequency of this mutation might suffice for a complete disappearance of p$^+$ cells in a few generations only. In the ARG8$^{\mu}$ system and in the absence of external arginine, p$^-$/p$^+$ cells cannot divide and are therefore expected to accumulate much less rapidly, provided that the systems in charge of mtDNA propagation/expression are still active and that retention of mtDNA is not detrimental to cellular viability.

In a first set of experiments, we analyzed the segregation of the $\delta$ subunit deletion and ARG8$^{\mu}$ markers upon sporulation of a heterozygous $\Delta\delta}$/y+ yeast strain (SDC13). Consistent with a previous study (15), we found that this strain had a reduced respiratory capacity (also observed in Ref. 50), presumably because of a partial mitochondrial uncoupling. In addition, we noticed the accumulation (20%) in SDC13 glucose cultures of “revertants” (called SDC13-G) with a good respiratory capacity. Upon sporulation, these revertants segregated the $\delta$ subunit deletion gene marker 2:2, but most (>90%) of the spores were respiratory competent. SDC13 subclones with a reduced respiratory capacity (SDC13-I) produced many aberrant tetrads as well, with two or three viable spores and a large excess of respiratory competent spores. Similar observations have been made by J. Velours and M.-F. Giraud. A possible explanation is that the partial mitochondrial uncoupling resulting from the reduced dosage of the $\delta$ subunit gene from 2 to 1 is so deleterious to the cell that it favors a rapid selection of mutations restoring the integrity of mitochondria, maybe by duplications of the remaining $\delta$ subunit gene or correction of the disrupted $\delta$ subunit gene by gene conversion.

Whatever the explanation for the genetic drift and aberrant meiotic segregation properties of $\Delta\delta}$/y+ yeast, a clear result emerged from these experiments. Indeed, in all tetrads exhibiting the expected segregation of the $\delta$ subunit deletion marker and respiratory deficiency, the $\Delta\delta$ spores were arginine-auxotrophic, and their early progenies were composed exclusively of p$^-$/p$^+$ cells. In addition, we could never observe germination of $\Delta\delta$ spores on media lacking arginine. These data indicate that $\Delta\delta$ yeast is strictly unable to propagate, or survive the presence of functional mtDNA.

In a second set of experiments, we found that mtDNA maintenance was possible in $\Delta\delta$ yeast when the ATP synthase F$_1$ component was inactivated either chemically by oligomycin or genetically by a nuclear mutation (Atp4$^{\mu}$) that prevents insertion of Atp6p in the ATP synthase. One possible explanation (model 1) is that the proton translocating activity of the F$_0$ is lethal to yeast lacking the ATP synthase $\delta$ subunit. A reasonable view is that the very strong weakening of the mitochondrial electrical potential ($\Delta\Psi$) observed after a block in the synthesis of the $\delta$ subunit (16) may result in a general defect in mitochondrial biogenesis. Consequently, essential reactions taking place in mitochondria can no longer proceed, leading to the death of the cell. Consistent with this idea, a recent study in yeast has shown that a collapsing of the mitochondrial $\Delta\Psi$ is followed by the onset of mitophagy and reduced cell viability (43). Accordingly, as previously discussed (16, 17), the p$^-$/p$^+$ mutation can be viewed as a rescuing event allowing survival of $\Delta\delta$ yeast. Indeed, after the loss of the mtDNA, the ATP synthase proton channel cannot be synthesized, and consequently a lack of $\delta$ subunit can no longer dissipate the mitochondrial $\Delta\Psi$. The inner membrane is believed to be sufficiently energized in p$^-$/p$^+$ $\Delta\delta$ yeast by the electrogenic activity of the ADP/ATP translocase (13). Thus, according to model 1, conversion of $\Delta\delta$ yeast into petites would preferentially proceed by selection of preexisting p$^-$/p$^+$ cells undergoing the loss of the $\delta$ subunit gene.

Alternatively (model 2), the F$_1$ activity would not kill $\Delta\delta$ cells but would induce petites at high frequencies (e.g. by preventing a sufficient transport into mitochondria of the systems needed for mtDNA propagation. Such a mechanism seems to occur in strains harboring $\epsilon$ subunit deletions, which induce many petites (70%), although $\rho^-$ cells lacking the $\epsilon$ subunit are viable (18).

The experiments performed with ATP4 are consistent with a petite selection scheme. Indeed, not a single p$^-$ cell was recovered by crossing with a p$^+$ tester hundreds of colonies formed (in the presence of arginine) by $\Delta\delta$ + Atp4$^{\mu}$ cells transformed with ATP4. This observation argues against model 2, since in that case, a number of the initial p$^-$ cells would probably have been recovered in the test crosses. Probably, the reconstituted $\Delta\delta$ colonies arose from $\Delta\delta$ + Atp4$^{\mu}$ cells that were already p$^-$/p$^+$ before the transformation with ATP4 (Fig. 4B shows that about 70% of the plated transformants were not p$^-$).

Normally, p$^-$ mtDNA segregates extremely efficiently into haploid spores. Assuming that it is also the case with $\Delta\delta}$/y+ cells, according to model 1, the deletion spores are expected to be killed. Even if they were largely underrepresented, viable $\Delta\delta$ spores could be recovered. However, because of the rapid genetic drift of $\Delta\delta}$/y+ (see above), it is difficult from these experiments to appreciate to what degree petite induction contributes to the conversion of $\Delta\delta$ yeast into populations lacking functional mtDNA.

Mutations in the $\alpha$-F$_1$ or $\beta$-F$_1$ subunit or in Atp11p, an F$_1$ assembly factor, have also been shown to suppress the formation of p$^-$/p$^+$ cells in

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7 J. Velours and M.-F. Giraud, personal communication.
strains deleted for the δ subunit (14). These genetic observations are in accordance with our conclusion that the loss of mtDNA in Δδ yeast is caused by an uncoupling of the ATP synthase. Indeed, as we have shown, the F₀ is absent in a yeast fmc1 mutant unable to assemble the αβγδ subcomplex of F₁ (48), and in a mutant lacking the β subunit (49). Thus, in cells lacking both the δ subunit and the catalytic unit of F₁, mtDNA maintenance is tolerated because it cannot result in a collapsing of the mitochondrial ΔΨ. Direct evidence has been provided earlier supporting this explanation (15).

The loss of the δ subunit gene has been reported to be viable in Kluyveromyces lactis, a “petite negative” yeast, which, despite a good fermenting capacity, does not survive the pᵢ/pᵣ mutation (19). Thus, contrary to S. cerevisiae, the mitochondrial genome is tolerated in K. lactis when the δ subunit is missing. This is a very interesting observation, indicating that elimination of the δ subunit does not have the same consequences in K. lactis than in S. cerevisiae. Indeed, as we have shown, the δ subunit is not required in S. cerevisiae for the assembly of the remaining ATP synthase subunits, but the resulting partial F₁F₀ complexes can still transport protons and dissipate the mitochondrial ΔΨ (16). As a result, in normal conditions, the synthesis of the δ subunit must be tightly regulated in S. cerevisiae to avoid any deficit in this protein. The ability of a Δδ mutant of K. lactis to remain pᵢ and viable suggests that a lack in the δ subunit does not prevent the maintenance of a sufficient mitochondrial ΔΨ. It might be that in K. lactis, the ATP synthase proton channel cannot assemble or function when the δ subunit is missing. Thus, it appears that different mechanisms may exist among species to avoid accumulation of aberrant ATP synthase assemblies that can uncouple the mitochondrion.

Within the ATP synthase, the δ subunit interacts with the e subunit to couple the αβγδ subcomplex of F₁ to the enzyme proton channel (4, 5). In S. cerevisiae, a null mutation of the γ subunit always results in 100% petites (14), and there is evidence that a failure in the expression of this subunit is followed by an F₀-mediated proton leak across the inner mitochondrial membrane (15). Thus, the pᵢ/pᵣ mutation might be for Δγ yeast, as it is for Δδ yeast, a rescuing event allowing cell survival. Interestingly, cultures of Δe yeast usually contain 30% pᵢ cells showing that mtDNA maintenance is not lethal to Δe yeast (18). Yet, mitochondria isolated from a Δe culture show defects in ΔΨ maintenance identical to those seen when the δ subunit is lacking. Experiments using a regulatable e subunit gene are ongoing to clarify this apparent contradiction.

Mutations of subunits belonging to ATP synthase components other than the central stalk were also found to have strong negative effects on the stability of the mtDNA (see Ref. 21 for a review). For example, a null mutation of the peripheral stalk Atp4p subunit usually resulted in 70% pᵢ/pᵣ cells (42). No F₀-mediated proton leak was detected in Δatp4δ mitochondria, which is not very surprising, since the Atp6p subunit cannot insert when Atp4p is missing (42) (this study). Thus, something else than an ATP synthase proton leak must be responsible for mtDNA destabilization in the Δatp4δ mutant.

A substantial loss of mtDNA was also observed for mutations of the ATP synthase having no major incidence on its proton translocating and ATP synthesis activities, such as null mutations of subunit e or g (44, 45). Each of these mutations results in about 40% pᵢ/pᵣ cells after growth in a lactate medium, and probably more would accumulate after growth on a fermentable carbon source. Abnormal mitochondrial morphologies characterized by numerous digitations and onion-like structures presumably corresponding to uncontrolled biogenesis and/or folding of the inner membrane were found in cells lacking subunit e or g (45). Such morphological defects may alter the association of the mtDNA to the inner membrane and/or transmission of the mtDNA to daughter cells. The importance of the mitochondrial structure for mtDNA maintenance is well illustrated by studies of mutants defective in systems directly controlling mitochondrial structure such as Fzo1p, whose absence leads to fragmentation of the mitochondrial network followed by a massive conversion into cells lacking mtDNA (46).

The present study, together with a report from Mueller and co-workers (14) provides a clear demonstration of a mutation causing a massive loss of mtDNA with no direct effect on the components in charge of the maintenance and inheritance of the mitochondrial genome. This is an interesting observation in view of the need for mtDNA maintenance of a huge number of mitochondrial proteins (>200) with no obvious link to the mtDNA (21). The pᵢ mtDNA nutritional base selection system we used in this study may help to clarify the connections between the immediate effects of mutations in these various proteins and mtDNA instability.

The observations reported in this study provide the basis for a genetic screen targeted on the F₀ component of the ATP synthase. Indeed, as we have shown, in order to survive, Δδ yeast needs to inactivate the ATP synthase proton channel. Thus, in a Δδ context, F₀-inactivating mutations can be positively selected. In the ARG₄₄™ system, the most frequent rescuing event, the pᵢ/pᵣ mutation, can be eliminated from the screen, by selecting the isolates in an arginine-lacking medium. We thus now have in hand a very powerful genetic system, which may help to better define the structural determinants essential for the activity of the ATP synthase proton channel and facilitate the study of the biogenesis of this channel, which is still largely unknown.

Acknowledgments—We are grateful to T. D. Fox, H. Mireau, and J. Velours for discussions and the generous gift of various strains, plasmids, and antibodies. Virginie Darrigade is warmly acknowledged for help in some experiments. We thank M. Herlan, J. Hermann, A. Kaniak, J. Rytkö, and C. Schwimmer for helpful discussions and critical reading of the manuscript.

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