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α and β subunits of F_1-ATPase are required for survival of petite mutants in *Saccharomyces cerevisiae*

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Abstract Although *Saccharomyces cerevisiae* can form petite mutants with deletions in mitochondrial DNA (mtDNA) (ρ^-) and can survive complete loss of the organellar genome (ρ^o), the genetic factor(s) that permit(s) survival of ρ^- and ρ^o mutants remain(s) unknown. In this report we show that a function associated with the F_1-ATPase, which is distinct from its role in energy transduction, is required for the petite-positive phenotype of *S. cerevisiae*. Inactivation of either the α or β subunit, but not the γ, δ, or ε subunit of F_1, renders cells petite-negative. The F_1 complex, or a subcomplex composed of the α and β subunits only, is essential for survival of ρ^o cells and those impaired in electron transport. The activity of F_1 that suppresses ρ^o lethality is independent of the membrane F_o complex, but is associated with an intrinsic ATPase activity. A further demonstration of the ability of F_1 subunits to suppress ρ^o lethality has been achieved by simultaneous expression of *S. cerevisiae* F_1 α and γ subunit genes in *Kluyveromyces lactis* – which allows this petite-negative yeast to survive the loss of its mtDNA. Consequently, *ATP1* and *ATP2*, in addition to the previously identified *AAC2*, *YME1* and *PEL1*/*PGS1* genes, are required for establishment of ρ^- or ρ^o mutations in *S. cerevisiae*.

Key words F_1-ATPase · Mitochondrial DNA · Petite mutation · ρ^o lethality · Yeast

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Introduction

The first description of the "petite colonie" mutation in baker's yeast (*Saccharomyces cerevisiae*) was published nearly half a century ago (Ephrussi 1953), but we still do not have a detailed understanding of this phenomenon despite the accumulation of considerable knowledge. The respiration-deficient petite mutation can arise either spontaneously or in response to DNA-targeting drugs, and is due to the formation of large deletions in mitochondrial DNA (mtDNA, Faye et al. 1973). Although mtDNA in baker's yeast encodes integral components of multiple enzyme complexes required for oxidative phosphorylation (for review, see Attardi and Schatz 1988; Grivell 1989), loss of the organelle genome is not lethal.

However, in contrast to *S. cerevisiae*, most yeast species (Bulder 1964a, 1964b; de Deken 1966; Clark-Walker et al. 1981) are unable to form viable mitochondrial genome deletion mutants, suggesting that mtDNA encodes an essential function. A direct demonstration that mtDNA is essential for the petite-negative yeast *Kluyveromyces lactis* has been achieved by disruption of a nuclear gene required for mtDNA maintenance, which resulted in lethality (Clark-Walker and Chen 1996). Hence a simple interpretation of ρ^o survival in *S. cerevisiae* and ρ^o lethality in *K. lactis* is that all mtDNA genes are dispensable in the former yeast but one or more genes are required in the latter species. An answer to the question as to which mitochondrial genes are needed by *K. lactis* would still leave unresolved the more fundamental issue of the underlying reason for the requirement for mtDNA gene products in petite-negative yeasts and their dispensability in petite-positive species.

A different perspective has been brought to the question of what factors are responsible for the petite mutation by the discovery of nuclear mutations that convert petite-negative yeasts into petite-positive forms (reviewed by Chen and Clark-Walker 1999). Such mutations have been found in *K. lactis* (Chen and Clark-Walker 1993) and *Schizosaccharomyces pombe* (Haffter

and Fox 1992). *K. lactis* cells carrying mutations designated *mgi* (for *M*itochondrial *G*enome *I*ntegrity), like wild type *S. cerevisiae*, are able to form deletions in mtDNA spontaneously and can lose their mitochondrial genome upon treatment with DNA-targeting drugs, without becoming inviable. Studies in *K. lactis* have shown that the three *MGI* genes encode the α, β and γ subunits of the mitochondrial F_1F_o-ATP synthase (Chen and Clark-Walker 1995, 1996).

The F_1F_o-ATP synthase is a multisubunit protein complex in the oxidative phosphorylation pathway, located on the inner membrane of mitochondria. The enzyme in yeast, like its counterparts in bacteria and chloroplasts, consists of two domains, namely, the extrinsic and intrinsic membrane complexes, F_1 and F_o. ATP is synthesised in the F_1 sector by using the energy transmitted from F_o as a result of proton movement from the intermembrane space to the matrix side of the inner membrane (for a recent review, see Boyer 1997). The F_1 portion is composed of five proteins with the stoichiometry $3\alpha{:}3\beta{:}1\gamma{:}1\delta{:}1\varepsilon$ and all five subunits are encoded by nuclear genes. The F_o sector consists of at least nine proteins (Arnold et al. 1998; Roudeau et al. 1999); the three genes for subunits 6, 8 and 9 are present in yeast mtDNA (Grivell 1989).

Following from our observations with *K. lactis*, we considered the possibility that the F_1-ATPase may be important for survival of *S. cerevisiae* lacking mtDNA. Consequently, we have examined the role of F_1 subunits in the viability of petite mutants of baker's yeast and asked whether wild-type *ATP1*, *2* and *3* genes of *S. cerevisiae* can support the formation of ρ^o mutants when transferred to *K. lactis*.

Materials and methods

Strains and media

Yeast strains used in this study are listed in Table 1. The complete medium used (GYP) contains 0.5% Bacto yeast extract, 1% Bacto Peptone and 2% glucose. GalYP and GlyYP media contained 2% galactose or 2% glycerol in place of glucose. Minimal medium (GMM) contains 0.67% Difco yeast nitrogen base without amino acids and 2% glucose. Ethidium bromide (EB) medium is GYP plus EB at 16 μg/ml. G418 medium is GYP plus G418 at 200 μg/ml.

Gene nomenclature

In our previous reports we described the isolation of three mitochondrial genome integrity loci, *MGI2*, *1* and *5* that encode the α, β and γ subunits of the mitochondrial F_1-ATPase (Chen and Clark-Walker 1993, 1995, 1996). To restore consistency with the nomenclature of the F_1-ATPase subunit genes in *S. cerevisiae*, we have renamed *MGI2*, *MGI1* and *MGI5* as *ATP1*, *ATP2* and *ATP3* (or *KlATP1*, *KlATP2* and *KlATP3*). Likewise, all the mutant alleles were renamed. For instance, the previously reported *mgi2-2* allele is now referred to as *atp1-2*, the *mgi1-1* allele is now designated as *atp2-1* and *mgi5-1* is referred as *atp3-1* etc.

Gene disruption and strain construction

Disruption of chromosomal genes was achieved by the one-step gene replacement procedure (Rothstein 1983). The five *S. cerevisiae* F_1 subunit genes were disrupted as depicted in Fig. 1A. The *ATP1* gene was disrupted by replacement of a 210-bp *Bgl*II segment within the coding sequence by a 1.4-kb *Bgl*II-*Xho*I fragment containing the *kan* expression module (Wach et al. 1994; Güldener et al. 1996) after end-filling by T_4 DNA polymerase. Likewise, *ATP2* was disrupted by insertion of *kan* in the unique *Kpn*I site within the ORF. *ATP3* was disrupted by replacement of a 440-bp *Bcl*I fragment spanning two-thirds of the coding sequence by *kan*. *ATPδ* was disrupted by replacement of a 440-bp *Nde*I-*Xba*I fragment covering the majority of the coding region by *kan*. *ATPε* was disrupted by insertion of *kan* into the *Nde*I site in codon 10 of the gene. The *kan*-containing cassettes were used to disrupt the five genes by transforming M2915-6A and selecting for stable $G418^R$ transformants to produce the strains CS113, 114, 115, 169 and 170. Disruption of *ATP1* and *ATP2* in W303-1B gave rise to CS110 and CS111. By using the same strategy as described above, *atp2*::*LEU2*, *atpδ*Δ::*URA3* and *atpε*::*LEU2* cassettes were constructed to disrupt the genes with the respective selectable markers. CS118 was constructed by disruption of *ATP2* in CS113 (*atp1*Δ::*kan*) using the *atp2*::*LEU2* cassette. CS229 and CS227/2 were constructed by successive disruption of *ATPδ* and *ATPε* in CS115 (*atp3*Δ::*kan*) and CS109 (*atp3*Δ::*kan*/+), using the *atpδϖ* Δ::*URA3* and *atpε*::*LEU2* cassettes. The *S. cerevisiae CYT1* gene was disrupted by replacement of an internal 455-bp *Nsi*I-*Kpn*I segment within the coding region by *kan*. The *COX4* gene was disrupted by insertion of *kan* into the *Cla*I site at codon 78 of the gene. The *ATP4* gene was disrupted by insertion of *kan* into the *Xho*I site at codon 130 of the ORF. The *cyt1*Δ::*kan*, *cox4*::*kan* and *atp4*::*kan* cassettes were used to transform M2915-6A to produce CS120, CS121 and CS171.

Plasmids pP159, pE430 and pE588 (kindly provided by S. Ackerman) were digested with *Eco*RI to release DNA fragments of 4.8 kb containing the *S. cerevisiae ATP2* gene with A192V, E222K and R293K mutations (Liang and Ackerman 1996), which were subsequently cloned into the *URA3*-based integrative vector pUC-URA3/4 (Chen and Fukuhara 1988). The resulting plasmids, pScATP2/159, pScATP2/430 and pScATP2/588, were linearised by cleavage with *Stu*I in the *URA3* gene and targeted onto the *ura3* locus of the *atp2*-disrupted mutant CS114 to produce CS162, CS163 and CS164. To introduce the *K. lactis atp1-2* allele (formerly *mgi2-2*) into *S. cerevisiae*, the plasmid pURA-Klatp1.2 was constructed by cloning of a 2.8-kb *Kpn*I-*Bam*HI fragment containing the *Klatp1-2* allele from pCXJ3-mgi2.2 (Chen et al. 1998) into pUC-URA3/4. pURA-Klatp1.2 was linearised by digestion with *Stu*I in the *URA3* gene and integrated into the *ura3* locus of CS113 to produce CS187.

Expression of *S. cerevisiae* F_1 genes in *K. lactis*

The plasmid pCXJ4 is a *K. lactis* integrative vector containing the *K. lactis LEU2* gene (X. J. Chen, unpublished) and was used to express *S. cerevisiae* F_1 genes in *K. lactis*. Simultaneous ligation of the 2.5-kb *Eco*RI-*Hin*dIII, the 2.45-kb *Hin*dIII-*Bam*HI and the 3.4-kb *Bam*HI-*Sph*I fragments, containing *S. cerevisiae* genes for F_1 β, γ and α subunits, respectively, into the *Eco*RI and *Sph*I sites of pCXJ4 produced the plasmid pCXJ4-ScABG. Likewise, the multiple fragment ligation strategy was used to construct pCXJ4-ScAG that contains *ScATP1* and *ScATP3*. These plasmids were then cleaved at the unique *Cla*I site in *KlLEU2* and targeted into the *leu2* locus of the *K. lactis atp1 atp2 atp3* triple mutant CK332/1 and *atp1 atp3* double disruptant CK333 by selecting for Leu^+.

ATPase activity determination

Precultures (50 ml), at late exponential phase, were inoculated into 500-ml portions of GalYP medium. Cells were grown at 30° C for 5 h before adding 2 g of chloramphenicol to inhibit mitochondrial protein synthesis. The cultures were allowed to grow for 15 h and mitochondrial extracts were prepared as described in a previous paper (Chen and Clark-Walker 1995). The assay for ATPase activity was performed essentially according to Law et al. (1995).

Table 1 Genotype and source of yeast strains

Strain	Relevant genotype	Source/reference
S. cerevisiae		
2262	*MATα*, *ade1*, *his5*, *leu2*, *lys11*, *ura1*, *gal1*, *can*^{s}	L. Hartwell
AH22	*MAT***a**, *leu2-3*, *112*, *his4-519*, *can1*	G. Fink
CH1462	*MATα*, *ade2*, *ade3*, *leu2*, *ura3*, *his3*, *can1*	C. Holm
CH1305	*MAT***a**, *ade2*, *ade3*, *leu2*, *ura3*, *lys2*, *can1*	C. Holm
CS25-6B	*Matα*, *his3*, *leu2*, *ura3*, *atp1*Δ::*LEU2*	This study
CS107-1D	*MAT***a**, *leu2*, *ura3*, *his4*	This study
CS5	*Mat***a**/*α*, *leu2*/*leu2*, *ura3*/*ura3*, *his4*/+, +/*ade2*	This study
CS109	Same as CS5, +/ *atp3*Δ::*kan*	This study
CS110	Same as W303-1B, *atp1*Δ::*kan*	This study
CS111	Same as W303-1B, *atp2*::*kan*	This study
CS113	Same as M2915-6A, *atp1*Δ::*kan*	This study
CS114	Same as M2915-6A, *atp2*::*kan*	This study
CS115	Same as M2915-6A, *atp3*Δ::*kan*	This study
CS118	*MAT***a**, *ade2-1*, *ura3-52*, *leu2-3*, *112*, *atp1*Δ::*kan*, *atp2*::*KlLEU2*	This study
CS120	Same as M2915-6A, *cyt1*Δ::*kan*	This study
CS121	Same as M2915-6A, *cox4*::*kan*	This study
CS143	diploid, W303-1B crossed to CS107–1D	This study
CS162	Same as CS114, *ura3*::*pScATP2*/*159*(*βA192 V*)	This study
CS163	Same as CS114, *ura3*::*pScATP2*/*430*(*βE222 K*)	This study
CS164	Same as CS114, *ura3*::*pScATP2*/*588*(*βR293 K*)	This study
CS169	Same as M2915-6A, *atpδ*Δ::*kan*	This study
CS170	Same as M2915-6A, *atpε*::*kan*	This study
CS171	Same as M2915-6A, *atp4*::*kan*	This study
CS178	Diploid, CS120 crossed to CS25-6A	This study
CS179	Diploid, CS121 crossed to CS25-6A	This study
CS180	Diploid, CS171 crossed to CS25-6A	This study
CS187	Same as CS113, *ura3*::*pURA-Klatp1.2*	This study
CS227/2	Same as CS5, +/*atp3*Δ::*kan*, +/*atpδ*Δ::*URA3*, +/*atpε*::*LEU2*	This study
CS229	Same as M2915-6A, *atp3*Δ::*kan*, *atpδ*Δ::*URA3*, *atpε*::*LEU2*	This study
D273–10B/A1	*MATα*, *met6*	F. Sherman
M2915–6A	*MAT***a**, *ade2-1*, *ura3-52*, *leu2-3*, *112*	This study
W303–1B	*MATα*, *ade2-1*, *his3-115*, *leu2-3*, *112*, *trp1-1*, *ura3-1*	R. Rothstein
K. lactis		
CK272-6C	*MAT***a**, Ade$^-$, *uraA1*, *lysA1*, *atp1*Δ::*URA3*	This study
CK273-4C	*MATα*, *ade1*, *uraA1*, *leu2*, *atp2*::*URA3*	This study
CK274-3C	*MAT***a**, *uraA1*, *leu2*, *atp3*::*URA3*	This study
CK282	Same as CK272-6C, *[pCXJ24-ScATP1]*	This study
CK283	Same as CK273-4C, [pCXJ24-ScATP2]	This study
CK284	Same as CK274-3C, [pCXJ24-ScATP3]	This study
CK332/1	*MAT***a**, *adeT-600*, *uraA1*, *leu2*, *atp1*Δ::*kan*, *atp2*::*URA3*, *atp3*::*URA3*	This study
CK333	*Matα*, *ade1*, *adeT-600*, *uraA1*, *leu2*, *atp1*Δ::*kan*, *atp3*::*URA3*	This study
CK334	Same as CK332/1, *leu2*::*pCXJ4-ScABG*	This study
CK340/5	Same as CK333, *leu2*::*pCXJ4-ScAG*	This study

Protein concentrations were determined using Biorad Protein Assay Kit 1.

Results

S. cerevisiae petite mutants require the α and β subunits of F_1-ATPase for survival

The *S. cerevisiae* genes *ATP1*, *ATP2*, *ATP3*, *ATPδ* (*ATP16*) and *ATPε* (*ATP15*), encoding the α, β, γ, δ and ε subunits of the F_1 complex (Takeda et al. 1985, 1986; Guélin et al. 1993; Giraud and Velours 1994; Paul et al. 1994), respectively, were individually disrupted in the strain M2915-6A, either by simple insertion of the kanamycin resistance gene cassette (*kan*) or by replacement of parts of their coding sequences by *kan* (Fig. 1A). In all cases viable colonies with a stable G418-resistant phenotype were obtained. Correct disruption of the genes was confirmed by Southern analysis of genomic DNA (data not shown). As expected, cells disrupted in any F_1-subunit gene are respiration deficient, as they failed to grow on glycerol (Fig. 1B), though residual respiratory growth on glycerol of cells disrupted in the ε-subunit gene is noticeable in some strains after prolonged incubation (not shown). Also noteworthy is the total conversion to the ρ^-/ρ^o state of strains disrupted in the γ- or δ-subunit genes, a 50% frequency of cytoplasmic petites among ε-deleted cells (data not shown; also see Guélin et al. 1993; Giraud and Velours 1994; Weber et al. 1995; Zhang et al. 1999), and significantly slower growth of γ- and δ-deleted mutants on glucose medium compared with those in which *ATP1* or *ATP2* was inactivated.

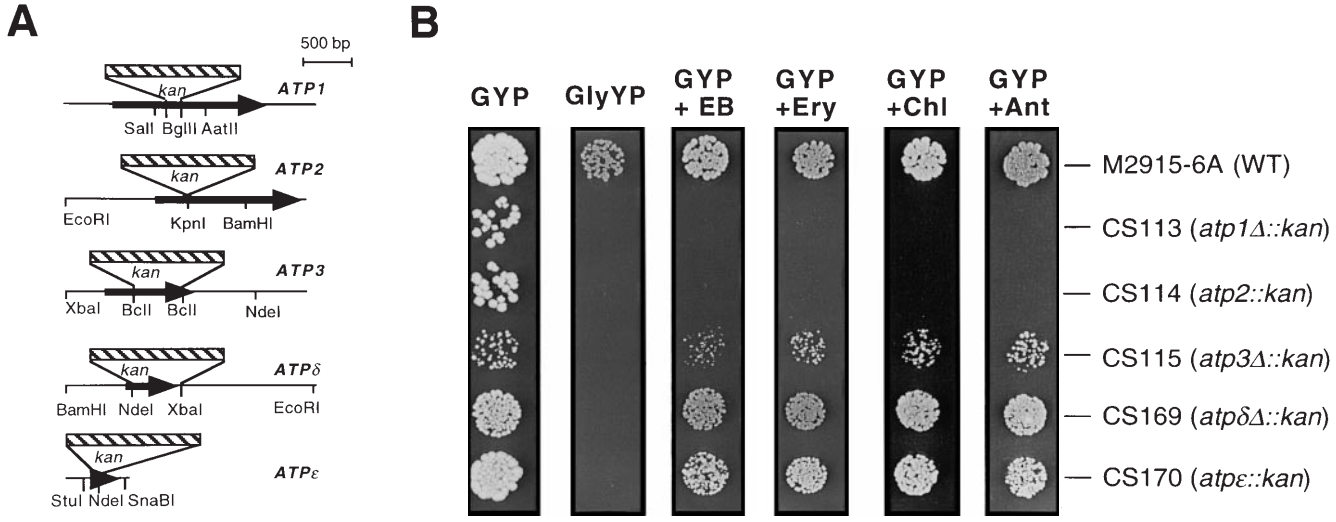

Fig. 1A, B Disruption of *S. cerevisiae* F_1 subunit genes and sensitivity of the F_1 mutants to various metabolic inhibitors. **A** Physical map of the region flanking the five *S. cerevisiae* F_1 subunit genes *ATP1*, *ATP2*, *ATP3*, *ATPδ* and *ATPε*, which encode the α, β, γ, δ and ε subunits of the complex, and strategies used for disruption of the genes by simple insertion of, or replacement of coding sequences by, the *kan*-expression module. The *arrows* represent the coding regions of the genes and indicate the direction of transcription. Restriction sites used for insertion of *kan* and for release of the disruption cassettes are indicated. **B** Sensitivity of the F_1 mutants to ethidium bromide (EB), erythromycin (Ery), chloramphenicol (Chl) and antimycin (Ant). Cells were grown to stationary phase, diluted in water and 10-μl aliquots were applied to plates of GYP, GlyYP and GYP plus EB, Ery, Chl or Ant at concentrations of 16 μg/ml, 5 mg/ml, 4 mg/ml and 0.5 μM, respectively. The plates were incubated at 30° C for 4 days before being photographed

The F_1-subunit disruptants were then examined for resistance to ethidium bromide (EB). As can be seen in Fig. 1B, CS113 and CS114, disrupted in α or β subunits, are sensitive to EB at 16 μg/ml on glucose. By contrast, CS115, CS169 and CS170, disrupted in the γ, δ and ε subunits, form viable colonies in the presence of EB. However, these strains show a significantly slower growth rate than the wild-type M2915-6A, with the γ-deleted strain being affected most. The EB^R cells lack mtDNA, as confirmed by Southern analysis of total DNA using *S. cerevisiae* mtDNA as a probe (not illustrated). These results indicate that the α and β subunits are required for viability of cells in which mtDNA has been eliminated by treatment with EB. To support this notion, we crossed an *ATP1*-disrupted mutant to a strain in which *MGM101*, a nuclear gene required for maintenance of the mitochondrial genome (Chen et al. 1993), had been inactivated. Synergistic lethality was found in the segregants that have a disruption in *ATP1* combined with inactivation of *MGM101* (data not shown).

Subsequently, to determine whether the lack of mtDNA-encoded proteins causes inviability of cells disrupted in the core F_1 complex, we examined the response of F_1 disruptants to chloramphenicol and erythromycin, which specifically inhibit mitochondrial protein synthesis. In agreement with expectations, strains lacking α or β subunits are unable to grow on glucose medium in the presence of these drugs, whereas those deficient in γ, δ and ε subunits can survive inhibition of mitochondrial protein synthesis (Fig. 1B).

As enzyme complexes for electron transport and ATP synthase contain protein subunits encoded by mtDNA, it was of interest to learn which function is required in strains lacking α or β subunits of F_1. Accordingly, nuclear genes for electron transport – *CYT1* (Sadler et al. 1984) and *COX4* (Maase et al. 1984) – or the F_o complex of ATP synthase (*ATP4*; Velours et al. 1988) were disrupted by insertion of *kan*. The three genes encode cytochrome c_1, cytochrome *c* oxidase subunit 4 and ATP synthase subunit b, which are integral components of the complexes III and IV and the F_o sector of complex V, respectively. The resulting strains, CS120, CS121 and CS171, were crossed to the *ATP1*-disrupted CS25-6B to produce the diploids CS178, CS179 and CS180, which were subsequently sporulated and dissected. Segregation of disrupted *CYT1*, *COX4* or *ATP4* was followed using the $G418^R$ marker, while disruption of *ATP1* was monitored by the Leu^+ phenotype. In 15 tetrads analysed for each diploid, no $G418^R$, Leu^+ spores were identified for CS178 and CS179. Instead, inviable spores were observed that were deduced to have cosegregated Leu^+ and $G418^R$ (Fig. 2). However, all segregants of CS180 formed viable colonies. These results clearly indicate that in the absence of the F_1 α-subunit, nuclear mutations affecting the electron transport chain are not tolerated. In agreement with these results, we found that CS113 and CS114, disrupted in the genes encoding the α and β subunits, are sensitive to antimycin, which specifically inhibits the function of the bc_1 complex in the electron transport chain (Fig. 1B).

To examine whether loss of viability of ρ^-/ρ^o mutants is a peculiarity of M2915-6A, we tested five additional strains of independent origin for their response to disruption of F_1. *ATP2* was disrupted in AH22, 2262, CH1462, CH1305 and D273-10B/A1 (Table 1). The resulting strains were respiration deficient and, like M2915-6A, were all sensitive to elimination of mtDNA by EB

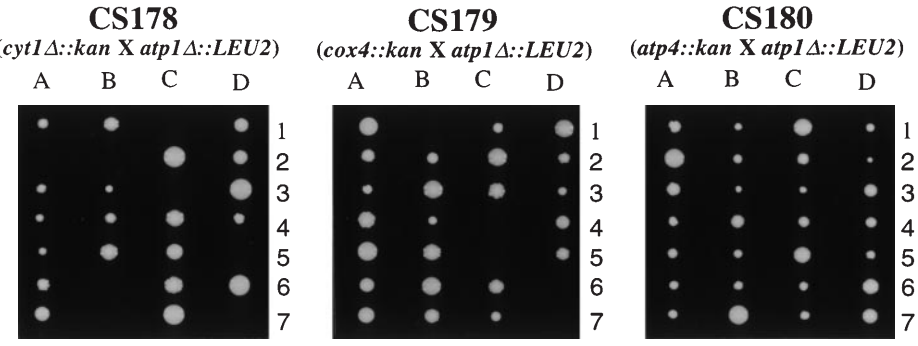

Fig. 2 Tetrad analysis showing that F_1 is required for viability of *S. cerevisiae* strains that are defective in the electron transport chain. Diploid strains CS178, CS179 and CS180 heterozygous for *atp1*Δ::*LEU2* and *cyt1*Δ::*kan* or *cox4*::*kan* or *atp4*::*kan* were sporulated and dissected on GYP plates. Plates were incubated at 30°C for 4 days

(not illustrated). However, when we disrupted the α or β subunit genes in a common laboratory strain, W303-1B, the resulting strains CS110 and CS111 were EB resistant and therefore petite-positive. To test whether (a) suppressor mutation(s) might be present in W303-1B that can overcome ρ^o lethality in the absence of F_1, we crossed CS111 (W303-1B disrupted in *ATP2*) to the wild-type CS107-1D. Analysis of 25 tetrads from the resulting diploid, CS143, revealed that a single nuclear gene is present in W303-1B that is responsible for the suppression of ρ^o lethality in the *ATP2*-disrupted spores (data not shown). Taken together, the above results lead us to conclude that a petite-negative phenotype can occur in most laboratory strains in the absence of F_1, with the exception of those with a W303-1B background.

The γ, δ and ε subunits of F_1 are dispensable for viability of ρ^o cells

From the examination of individually disrupted F_1 subunit genes, it seemed that a core complex, composed of α and β subunits, should be sufficient to suppress ρ^o lethality. To establish whether cells that lack the three smallest F_1 subunits can survive loss of mtDNA, we obtained the appropriate strains from a diploid, CS227/2, having insertions of the selectable marker genes *kan*, *URA3* and *LEU2* in the genes for γ, δ and ε subunits, respectively. Following sporulation, segregants harbouring disruptions in the loci *ATP3*, *ATPδ* and *ATPε* were identified by the presence of Kan^R, Ura^+ and Leu^+ phenotypes. Such strains, exemplified by CS229, can form colonies in the presence of EB (not illustrated). These results indicate that the core complex of F_1, lacking γ, δ and ε subunits, is sufficient for suppression of ρ^o lethality.

F_1-related ATP hydrolysis is required for suppression of ρ^o lethality but does not correlate with the ρ^o lethality suppressor activity

Earlier studies have demonstrated that mitochondria from *S. cerevisiae* ρ^o cells have a substantial oligomycin-insensitive ATPase activity (Kovac and Weissova 1968; Schatz 1968; Tzagoloff et al. 1973; our unpublished data). Consequently, we examined whether the intrinsic ability of F_1 to hydrolyse ATP contributes to the viability of ρ^o cells. Various respiration-deficient strains were constructed in the M2915-6A background to establish whether a correlation exists between a defect in ATP hydrolysis and sensitivity to mtDNA elimination. To mimic the state of F_1 in ρ^o strains, cells were grown in the presence of chloramphenicol to inhibit mitochondrial protein synthesis. Preliminary experiments had shown that in the presence of chloramphenicol, cells disrupted in F_1 α- or β-subunits can continue to divide for approximately 6–8 generations before ceasing to grow.

ATPase activity was assayed in mitochondrial extracts from the five strains disrupted in the F_1-subunit genes . We find that CS113 and CS114, disrupted in *ATP1* and *ATP2*, have an ATPase activity of 0.22 and 0.21 (Table 2). These values are comparable to the double mutant CS118 (0.20), which reflects a baseline, non-F_1 related, ATP hydrolysis in mitochondrial extracts. By contrast, mitochondria from strains with disruptions in the γ, δ and ε-subunits all retain detectable F_1-related ATP hydrolysis activity with values of 0.30, 2.07 and 2.71, respectively. Even CS229, a triple disruption of the genes for γ, δ and ε-subunits, has an ATPase activity of 0.32, which is above the baseline level. As strains with disruptions in the three smallest subunits and the triple disruptant are EB resistant and can form viable cells lacking mtDNA, it is suggested that an F_1-associated ATP hydrolysis activity is required for suppression of ρ^o lethality. Because CS115 and CS229 have ATPase levels of 0.30 and 0.32, which are higher than the base line level of 0.20, an F_1-related ATPase activity of 0.10 is correlated with the ρ^o lethality suppressor function.

To support the above notion, we extended the analysis to strains with point mutations in the F_1 β subunit. Isogenic strains were constructed with the A192V, E222K and R293K mutations in the β subunit that have been shown to be severely affected in catalytic properties but not in the assembly of F_1-ATPase (Liang and Ackerman 1996). Although all three strains harbouring β subunit mutations are respiration deficient (not illustrated), we find that one mutant, CS162, is resistant to EB and has a ρ^o lethality suppressor activity comparable with that of the wild-type M2915-6A, as judged by growth rate on EB

Table 2 Mitochondrial ATPase activity in *S. cerevisiae* strains

Strain[a]	Relevant genotype	Growth on GlyYP	Growth on EB[b]	Total ATPase activity[c]
M2915-6A	WT	+	+++	3.54 ± 0.06
CS118	*atp1*Δ::*kan atp2*::*kan*	–	–	0.20 ± 0.01
CS113	*atp1*Δ::*kan*	–	–	0.22 ± 0.01
CS114	*atp2*::*kan*	–	–	0.21 ± 0.01
CS115	*atp3*Δ::*kan*	–	+	0.30 ± 0.01
CS169	*atpδ*Δ::*kan*	–	++	2.07 ± 0.03
CS170	*atpε*::*kan*	–	++	2.71 ± 0.04
CS162	*β*A192V	–	++	0.34 ± 0.02
CS163	*β*E222K	–	–	0.20 ± 0.01
CS164	*β*R293K	–	–	0.20 ± 0.01
CS187	(*Kl*)αA333V (*Klatp1-2*)	–	++++	1.19 ± 0.03
CS229	*atp3*Δ::*kan atpδ*Δ::*URA3 atpε*::*LEU2*	–	++	0.32 ± 0.02

[a] All strains used are isogenic to M2915-6A
[b] ++++, very strong growth; +++, strong growth; ++, moderate growth; +, weak growth; –, no colony formation detected
[c] The assays were performed in triplicate. ATPase activities are expressed as μmol ATP hydrolysed/min/mg protein

medium (Fig. 3). Assay of ATPase activity revealed that CS162 expresses a detectable level of F_1-related ATP hydrolysis (0.34). The other two strains, CS163 and CS164, are sensitive to EB and do not form petite mutants (Fig. 3). These strains do not have a detectable F_1-related ATPase activity because the ATPase values are at the same level as in the control strain, CS118, disrupted in both *ATP1* and *ATP2* genes (Table 2).

Finally, we introduced the *K. lactis atp1-2* (*mgi2*-2) allele into the *S. cerevisiae ATP1*-disrupted strain, CS113. The *K. lactis* mutant allele contains an A333V mutation and confers a petite-positive phenotype on *K. lactis* (Chen and Clark-Walker 1995). Interestingly, the resulting strain CS187 has a ρ^o lethality suppressor activity significantly higher than the wild-type M2915-6A, as revealed by growth on EB medium (Fig. 3). However, CS187 has an ATPase value of only 1.19 compared with 3.54 in M2915-6A (Table 2). The combined evidence from the above experiments, as summarised in Table 2, strongly suggests that ATP hydrolysis activity of F_1 is needed for the suppression of ρ^o lethality, but under the experimental conditions that we used, no direct correlation between ρ^o lethality suppression and the ATPase level can be discerned.

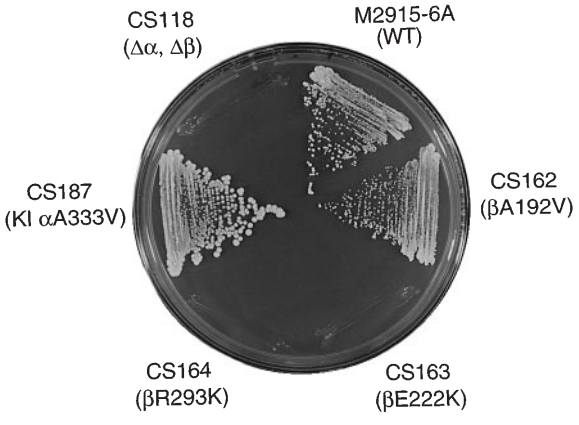

Fig. 3 F_1 mutations that abolish ATPase activity convert *S. cerevisiae* into a petite-negative yeast. Strains with the indicated genotypes were streaked on GYP plus EB and grown at 30° C for 4 days before being photographed

Suppression of ρ^o lethality in *K. lactis* by expression of *S. cerevisiae* F_1 subunits

The data described above, together with earlier observations showing the suppression of ρ^o lethality by specific mutations in F_1 subunits of *K. lactis*, strongly suggests that the difference between the responses of *S. cerevisiae* and *K. lactis* to loss of mtDNA may reside in the F_1 complex. To investigate this notion, we replaced the genes for the α, β and γ subunits of the *K. lactis* F_1 complex by their *S. cerevisiae* counterparts. If the *S. cerevisiae* F_1 subunits possess functional characteristics similar to those in *K. lactis* F_1 suppressor mutants, we would expect that expression of the *S. cerevisiae* genes should convert *K. lactis* into a petite-positive yeast.

The plasmid pCXJ4-ScABG, containing the *S. cerevisiae* genes *ATP1*, *ATP2* and *ATP3* in tandem, was targeted into the *leu2* locus of the *K. lactis* strain CK332/1, in which the three endogenous F_1 subunit genes, *KlATP1*, *KlATP2* and *KlATP3*, are disrupted. The resulting strain, CK334, was found to be petite-negative, as revealed by the sensitivity of cells to EB (not illustrated). However, we also found that CK334 is respiration deficient because cells do not grow on glycerol. This suggested that one or more *S. cerevisiae* F_1-subunits may not be compatible with other components for F_1 function or may not be recognised during the assembly of the complex. To investigate these possibilities, we replaced, individually, the α, β and γ-subunit genes of *K. lactis* with the *ATP1*, *2* and *3* genes of *S. cerevisiae*. We find that *ScATP1* and *ScATP3*, but not *ScATP2*, are able to complement the respiration-deficient phenotype of *K. lactis* strains disrupted in the corresponding genes, as CK282 and CK284 can grow on glycerol but CK283

cannot. Because both CK282 and CK284, in which *KlATP1* and *KlATP3*, respectively, are replaced by their *S. cerevisiae* counterparts, are sensitive to EB, we decided to replace both genes to see whether suppression of ρ^o lethality could be due to an interplay of *S. cerevisiae* F_1 subunits.

The *K. lactis* double mutant, CK333 (*Klatp1*Δ::*kan*, *Klatp3*::*URA3*), was transformed with the plasmid pCXJ4-ScAG which contains the *S. cerevisiae* genes encoding F_1 α and γ subunits (Fig. 4A). The resulting strain, CK340/5, containing pCXJ4-ScAG integrated at *leu2*, is respiration competent as these cells can grow on glycerol (not illustrated). Moreover, when exposed to EB in the margin-of-growth test as described in Fig. 4B, small colonies are produced at high frequency. As small colonies lack mtDNA (not illustrated) it can be concluded that expression of *S. cerevisiae* genes encoding α and γ subunits of F_1 can convert *K. lactis* into a petite-positive yeast and that the ability of baker's yeast to produce ρ^o is, in simple terms, due to a function that resides in these two subunits.

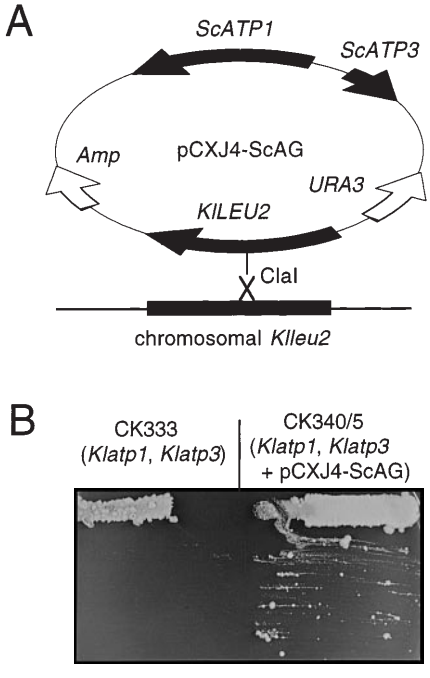

Fig. 4A, B Conversion of *K. lactis* into a petite-positive yeast by transformation with *S. cerevisiae ATP1* and *ATP3* genes. **A** Physical map of the *K. lactis* integrative plasmid pCXJ4-ScAG. The plasmid was linearised by digestion at the unique restriction site *Cla*I within the *KlLEU2* gene and targeted onto the *K. lactis* chromosome by transformation. Single-copy integration of the plasmid was confirmed by Southern analysis (not shown). **B** Production of petites from *K. lactis* CK340/5 (*Klatp1 Klatp3*) expressing *ScATP1* and *ScATP3* after treatment with EB. The *K. lactis* host strain CK333 and CK340/5 were grown to stationary phase in GYP and streaked across a 25-μl drop of EB (2.5 mg/ml). After incubation for 48 h at 28°C the surviving cells on the margin between growth and non-growth were substreaked, followed by incubation for 12 days before photography

Discussion

In this report we present evidence that the mitochondrial F_1-ATPase in *S. cerevisiae* has a second function, in addition to its primary role in energy transduction. The novel property of F_1 is required for survival of ρ^o and electron transport mutants. Furthermore, compelling data have been provided for a ρ^o lethal phenotype in cells disrupted in genes encoding F_1 α- and β-subunits. Supporting evidence for an active function of F_1 in ensuring the survival of ρ^o cells comes from our previous studies in *K. lactis*. In this petite-negative yeast, specific mutations in the α, β or γ subunits of F_1 confer on cells the ability to suppress ρ^o lethality (Chen and Clark-Walker 1995, 1996). We have previously suggested that the F_1-associated ρ^o-lethality suppressor activity is absent in wild-type *K. lactis* and that *S. cerevisiae* might be a natural mutant in F_1 that shares common properties with the *K. lactis atp* suppressor alleles. The present study provides strong support for this view, by showing that expression of *S. cerevisiae* F_1 α and γ subunits confers on *K. lactis* the ability to survive loss of mtDNA.

In *S. cerevisiae*, inactivation of genes encoding the three small subunits, γ, δ and ε, does not lead to a petite negative phenotype, indicating that these proteins are not essential for the ρ^o-lethality suppressor activity. The minimal functional unit for suppression is therefore either an α3β3 hexamer or an α/β dimer, although a partnership with non-F_1 proteins cannot be excluded. However, biochemical experiments have shown that F_1 is not properly assembled in the absence of γ or δ (Paul et al. 1994; Giraud and Velours 1997). The apparent lack of F_1 assembly observed in these studies is in contrast with the present genetic data which indicate that a core complex of F_1, composed of α and β subunits, has to be present for suppression of ρ^o lethality in the absence of γ, δ and ε. This notion is supported by the presence of an F_1-related ATPase activity in cells in which the three subunits were individually or simultaneously inactivated (Table 2). In this regard, it is noteworthy that ATPase activity of a complex composed only of α and β subunits has been observed in a bacterial F_1 that had been reconstituted in vitro (Gromet-Elhanan 1992; Kagawa et al. 1992). One can reconcile the above conflicting interpretations by assuming that some F_1 can be assembled in cells lacking γ or δ but that its detection is beyond the capacity of the currently employed biochemical methods which use detergents.

Because point mutations in the catalytic domain of the β subunit that abolish ATP hydrolysis activity cause loss of cell viability after elimination of mtDNA, ATPase activity appears to be essential for the ρ^o-lethality suppressor function. However, the novel F_1 function is independent of the membrane-bound F_o complex, as the three mtDNA-encoded F_o subunits, Atp6, 8 and 9, are absent in ρ^o cells. The observation that cells disrupted in the nuclear *ATP4* gene encoding the b subunit of F_o are viable after elimination of

mtDNA by EB (data not shown) is consistent with this interpretation. Further supporting evidence comes from studies showing that ρ^o lethality of *K. lactis* cells can be suppressed by specific F_1 mutations, in the absence of the six major F_o proteins, namely Atp4p, 5p, 6p, 7p, 8p and 9p (Chen et al. 1998). The combined evidence excludes any possibility that the ρ^o-lethality suppressor function of F_1 operates by activating proton back pumping or blocking proton flux through a residual F_o. In summary, it would appear that a core complex of F_1, composed of α and β subunits that retains some ATPase activity, is needed for suppression of ρ^o lethality and that this activity is independent of the role played by F_1 in energy transduction.

Several previous investigations have indicated that F_1-ATPase influences ρ^-/ρ^o production. In an early report, it was found that a strain defective in F_1-ATPase (the nature of the lesion was not identified) had a marked tendency to lose its mitochondrial genome (Ebner and Schatz 1973). The resulting double mutants display a slow growth phenotype and are unable to grow anaerobically. More recently, it has been shown that *S. cerevisiae* strains in which the genes encoding the γ and δ subunits of F_1-ATPase have been disrupted not only show a total conversion to ρ^-/ρ^o, but also grow poorly on glucose medium (Weber et al. 1995; Giraud and Velours 1997; Zhang et al. 1999). As far as the slow growth phenotype of $\rho^o\Delta\gamma$ and $\rho^o\Delta\delta$ cells is concerned, Zhang et al. (1999) suggested that it may be due to the inability of the ATP synthase inhibitor protein efficiently to control ATP hydrolysis by F_1 lacking the γ-subunit. As a result, the intracellular ATP level is decreased, resulting in slow growth. By contrast, Giraud and Velours (1997) explained the slow growth of cells lacking the δ-subunit by a defect in the assembly of F_1 that in turn is required for the maintenance of the mitochondrial membrane potential, $\Delta\psi$ (see below). In the light of the present study, the slow growth phenotype can be simply interpreted as being due to a low ρ^o-lethality suppressor activity of an F_1 complex lacking γ or δ. Because of the petite-negative nature of strains lacking F_1 function, ρ^-/ρ^o mutations are not expected to occur in strains disrupted in either α or β subunits or in the *ATP11* gene that is required for the assembly of the complex (Ackerman and Tzagoloff 1990; Ackerman et al. 1992), or in cells carrying the βE222K mutation (Zhang et al. 1999), regardless of the presence or absence of γ or δ.

What might be the nature of the ρ^o-lethality suppressor function of F_1? In attempting to answer this question it may be helpful to examine the functions of other genes required for ρ^o viability in *S. cerevisiae* and ask whether they share common properties with *ATP1* and *ATP2*. It has been found that mutations in the genes *AAC2*, *YME1* and *PEL1*/*PGS1* can convert *S. cerevisiae* into a petite-negative form. The products of the *AAC2* and *YME1* genes – an ADP/ATP translocase (Kovacova et al. 1968; Subik et al. 1972; Lawson and Douglas 1988; Lawson et al. 1990) and a putative ATP and zinc-dependent protease (Thorsness et al. 1993; Nakai et al. 1995) – are both localised to the mitochondrial inner membrane. The third gene, *PEL1*/*PGS1* (Janitor and Subik 1993), encodes the phosphatidylglycerolphosphate (PG-P) synthase that catalyses the synthesis of PG-P and cardiolipin (Chang et al. 1998), two anionic phospholipids in the mitochondrial inner membrane.

As mentioned above, the *AAC2* gene specifies the enzyme, ADP/ATP translocase, that is responsible for the reciprocal exchange of ADP for ATP across the mitochondrial inner membrane. Loss-of-function mutations in *AAC2*, such as *op1*, render cells unable to form petite mutants (Kovac et al. 1967; Kovacova et al. 1968). One explanation for the lack of petite mutants in an *op1* strain involves an inability to generate a voltage gradient, $\Delta\psi$, across the inner membrane, which is vital to cell survival (Neupert 1997). It is thought that petite mutants can generate $\Delta\psi$, in the absence of proton pumping by electron transport or reversal of F_1F_o-ATP synthase, by an electrogenic exchange of ADP from the matrix for external ATP (Kolarov and Klingenberg 1974; Klingenberg and Rottenberg 1977; Laris 1977; Klingenberg 1984; Dupont et al. 1985).

Imported ATP would be hydrolysed by F_1 in ρ^o cells to release ADP and inorganic phosphate. While the transport of phosphate across the inner membrane is basically electroneutral (reviewed by Wohlrab 1986), the electrogenic exchange of ADP for extra-mitochondrial ATP and the recycling of ATP to ADP in mitochondria by F_1-ATPase are thought to be the two important components of the $\Delta\psi$-generating pathway in ρ^o cells. Hence, mutations that inactivate *AAC2* would remove this alternative pathway used by petites to generate $\Delta\psi$. In addition, it has been suggested that slow growth of petite mutants in strains lacking the δ or ε subunits of F_1 reflects a low $\Delta\psi$ due to absence of an assembled F_1 (Giraud and Velours 1997). Viewed in this context, disruptions of *ATP1* and *ATP2* genes that abolish F_1-ATPase activity might be expected to cause petite mutants to be non-viable because ADP would not be produced for electrogenic exchange through the translocase. Also, petite-negative yeasts such as *K. lactis*, may not possess an F_1-mediated ATP hydrolysis activity in mitochondria following elimination of mtDNA.

Although we have found that some ATP hydrolysis activity of F_1 is needed for suppression of ρ^o lethality, the data do not fully support the idea that ATP hydrolysis is the primary function provided by F_1. First, strains CS169 and CS170, which lack the genes encoding the δ and ε subunits, respectively, have substantial F_1-related ATPase activity, reaching 56% and 75% of the level in the wild-type M2915-6A (Table 2). But these two strains display a significantly reduced ρ^o-lethality suppressor activity as judged from their growth rate on EB compared with that of their isogenic parent M2915-6A (Fig. 1B). By contrast, CS162, with the βA192V mutation, has an ATPase activity of only 0.34, while it has a growth rate on EB comparable to that of M2915-6A (Fig. 3). Finally, CS187, carrying the *K. lactis* α subunit with the A333V mutation, has a significantly higher ρ^o-

lethality suppressor activity (or growth rate on EB) than does M2915-6A (Fig. 3), but its F_1-related ATPase activity is only 30% of that of its parent (Table 2). These data clearly indicate that there is no direct correlation between the F_1-mediated ATP-hydrolysing activity and the ρ^o-lethality suppressor function. However, whether the ATP hydrolysis activity in vitro reflects ATP turnover rate in vivo has yet to be determined.

To account for the anomalies discussed above, an alternative hypothesis may be proposed: that ρ^o lethality is caused by loss of mitochondrial inner membrane integrity due to accumulation of unassembled nucleus-encoded proteins that lack mtDNA-specified partners. The F_1 complex could function as a monitor and be involved in the proper assembly of the inner membrane challenged by ρ^o conditions. For example, Weber et al. (1995) have shown that the ρ^o-lethal phenotype of *S. cerevisiae yme1* mutants can be suppressed by the Thr297Ala and Ile303Thr mutations in the γ subunit of F_1. Intriguingly, these two mutations are identical to those in the *Klatp3-1* and *3-2* alleles (formerly *mgi5-1* and *-2*) that suppress ρ^o lethality in *K. lactis* (Chen and Clark-Walker 1995; Clark-Walker et al., submitted). The *YME1* gene was initially identified by complementation of the yeast mtDNA escape phenotype (Thorsness and Fox 1993; Thorsness et al. 1993) and by complementation of a defect in the degradation of unassembled cytochrome *c* oxidase subunit 2 (Nakai et al. 1995). *YME1* encodes a putative ATP and zinc-dependent protease localised to the mitochondrial inner membrane (Schnall et al. 1994; Weber et al. 1996; Leonhard et al. 1996). The suppression of ρ^o lethality in *yme1* strains by the mutant F_1 seems not to be primarily due to ATP hydrolysis by F_1, as the wild-type complex, which is functionally active for ATP synthesis in a *yme1* background, is not capable of suppressing ρ^o lethality (Weber et al. 1995). The specific mutations in F_1 subunits might enhance the ρ^o-lethality suppressor function of F_1 that is required for viability of cells challenged by both inactivation of *YME1* and elimination of mtDNA. These observations strongly suggest that there could be a functional overlap between F_1 and Yme1p in the maintenance of mitochondrial inner membrane integrity. It is worthwhile to note that earlier reports have shown that the α subunit of F_1 shares sequence similarities with molecular chaperones (Luis et al. 1990; Alconada et al. 1994) and is required for normal function of the inner membrane in promoting efficient protein import (Yuan and Douglas 1992).

Another gene whose disruption leads to a ρ^o-lethal phenotype is *PEL1*/*PGS1* (Janitor and Subik 1993). Interestingly, the gene encodes the phosphatidylglycerolphosphate (PG-P) synthase that catalyses the formation of PG-P and cardiolipin, two anionic phospholipids on the mitochondrial inner membrane (Chang et al. 1998). The synergistic lethality created by mutation in *PEL1*/*PGS1* and loss of mtDNA again suggests a requirement for inner membrane integrity in cells challenged by loss of mtDNA. It will be interesting to determine whether specific F_1 mutations (*mgi*) can suppress ρ^o lethality in *pel1*/*pgs1* mutants.

In summary, this work describes an absolute requirement for an F_1-ATPase associated function in the establishment of the ρ^-/ρ^o state in *S. cerevisiae*, as disruption of the genes for the α and β subunits of F_1 gives rise to a lethal phenotype in cells lacking mtDNA or strains impaired in electron transport. Although we do not know the nature of the novel function of F_1, it would be interesting to test whether similar activities occur in other eukaryotes. Of particular concern is the molecular mechanism underlying the isolation of mammalian ρ^o cell lines that have been recovered after treatment with EB (Desjardins et al. 1985; King and Attardi 1989), since a role of F_1-ATPase for growth of human ρ^o cells has been proposed (Buchet and Godinot 1998).

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