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Subcellular relocalization of a long-chain fatty acid CoA ligase by a suppressor mutation alleviates a respiration deficiency in *Saccharomyces cerevisiae*

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We have isolated an extragenic suppressor, *FAM1-1*, which is able to restore respiratory growth to a deletion of the *CEM1* gene (mitochondrial β-keto-acyl synthase). The sequence of the suppressor strongly suggests that it encodes a long-chain fatty acid CoA ligase (fatty-acyl-CoA synthetase). We have also cloned and sequenced the wild-type *FAM1* gene, which is devoid of suppressor activity. The comparison of the two sequences shows that the suppressor mutation is an A→T transversion, which creates a new initiation codon and adds 18 amino acids to the N-terminus of the protein. This extension has all the characteristics of a mitochondrial targeting sequence, whilst the N-terminus of the wild-type protein has none of these characteristics. *In vitro* mitochondrial import experiments show that the N-terminal half of the suppressor protein, but not of the wild-type, is transported into mitochondria. Thus, we hypothesize that the suppressor acts by changing the subcellular localization of the protein and relocating at least some of the enzyme from the cytosol to the mitochondria. These results support the hypothesis that some form of fatty acid synthesis, specific for the mitochondria, is essential for the function of the organelle.

Key words: β-keto-acyl synthase/long-chain fatty acid CoA ligase/mitochondria/*S.cerevisiae*/suppressor

Introduction

The biogenesis of yeast mitochondria is complex, requiring components synthesized in both the cytoplasmic and mitochondrial compartments. A large number of nuclear *pet* mutations (nuclear *petite*), which disrupt this process and lead to a respiratory-negative phenotype, have been isolated and studied. The majority affect the expression of the mitochondrial genome and the subunits of the respiratory complexes [see Grivell (1989) and Tzagoloff and Dieckmann (1990) for reviews]. With the exception of heme, the possible existence and function of non-protein components necessary for respiration have been largely ignored. Recently, we have identified a nuclear gene, *CEM1*, which is essential for respiration and encodes a novel β-keto-acyl synthase (Harington *et al.*, 1993). Although the importance of lipids in mitochondrial biogenesis has been well established (Daum, 1985), it is generally believed that their synthesis occurs in the cytosol and is followed by transport into the organelle. Therefore, the finding of a specific link between fatty acid biosynthesis and respiration was unexpected.

Fatty acid synthetases can be of two types: type I is found in eukaryotes and all the enzymatic activities are carried on a single polypeptide chain; type II is found in prokaryotes and each enzymatic activity is carried by a separate polypeptide. In *Saccharomyces cerevisiae*, fatty acid synthesis is of type I; the fatty acid synthetase is a multifunctional enzyme formed of two subunits (encoded by the genes *FAS1* and *FAS2*), with an $(\alpha\beta)_6$ structure (Schüller *et al.*, 1992). The β-keto-acyl synthase encoded by the *CEM1* gene more closely resembles the prokaryotic type fatty acid synthetase, which is consistent with the mitochondrial function of the protein and the general idea of the endosymbiotic origin of the organelle. In fatty acid biosynthesis, the β-keto-acyl synthases carry out the condensation reaction which lengthens the carbon backbone of the fatty acid. For this they function with another protein, the acyl carrier protein (ACP), which is essential for all the reactions in fatty acid biosynthesis as it carries the growing fatty acid molecule.

Acyl carrier proteins of the prokaryotic type (type II) have been identified in the mitochondria of *Neurospora crassa* (Brody and Mikolajczyk, 1988), as well as the mitochondria of other eukaryotic micro-organisms (including *S.cerevisiae*) and plants (Chuman and Brody, 1989). These results suggested that some fatty acid biosynthesis may take place in the mitochondria. Experimental evidence for *de novo* mitochondrial synthesis of fatty acids was obtained in *N.crassa*, using the incorporation of [2-^{14}C]malonate (Mikolajczyk and Brody, 1990); this was subsequently confirmed by the isolation of fatty acids (C_6–C_{18}) covalently linked to the mitochondrial ACP (Zensen *et al.*, 1992). However, no specific function for the mitochondrially synthesized fatty acids was known, and the situation was further complicated when the *N.crassa* and beef heart mitochondrial ACPs were shown to be subunits of the NADH:ubiquinone oxidoreductase (complex I) (Runswick *et al.*, 1991; Sackmann *et al.*, 1991). In *S.cerevisiae*, a mitochondrial ACP has been detected immunologically (Chuman and Brody, 1989), but its localization within the organelle is unknown. However, it is unwise to assume that it will be associated with the NADH dehydrogenase complex, as the *S.cerevisiae* enzyme is very different and of a much simpler structure than the *N.crassa* or beef heart enzymes (Singer and Ramsay, 1992).

So far no genetic data are available from studies on the various ACPs to suggest a function for the mitochondrial cycle of fatty acid biosynthesis. The results obtained with

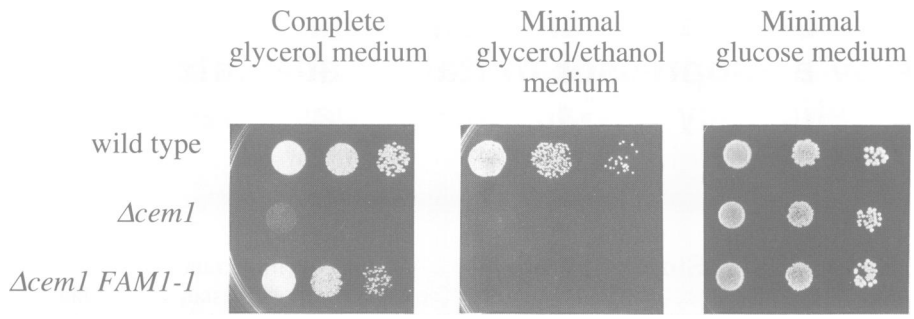

Fig. 1. Growth of the wild-type (CW04: *CEM1 FAM1*), *CEM1*-deleted (AHC001: Δ*cem1 FAM1*) and suppressor (AHC003: Δ*cem1 FAM1-1*) strains on complete (yeast extract and peptone) glycerol medium, minimal glycerol/ethanol and minimal glucose medium after 4 days incubation at 28°C. The strains were pre-cultured in complete glucose medium and serial dilutions were made in Ringer before plating.

the β-keto-acyl synthase encoded by the *CEM1* gene show that it is essential for respiration; when the gene is inactivated, the cells have no spectrally detectable cytochromes b, oxidase and no oxygen uptake. However, none of the principal mitochondrial functions, such as DNA replication, transcription and protein synthesis, are drastically affected. Also, an analysis of mitochondrial lipids and fatty acids indicated that Cem1p was not involved in the synthesis of the bulk mitochondrial fatty acids (Harington *et al.*, 1993).

To further our understanding of the role of Cem1p in mitochondrial biogenesis, we have decided to use the powerful and versatile genetics of *S.cerevisiae*. We report here the isolation of a suppressor of the *CEM1* deletion. The suppressor protein shows strong homology to long-chain fatty acid CoA ligases and casts new light on the possible function of mitochondrial fatty acid biosynthesis.

Results

Isolation of the suppressor

In an initial screen for multicopy suppressors, the respiratory deficient Δ*cem1* strain AHC001 was transformed with a bank of wild-type DNA cloned in the vector pFL44L and ~90 000 transformants were obtained. After replica plating onto complete respiratory glycerol medium 10 transformants grew well, these all contained the wild-type *CEM1* gene. After a prolonged incubation, a further five slow-growing transformants were obtained; restriction and Southern blot analyses of the plasmids harbored by these transformants showed that the inserts shared no common sequence (data not shown). Given the poor restoration of growth, the analysis of these plasmids was not pursued.

In order to obtain extragenic suppressors of the *CEM1* deletion, 10 independent subclones of AHC001 (Δ*cem1*) were grown to stationary phase in glucose medium. Approximately 10^9 cells from each culture were washed and spread on complete glycerol medium; after 14 days two independent revertant clones appeared. One of these (AHC003) grew comparatively well and was selected for further study (Figure 1). After confirmation of the presence of the deletion of the *CEM1* gene by Southern blot, the strain was subjected to a genetic analysis as described by Dujardin *et al.* (1980) and the suppressor was shown to be nuclear dominant. To confirm that a single gene was responsible for the suppression, tetrad analysis was carried out of diploids from the cross AHC003 (Δ*cem1*, SUP^{active})×CM1-17B (*cem1-1*, $SUP^{inactive}$). A 2:2 segregation of the glycerol-negative to glycerol-positive phenotype was observed, showing that the suppressor was monogenic.

Cloning of the suppressor gene

In order to clone the suppressor gene, strain AHC001 (Δ*cem1*) was transformed with a genomic bank of AHC003 DNA (Δ*cem1*, SUP^{active}) cloned in pFL44L (see Materials and methods). Approximately 200 000 transformants were replicated to complete glycerol medium and ~500 respiratory-competent clones were recovered; 10 of these were analysed further. A co-segregation analysis showed that the presence of the plasmid correlated with the capacity of the Δ*cem1* strain to grow on glycerol. The 10 plasmids were recovered and shown to contain two common *Bam*HI fragments. One plasmid, YEpAH033, was retransformed into AHC001 (Δ*cem1*) and shown to restore respiratory growth.

Using the marker insertion strategy of Herbert *et al.* (1992), the sequence of part of the suppressor ORF was obtained. This was compared to the available data banks and showed that the suppressor corresponded to a new gene. A 3 kb *Asp*718−*Sal*I fragment containing the insert flanked by some vector sequences was subcloned into the centromeric vector pFL38 and used to transform the strain AHC001 (Δ*cem1*). As expected, the transformed strain was able to grow on complete glycerol medium, indicating that the cloned suppressor is active when present in few copies or as a single copy. Finally, this suppressor was used to probe a blot of the five weak multicopy suppressors (see above). No hybridization was detected apart from the vector sequences, thus the active suppressor is not a mutated allele of one of the weak multicopy suppressors isolated previously.

Sequence of the suppressor gene

The 3 kb insert was sequenced as described in Materials and methods, and showed the presence of a single long open reading frame (ORF) capable of encoding a protein of 762 amino acids. The codon bias calculated according to Bennetzen and Hall (1982) is 0.131, which is low but significant. The suppressor compensates for the absence of the Cem1p and Getz *et al.* (personal communication) have shown that this protein is present in the mitochondria. Therefore, it might be expected that the suppressor protein would also be located in the mitochondria. The N-terminus

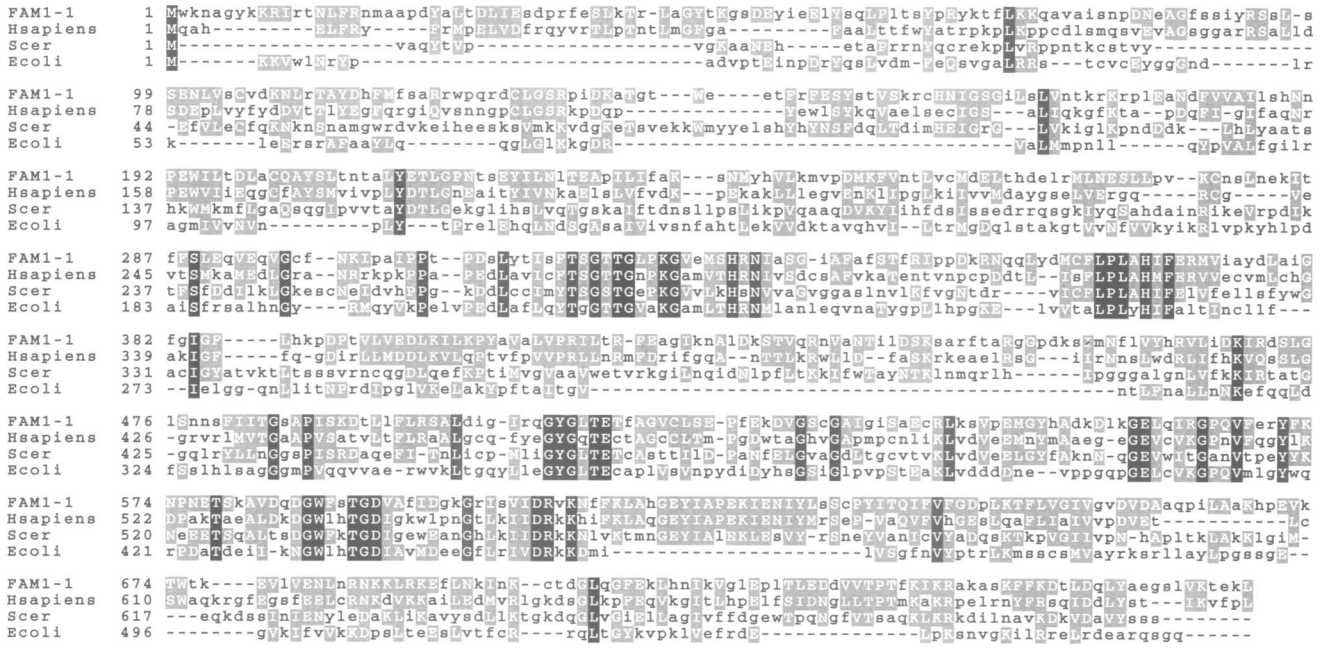

Fig. 2. A comparison of the Fam1-1p sequence and three other long-chain fatty acid CoA ligases: Hsapiens (human SWISSPROT:P33121), Scer (yeast Faa1p SWISSPROT:P30624) and Ecoli (*E.coli* SWISSPROT:P29212). The alignment was made using the CLUSTAL program of Higgins and Sharp (1988) with gap creation and prolongation penalties of 10. Residues identical to or similar to the Fam1-1p sequence are on a gray background, residues identical in all sequences are on a black background.

of the suppressor protein sequence, MwKnagyK-KRiRTnlfRn (positively charged and hydroxylated residues in capital letters), is typical of a mitochondrial import sequence (Pon and Schatz, 1991).

The deduced amino acid sequence was compared to the available data banks using the programs BLAST (Altschul *et al.*, 1990) and FASTA (Lipman and Pearson, 1985). The results showed a very strong homology to long-chain fatty acid CoA ligases and more limited similarity to coumarate-CoA ligases, luciferin mono-oxygenases and the gramicidin-S synthetase; in these cases the similarity is restricted to an AMP binding site. In view of the homology with long-chain fatty acid CoA ligases, we have called the suppressor gene *FAM1-1* for '*F*atty acid *A*ctivation with *M*itochondrial function'. The Fam1-1p and other long-chain fatty acid CoA ligases align easily, with few gaps, except for the N-terminal sections which are the least conserved. Figure 2 shows an alignment of several long-chain fatty acid CoA ligases.

Phenotypic analysis of the suppressor allele FAM1-1

The respiratory growth phenotype of the strain AHC003 (Δ*cem1 FAM1-1*) is presented in Figure 1 and shows that the suppressor restores a significant, but not wild-type level of growth to a *CEM1*-deleted strain on complete (yeast extract with peptone) respiratory medium. In order to further understand the physiology of the suppressor, we have examined several parameters related to respiration and mitochondrial function. First, we determined the level of oxygen uptake in wild-type, *CEM1*-deleted and suppressor (Δ*cem1*, *FAM1-1*) cells. The results in Table I show that the suppressor strain has a significantly depressed rate of endogenous respiration (seven times less than the control, approximately equivalent to the *CEM1*-deleted strain). In the presence of ethanol, oxygen consumption increases significantly in the suppressor cells compared to the *CEM1*-deleted cells, but is still only ~50% of the wild-type level. Oxygen consumption by the suppressor cells in the presence of lactate is ~20% of the wild-type level. The differences in oxygen consumption between the wild-type and suppressor cells presented in Table I are consistent with the differences seen in respiratory growth, shown in Figure 1.

Table I. Oxygen consumption in wild-type (CW04: *CEM1 FAM1*), *CEM1*-deleted (AHC001: Δ*cem1 FAM1*) and suppressor (AHC003: Δ*cem1 FAM1-1*) cells

Substrate	CW04 (wild type)	AHC001 (Δ*cem1*)	AHC003 (Δ*cem1 FAM1-1*)
Endogenous	14.0	1.4	1.9
Ethanol	30.6	4.5	12.6
Lactate	26.0	2.9	5.7

The oxygen consumption is expressed in nmol of oxygen consumed/min/5×10^7 cells; ethanol was added at 2% and lactate at 50 mM.

We have previously shown that mitochondrially synthesized cytochromes are not detectable spectrally in the *CEM1*-deleted strain (Harington *et al.*, 1993). To determine the effect of the *FAM1-1* suppressor allele on mitochondrial cytochromes, we recorded low-temperature whole-cell cytochrome spectra. The results, presented in Figure 3, show a complete restoration of the cytochromes b and oxidase. As it was not known at which level mitochondrial cytochrome biogenesis was affected in the *CEM1*-deleted strain, we examined mitochondrial protein synthesis in wild-type, *CEM1*-deleted and suppressor (Δ*cem1*, *FAM1-1*) cells, using $^{35}SO_4^{2-}$ labeling after blocking cytoplasmic protein synthesis with cycloheximide. All the

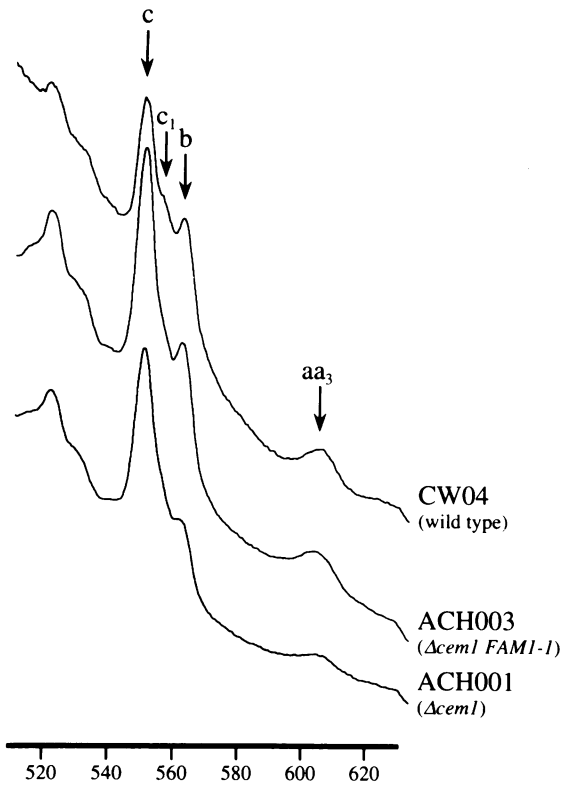

Fig. 3. Low-temperature cytochrome spectra of the wild-type (CW04: *CEM1 FAM1*), *CEM1*-deleted (AHC001: Δ*cem1 FAM1*) and suppressor (AHC003: Δ*cem1 FAM1-1*) strains. The cytochrome absorption maxima are marked.

major mitochondrial proteins were synthesized normally in all three strains (data not shown). Thus, in the *CEM1*-deleted strain cytochromes b and oxidase are spectrally absent even though the mitochondrial subunits are synthesized normally.

The sequence homologies shown in Figure 2 strongly suggest that the Fam1p is a long-chain fatty acid CoA ligase. The Cem1p is thought to be a β-keto-acyl synthase, thus the product of the biosynthetic cycle in which it participates is likely to be the CoA derivative of a fatty acid or similar molecule. Taken together this may suggest that the suppressor acts by forming the CoA derivative of a molecule identical to or similar to the product of the Cem1p biosynthetic cycle. As the suppressor was isolated on complete glycerol medium, it is possible that this molecule is present in the medium. To test this hypothesis the wild-type, *CEM1*-deleted and suppressor strains (Δ*cem1*, *FAM1-1*) were grown in complete glucose medium, washed and plated on complete glycerol, minimal glycerol/ethanol and minimal glucose medium. After 4 days incubation at 28°C, the suppressor strain grows well on complete glycerol medium, but shows no detectable growth on minimal glycerol/ethanol medium (Figure 1). In an attempt to determine whether the difference in growth is due to the absence of a fatty acid, we supplemented minimal glycerol/ethanol medium with mixtures of fatty acids (Tween 20 and Tween 80), specific saturated and unsaturated fatty acids (see Materials and methods), lipoic acid and lipoamide. None of these supplements were able to restore the growth of the suppressor strain (Δ*cem1*, *FAM1-1*) on minimal glycerol/ethanol medium (data not shown).

Inactivation of the chromosomal FAM1 and FAM1-1 genes

As the *FAM1-1* suppressor is an allele of a new gene, no information is available concerning the role of the wild-type allele in the cell. To determine whether the wild-type *FAM1* gene is necessary for respiratory growth, we decided to create a null allele (Rothstein, 1991). Briefly, a 2432 bp *Bsa*AI and *Xho*I fragment which contains most of the *FAM1-1* ORF and 273 nucleotides 5′ to the initiation codon was replaced by the *URA3* gene; in this construction, only the last 148 bp of the *FAM1-1* ORF remain. The resulting plasmid, pAH039, was digested to liberate the insert and used to transform the strains CW04 (wild-type), AHC001 (Δ*cem1*) and AHC003 (Δ*cem1*, *FAM1-1*). Uracil prototrophs were selected and their genomic DNA analysed by Southern blot. In all cases we were able to recover haploid clones with an inactivated allele (Δ*::URA3*) of the *FAM1* gene. This demonstrates that the *FAM1* gene is not essential for cellular viability.

The ability of the inactivated strains to grow on complete glycerol medium was determined; the results (Figure 4) show that, as expected, the deletion of the suppressor gene in a Δ*cem1* background resulted in a glycerol-negative phenotype (AHC004 Δ*cem1*, Δ*fam1-1::URA3*). This strain, AHC004, was crossed to the *rho*° tester strain KL14-4A/60, the resulting diploids were able to grow on glycerol; therefore, AHC004 had not lost its mitochondrial DNA. Interestingly, the deletion of the *FAM1* gene in a wild-type background did not affect growth on glycerol (AHC005 Δ*fam1::URA3*), this demonstrates that the wild-type *FAM1* gene is not essential for respiratory growth.

Isolation of the wild-type FAM1 gene and identification of the nature of the suppressor mutation

In order to determine the precise nature of the change which confers the suppressor activity on Fam1-1p, we decided to clone and sequence the wild-type *FAM1* gene. For this a bank of CW04 (the wild-type strain from which the Δ*cem1* and *FAM1-1* strains were isolated) was constructed in the vector λEMBL3. A clone containing the *FAM1* gene was identified by hybridization with a *FAM1-1* probe. From this λ clone, a 2.7 kb *Eco*RI–*Hin*dIII fragment which contained all the *FAM1* ORF except the termination codon was subcloned and sequenced (see Materials and methods). The sequences determined in this study are available in the EMBL bank under accession number X82364.

A comparison of this sequence with the *FAM1-1* sequence revealed the presence of a single A→T transversion. Interestingly, this transversion is in the ATG initiation codon of the *FAM1-1* gene. In fact, the effect of the mutation is to change an AAG triplet, located upstream and in phase with the initiation codon of the wild-type gene, into an ATG codon. Thus, the suppressor mutation creates a new initiation codon which results in an 18 amino acid extension to the N-terminus of the wild-type Fam1p (Figure 5).

In order to confirm that the ability of Fam1-1p to suppress the Δ*cem1* mutation was due to the creation of

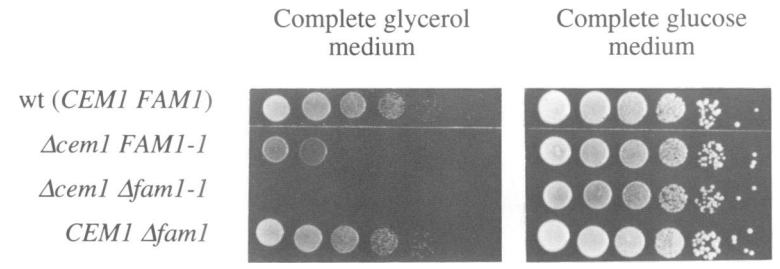

Fig. 4. Effect of deletion of the wild-type (*FAM1*) and suppressor (*FAM1-1*) genes. The wild-type (CW04: *CEM1 FAM1*), suppressor (AHC003: *Δcem1 FAM1-1*), *FAM1-1*-deleted (AHC004: *Δcem1 Δfam1-1*) and *FAM1*-deleted (AHC005: *CEM1 Δfam1*) strains were grown on glycerol and glucose complete medium for 3 days at 28°C. The strains were pre-cultured in complete glucose medium and serial dilutions were made in Ringer before plating.

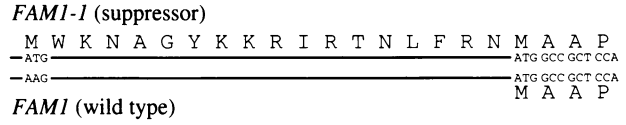

Fig. 5. Schematic representation of the N-termini of Fam1-1p and Fam1p and the corresponding nucleotide sequences. The A→T transversion which creates the new initiation codon in the suppressor *FAM1-1* allele is shown.

a new initiation codon, we decided to clone this region of the wild-type gene *in vivo* by gap repair. In this process, part of a sequence is removed from a replicative plasmid, the linear DNA is then transformed in yeast and the DNA repair/recombination machinery 'fills in' the gap using the chromosomal sequence (Rothstein, 1991). For this we used two plasmids with a *URA3* marker which carry the *FAM1-1* gene (YCpAH036 and YCpAH037; centromeric and multicopy, respectively). These plasmids were digested with *Sfi*I and *Rsr*II to remove a 728 bp fragment which carries the mutation. The gapped plasmids were gel purified and 2 μg of each used to transform AHC001 (*Δcem1*, *FAM1*); ~4000 uracil-positive transformants were obtained. These transformants were replicated onto glycerol medium to eliminate any background due to partially digested plasmids, and onto 5-fluoro-orotic acid medium to identify transformants that harbored a replicative plasmid and were therefore able to lose the *URA3* marker (Boeke *et al.*, 1984). Approximately 95% of the transformants passed these controls, six from each transformation were selected and the plasmids were isolated after a transformation of *Escherichia coli* by total yeast DNA. A preliminary analysis showed that in each case the plasmid carried the wild-type *FAM1* sequence, one plasmid from each group was selected and the region between the two restriction sites was sequenced (YCpAH052 centromeric and YEpAH053 multicopy). This confirmed that no secondary mutations had been introduced during the gap repair. These plasmids were also digested by a series of restriction enzymes to confirm that the overall structure of the cloned fragment had not been rearranged. Finally, plasmids YCpAH052 and YEpAH053 were retransformed into AHC001 (*Δcem1*), as expected neither plasmid was able to overcome the effect of the *CEM1* deletion and permit growth on complete glycerol medium.

In vitro mitochondrial import of the Fam1-1p

The results described above strongly suggest that the effect of the suppressor mutation is to create a mitochondrial address signal and relocate the Fam1p long-chain fatty acid CoA ligase activity within the mitochondria. To test this hypothesis, truncated proteins corresponding to the N-terminal sections of Fam1p (357 amino acids, 40.4 kDa) and Fam1-1p (375 amino acids, 42.7 kDa) were synthesized by *in vitro* transcription and translation, and used in an *in vitro* mitochondrial import assay. The results shown in Figure 6 clearly show that the wild-type Fam1p is not accumulated in the mitochondria. However, the suppressor Fam1-1p is accumulated in mitochondria and, furthermore, this accumulation is dependent upon the presence of a mitochondrial membrane potential as it is abolished by the addition of valinomycin. Therefore, we conclude that the effect of the suppressor mutation is to change the subcellular location of a long-chain fatty acid CoA ligase.

Discussion

Until recently, it has been assumed that the mitochondrion is not involved in the *de novo* synthesis of fatty acids. However, the discovery of a mitochondrial acyl carrier protein in several systems (Brody and Mikolajczyk, 1989; Chuman and Brody, 1989; Runswick *et al.*, 1991) suggested that some fatty acid synthesis may take place in the organelle, a hypothesis which was subsequently confirmed in *N.crassa* (Mikolajczyk and Brody, 1990; Zensen *et al.*, 1992).

We have previously identified a β-keto-acyl synthase which is essential for respiration in *S.cerevisiae* (Harington *et al.*, 1993). Although the archetype of this family of enzymes is involved in fatty acid biosynthesis, β-keto-acyl synthases are involved in a variety of processes, including the biosynthesis of polyketide antibiotics and pigments in *Streptomyces* (Davis and Chater, 1990; Hopwood and Sherman, 1990), and the synthesis of a highly unsaturated fatty acid which forms part of a lipo-oligosaccharide involved in host recognition in *Rhizobium* (Spaink *et al.*, 1991). The identity and function of the molecule synthesized by Cem1p remain unknown. Thus, in order to extend our understanding of this system, we have isolated a suppressor, *FAM1-1*, which is able to restore respiratory growth to a *CEM1*-deleted strain.

Fam1-1p is able to compensate for the absence of Cem1p and restore respiratory growth (Figures 1 and 3). The alignment in Figure 2 strongly suggests that Fam1-1p is a long-chain fatty acid CoA ligase. These enzymes have previously been identified in animals, fungi and

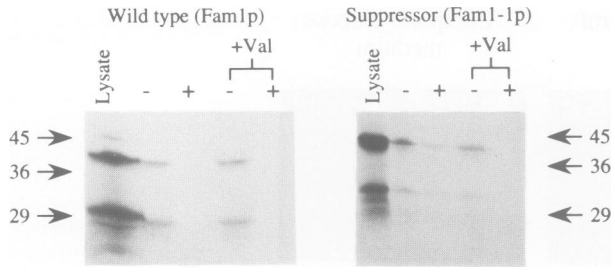

Fig. 6. *In vitro* mitochondrial import of truncated forms of the Fam1p (40.4 kDa) and Fam1-1p (42.7 kDa). The labeled proteins were produced as described in Materials and methods. After import, half of the sample was treated with proteinase K before electrophoresis (+), whereas the other half remained untreated (−). Val indicates that the import assay was carried out in the presence of valinomycin (2 μm). The positions of the molecular weight markers (kDa) are indicated by arrows.

bacteria, and there is often more than one type in the cell. The related forms of the same enzyme are encoded by different genes, and have different functions and/or intracellular locations. In *S.cerevisiae*, at least two long-chain fatty acid CoA ligases have been shown to exist (Kamiryo *et al.*, 1976). A gene (*FAA1*) encoding a long-chain fatty acid CoA ligase has been identified which is essential for protein myristoylation and the synthesis of certain phospholipids in the absence of *de novo* fatty acid synthesis (Duronio *et al.*, 1992). An analysis of the *FAA1* disruptant phenotype led these authors to predict the existence of a second long-chain fatty acid CoA ligase, but the precise function of this enzyme was not established. Interestingly, in pairwise comparisons the Fam1p more closely resembles the human and rat enzyme than the yeast Faa1p. When the *Z* score, or statistical significance, was calculated after 100 randomizations using the wild-type Fam1p as reference, the values were: human $Z = 109$, rat $Z = 100$, yeast $Z = 53$ and *E.coli* $Z = 14$ [Pearson and Lipman, 1988; see Slonimski and Brouillet (1993) for a discussion of the significance of the *Z* score]. Unfortunately, not enough is known of the biochemistry of these enzymes to allow the deduction of functional conclusions from these observations.

A comparison of the wild-type *FAM1* and suppressor *FAM1-1* allele sequences showed that the suppressor mutation resulted in the creation of a new initiation codon and the addition of 18 amino acids to the N-terminus of the protein (Figure 5). *In vitro* mitochondrial import experiments with truncated forms of Fam1p and Fam1-1p have shown that this N-terminal extension on the suppressor protein acts as a mitochondrial address signal (Figure 6). Thus, at least some of the Fam1-1p long-chain fatty acid CoA ligase activity will be relocated in the mitochondria.

Our results suggest that both Cem1p and Fam1p are involved in the synthesis of a fatty acid, or fatty acid-like molecule. However, the function of this, or these, molecules in mitochondrial metabolism remains unclear. They are essential for respiratory growth, but are not needed for the major mitochondrial functions such as DNA replication, transcription, pre-mRNA splicing and translation, as mitochondrial protein synthesis is normal in the *CEM1*-deleted strain. Also, this strain does not produce an elevated level of *petites* (data not shown). *A priori* two types of hypothesis may be formulated: the first is that Cem1p is involved in the synthesis of a specific molecule or cofactor, which is essential for respiration; the second is that it is has a more general role in mitochondrial metabolism.

If we consider the first hypothesis, the most obvious candidate for a 'fatty acid-like' molecule essential for respiration is lipoic acid; this is a cofactor of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes. Lipoic acid-deficient strains have a *pet* phenotype. To date, four complementation groups have been isolated, all of which have spectrally detectable cytochromes (Tzagoloff and Dieckmann, 1990). Therefore, the phenotype of the *CEM1* deletion is different from that of the known lipoic acid-deficient mutants. Also, we have found that the addition of lipoic acid or lipoamide to the medium cannot restore respiratory growth to a *CEM1*-deleted strain (data not shown). Thus we conclude that Cem1p is not involved in the synthesis of lipoic acid.

Considering the second hypothesis, the most striking observation concerning the *CEM1* deletion strain is that mitochondrial apo-cytochromes are synthesized, but holo-cytochromes are spectrally absent. This may suggest that the defect is either in the addition of heme, or at the level of the insertion of the proteins into the membrane. It is becoming increasingly clear that the covalent addition of fatty acid-like molecules to proteins can be essential for their functional activity. The majority of these modifications fall into three groups (see Towler *et al.*, 1988): palmitoylation, myristoylation and the addition of a phosphatidyl inositol glycan moiety. As with protein prenylation (see Paltauf *et al.*, 1992), these modifications permit the anchorage of the proteins in the membrane. If these additions cannot be made, or are inefficiently made, the result can be the abolition or impairment of protein function, as is the case for non-palmitoylated yeast RAS proteins (James and Olson, 1990). Thus, it is plausible that Cem1p is involved in the synthesis of a fatty acid (perhaps a specialized fatty acid), which is attached to at least some proteins of the respiratory complexes and assures their correct positioning in the membrane. Obviously, this hypothesis can be extended to include the correct association of different proteins within a complex.

By analogy with cytoplasmic fatty acid biosynthesis, the product of the biosynthetic cycle in which Cem1p participates would be a CoA derivative. Thus, the Fam1-1p could circumvent the absence of the Cem1p by forming the CoA derivative of an exogenous molecule. The observation that the suppressor is only active on complete respiratory medium (i.e. containing yeast extract), but not on synthetic respiratory medium, also suggests that an exogenous compound is needed for the suppressor activity.

Attempts to restore the growth of the suppressor strain on chemically defined respiratory medium by the addition of simple fatty acid mixtures, or purified fatty acids, were unsuccessful. In *N.crassa*, Zensen *et al.* (1992) have detected C_6-C_{18} saturated fatty acids and oleic acid associated with the mitochondrial acyl carrier protein. If the *S.cerevisiae* system is similar, we would have expected fatty acid supplementation to restore growth on synthetic respiratory medium. However, it should be remembered that cytoplasmic fatty acid synthesis is not affected in our strains, thus it is possible that the transport systems

necessary for the uptake of individual fatty acids by cells on synthetic medium would not be induced.

A priori suppressor mutations may act in many ways, those functioning in biosynthetic pathways normally result in the replacement of the missing activity, or by providing a bypass or alternative pathway. To our knowledge, the *FAM1-1* suppressor allele is the first example of a suppressor where the alternative pathway is provided by changing the subcellular localization of an enzyme by creating a new initiation codon.

Materials and methods

Strains, media and genetic methods

The yeast strains used in this study were: CWO4 (*MATα leu2-3,112 his3-11,15 trp1-1 can1-100 ade2-1 ura3-1 rho*$^+$ 777-3A), AHC001 (*MATα leu2-3,112 his3-11,15 trp1-1 can1-100 ade2-1 ura3-1 cem1-Δ1::LEU2 rho*$^+$ 777-3A), CM1-17B (*MATa leu2-3,112 ade2-1 trp1 his can1-100 cem1-1 rho*$^+$ 777-3A) and KL14-4A/60 (*MATa his1 trp2 rho°*). Other yeast strains were derived from these as described in the text. The *E.coli* strains used were XL1 blue (*recA1 endA1 gyrA96 thi-1 supE44 hsdR17 relA1 lac F'* [*proA*$^+$*B*$^+$ *lacI*^{Q} *lacZ*ΔM15 Tn*10 (tet* ^{r})]) and JA221 (*recA hsdR M*$^+$ *leuB6 trpE5 lacY*). The media used for *E.coli* were as described in Maniatis *et al.* (1982). With the exception of minimal glycerol ethanol medium, the media and genetic methods used for yeast were as described in Dujardin *et al.* (1980). Minimal glycerol ethanol medium contained 0.67% yeast nitrogen base (Difco), 1% glycerol, 2% ethanol, 2.5% agar and the appropriate supplements. Fatty acid supplements were dissolved in ethanol:tergitol NP40 1:1, 100 μl of this were added to 10 ml of medium to give final fatty acid concentrations of 50 or 10 μg/ml. The supplements used were Tween 20 (a mixture of lauric, myristic, palmitic and stearic acids), Tween 80 (70% oleic acid, the rest being linoleic, palmitic and stearic acids), elaidic, linoleic, linolenic, oleic, palmitic, palmitoleic, petroselenic, lauric, myristic, stearic, octanoic and lipoic acids, and lipoamide.

DNA manipulations

Enzymes were used in accordance with the manufacturer's instructions. Southern blot, plasmid preparation and other routine DNA manipulations were performed as described in Maniatis *et al.* (1982). *Escherichia coli* were transformed by electroporation using the method of Dower *et al.* (1988) and yeast were transformed using the method of Chen *et al.* (1992). The bank of AHC003 genomic DNA was made by partially digesting cesium chloride-purified genomic DNA by *Sau*3A to yield a maximum of fragments in the 3–6 kb range. The purified fragments were ligated to *Bam*HI-digested, CIP dephosphorylated pFL44L and transformed into *E.coli* by electroporation. After propagation on plates, plasmid DNA was extracted by alkaline lysis.

DNA sequencing

The sequence of one strand of the *FAM1-1* gene was determined by making nested Exonuclease III deletions. These were then sequenced using fluorescently labeled universal primer and the Pharmacia ALF DNA sequencer. The sequence of the other strand was determined using synthetic oligonucleotides and fluorescently labeled dATP. The sequence of the wild-type *FAM1* gene was determined using the same set of oligonucleotides; the mutation was confirmed by synthesizing another oligonucleotide and sequencing the second strand.

Cytochrome spectra, respiration and mitochondrial protein synthesis

Low-temperature cytochrome absorption spectra were performed as described by Claisse and Pajot (1974). Oxygen consumption by whole cells was measured as described by Harington *et al.* (1993). Mitochondrial proteins were labeled in cycloheximide-blocked cells according to Claisse *et al.* (1980).

Synthesis of radiolabeled proteins and in vitro mitochondrial import

Templates for *in vitro* transcription by SP6 RNA polymerase were prepared by polymerase chain reaction (PCR) using oligonucleotide primers. The primers were designed so that an SP6 promoter was added in the appropriate position in front of the initiation codon and a stop codon was created in the position corresponding to residue 358 of Fam1p and residue 376 of Fam1-1p. The transcription products were translated *in vitro* using a reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. *In vitro* mitochondrial import assays were performed as described in Schwarz *et al.* (1993).

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Note added in proof

Since the completion of this study, the sequence of an identical long-chain fatty acid CoA ligase (acyl-CoA synthetase) has been deposited in the EMBL data bank under the accession number X77783.