Curr Genet (1997) 32: 384–388 © Springer-Verlag 1997

ORIGINAL PAPER

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Two genes of the putative mitochondrial fatty acid synthase in the genome of *Saccharomyces cerevisiae*

Received: 25 April / 16 September 1997

Abstract In order to find further genes of the mitochondrial fatty acid synthase, we searched the genome of *Saccharomyces cerevisiae* for sequences that are homologous to conserved regions of bacterial fatty acid synthase genes. We found the gene products of ORF YKL055c (EMBL Accession No. X75781) and of YOR221C (EMBL Accession No. X92441) to be homologous to bacterial 3-oxoacyl-(acyl carrier protein) reductases and to malonyl-CoA:ACP-transferases, respectively. We disrupted these two genes which in both cases led to a respiratory deficient phenotype, as is the case for the genes encoding a mitochondrial acyl carrier protein and a β-ketoacyl-ACP synthase. We propose to call the above mentioned genes *OAR1* [3-*o*xo-acyl-(*a*cyl carrier protein) *r*eductase] and *MCT1* (*m*alonyl-*C*oA:ACP *t*ransferase). They are presumed to be part of a type-II mitochondrial fatty acid synthase, a relic of the endosymbiontic origin of mitochondria, delivering substrates for phospholipid re-modelling and/or repair.

Key words Mitochondrial fatty acid synthase · Respiration · *PET* gene · Oxidative phosphorylation

Introduction

The fatty acid synthase (FAS) complex exists either in a procaryotic or in a eucaryotic structural form (McCarthy and Hardie 1984). Discrete proteins are present in the type-II FAS of bacteria and chloroplasts. In the type-I FAS, found in the cytosol of animals and fungi, all enzymatic activities are located on one, or two, polypeptide chain(s). A first hint for the existence of a type-II FAS in mitochondria was the discovery of an acyl carrier protein (ACP) of the procaryotic type in the mitochondria of *Neurospora crassa* (Brody and Mikolajczyk 1988). Later, the mitochondrial ACP was identified as a subunit of the respiratory NADH:ubiquinone oxidoreductase (complex I) in *N. crassa* and *Bos taurus* (Runswick et al. 1991; Sackmann et al. 1991). Genes for a mitochondrial ACP were found in *Saccharomyces cerevisiae*, *Arabidopsis thaliana* and *Caenorhabditis elegans* (Chéret et al. 1993; Shintani and Ohlrogge 1994; Wilson et al. 1994). All mitochondrial ACPs possess a characteristic mitochondrial import sequence.

In *S. cerevisiae,* the mitochondrial ACP is not bound as a subunit to the respiratory complex I, due to the lack of this complex (de Vries and Grivell 1988).The *S. cerevisiae* mutant *Δacp1* showed a respiratory deficient *pet* phenotype with complexes III and IV being absent (Schneider et al. 1995). The gene of a mitochondrial *β*-ketoacyl-ACP synthase (*CEM1*) was found in *S. cerevisiae,* and gene disruption led to a respiratory deficient *pet* phenotype very similar to that of the *Δacp1* disruption mutant (Harington et al. 1993). The mutant *Δcem1* could be complemented by a long-chain acyl co-enzyme-A ligase (*FAM1-1*) redirected to mitochondria by a mutation that provided the enzyme with a mitochondrial import sequence (Harington et al. 1994). However, this complementation works only when yeast is grown in rich medium.

Here we report the identification of the genes for two new putative components of the mitochondrial type-II FAS. We show that they are essential for mitochondrial respiration.

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Communicated by L. A. Grivell

Materials and methods

Strains, plasmids and media. Escherichia coli DH5α was used for transformation and propagation of plasmids (Hanahan 1983). *S. cerevisiae* strain VW1 [*MAT***a**, *ura3-52*, *leu2-3*, *112*, *trp1-289*, *Δhis3*] was used for transformation (a gift of K. D. Entian). The maintenance of the mitochondrial genome was checked by growth of diploids obtained after crossing with a rho^0 strain on glycerol. Plasmids

YEp351 and YEp352 were described by Hill et al. (1986); YCp50 was described by Rose et al. (1987). pT7T3 18U was obtained from Pharmacia (Mead et al. 1987). Complete or minimal media with the appropriate supplements and containing glucose (2%), glycerol (3%) or galactose (3%) were used as described by Sherman et al. (1986).

Nucleic acid manipulations and molecular cloning. Standard methods for DNA manipulations and *E. coli* transformation were employed (Sambrook et al. 1989). Yeast strains were transformed by means of the lithium-acetate method (Soni et al. 1993). Genomic DNA of *S. cerevisiae* was extracted according to Philippsen et al. (1991). DNA amplification by PCR was performed with a Perkin-Elmer GeneAmp PCR System 2400 thermo cycler using Prime-Zyme™ (Biometra). *OAR1* was amplified with primers scoarn (5′ CAATACTATGAATTCCAACAACAACAGC 3′) and scoarc (5′ TCTTGCACTAAGCTTTAACAGCCTTCATG 3′). *MCT1* was amplified with primers scmctn (5′ GCCGCAATGGAGATCTTCTG-CGTAG 3′) and scmctc (5′ CGGAGGGAATAACTCCCTACTTG-AC 3′). Primer leu2r (5′ GTCAGAAACGGCCTTAACGACGTAC 3′) was used to prove one-step gene disruption.

Disruption of the genes OAR1 and MCT1. A 2-kb DNA fragment containing the *OAR1* gene and flanking regions was amplified from genomic DNA by PCR. The primers scoarn and scoarc (each with one mismatch to insert restriction sites for *Hin*dIII and *Eco*RI) were used (see Fig. 2 a). For the construction of the replacement vector, the PCR product was digested with *Hin*dIII and *Eco*RI and inserted in *Eco*RI/*Hin*dIII-cut pT7T3 18U to give pOAR-EH. A 426-bp *Nsi*I/*Hpa*I fragment encoding the N-terminal half of the polypeptide was deleted. The *Pst*I/*Hpa*I fragment of YEp351, containing the *LEU2* gene, was inserted to give pOAR-TV. Sequence verification was carried out by restriction analysis and PCR. Transformation of *S. cerevisiae* was performed with a 3.1-kb *Nde*I/*Sac*I fragment containing the deleted gene and flanking regions (see Fig. 2). For complementation, *Δoar1* was transformed with the *OAR1* gene as an *Eco*RI/*Hin*dIII fragment from pOAR-EH inserted in *Eco*RI/*Hin*dIII-cut YCp50 (centromeric) and in *Eco*RI/*Hin*dIII-cut YEp352 (multicopy). To inactivate *MCT1* a 3-kb DNA fragment containing the gene and flanking DNA was amplified with scmctn and scmctc (Fig. 2 b). The PCR product was digested with *Xba*I and *Pst*I and inserted in *Xba*I/*Pst*I-cut pT7T3 18U to give pMCTXP. A 1076-bp *Cla*I/*Eco*RV fragment was removed and the *LEU2* gene was inserted as a *Nar*I/*Hpa*I fragment giving pMCT-TV. For transformation of *S. cerevisiae,* a 3.6-kb *Pst*I/*Xba*I fragment was employed. For complementation, *Δmct1* was transformed with the *MCT1* gene as an *Eco*RV/*Bgl*II fragment of pMCTXP inserted in *Nru*I/*Bam*HI-cut YCp50 and as a *Xba*I/*Bgl*II fragment of pMCTXP inserted in *Xba*I/*Bam*HI-cut YEp352.

Determination of mitochondrial cytochrome contents. Isolation and spectroscopic analysis of *S. cerevisiae* mitochondria was performed as described by Rickwood et al. (1990).

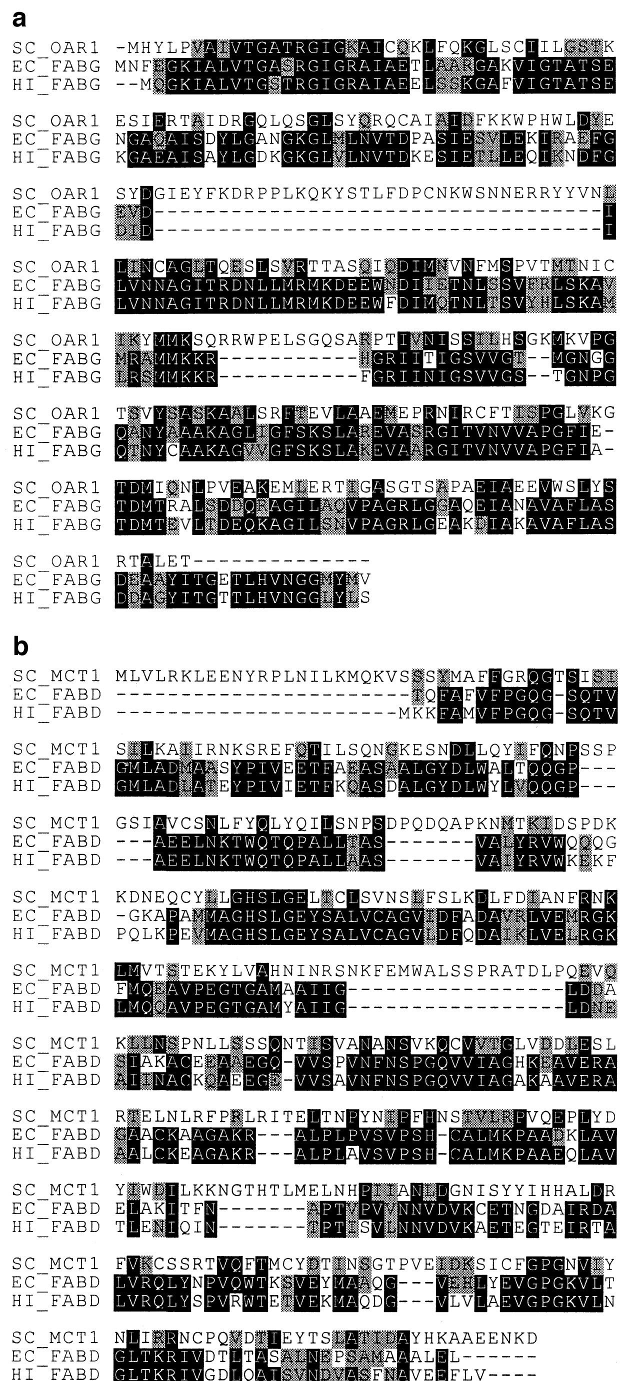

Fig. 1 a, b Sequence comparison of the *S. cerevisiae* gene products OAR1 (**a**) and MCT1 (**b**) with the corresponding homologues of *E. coli* and *Haemophilus influenzae*. Identical residues are shown in *reverse print*, similar residues are *shaded in light gray*. Sequence alignment was performed with the program CLUSTAL W (Thompson et al. 1994). Accession Nos: (**a**) SC OAR1, X75781; EC FABG, P25716; HI FABG, P43713. (**b**) SC MCT1, X92441; EC FABD, P25715; HI FABD, 43712

Results and discussion

Genes encoding components of type-II FAS genes in the genome of *S. cerevisiae*

We searched the *S. cerevisiae* genome for homologues of bacterial FAS type-II genes using the program BLAST (Altschul et al. 1990). Two genes that encode proteins homologous to the bacterial FAS type II (Fig. 1) were found: ORF YKL055c encodes a putative 3-oxoacyl-(acyl carrier protein) reductase, and YOR221C encodes a malonyl-CoA:ACP transferase (Rasmussen 1994; Galisson and Dujon 1996). We propose to call these genes *OAR1* [3-*o*xo-acyl-(*a*cyl carrier protein) *r*eductase] and *MCT1* (*m*alonyl-*C*oA:ACP *t*ransferase). There is no consensus for a cleavable N-terminal mitochondrial import sequence in the *OAR1* product. The N-terminal region of the *MCT1* product contains an arginine in position –10 belonging to a pu-

tative cleavage site, followed by a leucine in position –8. However, there seems to be no amphiphilic helix in the presequence.

Inactivation and complementation of *OAR1* and *MCT1*

The one-step procedure described by Rothstein (1991) was applied to inactivate the genes *OAR1* and *MCT1* in *S. cerevisiae*. The genes including flanking DNA regions were amplified by PCR and the products were used for plasmid construction (Fig. 2). Parts of *OAR1* and *MCT1* were deleted and replaced by the *LEU2* marker gene in the strain VW1. Haploid *S. cerevisiae* VW1 was transformed with linear fragments according to Fig. 2. Genomic DNA of leucine prototrophs was isolated and inactivation of *OAR1* and *MCT1* was checked by PCR using a primer internal to the *LEU2* gene (leu2r) and a primer external to the fragment used for transformation (Figs. 2 and 3). For complementation of the disruptants *Δoar1* and *Δmct1,* the genes *OAR1* and *MCT1* were inserted in YEp352 or YCp50 (data not shown). Transformation with either of these plasmids restores the wild-type phenotype (Fig. 4).

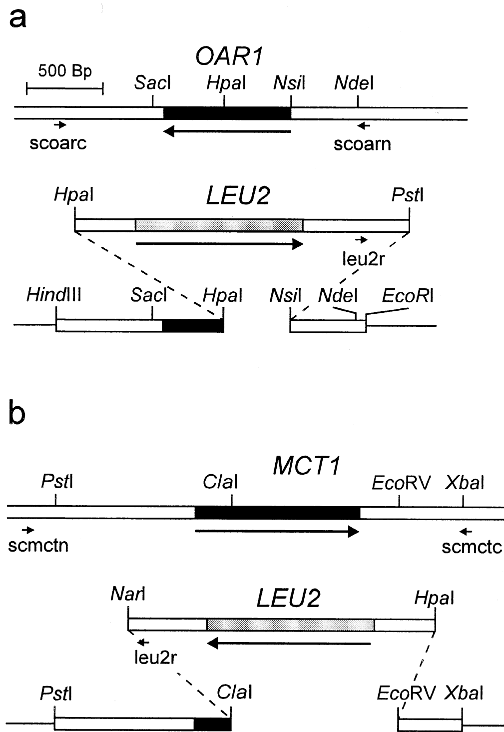

Fig. 2a, b Construction of the replacement vectors for the inactivation of the genes *OAR1* and *MCT1*. **a** for the disruption of *OAR1*, a 2-kb fragment was amplified with the primers scoarn and scoarc. Two bases were changed to insert restriction sites for *Eco*RI and *Hin*dIII. The fragment was digested with *Eco*RI/*Hin*dIII and ligated in *Eco*RI/*Hind*III-cut pT7T3 18U. A 426-bp *Nsi*I/*Hpa*I fragment was replaced by a *Pst*I/*Hpa*I fragment of YEp351 containing the *LEU2* gene. Transformation was performed with a 3.1-kb *Nde*I/*Sac*I fragment containing the disrupted gene and flanking regions. **b** for the disruption of *MCT1*, a 3-kb fragment was amplified with the primers scmctn and scmctc. The PCR product was digested with *Xba*I and *Pst*I and inserted in *Xba*I/*Pst*I-cut pT7T3 18U. A 1076-bp *Cla*I/*Eco*RV fragment was deleted and replaced by a *Nar*I/*Hpa*I fragment of the plasmid YEp351 containing the *LEU2* gene. Transformation was performed with a 3.6-kb *Xba*I/*Pst*I fragment containing the disrupted gene and flanking DNA

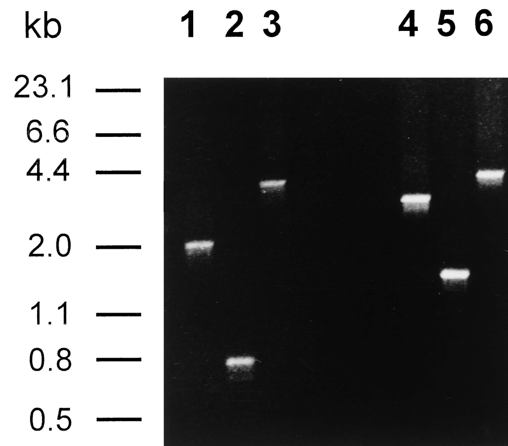

Fig. 3 PCR-analysis of genomic DNA from the disruptant strains *Δoar1* and *Δmct1* and from a VW1 wild-type strain was used as template. *Lanes 1 and 4,* wild-type; *lanes 2 and 3*, *Δoar1*; *lanes 5 and 6*, *Δmct1*. The following primer combinations were used for amplification: *Lanes 1 and 3*, scoarn and scoarc; *lane 2*, scoarn and leu2r; *lanes 4 and 6*, scmctn and scmctc; *lane 5*, scmctn and leu2r. Predicted lengths of PCR fragments (*lanes 1–6*): 2.0 kb, 0.7 kb , 3.8 kb, 3.0 kb, 1.5 kb, and 3.9 kb

Growth

The mutants *Δoar1* and *Δmct1* are unable to grow on glycerol as a sole carbon source. The cytochromes aa_3 and *b* are absent in the mitochondria of the deleted strains and the content of cytochrome *c* is decreased (Fig. 5). The mutants *Δoar1* and *Δmct1* were crossed to a rho^0 tester strain to check the integrity of mitochondrial DNA. The mutants *Δoar1* and *Δmct1* show a rapid loss of mitochondrial DNA integrity. About 30 % became cytoplasmic petite.

Necessity of mitochondrial FAS for oxidative phosphorylation

S. cerevisiae is now known to contain four genes encoding components of a mitochondrial FAS of type II, namely the genes *ACP1* (Schneider et al. 1995), *CEM1* (Harington et al. 1993), and the genes for the above described malonyl-CoA:ACP transferase and 3-oxoacyl-ACP reductase. Genes encoding 3-hydroxy-acyl dehydratases cannot be detected yet in the *S. cerevisiae* database due to the small number of species from which the corresponding genes have been sequenced. In the case of the enoyl reductase, homology between proteins from different species is rather low. There seem to be no blocks of conserved residues in

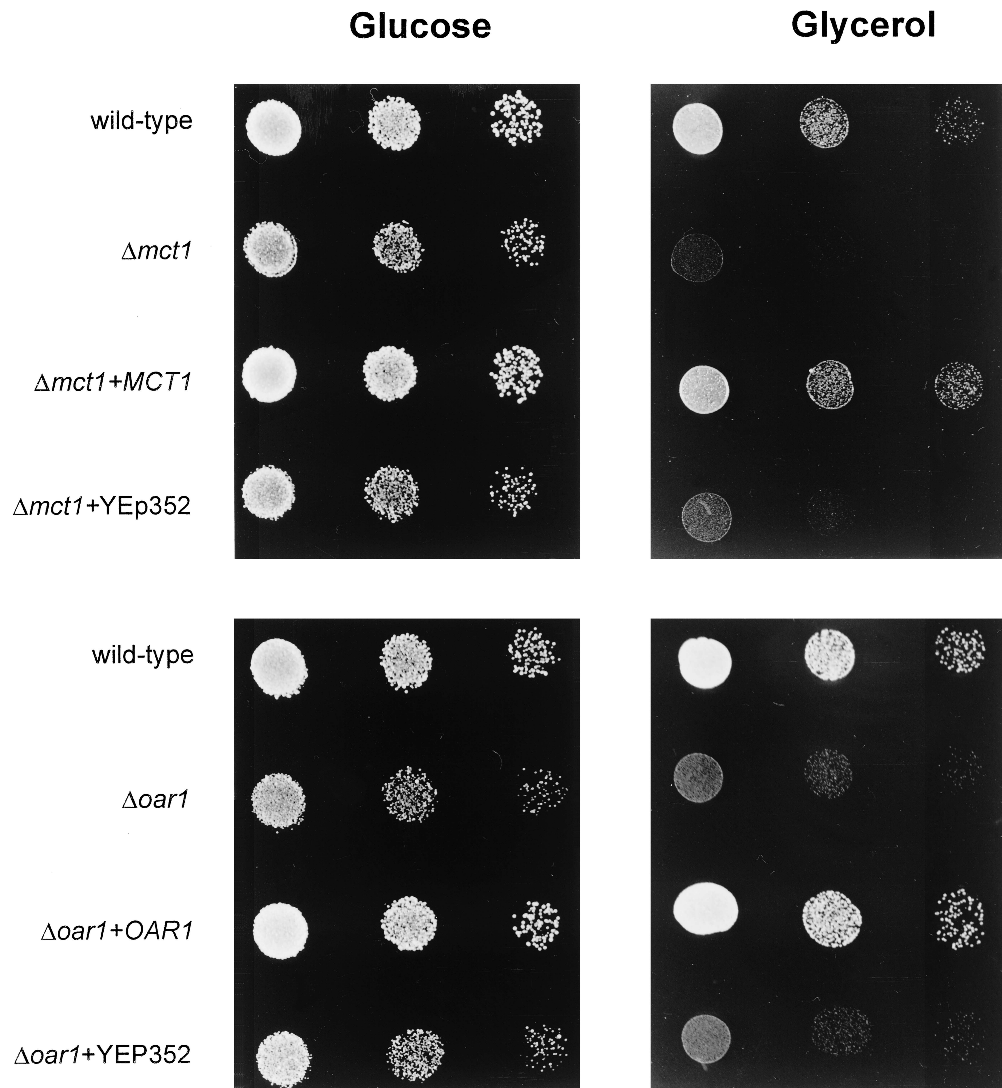

Fig. 4 Growth of wild-type (VW1), *Δoar1*, *Δmct1*, and complemented strains (multicopy). First the strains were grown in liquid minimal medium, serial dilutions were then spotted onto complete glucose and glycerol plates and incubated at 30°C. Transformation with *OAR1* and *MCT1* inserted in centromeric YCp50 also restored the wild-type phenotype (data not shown)

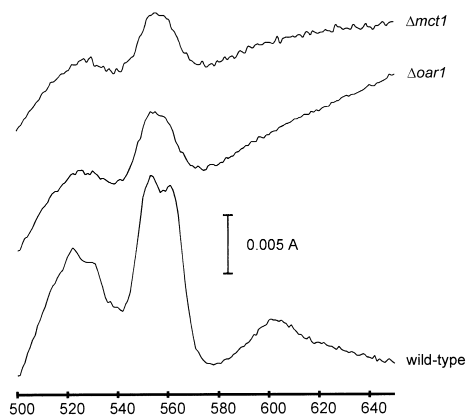

Fig. 5 Difference spectra of wavelength/nm dithionite-reduced minus air-oxidized mitochondria of *S. cerevisiae* wild-type and mutants *Δoar1* and *Δmct1*

the primary structure that would allow the corresponding gene to be identified in a genome database. The mutants *Δacp1*, *Δcem1*, *Δaor1* and *Δmct1* are characterized by their respiratory deficiency, virtually without any cytochromes (Fig. 5). This points to the necessity of the mitochondrial FAS for respiration and/or oxidative phosphorylation. However, it is unlikely that the mitochondrial FAS participates directly in the biosynthesis of the respiratory complexes and/or the ATPase. In the obligate aerobic fungus *N. crassa,* disruption of the mitochondrial ACP – which in fungi as in animals is a subunit of the respiratory complex I – has no effect on the formation of the respiratory complexes III and IV. The content of cytochromes *a*, *b* and *c* is normal in the *N. crassa* mutant, and it respires as actively as the wild-type using an alternative NADH:ubiquinone oxidoreductase in place of complex I. A phenotypic feature of the mutant was a four-fold increase in the mito-

chondrial lysophospholipid content (Schneider et al. 1995). We therefore suppose that the mitochondrial FAS provides fatty acids required for phospholipid re-modelling and/or repair. If this function is absent in *S. cerevisiae,* the yeast inner mitochondrial membranes are incapable of coupling respiration with ATP synthesis by means of the proton motive force. This can be overcome by *S. cerevisiae* by switching to fermentation, thus abolishing oxidative phosphorylation and resulting in a *pet* phenotype.

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. We are grateful to Dr. K. D. Entian for the gift of the *S. cerevisiae* strain VW1.

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