The peroxisomal transporter gene *ANT1* is regulated by a deviant oleate response element (ORE): characterization of the signal for fatty acid induction

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Saccharomyces cerevisiae *ANT1/YPR128c* encodes the peroxisomal adenine nucleotide transporter that provides ATP for intra-peroxisomal activation of medium-chain fatty acids. A *lacZ* reporter construct comprising the *ANT1* promoter was shown to be comparatively more highly expressed in a wild-type strain grown on oleic acid, a long-chain fatty acid, than in *pip2Δoaf1Δ* mutant cells that are defective in fatty acid induction. The *ANT1* promoter was demonstrated to contain a deviant oleate response element (ORE) that could bind the Pip2p-Oaf1p transcription factor and confer activation on a basal *CYC1-lacZ* reporter gene. Expression of Ant1p as well as other enzymes whose genes are known to be regulated by a canonical ORE was found to be increased in cells grown on lauric acid, a medium-chain fatty acid. We concluded that the signal for induction does not differentiate between long- and medium-chain fatty acids. This

signal was independent of β -oxidation or the biogenesis of the peroxisomal compartment where this process occurs, since a *pox1Δ* strain blocked in the first and rate-limiting step of β -oxidation as well as various *pex* mutant cells devoid of intact peroxisomes produced sufficient amounts of Pip2p-Oaf1p for binding OREs *in vitro* and for expressing an ORE-driven reporter gene. The signal's durability was shown to be related to the concentration of fatty acids in the medium, since a *pex6Δ* strain expressed an ORE-driven reporter gene at high levels for a longer period than did isogenic wild-type cells. Generation of the signal was also independent of protein synthesis, as demonstrated by cycloheximide treatment.

Key words: ORE consensus, peroxisomal adenine nucleotide transporter, Pip2p-Oaf1p, Ypr128cp/Ant1p.

INTRODUCTION

In *Saccharomyces cerevisiae*, peroxisomes serve as the exclusive site for the β -oxidation of fatty acids [1]. Growth of *S. cerevisiae* cells on oleic acid as a sole carbon source causes a 10-fold induction of the enzymes of the β -oxidation spiral, and is accompanied by a massive expansion of the peroxisomal compartment [2]. The simultaneous transcriptional up-regulation of the corresponding β -oxidation enzyme genes has now been revealed. A pair of zinc-cluster proteins, Pip2p and Oaf1p [3,4], combine to form a transcription factor that binds to oleate response elements (OREs) in the promoters of the relevant genes [5,6]. OREs consist of two CGG triplets arranged in an inverted array, with 15–18 intervening nucleotides [7–9]. All known OREs contain at least one half site with conserved T and A residues (CGGN₃TNA) that appear to be important for activation [5,6]. In addition, several ORE-dependent genes are also regulated by the Adr1p transcription factor, including those coding for the β -oxidation spiral enzymes Pox1p and Pot1p/Fox3p, the β -oxidation auxiliary enzyme Sps1p and peroxisomal catalase A Cta1p [10–13]. OREs and Adr1p-binding elements (upstream activation sequences; UAS1s) appear to act synergistically, since both are required to induce fully the transcription of *CTA1* on oleic acid [13].

Activation of the Pip2p-Oaf1p transcription factor requires the presence of fatty acids in the growth medium [14]. These convert Oaf1p into a transcriptional activator by an unknown

mechanism; conversion of Oaf1p in turn is thought to affect the activation domain of Pip2p [14]. In addition, expression of Pip2p is itself inducible by oleic acid [3,4], which appears to be a most convenient way to regulate the amount of transcription factor. The emerging picture of the mechanism of fatty acid activation notwithstanding, little is known about the nature of the signal that actually activates Oaf1p.

The fatty acid signalling pathway affecting β -oxidation enzymes is also thought to regulate the expression of yeast peroxisomal membrane proteins. Oleic acid-dependent transcriptional activation by Pip2p-Oaf1p has been demonstrated for the peroxisomal membrane proteins Pex11p, Pat1p/Pxa2p and Pat2p/Pxa1p [7–9], although in the case of the *PAT2/PXA1* gene the mechanism for this is not yet clear since the corresponding promoter lacks an ORE consensus sequence [8]. Similarly, the regulation of *ANT1/YPR128c* encoding the recently identified adenine nucleotide transporter of the peroxisomal membrane [15,16] has also remained hitherto uncertain, since despite the absence of a clear-cut canonical ORE in the corresponding promoter (Figure 1), the abundance of Ant1p increases in the presence of oleic acid [15,16].

Here we determined whether Pip2p-Oaf1p is required for the increased levels of Ant1p seen in cells grown on fatty acids, and probed the functionality of the ORE-like element in the *ANT1* promoter. In addition, the general signal triggering ORE activation was studied with regard to the identity of the fatty acids used for induction, the requirement for effective β -oxidation and

Abbreviations used: ORE, oleate response element; UAS, upstream activation sequence; EMSA, electrophoretic mobility-shift assay; MCFA, medium-chain fatty acid.

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Figure 1 A section of the *ANT1* promoter

The terminal nucleotides depicted to the right are situated 130 bp 5' to the ATG start site. Sequences similar to OREs (CGGN₃TNAN₉₋₁₂CCG) or UAS1s (CYCCRDN₄₋₃₆HYGGRG) are indicated as boxes below the nucleotide sequence. Conserved CGG triplets as well as A and T nucleotides are shown in bold. Asterisks indicate mismatches to the UAS1 consensus. The overlapping ORE/UAS1 arrangement in *ANT1* is similar to that found in *POX1* [13].

peroxisome biogenesis, and protein synthesis. Finally, the cause of termination of the signal was also investigated. The results are discussed in terms of a co-ordinated response to carbon-source availability.

MATERIALS AND METHODS

Strains and plasmids

The *S. cerevisiae* strains and plasmids used are listed in Tables 1 and 2, respectively. The strains harbouring the MF24-6x reporter construct were generated using a *Stu*I-linearized plasmid pMF24-6x as described in [6]. *Escherichia coli* strain DH10B was used for all plasmid amplifications and isolations.

Media and growth conditions

For β -galactosidase measurements, cells were induced in oleic acid medium as described previously [3]. Briefly, late exponential-phase cells from overnight pre-cultures grown in medium consisting of 1 % (w/v) yeast extract/2 % (w/v) peptone (YP medium)

and 5 % (w/v) D-glucose were transferred to 100 ml conical flasks containing 50 ml of YP medium plus 0.2 % (w/v) oleic acid, 0.02 % (w/v) Tween 80 (adjusted to pH 7 with NaOH), 0.05 % (w/v) D-glucose and 75 μ g/ml ampicillin, to an absorbance of A_{600} = 0.2. The cultures were grown at 30 °C with vigorous agitation at 170 rev./min for the periods indicated. Comparison of fatty acid induction using lauric acid or oleic acid was performed on cells grown in fatty acid media that additionally contained 0.05 % (w/v) Tween 40, 0.17 % (w/v) yeast nitrogen base without amino acids and ammonium sulphate, 0.5 % (w/v) ammonium sulphate, 0.1 % (w/v) yeast extract and 20 mg/l amino acids, as required, all adjusted to pH 6.

Plasmid construction

The ORE in the *ANT1* promoter was generated from synthetic oligonucleotides (Table 2). The resultant double-stranded DNA fragment, which was delineated by *Sal*I and *Eco*RI sites, was ligated into the appropriate sites in the plasmid vector, pBlue-script® SK(+) (hereon referred to as pSK; Stratagene, La Jolla,

Table 1 *S. cerevisiae* strains used in this study

The numbers in superscript following the strains' designation refer to their parental genotypes; e.g. BJ1991*adr1* Δ ¹ was derived from 1 BJ1991.

<i>S. cerevisiae</i> strain	Description	Source or reference
1 BJ1991	<i>MATα leu2 ura3-52 trp1 pep4-3 prb1-1122 gal2</i>	[44]
2 BJ1991 <i>pip2</i> Δ <i>oaf1</i> Δ ^{1a}	<i>pip2</i> Δ :: <i>kanMX oaf1</i> Δ :: <i>LEU2</i>	[3]
BJ1991 <i>adr1</i> Δ ¹	<i>adr1</i> Δ :: <i>LEU2</i>	Laboratory of Dr H. F. Tabak
BJ1991 <i>pox1</i> Δ ¹	<i>pox1</i> Δ :: <i>LEU2</i>	[45]
BJ1991 <i>pex5</i> Δ ¹	<i>pex5</i> Δ :: <i>LEU2</i>	Laboratory of Dr H. F. Tabak
3 BJ1991 <i>pex6</i> Δ ¹	<i>pex6</i> Δ :: <i>LEU2</i>	[45]
yHPR111 ¹	<i>FOX3 ORE-CYC1-lacZ</i>	[14]
yHPR112 ¹	<i>FOX3 ORE-CYC1-lacZ ADH2-PIP2</i> :: <i>TRP1</i>	[14]
yHPR113 ¹	<i>FOX3 ORE-CYC1-lacZ pip2</i> Δ :: <i>kanMX</i>	[14]
yAG1046 ¹	Harbouring the pLacZi plasmid vector	This study
yHPR290 ¹	<i>ANT1 ORE</i> :: <i>CYC1-lacZ</i> reporter gene (pHPR154)	This study
yHPR291 ²	<i>ANT1 ORE</i> :: <i>CYC1-lacZ</i> reporter gene (pHPR154)	This study
yHPR320 ¹	Expressing <i>ANT1-lacZ</i> from pHPR161	This study
yHPR321 ¹	Expressing <i>ANT1-lacZ</i> from pHPR162	This study
yHPR322 ²	Expressing <i>ANT1-lacZ</i> from pHPR162	This study
yHPR339 ³	<i>ANT1 ORE</i> :: <i>CYC1-lacZ</i> reporter gene (pHPR154)	This study
4 MF24-6x	<i>MATα leu2 ura3-52 trp1 his3 ctt1 gal2</i>	
MF24-6x <i>pip2</i> Δ <i>oaf1</i> Δ ⁴	<i>MF24-6x-CYC1-lacZ</i> :: <i>URA3</i>	[6]
yHPR103 ⁴	<i>pip2</i> Δ :: <i>kanMX oaf1</i> Δ :: <i>LEU2</i>	[3]
yHPR104 ⁴	<i>pex5</i> Δ :: <i>LEU2</i>	This study
yHPR105 ⁴	<i>pex6</i> Δ :: <i>LEU2</i>	This study
5 A232-4A	<i>MATα leu2 ura3-52 trp1</i>	[31]
6 A232-4A-23 ⁵	<i>pox1</i> Δ :: <i>LEU2</i>	[31]
yHPR101 ⁵	<i>POX1 MF24-6x-CYC1-lacZ</i> :: <i>URA3</i>	This study
yHPR102 ⁶	<i>pox1</i> Δ :: <i>LEU2 MF24-6x-CYC1-lacZ</i> :: <i>URA3</i>	This study
BY4742	<i>MATα his3</i> Δ <i>1 leu2</i> Δ <i>0 lys2</i> Δ <i>0 ura3</i> Δ <i>0</i>	[46]

Table 2 Plasmids and oligonucleotides used in this study

Plasmid or oligonucleotide	Description	Source or reference
Plasmid		
pHPR123	<i>ANT1</i> promoter in pSK/ <i>EcoRV</i>	This study
pHPR142	<i>ANT1</i> ORE in pSK/ <i>SaII-EcoRI</i>	This study
pHPR154	pLacZi-based <i>ANT1</i> ORE:: <i>CYC1-lacZ</i>	This study
pHPR161	<i>ANT1-lacZ</i> in Ylp356	This study
pHPR162	<i>ANT1-lacZ</i> in YEp356	This study
pMF24-6x	<i>CTA1</i> ORE(MF24-6x)- <i>CYC1-lacZ</i>	[6]
pBluescript® SK(+)	Cloning vector (pSK)	Stratagene
pLacZi	MATCHMAKER one-hybrid vector	Clontech
Ylp/YEp356/8	<i>URA3</i> -marked integrative or episomal plasmids	[47]
Oligonucleotide		
FOX3ORE1	5'-TCGACCATCTCGGGGATAATAGTATTAACACCGAGATGG-3'	[48]
FOX3ORE2	5'-TCGACCATCTCGGTGTTAATACTATTATCCCGAGATGG-3'	[48]
POX1ORE1	5'-AATTCATTACGGTATTAGTTGATTAACCTCGAAGCG-3'	[7]
POX1ORE2	5'-GATCCGCTTCGGAGTTTAATCAACTAATACCGTAAATG-3'	[7]
CTA1ORE1	5'-TCGACAAGGACGGCTTTAACAATAATAAATCCGAAG-3'	[7]
CTA1ORE2	5'-TCGACTTCCGAGTTTATATTGTTAAAGCCGTCCTTC-3'	[7]
CTA1mutORE1	5'-TCGACAAGGACGCTTTAACAATAATAAATGTCAG-3'	[7]
CTA1mutORE2	5'-AATCTTGACACTTTATATTGTTAAAGGTCCTCTG-3'	[7]
ANT1ORE1 (RE311)	5'-AATTCCTCTCGGATCGAGTTTAAATCCGATTTTG-3'	This study
ANT1ORE2 (RE312)	5'-TCGACAAAAATCGGATTTAAATCCGATCCGAGGAGG-3'	This study
RE195	5'-GAATTCGCGGGTACAGTTTAATT-3'	This study
RE196	5'-GCGGCCGCTAGTGAAGCCAGCTTGCG-3'	This study
LacZi	5'-AATGTGAGCGAGTAACAAC-3'	This study

CA, U.S.A.), giving rise to plasmid pHPR142. The nucleotides of the insert were confirmed by automatic sequencing. To insert the *ANT1* ORE into a basal *CYC1-lacZ* reporter gene, plasmid pHPR142 was digested with *EcoRI* and *XhoI*, and the fragment containing the *ANT1* ORE was ligated into a similarly digested MATCHMAKER one-hybrid vector pLacZi (Clontech, Palo Alto, CA, U.S.A.). This resulted in plasmid pHPR154 (*ANT1* ORE:: *CYC1-lacZ*).

To fuse the native promoter of *ANT1* to *lacZ*, an *EcoRI*- and *NotI*-delineated promoter fragment spanning 386 bp 5' of the ATG start site was amplified by PCR using oligonucleotides RE195 and RE196. The resulting single amplification product was inserted into an *EcoRV*-digested pSK vector, resulting in plasmid pHPR123. The promoter fragment was released from this plasmid following digestion with *EcoRI* and *PstI*, and inserted into similarly digested YIp356 and YEp356 vectors (Table 2) to yield plasmids pHPR161 and pHPR162, respectively.

Immunoblotting

Whole-cell extracts were prepared according to a published protocol [17]. Monoclonal anti-yeast 3-phosphoglycerate kinase (Pgk1p) antibodies were obtained from Molecular Probes (Eugene, OR, U.S.A.). The antibodies against Ant1p, Pot1p/Fox3p and Kar2p have been described previously [16], whereas that against Cta1p was a gift from the laboratory of Dr H. F. Tabak (Department of Biochemistry, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands). Immunoreactive complexes were visualized using anti-rabbit or anti-mouse IgG-coupled horseradish peroxidase in combination with the ECLTM system from Amersham Bioscience (Uppsala, Sweden).

Electrophoretic mobility-shift assay (EMSA)

Protein–DNA binding experiments were performed according to a published protocol [7]. The assays were carried out in the presence of 4 mM Tris/HCl (pH 8.0), 100 mM NaCl, 4 mM

MgCl₂, 25 ng/μl poly[(dI-dC)-(dI-dC)], 10% (w/v) glycerol, 1–2 μl of protein sample and 0.2 ng of labelled DNA probe in a total reaction volume of 20 μl. The reactions were incubated at room temperature for 25 min prior to loading, and free and bound DNA was separated by electrophoresis in 5% (w/v) polyacrylamide gels in 45 mM Tris/45 mM boric acid/1 mM EDTA, pH 8.5 (0.5 × TBE) for 1 h (135 V) at room temperature. For competition experiments, 2–10 ng of double-stranded oligonucleotides (10–50-fold excess) were added.

Enzyme assays

β-Galactosidase activities were assayed in soluble-protein extracts prepared by breaking cells with glass beads [18], and expressed as nmol of *O*-nitrophenyl β-D-galactopyranoside hydrolysed/min per mg of protein. Alternatively, β-galactosidase activities were assayed in cells that were broken following three freeze–thaw cycles, and were expressed as μmol of chlorophenol red β-D-galactopyranoside hydrolysed/min per 10⁷ cells [18]. Catalase measurements were performed as described in [19]. Unless stated otherwise, values reported here are means ± S.D. from three experiments.

Cycloheximide treatment

Cultures of the strains yHPR111 (BJ1991), yHPR112 (BJ1991 + *ADH2-PIP2*) and yHPR113 (BJ1991 *pip2Δ*) that were propagated in YP medium containing 2% (v/v) ethanol were split and each half was treated with 100 μg/ml cycloheximide. Following a further incubation for 30 min, 0.2% (w/v) oleic acid and 0.02% (w/v) Tween 80 were added to all flasks and samples were taken at the zero time point, after 30 min and after 2 h. RNA was prepared as described in [20]. Northern blots were probed with *POT1/FOX3* and *ACT1*, and band intensities were quantified using a PhosphorImager. *POT1/FOX3* mRNA levels were corrected using *ACT1* mRNA as an internal control and are presented relative to that of untreated, non-induced wild-type cells.

Miscellaneous

Nucleic acid manipulations [21], yeast transformation [22], verification of single-plasmid integration [23], determination of protein concentration [24] and electrophoresis [25] were performed according to published methods. Fragments containing *FOX3* ORE or *ANT1* ORE were isolated from the respective plasmids pSKFOX3ORE and pHPR143 following digestion with *EcoRI* and *XhoI*.

RESULTS

Peroxisomal adenine nucleotide transporter Ant1p is inducible by oleic acid via a deviant ORE

Intra-peroxisomal activation of medium-chain fatty acids (MCFAs) is mediated by fatty acyl-CoA synthetase 2 (Faa2p) in an ATP-consuming reaction [26]. *FAA2*, which is moderately up-regulated in cells grown on oleic acid [8] contains an ORE in its promoter. The requisite ATP for this activation process is transported into the peroxisomes by Ant1p [15,16]. The *ANT1* promoter has previously been reported to contain an element resembling an ORE [9], but which falls short of the N₁₅₋₁₈ consensus by a single intervening nucleotide (i.e. N₁₄; Figure 1). Regulation of *ANT1* is controversial since a previous Northern analysis that could detect oleic acid-dependent transcriptional up-regulation of the known ORE-regulated genes *ECII* and *DCII* [8,27–30] failed to demonstrate a similar up-regulation for *ANT1* [9], whereas at the protein level, an increase in the amount

of Ant1p was shown with time when cells were shifted from low-glucose medium to that containing oleic acid [16].

To determine whether levels of Ant1p increase in oleic acid-grown cells in a manner depending on Pip2p-Oaf1p, Ant1p was monitored by immunoblot analysis in wild-type cells as well as *pip2Δoaf1Δ* mutants that are characterized by a specific defect in oleic acid-dependent transcriptional activation [3,4]. As a control for oleic acid induction, the amount of Pot1p/Fox3p was also analysed. The results demonstrated that when oleic acid was added to the medium, an increase in the amount of Ant1p was observed in wild-type cells (Figure 2), but in contrast to Pot1p/Fox3p, Ant1p was clearly detectable in the presence of ethanol. This additional increase in Ant1p abundance on oleic acid did not occur in the *pip2Δoaf1Δ* mutant strain, indicating that the Pip2p-Oaf1p transcription factor might be involved in up-regulating *ANT1*.

To investigate whether the oleic acid-dependent increase in protein levels correlates with transcriptional up-regulation of *ANT1*, a *lacZ* reporter gene was used that consisted of 386 bp of the *ANT1* promoter region. This *ANT1-lacZ* reporter gene was loaded on to integrative- as well as episomal plasmids. Wild-type cells and *pip2Δoaf1Δ* mutants were transformed with either plasmid, grown to late exponential phase in rich-glucose medium, and transferred to media containing ethanol or oleic acid for 18 h. The results demonstrated that as a single integrative copy, the reporter construct was not expressed to levels that were above the detection limit of the assay. However, expression of *ANT1-lacZ* loaded on the episomal counterpart resulted in clearly detectable β -galactosidase activities that were higher in the oleic acid-grown wild-type strain compared with the ethanol-grown strain (Table 3). In the *pip2Δoaf1Δ* mutant, this induction was abolished, indicating that the ORE-like sequence in *ANT1* might be functional. To test whether this putative element (tentatively called *ANT1* ORE) was sufficient to confer transcriptional activation, it was inserted into a promoterless *CYC1-lacZ* reporter gene and expressed from a single copy in the wild-type strain or the corresponding *pip2Δoaf1Δ* mutant. Measurements of β -galactosidase activities demonstrated a 6-fold increase in reporter expression in the wild-type strain grown under oleic acid-medium conditions compared with ethanol, whereas no up-regulation was detectable in the mutant strain (Table 3). These results implicated Pip2p-Oaf1p in the activation of the ORE-like element in the *ANT1* promoter.

To examine whether the element represented a binding target for the Pip2p-Oaf1p transcription factor, EMSAs were performed. Soluble protein extract from a wild-type strain was mixed with labelled *POT1/FOX3* ORE, and this resulted in the formation of a complex with a high molecular mass (Figure 3A, lane 8) that was missing from the lane containing

Figure 2 Induced expression of Ant1p depends on Pip2p-Oaf1p

Immunoblotting using whole-cell extracts from the strains BJ1991 wild type or BJ1991 *pip2Δoaf1Δ* grown on the indicated carbon sources.

Table 3 Reporter-gene activities under ethanol or oleic acid medium conditions

Data are means $\pm$ S.D. ($n = 3$).

Strain	β -Galactosidase activity ($\mu\text{mol}/\text{min}$ per 10^7 cells)		Relative level on oleic acid (%)
	Ethanol	Oleic acid	
<i>ANT1-lacZ</i> fusion on an episomal plasmid			
BJ1991	0.3 ± 0.1	11.5 ± 2.5	100.0
BJ1991 <i>pip2</i> Δ <i>oaf1</i> Δ	1.8 ± 0.3	1.2 ± 0.1	10.4
<i>ANT1</i> ORE:: <i>CYC1-lacZ</i> fusion on an integrative plasmid			
BJ1991	2.0 ± 0.7	12.9 ± 1.3	100.0
BJ1991 <i>pip2</i> Δ <i>oaf1</i> Δ	2.0 ± 0.8	1.6 ± 0.7	12.4

Figure 3 The *ANT1* ORE is a binding target for Pip2p-Oaf1p

EMSA using the OREs of *POT1/FOX3* (A) and *ANT1* (B). (A) The *ANT1* ORE competes with the *POT1/FOX3* ORE for binding Pip2p-Oaf1p. A Pip2p-Oaf1p complex forms with a labelled DNA fragment containing the *POT1/FOX3* ORE using protein extracts from wild-type cells (lane 8) but is absent from extracts obtained from *pip2Δoaf1Δ* mutant cells (lane 9). Excess (25-fold) competitor DNA (Table 2), including *ANT1* ORE (lane 5), was added as indicated. (B) Pip2p-Oaf1p binds directly to *ANT1* ORE. Binding of Pip2p-Oaf1p to radiolabelled *ANT1* ORE could be competed with by the *POX1* ORE and *CTA1* ORE, but not by the non-functional *CTA1* OREmut.

pip2Δoaf1Δ extract (Figure 3A, lane 9). The intensity of this complex could be shown to diminish following the addition of unlabelled control DNA representing *POT1/FOX3* ORE (self competition), *POX1/FOX1* ORE or *CTA1* ORE (Figure 3A, lanes 2, 4 and 6), but not with *CTA1* OREmut (Figure 3A, lane 7), a mutated inactive variant of the *CTA1* ORE [7]. Since the intensity of the Pip2p-Oaf1p complex was also diminished when

unlabelled *ANT1* ORE was added to the incubation mixture (Figure 3A, lane 5), the presumptive *ANT1* ORE was therefore able to recruit Pip2p-Oaf1p. When labelled *ANT1* ORE was used in this assay (Figure 3B), a Pip2p-Oaf1p complex appeared in the lane containing wild-type extract (Figure 3B, lane 2), but not when using an extract obtained from the *pip2Δoaf1Δ* mutant (Figure 3B, lane 7). Complex formation was diminished using competitor DNA representing various OREs, as indicated. The combined data demonstrated that *ANT1* is an oleic acid-inducible gene controlled by a promoter element that represents a *bona fide* ORE despite containing only 14 intervening nucleotides.

Comparison of ORE activation in cells grown on different fatty acid substrates

Although expression of Ant1p was enhanced in cells grown on a long-chain fatty acid such as oleic acid (Figure 2), the protein predominantly functions in the activation of MCFAs such as lauric acid. To determine whether lauric acid would be more efficient than oleic acid in activating gene expression, the two fatty acids were compared as inducers of the general response of cells to this sort of carbon source. Wild-type cells were grown under low-glucose medium conditions and transferred to Tween 40-containing media to which lauric acid or oleic acid was added. Expression levels of Ant1p were compared with those of Pot1p/Fox3p and Cta1p, peroxisomal proteins that are encoded by ORE-regulated genes. The results showed that Ant1p expression could be induced by each of the fatty acids used (Figure 4A, upper panel) in a manner that was similar to Cta1p and Pot1p/Fox3p (Figure 4A, lower panel). Additionally the results showed that at high concentrations (0.007 and 0.035 %, w/v) lauric acid failed to induce the ORE-regulated genes (Figure 4A). This was probably due to the toxicity of this fatty acid, since an overall decrease in protein content was observed at these concentrations (results not shown).

To compare the efficiency of induction between lauric and oleic acids, increasing concentrations of these compounds were used that ranged from 0.0001 to 0.1 % (w/v). At low concentrations (0.0001 and 0.001 %, but not 0.01 %), lauric acid was as efficient as oleic acid in inducing the expression of Pot1/Fox3p (Figure 4B, lower panel), indicating that the responses triggered by the two fatty acids may be similar to one another. This postulate was supported by the demonstration that Pip2p-Oaf1p, which is required for the induction of OREs by oleic acid, also governed lauric acid-mediated induction (Figure 4C). As anticipated, the controls demonstrated that oleic acid-induced expression of Cta1p and Pot1p/Fox3p was not observed in a *pip2Δoaf1Δ* mutant. In addition, when lauric acid was used as the inducer, the *pip2Δoaf1Δ* mutant strain failed to express the above-mentioned two control proteins to a greater extent than under low-glucose medium conditions (Figure 4C). The combined results demonstrated that ORE-regulated genes were inducible also by MCFAs and, importantly, this induction was likely to be mediated exclusively by Pip2p-Oaf1p.

Oleic acid induction is independent of β -oxidation or intact peroxisome biogenesis

The above findings raised the question of whether fatty acids from different classes might first be converted into a common compound that in turn activates the Pip2p-Oaf1p transcription factor. Were this compound to represent a β -oxidation breakdown intermediary, blockage in fatty acid degradation due to impaired β -oxidation or peroxisomal structures would ostensibly prevent the generation of this putative metabolite and subsequent

Figure 4 Lauric acid, a MCFA, is a potent inducer of OREs

(A) Whole-cell extracts obtained from a BY4742 wild type that was propagated on media supplemented with the indicated concentrations of lauric acid or oleic acid were analysed for the presence of Kar2p, Cta1p, Pot1p/Fox3p and Ant1p by immunoblotting. (B) Concentration range (w/v) of fatty acids for induction. The BY4742 wild type was induced with increasing concentrations of lauric acid or oleic acid, and analysis of Pot1p/Fox3p abundance was performed by immunoblot analysis. (C) Dependence of lauric acid induction on Pip2p-Oaf1p. Concentrations of 0.01% oleic acid or 0.001% lauric acid were used to induce BJ1991 wild type and an otherwise isogenic *pip2Δoaf1Δ* mutant for 12 h. Immunoblot analysis of whole-cell extracts was performed using antibodies against Kar2p, Cta1p and Pot1p/Fox3p.

induction of OREs. This possibility was examined using a *CYC1-lacZ* reporter gene that contains six copies of the *CTA1* ORE half site (pMF24-6x; Table 2), which is routinely used to examine oleic acid induction in yeast cells [6].

The reporter gene was inserted as a single copy into the genomes of *pox1Δ* cells that were blocked in the first and rate-limiting step of β -oxidation [31] or those lacking Pex5p [32], Pex6p [33] or Pex7p [34]. The rationale for using such *pex* mutants was that although they express all the requisite enzymes for a functional β -oxidation process, they fail to grow on oleic acid due to impaired peroxisome biogenesis [35]. In addition, the reporter gene was also introduced into the corresponding control wild-type strain as well as mutant cells devoid of Pip2p-Oaf1p [7,36]. The result of growing the strains on oleic acid demonstrated that with the exception of cells lacking Pip2p-Oaf1p, all of the strains expressed high levels of β -galactosidase activities (Table 4). An EMSA provided further supporting evidence for unimpaired Pip2p-Oaf1p interaction with OREs in these strains since they all produced ample levels of the corresponding signal

Table 4 Effect of *pox1Δ*, *pex5Δ*, *pex6Δ*, *pex7Δ* and *pip2Δoaf1Δ* deletions on the expression of a *CTA1* ORE::CYC1-lacZ reporter gene under oleic acid medium conditions

Data are means $\pm$ S.D. ($n = 3$).

Strain	Genotype	β -Galactosidase activity (nmol/min per mg of protein)
yHPR102	<i>pox1Δ</i>	253 $\pm$ 26
yHPR101	Wild type	194 $\pm$ 12
yHPR103	<i>pex5Δ</i>	236 $\pm$ 37
yHPR104	<i>pex6Δ</i>	242 $\pm$ 5
yHPR105	<i>pex7Δ</i>	223 $\pm$ 14
MF24-6x	Wild type	235 $\pm$ 25
MF24-6x	<i>pip2Δoaf1Δ</i>	17 $\pm$ 1

Figure 5 Pox1p, Pex5p and Pex6p are dispensable for Pip2p-Oaf1p binding to *POT1/FOX3* ORE

The dependence of the binding of Pip2p-Oaf1p to the *POT1/FOX3* ORE on a functional β -oxidation system and proper peroxisome biogenesis was scrutinized by EMSA, which was performed with soluble protein extracts from BJ1991-derived yeast cells, as indicated. The asterisk denotes a non-specific complex.

(Figure 5). This indicated that transcriptional induction of ORE-regulated genes was not abrogated by a disruption of β -oxidation and peroxisome biogenesis, and, as a consequence, was unlikely to be mediated by a common molecule originating from the β -oxidation of fatty acids.

To examine whether ORE activation would be extended in cells with impaired peroxisomes, expression of the *ANT1* ORE reporter was monitored in wild-type and *pex6Δ* cells incubated for prolonged periods in a medium consisting of low concentrations of oleic acid (0.02%, w/v) supplemented with an alternative carbon source (2%, v/v, ethanol). The *pip2Δoaf1Δ* strain again served as an induction-deficient control.

The results demonstrated that following 26 h growth in oleic acid medium, β -galactosidase activity in the wild-type strain was

Table 5 Effect of the *pex6Δ* deletion on the endurance of the oleic acid-induction signal in cells grown on medium supplemented with 0.02% oleic acid and 2% ethanolData are means $\pm$ S.D. ($n = 3$).

		β -Galactosidase activity (μ mol/min per 10^7 cells)	
Strain	Time into induction ...	12 h	26 h
<i>ANT1</i> ORE:: <i>CYC1-lacZ</i>			
yHPR290 (wild type)		69.2 $\pm$ 18	21.4 $\pm$ 1
yHPR339 (<i>pex6</i> Δ)		59.2 $\pm$ 6	53.4 $\pm$ 5
yHPR291 (<i>pip2</i> Δ <i>oaf1</i> Δ)		4.9 $\pm$ 0.5	5.1 $\pm$ 0.2

reduced compared with the earlier 12 h time point (Table 5). This loss of induction correlated with the fatty acid concentration in the medium; the turbidity of the medium caused by the dispersed oleic acid vanished after 26 h due to cellular uptake of the fatty acid from the medium. On the other hand, *pex6Δ* mutant cells, which failed to clear up the medium from oleic acid because they cannot degrade fatty acids, continued to express β -galactosidase activity relatively unabated. This indicated that protraction of the induction signal in cells was related directly to the high levels of oleic acid lingering in the medium.

Oleic acid induction does not require protein synthesis

To gain further insight into the molecular nature of the signal responsible for activating ORE-dependent genes, it was examined whether an increase in transcription levels (exemplified by *POT1/FOX3* mRNA) would still be mediated in the absence of *de novo* protein synthesis. Cycloheximide was used to block protein synthesis in a wild-type strain as well as in a strain supplemented with excess Pip2p using a construct in which *PIP2* was expressed from the *ADH2* promoter. As a control, a *pip2Δ* strain was used [7].

The results showed that, in untreated wild-type cells, oleic acid caused a fast transcriptional response, with *POT1/FOX3* mRNA levels becoming induced 3.4-fold as early as 30 min after the addition of oleic acid (Table 6). This induction was only slightly higher in the strain containing excess Pip2p (4.2-fold), indicating that in the wild-type strain normal amounts of Pip2p were already sufficient to saturate binding to OREs within the first 30 min. Cycloheximide treatment of the wild-type strain demonstrated that it was still able to induce 1.6-fold *POT1/FOX3* mRNA levels at 30 min, whereas a further increase in mRNA (3.5-fold) could be obtained following 2 h of oleic acid induction. Inhibition of protein synthesis at the 2 h time point was verified since expression of a *POT1/FOX3* ORE::CYC1-lacZ reporter gene in the treated strain was 8-fold less compared with the situation in the untreated strain (β -galactosidase activity of 7 nmol/min per mg of protein compared with 56 nmol/min per mg of protein). Hence, protein synthesis *de novo* was not required to relay the signal generated by the fatty acid inducer to the transcriptional machinery. Moreover, the amount of Pip2p present in wild-type cells under ethanol conditions was sufficient to generate an attenuated response. Nevertheless, when induction levels were compared with those of the cycloheximide-treated strain over-expressing Pip2p (2.5-fold after 30 min and 6.3-fold after 2 h compared with the respective values of 1.6-fold and 3.5-fold in the wild type), it became clear that, in wild-type cells,

Table 6 Relative levels of *POT1/FOX3* mRNA following growth of cells on oleic acid for the indicated periods with or without added cycloheximide

Strain	Relative level of <i>POT1/FOX3</i> mRNA		
	0 min	30 min	120 min
Without cycloheximide			
yHPR111 (wild type)	1.0	3.43	5.62
yHPR112 (overexpressing Pip2p)	1.16	4.2	5.77
yHPR113 (<i>pip2Δ</i>)	1.05	0.98	0.96
With cycloheximide			
yHPR111 (wild type)	1.1	1.57	3.49
yHPR112 (overexpressing Pip2p)	1.01	2.54	6.28
yHPR113 (<i>pip2Δ</i>)	1.06	0.6	0.96

subsequent induction of Pip2p expression by auto-regulation [14] was required for a full response.

DISCUSSION

Oleic acid induction in *S. cerevisiae* is an exquisitely regulated process involving both the up-regulated expression of catalytic enzymes and the expansion of the peroxisomal compartment. We demonstrated here that, at least at the transcriptional and protein levels, induced transport of ATP into peroxisomes is co-ordinated with this programme. This co-ordination is due to the *ANT1* gene, coding for the requisite adenine nucleotide transporter [15,16], being inducible under oleic acid-medium conditions via an N_{14} -spaced ORE that could bind Pip2p-Oaf1p. The *ANT1* ORE contains a CCGNNNTNA half site, which abides to the requirement for conserved A and T residues in other functional OREs. Since the isolated *ANT1* ORE was sufficient to confer oleic acid-dependent transcription on a basal promoter, we propose CCGN₁₄₋₁₈CCG (instead of ... N₁₅₋₁₈ ...) as a revised minimal consensus sequence for OREs.

Peroxisomal membrane proteins have been shown previously to be co-induced with the enzymes of β -oxidation in the presence of oleic acid, e.g. the heterodimeric ABC transporter comprising Pat1p (Pxa2p) and Pat2p (Pxa1p) [8,26,37,38]. Our data show that induction of peroxisomal functions was not limited to oleic acid, as it could also be mediated by lauric acid, an MCFA. Although *S. cerevisiae* tolerated the latter compound only at low concentrations, the response provoked by lauric acid was comparable with that of oleic acid. Furthermore, no evidence was found for favoured activation by lauric acid of genes that are preferentially required for MCFA utilization, such as *ANT1*. This non-specific mechanism relates to a previous observation made with the β -oxidation auxiliary enzyme Sps19p, representing peroxisomal 2,4-dienoyl-CoA reductase. Despite being solely required for breaking down unsaturated fatty acids with *cis* double bonds at even-numbered positions, such as petroselinic acid [39,40], Sps19p is transcriptionally activated by oleic acid, which contains a *cis* double bond at an odd-numbered position. Peroxisomal enzymes are therefore likely to be induced *en bloc* in the presence of a variety of fatty acids, irrespective of the role single enzymes might play in the degradation of the fatty acid present in the medium [39]. This conclusion is in agreement with pioneering work on peroxisomal catalase A and acyl-CoA oxidase in *S. cerevisiae*, which proposed that fatty acids with a chain length ranging from 8 to 18 carbon atoms, with a peak at C₁₂, could activate the expression of these enzymes [41]. The observation that Pip2p-Oaf1p is probably the exclusive sensor for a variety of fatty acids is reminiscent of the situation in higher

eukaryotic cells. Peroxisome-proliferator-activated receptor α , the key nuclear receptor that regulates peroxisomal lipid homeostasis, is able to interact with fatty acids that vary in both chain length and saturation [42,43], and it has been reasoned that such promiscuity of binding may reflect the necessity for a physiological sensor of lipid levels.

The issue of whether fatty acids or their respective CoA esters bind directly to the responsive partner (Oaf1p) of the Pip2p-Oaf1p transcription factor upon activation remains open [14]. Nevertheless, our findings favour such a direct mechanism. First, the response to oleic acid was fast, since a significant increase in *POT1/FOX3* mRNA could be observed within 30 min. Secondly, this response was still feasible in the absence of protein synthesis. The attenuated rate of induction observed in cycloheximide-treated wild-type cells could be suppressed by ectopically expressing Pip2p prior to blockage, indicating that no other proteins had to be newly synthesized for generating the activating signal. Thirdly, fatty acid signalling remained proficient in the absence of peroxisomes or a functional β -oxidation system. Finally, in *pex* mutant cells, the signal relaying the availability of fatty acids as a sole carbon source was protracted, probably due to the persistence of oleic acid in the medium.

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