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A heterodimer of the Zn₂Cys₆ transcription factors Pip2p and Oaf1p controls induction of genes encoding peroxisomal proteins in Saccharomyces cerevisiae

Hanspeter ROTTENSTEINER¹, Arnoud J. KAL², Barbara HAMILTON¹, Helmut RUIS¹ and Henk F. TABAK²

¹ Vienna Biocenter, Institut für Biochemie und Molekulare Zellbiologie der Universität Wien and Ludwig Boltzmann-Forschungstelle für Biochemie, Wien, Austria
² Department of Biochemistry, Academic Medical Center of the University of Amsterdam, Amsterdam, The Netherlands

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In the yeast Saccharomyces cerevisiae, two transcriptional activators belonging to the Zn₂Cys₆ protein family, Pip2p and Oaf1p, are involved in fatty-acid-dependent induction of genes encoding peroxisomal proteins. This induction is mediated via an upstream activation sequence called the oleate-response element (ORE). DNA-bandshift experiments with ORE probes and epitope-tagged proteins showed that two binary complexes occurred: in wild-type cells the major complex consisted of a Pip2p·Oaf1p heterodimer, but in cells in which Oaf1p was overexpressed an Oaf1p homodimer was also observed. The genes encoding Oaf1p and Pip2p were controlled in different ways. The OAF1 gene was constitutively expressed, while the PIP2 gene was induced upon growth on oleate, giving rise to positive autoregulatory control. We have shown that the Pip2p·Oaf1p heterodimer is responsible for the strong expression of the genes encoding peroxisomal proteins upon growth on oleate. Pip2p and Oaf1p form an example of a heterodimer of yeast Zn₂Cys₆ zinc-finger proteins binding to DNA.

Keywords: PIP2; OAF1; peroxisome; oleate-response element; Saccharomyces cerevisiae.

Peroxisomes are organelles present in almost all eukaryotic cells. Depending on the species they may harbour enzymes for diverse catabolic and anabolic processes, such as the β-oxidation of fatty acids and the first steps leading to ether-phospholipid synthesis (Van den Bosch et al., 1992; Reddy and Mannaerts, 1994). In the yeast Saccharomyces cerevisiae, the exclusive site for β-oxidation of fatty acids. When cells are grown on glucose medium, peroxisomal functions are less in demand and transcription of genes encoding peroxisomal proteins is repressed. On non-fermentable carbon sources such as ethanol or glycerol, derepression occurs. Full induction takes place when cells are grown on the fatty acid oleate as sole carbon source (Veenhuis et al., 1987). Induction of many of these genes is exerted via an upstream activation sequence called the oleate-response element (ORE), an inverted repeat of CGG triplets with a spacing of 15–18 nucleotides (CGGN₁₁₋₁₈CGG) (Einerhand et al., 1993; Filipits et al., 1993). ORE have been found in upstream regions of genes encoding peroxisomal matrix proteins, such as fatty acyl-CoA oxidase (Fox1p), thiolase (Fox3p), catalase A (Cat1p) and malate dehydrogenase (Mdh3p), in upstream regions of genes encoding certain peroxisomal membrane proteins, such as Pex11p and Pat1p, and in the upstream region of the gene encoding the transcriptional activator Pip2p (Wang et al., 1994; Einerhand et al., 1993; Filipits et al., 1993; Hiltunen et al., 1992; Erdmann and Blobel, 1995; Hettema et al., 1996; Distel et al., 1996; Rottensteiner et al., 1996).

Pip2p is a protein essential for induction of peroxisomal matrix proteins and for the proliferation of the peroxisomal compartment itself. Pip2p interacts directly with the ORE and mediates oleate-specific induction of ORE-dependent transcription (Rottensteiner et al., 1996). A second transcription factor, Oaf1p, was purified and cloned on the basis of its interaction with the FOX1 ORE (Luo et al., 1996). Mutants of the PIP2 or OAF1 genes failed to grow on oleate as sole carbon source, and cell extracts of deletion strains showed no binding activity towards ORE in a DNA-bandshift assay. The PIP2 and OAF1 genes encode proteins belonging to the Zn₂Cys₆ protein family of fungal transcriptional activators. Several members of this family have been shown to bind to palindromic CGG triplets, spaced by a certain number of residues, which defines the binding site for a particular member of the family. For instance, palindromic CGG triplets spaced by 11 bp define a binding site for Gal4p, while a 6-bp spacing defines the Ppr1p-binding site. The well-studied members of this typical fungal transcription-factor family of 56 reported Zn₂Cys₆ proteins interact with their DNA-binding sites as homodimers. Gal4p, Ppr1p, Put3p, Cha4p and Tea1p bind to palindromic CGG repeats (Reece and Pusheine, 1993; Marmorstein and Harrison, 1994; Holmberg and Schjerling, 1996; Gray and Fassler, 1996); Leu3p and Pdr3p bind to everted CGG repeats (Hellauer et al., 1996), and Hap1 binds to directly repeated CGG triplets (Zhang and Guarante, 1994).

The high similarity of Pip2p and Oaf1p and the similar phe-notypes of pip2Δ and oaf1Δ strains suggest that the proteins are
involved in the same process required for activation of genes encoding peroxisomal proteins. Therefore, we carried out experiments to study in more detail the involvement of Pip2p and Oaflp in the induction of genes encoding peroxisomal proteins. Here we show that the PIP2 and OAF1 genes are regulated differentially: the PIP2 gene is positively autoregulated, while the OAF1 gene is constitutively expressed. In the absence of Pip2p, overexpressed Oaflp binds ORE, probably as a homodimer. The most important finding of this investigation is that under physiological circumstances Pip2p and Oaflp form a functional heterodimer that binds to ORE.

MATERIALS AND METHODS

Yeast strains, media and culture conditions. The following S. cerevisiae strains were used in this study: BJ1991 (MATα, leu2 trpl ura3-52 pep4-3 prb1-1122) (Jones, 1977); and MF24-6x [MATα, leu2 his3 trpl1 c1-1 gal2 URA3::(CTA1-184-198) CYC1-lacZ] (Rottensteiner et al., 1996). BJ1991pip2A, BJ1991oaf1A, BJ1991pip2Aoaf1A, MF24-6x pip2A, MF24-6x oaf1A and MF24-6x pip2Aoaf1A were derived from the two parental strains by gene disruption of the PIP2 or OAF1 loci (Rottensteiner et al., 1996; this study).

For expression of Pip2p and the triple-haemagglutinin-epitope (HA)-tagged variant Pip2p-HA, under control of the TRP1 promoter, the required strains were transformed with centromeric plasmids pTP1-Pip2p and pTP1-Pip2HA3, respectively. For expression of Oaflp and the variant tagged with nine Myc epitopes, Oaflp-Myc, under control of the ADH2 promoter, integrative plasmids pADH2-OAFl and pADH2-OAFlMYC9T, respectively, were digested with BstXI and integrated in the TRP1 locus of the required strains. BJ1991pip2Aoaf1A expressing Pip2p-HA, and Oaflp-Myc, was prepared by transformation with centromeric plasmid pTP1-Pip2HA3 and integration of Sall-digested pADH2-OAFlMYC9U into the URA3 locus.

For catalase A assays, β-galactosidase assays, luciferase assays and bandshift assays, cells were cultured overnight on minimal medium containing 0.67% yeast nitrogen base (Difco), 2% glucose and 20 mg/ml amino acids as required. They were then incubated in minimal medium containing 0.3% glucose or 0.2% oleic acid/O.02% Tween-80 for 18 h until the cultures reached an A600 of 1.0–1.5.

Cloning procedures and gene disruptions. Standard techniques for DNA manipulations were used throughout this study (Sambrook et al., 1989). Yeast transformation was carried out as described (Ito et al., 1983).

To clone the FOX1 ORE, annealed oligonucleotides (5’-CATGAGGCGGCCGCTG-3’) and Notl-2 (5’-CATGACGCGCCGCCGCTG-3’) were ligated into the NotI site of pADH2-OAF1. The resulting construct was cloned into the NcoI site of pADH2-OAF1MYC9T, resulting in the NcoI site of pSKMP3-Norl. The newly generated NotI site was used to insert a 111-bp NotI triple-HA-epitope cassette (Tysers et al., 1993), resulting in pSKMP3-HA3. The 1.2-kb SalI-BgIII fragment of pTP1-Pip2p was replaced by the 1.3-kb SalI-BgIII fragment of pSKMP3-HA3.

The Oaflp overexpression plasmid pADH2-OAF1 was generated by inserting the 3.2-kb HindIII-Norl fragment from pAK56 and a PCR product containing the 744-bp NcoI-EcoRI fragment of the ADH2 promoter into an EcoRI-HindIII-digested pYEp358R (Myers et al., 1986). The resulting vector was cloned into the NotI site of pADH2-OAF1.

Enzyme activities. Activities of β-galactosidase, catalase A and luciferase were measured as described (Rottensteiner et al., 1996). Protein concentrations were measured by the method of Bradford (1976). All enzyme activities presented are the average of results obtained in at least two experiments.
Antibodies and western blotting. Tissue culture supernatants containing anti-Myc and anti-HA (cell lines 9E10 and 12CA5, respectively) mouse mAb were a gift from Anton Gartner. For western blotting, protein extracts were separated by SDS/PAGE and blotted to nitrocellulose filters. Secondary anti-mouse horseradish peroxidase antibodies and SuperSignal (Pierce) were used to visualise HA-tagged and Myc-tagged proteins.

Bandshift assays. Bandshift assays, DNA labelling and isolation of the FOX3 ORE and CTAl ORE were described previously (Rottensteiner et al., 1996). The FOX3 ORE probe was 85 bp in length, the FOX1 and CTAl ORE probes were 38 bp and 37 bp, respectively. The FOX1 ORE was isolated from pAK77 by digestion with EcoRI and BamHI. Bandshift reactions with 2 μl protein samples (30 μg) and 0.2 ng 32P-labelled DNA probes were carried out in the presence of 4 mM Tris/HCl, pH 8, 100 mM NaCl, 4 mM MgCl2, 25 ng/μl poly[d(I-C)·d(C-I)] and 4% glycerol in 20 μl. Bandshift reactions were incubated for 25 min at room temperature. Where protein−DNA complexes were supershifted, 1 μl mAb against HA (diluted 10 fold) or Myc-epitope (undiluted) was added to the bandshift reactions after 5 min incubation and reactions were further incubated for 20 min at room temperature.

RESULTS

Pip2p and Oaf1p are both required for the oleate-induction process and for ORE binding. We constructed a pip2Δaof1Δ double-deletion strain, which showed essentially the same phenotype as the strains with the single pip2Δ or aof1Δ deletions; no growth deficiencies were found on glucose, ethanol, glycerol or acetate media, but growth on oleate medium was impaired (data not shown).

To study the DNA-binding activities towards ORE, we performed DNA-bandshift assays with the 32P-labelled FOX3 ORE as a probe with extracts from wild-type, pip2Δ, aof1Δ and pip2Δaof1Δ cells grown on glucose or oleate (Fig. 1). The ORE-binding complex was present in extracts from wild-type cells grown on glucose and was induced in cells grown on oleate (Fig. 1), as described before (Rottensteiner et al., 1996). The ORE-binding complex was not present in extracts from pip2Δ, aof1Δ and pip2Δaof1Δ cells (lanes 3–5 and 7–9). The same results were obtained for ORE from CTAl and FOX1 (data not shown).

Despite the similarities between pip2Δ and aof1Δ strains, a subtle difference in phenotype was found when catalase A activity was measured in cells grown on oleate (Table 1). The amount of residual catalase A activity measured in aof1Δ cells was about 2−3-fold lower than in the pip2Δ cells. Similar results were obtained when luciferase activities were measured in a different strain transformed with reporter plasmid YTL985 expressing the firefly luciferase gene driven by the FOX3 promoter (Einerhand et al., 1991; Table 2). This indicated that Oaf1p in the absence of Pip2p could activate transcription of CTAl and FOX3 genes at a low level. The difference between luciferase activities in pip2Δ and aof1Δ cells was not detected when one of the CGG triplets of the ORE in the FOX3 promoter was mutated [plasmid YTL985 (Einerhand et al., 1991)], which is consistent with previous conclusions that the ORE is critical in transcription activation by Pip2p and Oaf1p (Rottensteiner et al., 1996; Luo et al., 1996).

Table 1. Determination of catalase A activity in wild-type and mutant cells. Catalase A activity in oleate-grown cells. Catalase A activity in wild-type cells was set at 100%.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Catalase A activity (%)</th>
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<tbody>
<tr>
<td>MF24-6x</td>
<td>100.0 ± 6.0</td>
</tr>
<tr>
<td>MF24-6x pip2Δ</td>
<td>26 ± 0.5</td>
</tr>
<tr>
<td>MF24-6x oaf1Δ</td>
<td>8.2 ± 1.6</td>
</tr>
<tr>
<td>MF24-6x pip2Δoaf1Δ</td>
<td>11.4 ± 1.9</td>
</tr>
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</table>

Table 2. Determination of luciferase activity in wild-type and mutant cells. Luciferase activity in oleate-grown cells. Luciferase activity in wild-type cells transformed with YTL985 was set at 100%.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Luciferase activity in cells transformed with YTL985 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJ1991</td>
<td>100.0 ± 6.6</td>
</tr>
<tr>
<td>BJ1991 pip2Δ</td>
<td>12.2 ± 2.2</td>
</tr>
<tr>
<td>BJ1991 oaf1Δ</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>BJ1991 pip2Δoaf1Δ</td>
<td>7.2 ± 0.4</td>
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</table>
ethanol, β-galactosidase was expressed at low levels and was induced 10–20-fold on oleate. In pip2Δ, oaf1Δ and pip2Δoaf1Δ this induction was absent. These results indicate that the PIP2 gene is not only under control of Pip2p itself but is also dependent on the presence of Oaflp.

To study whether or not the OAF1 gene is controlled in a carbon-source-dependent manner, similar experiments as described above were performed with an OAF1-lacZ reporter plasmid (Fig. 2B). We found that the OAF1 gene was constitutively expressed on all carbon sources tested and that the expression was independent of Pip2p. Deletion of the OAF1 gene resulted in a twofold increase in the expression of the lacZ reporter, indicating that Oaflp may have some negative effect on its own expression. That no ORE could be detected in the OAF1 promoter is in accordance with the lack of oleate induction. The finding of a progressive increase in the specific ORE-complex formation upon induction on oleate (Fig. 1; Luo et al., 1996; Rottensteiner et al., 1996) is therefore due to the induction of PIP2 expression.

**Pip2p and Oaflp are present in the ORE-binding complex.** We expressed Pip2p and Oaflp from heterologous promoters to ensure expression on oleate medium, even in deletion strains. This was especially important for expression of the PIP2 gene since its expression was low in the oaf1Δ strain. For Pip2p we applied the triose phosphate isomerase (TPZ1) promoter, and for Oaflp the alcohol dehydrogenase II (ADH2) promoter. We also epitope tagged Pip2p with a triple-HA epitope (HA3) and Oaflp with nine copies of a Myc epitope (Myc9). We subsequently tested all constructs for functionality (data not shown). The constructs complemented the mutant cells for growth on oleate plates and for catalase A activity. Moreover, the tagged versions were stably expressed in pip2Δ and oaf1Δ strains, as shown by western blotting (data not shown). In pip2Δ cells transformed with the TPZ1-PIP2 construct, catalase A activity was restored to 75% of the wild-type level. Expression of Oaflp in the pip2Δ and oaf1Δ strains restored catalase A activity to 74% and 139%, respectively, whereas no such enhanced levels were observed in oaf1Δ cells expressing Pip2p (Table 3). Apparently, Oaflp when overexpressed could act independently of Pip2p in activation of at least some genes encoding peroxisomal proteins.

**Fig. 2. Regulation of the PIP2 and OAF1 genes.** Reporter plasmids pPIP2lacZ (A) or pOAF1lacZ (B) were transformed into wild type, pip2Δ, oaf1Δ and pip2Δoaf1Δ cells. β-Galactosidase activity was measured after growth on the carbon source as indicated.

**Fig. 3. Analysis of ORE-binding complexes.** Extracts from oleate-grown cells were tested in the DNA-bandshift assay with the FOX3 ORE (upper panel) or the FOX1 ORE (lower panel) as a probe. For the FOX1 ORE, only the shifted complexes are shown. Lane 1, no extract added; lane 2, extract from wild-type cells; lanes 3–7, extracts from pip2Δ cells expressing Pip2p and Oaflp and their epitope-tagged variants as indicated; lanes 8–12, extracts from oaf1Δ cells expressing Pip2p and Oaflp and their epitope-tagged variants as indicated.

**Table 3. Catalase A activities in oleate-grown cells.** Determination of catalase A activities in oleate-grown wild-type and mutant cells expressing PIP2 and OAF1 genes from TPZ1 and ADH2 promoters, respectively. Catalase A activity in wild-type cells was set at 100%.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Catalase A activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF24-6x</td>
<td>100.0 ± 6.0</td>
</tr>
<tr>
<td>MF24-6x pip2Δ TPZ1-PIP2</td>
<td>75.0 ± 7.1</td>
</tr>
<tr>
<td>MF24-6x oaf1Δ TPZ1-PIP2</td>
<td>6.8 ± 0.3</td>
</tr>
<tr>
<td>MF24-6x oaf1Δ ADH2-OAF1</td>
<td>139.0 ± 2.8</td>
</tr>
<tr>
<td>MF24-6x pip2Δ ADH2-OAF1</td>
<td>74.0 ± 5.2</td>
</tr>
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</table>
When Oaf1p was expressed in the pip2A strain at high levels from the ADH2 promoter, the complex seen in extracts from wild-type cells was not restored. However, a different retarded complex was present that was located at a higher position in the gel than the wild-type complex (Fig. 3; complex B). Addition of the large Myc tag to Oaf1p shifted this complex to an even higher position complex C. Because Pip2p was absent in these extracts, we suggest that complexes B and C consist of an Oaf1p homodimer. The homodimers of Oaf1p migrated at a different position compared with that of complex A in wild-type cells.

Because Pip2p and Oaf1p and/or a different structure of the Oaf1p homodimer compared with that of complex A may explain this observation of different migration on the native gel.

The same set of incubations was carried out with protein extracts derived from an oaf1Δ strain. In oaf1Δ cell extracts no complex is visible (Fig. 3). Expression of Pip2p or Pip2p-HA, in the oaf1Δ strain did not restore formation of the complex, despite that the expressed Pip2p was present in these cells as shown by western blotting (data not shown). We conclude that expression of Pip2p did not lead to a homodimer complex of Pip2p capable of binding ORE as we observed for Oaf1p (compare lanes 9 and 10 with 6 and 7). Overexpression of Oaf1p in the oaf1Δ strain (i.e. in the presence of Pip2p) resulted in the formation of two complexes (complexes A and B). The complex at the lower position (complex A) migrated to the same position as the complex seen in wild-type extracts. Formation of this complex was dependent on Pip2p and, therefore, probably consisted of a Pip2p-Oaf1p heterodimer. The higher complex (complex B) was identical to the Oaf1p homodimer in lane 6. Both complexes shifted to a higher position in the gel when protein extracts were used containing the Myc-tagged Oaf1p (complexes A and B shift to D and C, respectively). This is consistent with the presence of Oaf1p in both complexes. Similar results were obtained with the FOX1 ORE (Fig. 3).

To show that Pip2p and Oaf1p bind to ORE as a heterodimer, we expressed the tagged proteins in the pip2Δoaf1Δ double-deletion strain and tested whether the complexes observed could be influenced in their migration by incubation with mAb directed against the epitope tags when the FOX3 ORE and FOX1 ORE were used as probes (Fig. 4). Addition of a protein extract from wild-type cells showed the ORE-binding complex (complex A), which was absent in the untransformed pip2Δoaf1Δ strain. When Pip2p-HA, was expressed alone in the pip2Δoaf1Δ strain no complex was observed. Expression of Oaf1p-Myc, resulted in formation of a complex higher up in the gel (complex C). When both tagged proteins were expressed, two complexes became visible (lane 6a).

The expression of both tagged proteins formed the basis for the identification of the proteins in the complexes. Addition of anti-HA mAb directed against Pip2p-HA, to the bandshift reaction supershifted only the lower complex (complex B) to a higher position in the gel (complex C), coinciding with the upper band, which indicates that Pip2p-HA, is present in complex B. Oaf1p cells expressing only Pip2p-HA, lacked the upper complex, and for the lower complex the same shift was observed upon addition of the anti-HA mAb (data not shown), excluding the possibility that the anti-HA mAb interfere with ORE recognition by Pip2p-HA.

When Oaf1p-Myc, was expressed at a low level from the OAF1 promoter instead of the ADH2 promoter, only complex B was present and addition of anti-Myc mAb supershifted complex B to a position coinciding with complex C (data not shown). Addition of anti-Myc mAb to a bandshift reaction (lane 6a) supershifted both complexes B and C (lane 6c). Complex B was shifted to a position coinciding with complex C, and complex C was shifted to complex D. Thus, this experiment revealed the presence of Oaf1p-Myc, in complexes B and C. A complete supershift with the anti-Myc mAb was not possible due to their limited concentration in the tissue-culture supernatants.

When both mAb were added, complex B was supershifted to complex C when anti-Myc or anti-HA mAb were bound. This complex was further supershifted to complex D when both antibodies bound simultaneously. Complex C was supershifted to complex D, to a comparable extent as in the presence of anti-Myc mAb (lane 6c). The resolution of the native bandshift gels was not sufficient to separate the high-molecular-mass complexes that form complex D (i.e. ORE DNA · Pip2p-HA, · anti-HA · Oaf1p-Myc, · anti-Myc and ORE DNA · Oaf1p-Myc, · Oaf1p-Myc, · anti-Myc). A complex of ORE DNA · Oaf1p-Myc, · anti-Myc · Oaf1p-Myc, · anti-Myc was not seen due to
either insufficient resolution of the bandshift gel or to the limited concentration of anti-Myc mAb in the tissue culture supernatants. From the observations we conclude that the upper complex (complex C) was an Oaf1p-Myc, homodimer and that the lower complex (complex B) was a heterodimer of Pip2p-HA, and Oaf1p-Myc+. Together, the experiments shown in Figs 3 and 4 allowed us to conclude that the complex present in wild-type cell extracts consisted of a heterodimer of Pip2p and Oaf1p. In extracts prepared from cells overexpressing Oaf1p an Oaf1p homodimer complex could be formed. When the relative amounts of the Pip2p-Oaf1p heterodimer and the Oaf1p homodimer binding to certain ORE were compared (Fig. 4), it appeared that the homodimer had a higher affinity for the FOX1 ORE than for the FOX3 ORE. This indicated that the type of ORE and the homodimeric and heteromeric complexes might influence promoter recognition. However, under physiological conditions in the presence of oleate, all Oaf1p was present in the heterodimer complex with Pip2p.

In a previous study (Rottensteiner et al., 1996) we reported that Pip2p was likely to bind ORE as a homodimer, based on gel-filtration experiments and binding of Escherichia coli-produced glutathione S-transferase(GST)-Pip2p-(1-179)-peptide in DNA-bandshift assays. The previous gel-filtration studies are in accordance with the present conclusion that Pip2p and Oaf1p are in a heteromeric complex, since Pip2p and Oaf1p are very similar in molecular mass (Pip2p 114.6 kDa; Oaf1p 118.8 kDa). The results from the GST-Pip2p fusion protein may in hindsight be explained by that GST is a dimeric protein (Ji et al., 1992). The GST part of the GST-Pip2p fusion protein might have been responsible for dimerisation, leading to functional ORE binding.

**DISCUSSION**

Peroxisomes form a flexible compartment in the eukaryotic cell and their number may vary from one to more than a hundred. The number, volume and morphology of peroxisomes, and the extent of transcription of genes encoding peroxisomal proteins are determined largely by the differentiation state of the cells and by their environment. For instance, administration of certain xenobiotics, such as hypolipidemic drugs or plasticizers, results in proliferation of peroxisomes in rodent tissues (Lock et al., 1989). These compounds act via peroxisome proliferation-activating receptors (PPAR), transcription factors belonging to the nuclear hormone-receptor family. Studies on PPARα knockout mice showed that PPARα is a crucial transcription factor involved in the control of the peroxisomal compartment size (Lee et al., 1995). The active DNA-binding complex consists of a heterodimer of PPAR and the retinoid-X receptor, which exerts its effect via peroxisome-proliferator-response elements (Schoonjans et al., 1996).

*S. cerevisiae* uses different types of transcription factors for the same purpose. Pip2p and Oaf1p have been described as transcription factors for the induction of many genes encoding peroxisomal proteins and for the enlargement of the peroxisomal compartment as a whole (Luo et al., 1996; Rottensteiner et al., 1996). The Pip2p gene (YOR362c) is localised on chromosome XV and the Oaf1p gene (YAL051w) on chromosome 1. They lie in regions that are likely to originate from an early genome duplication (Wolfe and Shields, 1997). Both proteins are highly similar and belong to the Zn,Cys family of transcription factors, of which Gal4p and Ppr1p are well-known examples. Pip2p and Oaf1p bind to ORE present in the upstream region of many peroxisomal protein-encoding genes. Strains in which either the *Pip2* or *Oaf1* gene is deleted show the same phenotype: inability to grow on the fatty acid oleate as sole carbon source.

Recently, Karpichev et al. (1997) reported that Pip2p (which they called Oaf2p) and Oaf1p can be coimmunoprecipitated, suggesting that heterodimeric complexes bound to ORE. Here we describe in more detail how Pip2p and Oaf1p function in the activation of promoters containing ORE. Upon addition of ORE-containing DNA to protein extracts derived from oleate-grown cells, a single shifted DNA-protein complex was formed (Fig. 1). By means of functional epitope-tagged derivatives of Pip2p and Oaf1p introduced into mutants deleted for their endogenous wild-type *PIP2* and/or *OAF1* genes, we could demonstrate that this shifted complex consisted of a heterodimer comprising Pip2p and Oaf1p (Figs 3 and 4). In singly deleted strains no evidence was found for the presence of Pip2p homodimers in the *oaf1Δ* strain, but in the *pip2Δ* strain a weak signal of a putative Oaf1p-homodimer-shifted complex was observed after long exposure of the gels (data not shown). Catalase A and luciferase measurements (Tables 1 and 2) suggested that Oaf1p alone may mediate a low level of *CTA1* and *FOX3* transcription. In a *pip2Δ* strain overexpressing Oaf1p, the presence of the Oaf1p homodimer could be demonstrated in DNA-bandshift experiments (Figs 3 and 4). This Oaf1p homodimer was transcriptionally active, as shown by measurement of catalase A activity in *pip2Δ* and *oaf1Δ* mutant strains overexpressing Oaf1p (Table 3).

Indications for the functional significance of the Pip2p-Oaf1p heterodimer and the Oaf1p-Oaf1p homodimer were obtained by studying the expression of the genes encoding the transcription factors (Fig. 2). This was carried out by replacing the corresponding reading frames with that of the gene encoding β-galactosidase and using this enzyme as a reporter for changes in gene expression. The *OAF1* gene was constitutively expressed under all conditions tested: glucose repression; ethanol derepression; and oleate induction. On the contrary, the *Pip2* gene was strictly controlled by carbon source; it was glucose-repressed and fully induced when cells were grown on oleate. This is consistent with the presence of an ORE in the promoter of the *PIP2* gene (Rottensteiner et al., 1996) and our observations that Pip2p stimulated its own synthesis. Therefore, we propose that the increasing amount of Pip2p in oleate-induced cells recruits the Oaf1p in the heterodimer that binds to the ORE. Combined with the results that heterodimer formation was preferred over Oaf1p-homodimer formation, we would like to suggest the following role for the individual transcription factors.

Under glucose conditions (i.e. non-inducing conditions for *PIP2*) the amount of Oaf1p exceeds that of Pip2p, and Oaf1p could exist as a homodimer. In addition to Pip2p, two Zn,Cys, proteins of unknown function and with high similarity to Oaf1p are present in *S. cerevisiae* (YIL054c and YCR106w). Therefore, we cannot exclude the possibility that Oaf1p also forms a heterodimer with another Zn,Cys, protein, recognising a binding site different from the ORE. When cells grow on oleate as sole carbon source they induce expression of Pip2p, thus mobilising Oaf1p into the heterodimeric complex at the expense of the homodimer Oaf1p complex. This apparent preference for heterodimer formation is similar to the behaviour of certain mammalian transcription factors, such as Fos and Jun. Fos and Jun contain single leucine zippers, a protein motif responsible for mediating protein interactions. The favoured interaction is between the zippers of Fos and Jun, resulting in Fos-Jun heterodimer formation. Self-interaction is observed between the zippers of Jun giving rise to Jun homodimer formation, while Fos only weakly interacts with itself (O'Shea et al., 1989, 1992; Smeal et al., 1989).

Combinatorial interactions enlarge the repertoire of transcriptional control using a limited number of different transcription factors. In higher eukaryotes, for instance, the superfamily of nuclear hormone receptors and the Fos-Jun and activating
transcription factor/cAMP-responsive-element-binding protein families are well known for this behaviour (Chambon, 1996; Lamb and McKnight, 1991). It was not clear whether this principle was also used in the Zn,Cys, proteins of S. cerevisiae. The well-studied members of this family interact with their DNA-binding sites as homodimers. The Pip2p-Oaf1p heterodimer is an exception to this rule. Indirect evidence suggests that a situation similar to Pip2p and Oaf1p may hold also for the related family members Pdr1p and Pdr3p, which are involved in multidrug-resistance processes in yeast. The PDR3 gene is positively autoregulated by its own product by binding to sequences in the 5' region of the gene. Moreover, activation of the PDR3 gene is tuned by a combination of the sequence of the ORE and the type of dimeric transcription complex that is bound to it.

If the situation we described here for Pip2p and Oaf1p applies to more Zn,Cys, family members, there is a possibility that factors that were described to homodimerise under overexpression conditions may form heterodimers under physiological conditions.

Additional work is needed to see to what extent this principle of combinatorial control occurs in S. cerevisiae. A first positive indication was observed in experiments in which we overproduced Pip2p and Oaf1p and used extracts from such cells to study the formation of gel-retarded complexes using ORE from different promoters. The ORE of the FOX3 gene gave rise to equal amounts of homodimeric Oaf1p and heterodimeric Pip2p. Oaf1p complexes; with the ORE of the FOX1 gene this ratio was different and much less of the heterodimeric complex was found (Fig. 4). These results might indicate that gene expression is tuned by a combination of the sequence of the ORE and the type of dimeric transcription complex that is bound to it.

Questions that need to be resolved are how the primary signal elicited by the presence of oleate in the growth medium is relayed to the Pip2p · Oaf1p complex and which of the two proteins receives the signal. Further research is needed to distinguish between several types of regulatory mechanisms. One possibility is direct stimulation by low-molecular-mass ligands such as acyl-CoA esters, a concept proposed for the activation of PPARα in mammals. In E. coli, direct binding of acyl-CoA to the transcriptional repressor FadR has been demonstrated, relieving genes encoding fatty acid degradative enzymes from repression (Raman and DiRusso, 1995). A different mechanism could be a relay via a protein-kinase signal-transduction cascade. In this connection the serine/threonine protein kinase Snf1p is somehow involved in the oleate-induction process and in general derepression events (Celenza and Carlson, 1986; Einerhand et al., 1992; Simon et al., 1992). Alternatively, in analogy with Gal80p controlling the action of Gal4p (Johnston et al., 1987; Lee et al., 1987), additional proteins may be involved in mediating the response elicited by the presence of fatty acids.

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