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A Complex Containing Two Transcription Factors Regulates Peroxisome Proliferation and the Coordinate Induction of β-Oxidation Enzymes in *Saccharomyces cerevisiae*

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Expression of the *POX1* gene, which encodes peroxisomal acyl coenzyme A oxidase in the yeast *Saccharomyces cerevisiae*, is tightly regulated and can be induced by fatty acids such as oleate. Previously we have shown that this regulation is brought about by interactions between *trans*-acting factor(s) and an upstream activating sequence (UAS1) in the *POX1* promoter. We recently identified and isolated a transcription factor, Oaf1p, that binds to the UAS1 of *POX1* and mediates its induction. A screening strategy has been developed and used to identify eight *S. cerevisiae* mutants, from three complementation groups, that are defective in the oleate induction of *POX1*. Characterization of one such mutant led to the identification of Oaf2p, a protein that is 39% identical to Oaf1p. Oaf1p and Oaf2p form a protein complex that is required for the activation of *POX1* and *FOX3* and for proliferation of peroxisomes. We propose a model in which these two transcription factors heterodimerize and mediate this activation process. The mutants that we have isolated, and further identification of the corresponding defective genes, provide us with an opportunity to characterize the mechanisms involved in the coordinate regulation of peroxisomal β-oxidation enzymes.

Peroxisomes are organelles that play important roles in cellular respiration and lipid metabolism. In most organisms, peroxisomes contain enzymes involved in fatty acid oxidation (β-oxidation) and catalase which decomposes the hydrogen peroxide generated from this process (for a review, see reference 21). Peroxisomes are essential for human survival, as demonstrated by the fact that the genetic disorder Zellweger syndrome, in which there is a lack of functional peroxisomes, is lethal (15, 22).

The size, abundance, and enzyme content of peroxisomes vary according to the cell type, organism, and metabolic requirements. In the yeast *Saccharomyces cerevisiae*, levels of peroxisomal β-oxidation enzymes are regulated by the available carbon source. The rate-limiting enzyme in the β-oxidation pathway is acyl coenzyme A (acyl-CoA) oxidase, which is encoded by a single-copy gene, *POX1* (9). We have previously shown that *POX1* expression is induced when *S. cerevisiae* is grown in the presence of oleic acid and that this transcriptional regulation is brought about by a protein, or proteins, binding to a specific upstream activating sequence (UAS1) in the *POX1* promoter (41, 42). UAS1-like sequences (also called oleate response elements [OREs]) (11, 14) are present in genes encoding many peroxisomal proteins, including the other enzymes of the peroxisomal β-oxidation cycle (10).

We recently purified one protein, Oaf1p, that binds to UAS1 when cells are grown in oleate medium (25). Furthermore, by cloning and subsequently disrupting the gene encoding Oaf1p, we demonstrated that this protein is required for the oleate induction of POX1. The deduced amino acid sequence of Oaf1p reveals a C_6 zinc cluster motif, placing it in the same family of transcription factors as Gal4p and Cyp1p (7, 20).

The genes *RTG1* and *RTG2* have been shown to be essential for full oleic acid induction of *POX1*, as well as of genes encoding peroxisomal catalase and a peroxisomal membrane protein, Pmp27 (6). The *RTG1* product is a basic helix-loop-helix transcription factor that binds upstream of the peroxisomal citrate synthase gene *CIT2* (23, 29) and is essential for its expression (24). The function of the Rtg2 protein is unknown. The fact that there are a number of genes required for the oleate induction of peroxisomal enzymes leads us to believe that there is a multiplicity of factors involved in the signaling pathway that regulates expression of peroxisomal β-oxidation enzymes.

To fully understand the mechanisms involved in the oleate induction of peroxisomal proteins in *S. cerevisiae*, we have taken a genetic approach to isolate mutants in which acyl-CoA oxidase is not induced when cells are grown in the presence of oleate. A double-screening strategy (described in Materials and Methods) has enabled us to screen for loss of *POX1* expression due to mutation in a *trans*-acting factor(s) involved in the pathway leading to *POX1* induction. Here we describe the use of this strategy to isolate oleate induction mutants belonging to three different complementation groups. Strains from one of these groups carry a mutation in *OAF2*, which encodes a transcription factor that is highly homologous to Oaf1p. These two proteins appear to form a complex that is required for the activation of genes encoding peroxisomal β-oxidation enzymes. A model for the possible mode of action of these two transcription factors in regulating peroxisomal enzymes is proposed.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this study are described in Table 1. Yeast strains were grown in either YPD (1% yeast extract, 2% peptone, 2% glucose), SD (0.67% yeast nitrogen base without amino acids, 2% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol), YPGO (0.1% [wt/vol] oleic acid and 0.25% [vol/vol] Tween 40 added to YPG), or YPRO (1% yeast extract, 2% peptone, 2% raffinose, 0.1% [wt/vol] oleic acid, 0.25% [vol/vol] Tween 40). Auxotrophic supplements were added to 20 μg/ml (40 μg/ml in the case of leucine) as required. When 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 5-fluoro-orotic acid (5-FOA) were used, they were added to give final concentrations of 0.02 and 0.1%, respectively.

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TABLE 1. *S. cerevisiae* strains used

Strain	Genotype	Reference
W3031A	*MAT***a** *leu2 ura3 trp1 ade2 his3*	38
W3031B	*MAT*α *leu2 ura3 trp1 ade2 his3*	38
OA1	*MAT***a** *leu2 ura3 trp1* [pRS304-*TRP1-POX1-lacZ ade2 his3 oaf1*::*HIS3*	25
OA2	*MAT*α *leu2 ura3 trp1*, *ade2 his3 oaf2*::*HIS3*	This study
W303-PU	*MAT***a** *leu2* [YIp367R-*LEU2-POX1-URA3*] *ura3 trp1* [pRS304-*TRP1-POX1-lacZ*] *ade2 his3*	This study
W303B-P	*MAT*α *leu2 ura3 trp1 TRP1*::*POX1-lacZ ade2 his3* [pRS303-*HIS3*]	This study
m7OA1	*MAT***a**/α *leu2*/*leu2* [YIp367R-*LEU2-POX1-URA3*] *trp1* [pRS304-*TRP1-POX1-lacZ*]/*trp1*[pRS304-*TRP1-POX1-lacZ*] *ade2*/*ade2 his3 oaf1*::*HIS3*/*his3*	This study
m65OA2	*MAT***a**/α *leu2* [YIp367R-*LEU2-POX1-URA3*/*leu2 ura3*/*ura3 trp1* [pRS304-*TRP1-POX1-lacZ*]/*trp1 ade2*/*ade2 his3*/*his3 oaf2*::*HIS3*	This study
$OA1HA_3$	*MAT***a** *leu2* [pRS305-*LEU2-OAF1*HA_3] *ura3 trp1* [pRS304-*TRP1-POX1-lacZ*] *ade2 his3 oaf1*::*HIS3*	This study
$OA2HA_3$	*MAT*α *leu2* [pRS305-*LEU2-OAF1*HA_3] *ura3 trp1* [pRS304-*TRP1-POX1-lacZ*] *ade2 his3 oaf2*::*HIS3*	This study
OA2myc	*MAT*α *leu2 ura3* [YIp357-*URA3-POX1-lacZ*] *ade2 his3 oaf2*::*HIS3 trp1* [pRS304-*TRP1-OAF2*myc]	This study
$OA2HA_3$myc	*MAT*α *leu2* [pRS305-*LEU2-OAF1*HA_3] *ura3* [YIp357-*URA3-POX1-lacZ*] *his3 oaf2*::*HIS3 trp1* [pRS304-*TRP1-OAF2*myc] *ade2*	This study

Mutant isolation and genetic analysis. A double-screening strategy was used to isolate mutants in which *POX1* is not induced in the presence of oleate. The first screen used the expression of the *lacZ* gene under the control of the *POX1* promoter (p*POX1*lz), resulting in β-galactosidase induction when cells are grown on plates containing carbon sources such as glycerol or raffinose, together with oleate. This leads to formation of blue colonies when the plates contain X-Gal. Following mutagenesis, cells that fail to turn blue could carry mutations either in the *lacZ* gene, in the *POX1* promoter of the plasmid, or in *trans*-acting factors involved in the pathway leading to *POX1* induction. To distinguish among these types of mutants, we created a plasmid which contains the *URA3* gene under the control of the *POX1* promoter (p*POX1*U). Yeast strains that contain a wild-type copy of the *URA3* gene are unable to grow in the presence of the pyrimidine analog 5-FOA (1). Cells transformed with this plasmid and that are able to grow on plates containing 5-FOA could carry mutations in the *URA3* gene, in the *POX1* promoter of this plasmid, or in *trans*-acting factors in the oleate induction pathway as described above. To enrich for this latter category of mutants, a strain (W303-PU) in which both of the plasmids described above were integrated into the *S. cerevisiae* genome was prepared. When this strain is grown on YPGO plates containing X-Gal and 5-FOA, it grows poorly because the *URA3* gene is expressed from the *POX1* promoter (Fig. 1). Following mutagenesis, strains that are able to grow in the presence of 5-FOA, and are white or pale blue, were selected as candidates for carrying mutations in genes encoding *trans*-acting factors in the oleate induction pathway.

Mutagenesis with 3% ethylmethanesulfonate was carried out with 60% mortality as described previously (33). The cells were grown at 30°C for 5 to 7 days on plates containing YPRO and X-Gal. White and pale blue colonies were selected and streaked, in duplicate, onto YPGO plates, one supplemented with X-Gal and the other supplemented with X-Gal and 5-FOA. Cells were grown at 30°C for 2 days, and putative mutants were selected (Fig. 1).

Each of the mutant clones selected was crossed with a tester strain, W303B-P, which was constructed as follows. W3031B, a strain isogeneic to W3031A but of the opposite mating type, was transformed with two plasmids. First, we integrated p*POX1*lz into the *TRP1* site of W3031B so that *lacZ*, under control of the *POX1* promoter, will be induced when this strain is grown in YPGO. In addition, we integrated the plasmid pRS303 (34) into the *his3* locus of this strain so that it would carry a wild-type copy of the *HIS3* gene for future selection of diploids.

Diploids resulting from the crossing of mutants with W303B-P were selected on plates lacking leucine and histidine. They were then grown on YPRO plates containing X-Gal. All diploids gave a control phenotype in that they produced dark blue colonies, indicating that the mutations in each mutant strain are recessive. This was also confirmed by Northern blot analysis. The diploids obtained from each cross were sporulated, and 10 to 12 tetrads from each were dissected. All showed a 2:2 segregation pattern for *POX1* induction by oleate, measured both by β-galactosidase activity expressed from the *POX1-lacZ* construct and by Western blot analysis using an antibody to acyl-CoA oxidase (generously supplied by Joel Goodman). This result demonstrates that there is a single gene defect in each mutant strain.

The mutants were assigned to complementation groups by crossing strains with appropriate phenotypes, obtained from tetrad analysis performed as described previously (33). Diploids were selected on minimal plates with the appropriate auxotrophic supplements. The diploids strains were then grown in YPGO medium and analyzed for acyl-CoA oxidase induction by Western blot analysis.

RNA purification and Northern analysis. Total yeast RNA was isolated, resolved, and transferred to membranes as previously described (25). The membranes were prehybridized for 1 h and then hybridized overnight in the presence of [α-^{32}P]dATP-labeled random-primed probes (10^7 cpm/ml) at 65°C, using standard conditions (32). After extensive washing, the filters were exposed to a PhosphorImager screen and scanned. The intensities of the corresponding bands were determined by using Molecular Dynamics PhosphorImager software (Imagequant); values were normalized by using 3-phosphoglycerate kinase (*PGK1*) mRNA expression levels as an internal control for loading.

Plasmids (i) p*POX1*lz. Plasmid p13570, which contains the *POX1* promoter fused, in frame, with the *lacZ* gene (42), was digested with *Kpn*I and *Hin*dIII and cloned into *Kpn*I/*Hin*dIII-digested YIp357 (26), to create a plasmid named YIpKH. Next, YIpKH was digested with *Nco*I, the ends were rendered blunt by treatment with T4 DNA polymerase, and the plasmid was then digested with *Kpn*I. The resultant 4.5-kb fragment was cloned into pRS304 (34) which was previously digested with *Kpn*I and *Nea*I.

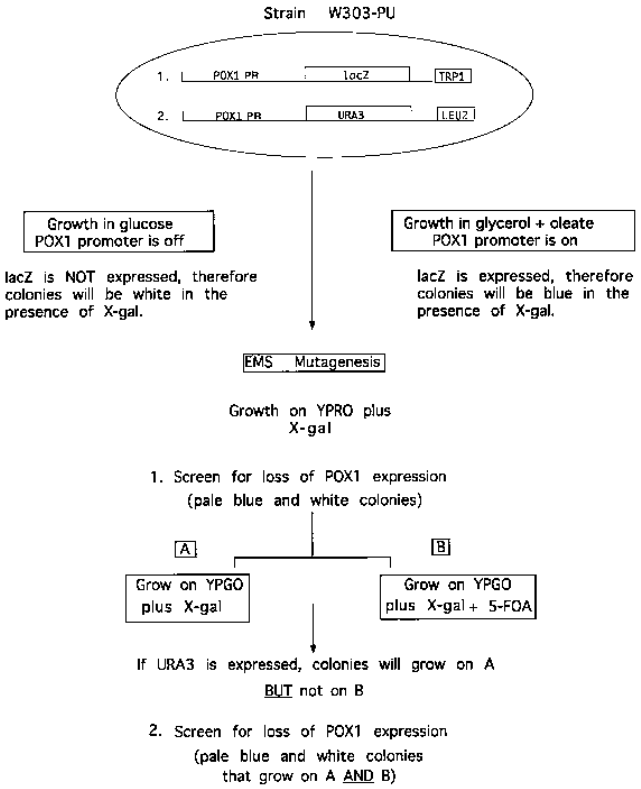

FIG. 1. Strategy for the isolation of yeast strains that carry mutations in the pathway leading to the induction of peroxisomal acyl-CoA oxidase by oleate. Plasmid 1 consists of the *lacZ* gene under control of the *POX1* promoter (PR), integrated at the *trp1* locus. Plasmid 2 consists of the *URA3* gene under control of the *POX1* promoter, integrated at the *leu2* locus. (See text for details of the screening strategy.)

(ii) p*POX1*U. The vector YIp367R (26) was digested with *Pst*I and *Sma*I, and a *Pst*I/*Sma*I fragment containing the *S. cerevisiae URA3* gene was cloned into this vector to create a plasmid named YIpURA3. Two DNA oligonucleotide primers, 5′-GCGCGCGAATTCAGATCTCGACCAA-3′ and 5′-GCGCGCGGATCCATCGCAATACTAATTT-3′, were used in a PCR with plasmid DNA prepared from p13570. The amplified product was cloned into pT7Blue to create a plasmid named pR1T7B. Next, YIpURA3 was digested with *Pst*I, and the ends were rendered blunt with T4 DNA polymerase. The plasmid was then digested with *Hin*dIII. A 450-bp *Hin*dIII/*Sma*I fragment, containing the *POX1* promoter, was excised from pR1T7B and was subcloned into YIpURA3 to create a plasmid named p*POX1*U.

Epitope tagging. The 10-amino-acid epitope from human c-myc (EQKLISEEDL), which can be recognized by the monoclonal antibody 9E10 (18), was used for tagging the *OAF2* gene product. The myc epitope sequence was added to the extreme carboxyl terminus of the *OAF2* gene by PCR amplification with primers y130 (5′-**CTA**CAAGTCTTCTTCAGAAATAAGCTTTTGTTCGTCGTTCTGGAAAAGTA-3′; underlined bases are complementary to the plus strand encoding the myc epitope, with the stop codon in boldface) and y131 (5′-TTAAAAACTACTATGAC-3′, nucleotides 2876 to 2896 of *OAF2*). The resultant 110-bp DNA fragment contained the 3′ end of the *OAF2* open reading frame (minus its stop codon) followed by the myc sequence. This fragment was blunt ended, digested with *Nco*I, and subcloned into the *Nco*I/blunt-ended *Sal*I sites in pOAF27B to create pOAF2-myc. A DNA fragment encoding the complete OAF2-myc protein was released by digestion with *Sac*I and *Pst*I and was subcloned into the corresponding sites in pRS304 to create pRSOAF2-myc.

To obtain a genomic copy of the *OAF1* gene, we screened a yeast genomic library (28) with a PCR product encoding *OAF1* (25). A positive clone was identified, and a 4.8-kb *Hin*cII fragment containing the *OAF1* gene was subcloned into the *Eco*RV site of pT7Blue (Novagen) to create pOAF1.

The nine-amino-acid epitope of influenza virus hemagglutinin (HA) (YPYDVPDYA), which can be recognized by monoclonal antibody 12CA5 (18), was used for tagging the *OAF1* gene product. Three tandem copies of the HA epitope were added to the carboxyl terminus of the *OAF1* product by the PCR. Primers y124 (5′-CATTCGGAAATGCTGTTCC-3′) and y125 (5′-TGGGACGTCGTATGGGTAAGCAAAGTCATTGCCAAACAAAA-3′) were used to synthesize a 345-bp DNA fragment containing the 3′ open reading frame of *OAF1* (minus its stop codon) followed by the DNA sequence encoding six amino acids of the HA epitope. The underlined bases are complementary to the plus strand encoding HA. The PCR fragment was blunt ended, digested with *Eag*I, and subcloned into the *Eag*I/*Sma*I sites in pOAF1. The resulting construct was then digested with *Aat*II and *Hin*cII, and the released DNA was subcloned into the corresponding sites in pT7pebHA$_3$ (45). This produced pOAF1-HA$_3$ which contains the *OAF1* gene followed by DNA sequence encoding three copies of the HA epitope. A DNA fragment encoding OAF1-HA$_3$ was released from this plasmid by digestion with *Hin*dIII/*Sac*I and was subsequently subcloned into the corresponding sites in pRS305 to create pRSOAF-1HA$_3$.

Coimmunoprecipitation. Cellular extract was prepared as described previously (41), and 100 μl of the extract (approximately 500 μg of protein) was diluted with 500 μl of buffer H (25 mM Tris-HCl [pH 7.4], 15 mM $MgCl_2$, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 0.1 μg of sodium metabisulfite per μl, 1 mM phenylmethylsulfonylfluoride, 0.1 μg each of chymostatin, pepstatin, leupeptin, and antipain per μl). Two micrograms of monoclonal antibody 12CA5, which recognizes the HA epitope (18), or 9E10, which recognizes the myc epitope (18), was added to the extract. The mixture was incubated at 4°C for 2 h with gentle rotation. Then 50 μl of protein G-agarose (Boehringer Mannheim) was added, and the mixture was incubated for a further 2 h. The agarose beads were collected by centrifugation and were washed three times with buffer H. Bound proteins were eluted by boiling the sample in sodium dodecyl sulfate (SDS) gel loading buffer. The beads were pelleted by centrifugation; the supernatant was loaded on an SDS-polyacrylamide gel and was then subjected to polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Construction of an *OAF2* disruption strain. To disrupt the wild-type *OAF2* gene, a 6.1-kb *Sal*I/*Sac*I fragment containing the *OAF2* gene was subcloned into the *Sal*I/*Sac*I sites of pT7Blue (Novagen), resulting in a plasmid named pOAF27B. Next, a 1.7-kb *Bam*HI/*Cla*I fragment containing the *S. cerevisiae HIS3* gene was ligated into the *Bam*HI/*Cla*I-digested pOAF27B. This led to a deletion of 538 bp of the *OAF2* open reading frame. The resulting plasmid, named pOaf2::HIS37B, was digested with *Sal*I and *Sac*I to yield a 7.25-kb fragment composed of the *HIS3* gene flanked by *OAF2* 5′ and 3′ sequences (see Fig. 5a). This fragment was transformed into *S. cerevisiae* W3031B. Selected transformants were screened for correctly targeted genomic integration by Southern blot analysis of total DNA isolated from the transformants. The transformants were also screened for oleate induction of acyl-CoA oxidase by preparing cell extracts from YPGO-grown cells and performing Western blot analysis. One *OAF2*-disrupted strain, named OA2, was selected for further studies.

Immunoelectron microscopy. The electron microscope immunolocalization with gold particles was carried out as described previously (43), using 10-324 at 1:100 and protein A conjugated to 15-nm gold at 1:50. 10-324 is a rabbit antiserum raised against total peroxisomal proteins from *Candida tropicalis* (36) which cross-reacts with a number of peroxisomal proteins from *S. cerevisiae* (unpublished data and reference 44).

TABLE 2. β-Galactosidase activities and assigned complementation groups of the mutant strains

Strain	β-galactosidase activity (U/mg of protein)		Complementation group
	YPG	YPGO	
m7	0.061	0.082	1
m10	0.023	0.022	2
m16	0.057	0.057	2
m62	0.077	0.071	2
m64	0.040	0.066	2
m65	0.052	0.072	2
m85	0.018	0.091	2
m50	0.012	0.177	3
W303-PU	0.128	1.67	

Other methods. DNA band shift, protein, and β-galactosidase assays were performed as described previously (41). Western and Southern blot analyses were carried out by using standard procedures (32). DNA recombination techniques were generally as described by Sambrook et al. (32), and yeast genetics were as specified by Sherman et al. (33).

RESULTS

Isolation of oleate induction mutants. We used the mutagenesis and screening strategy described above (Fig. 1) to isolate mutants that were unable to induce *POX1*. Mutagenized W303-PU cells, in which both *lacZ* and *URA3* are under the control of the *POX1* promoter, were grown on YPRO plates in the presence of X-Gal. YPRO was used in place of YPGO for this screen because *POX1* expression in the presence of raffinose is lower than in glycerol (unpublished data), resulting in more distinct differencies between white (loss of the *POX1-lacZ* plasmid), pale blue (possible mutants of interest), and dark blue (wild-type) colonies, due to oleate induction.

Of 10^5 colonies, approximately 500 appeared pale blue. These colonies were then grown on YPGO plates containing X-Gal in the presence and absence of 5-FOA (Fig. 1). Those strains that were able to grow well in the presence of this drug and that were pale blue (approximately 100) were selected for further analysis. The selected colonies were then tested for levels of β-galactosidase activity in extracts from cells grown in either YPG or YPGO medium. Some of these candidates displayed no β-galactosidase activity in either medium, some showed low activity when grown in YPG medium and a 10-fold increase in activity when grown in YPGO medium (representing levels in parental strain), and others had low activity when grown in YPG or in YPGO. The latter group were candidates for being defective in the oleate induction pathway. RNA was extracted from each of these strains, and acyl-CoA oxidase mRNA levels were examined by Northern blotting. The eight mutants in which *POX1* expression was not induced, or was markedly lower than in the wild-type strain grown in the presence of oleate, were further analyzed.

Relative β-galactosidase activities in these eight mutants compared to the control strain are shown in Table 2. The levels of β-galactosidase activity in the mutants grown in YPG medium were variable and usually slightly lower than that of the parental strain. However, the activities in the mutant strains grown in the presence of oleate were induced only in m50, confirming that most of these mutants lacked the capacity for oleate induction of *POX1*. In the case of m50, the absolute level of β-galactosidase was lower than in the control (sevenfold in oleate-grown cells); however, the extent of induction by oleate was similar to the control value (Table 2).

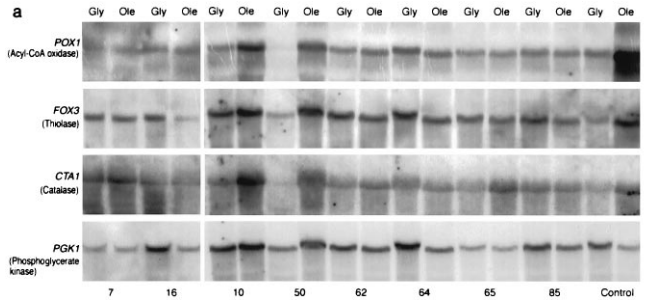

FIG. 2. Levels of acyl-CoA oxidase, thiolase, and catalase mRNAs in wild-type and mutant strains. (a) Northern analysis of *POX1*, *FOX3*, *CTA1*, and *PGK1* expression in a control (W303-PU) and eight mutant strains grown in YPG (Gly) or YPGO (Ole) medium. (b to d) Quantitation of the mRNA levels of *POX1* (b), *FOX3* (c), and *CTA1* (d) in the strains shown in panel a, using a PhosphorImager and Imagequant software. In each case, the message was normalized by using *PGK1* levels as an internal control for loading. The results are expressed as percentages of the level in the control strain grown in the presence of oleate, set at 100%.

All eight mutants selected carried single gene defects and displayed recessive phenotypes as determined by genetic analysis (see Materials and Methods). The mutants were categorized into three complementation groups (Table 2).

Expression of peroxisomal proteins in the oleate induction mutants. The levels of acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, and catalase A were determined by Northern blot analysis of total RNA isolated from mutant and control cells grown in YPG or YPGO medium (Fig. 2a). The intensity of signal for each message was quantitated, and the fold induction was normalized to 3-phosphoglycerate kinase (*PGK1*) levels as described in Materials and Methods. Although expression of *PGK1* is known to be induced severalfold by growth of cells in glucose (5), we have found that, in general, the mRNA levels are similar in cells grown in YPG or YPGO; therefore, we use it as an internal control for loading.

In control cells, the *POX1* message was induced approximately 14-fold in oleate-grown cells compared to the level in cells grown in the presence of glycerol (Fig. 2a and b). As shown in Fig. 2b, expression of *POX1* in most of the YPG-grown mutants was approximately equal to the level in YPGO-grown cells. However, in the cases of m16 and m50, there was approximately a threefold increase in expression in YPGO-grown cells compared to those grown in YPG. These values are taken from a single experiment. Northern analysis and quantitation have been performed on each mutant at least twice, and while the actual numbers vary slightly, the differences in expression levels are reproducible.

The level of thiolase (*FOX3*) mRNA was increased eightfold in control cells grown in oleate medium compared to those grown in glycerol medium (Fig. 2a and c). In mutants m7, m10, m16, and m62, the level of thiolase expression was the same in cells grown in YPG or YPGO medium. In m64, m65, and m85, there appeared to be a slight induction of thiolase message in cells grown in the presence of oleate. In the case of m50, the relative induction of thiolase was the same as in control cells, but the absolute level of expression was fivefold lower.

Catalase expression was induced fourfold in control cells grown in oleate medium (Fig. 2a and d). The catalase expression levels in mutants m10, m16, m50, m62, m64, and m85 grown in glycerol were approximately the same as in control cells. In m65 and m7, the expression levels of catalase message in glycerol-grown cells were higher than in the control strain by three- and fourfold, respectively. Catalase was marginally induced in mutants m7, m10, and m62 and was approximately doubled in the remaining mutants grown in the presence of oleate.

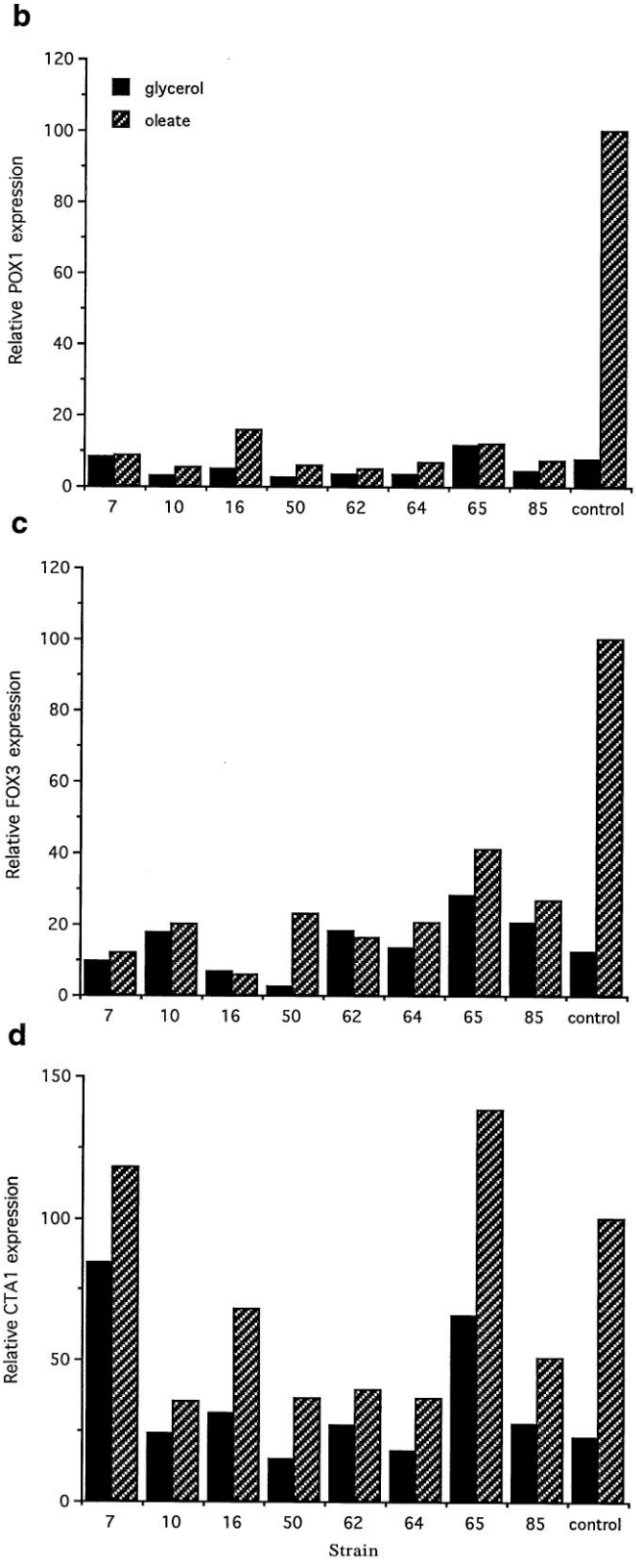

TABLE 3. Number of cells containing visible peroxisomes in different strains

Sample	Growth medium	No. of cells with peroxisomes (100 cells counted)
W3031A	YPGO	67
		66
		68
W3031A	YPG	29
		37
		31
OA1	YPGO	14
		10
		8
m65	YPGO	29
		31
		18
m50	YPGO	45
		33
		43

In summary, all of the mutant strains except m50 were defective in the induction of *POX1* and of *FOX3*. In m50, both of these genes are expressed at low levels in both media but the fold induction resembles that of wild-type cells. This is similar to the pattern seen for β-galactosidase activity in this strain. Expression of *CTA1* in cells grown in either medium appears to be variable.

Morphology of the oleate induction mutants. The mutant strains deficient in *POX1* inducibility by oleate were examined by immunoelectron microscopy in order to compare the morphologies of the peroxisomes between these strains and a control strain. Samples were prepared by preculturing cells in YPD medium, then transferring them to either YPG or YPGO, and culturing them for a further 18 h. The number and size of peroxisomes varied for the different samples. A random sample of 100 cells was examined on three separate occasions for wild-type cells (W3031A) grown in YPG or YPGO and for mutants m65 and m50 and a strain in which *OAF1* was disrupted (OA1), each grown in YPGO medium (Table 3).

In the wild-type strain grown in YPGO, numerous peroxisomes were easily identified and were often found in clusters (Fig. 3a). Upon immunostaining, the peroxisomes were labeled, often densely, with gold particles (Fig. 3a and b). In contrast, when this strain was grown in YPG medium, there were fewer peroxisomes and the labeling was weak (Table 3; Fig. 3c). Mutant m50 closely resembled the wild-type strain morphologically, in that peroxisomes were easily identifiable and were clearly labeled by the immunogold procedure, although not as densely as the wild-type strain (Fig. 3d).

Strain OA1 contained very few identifiable peroxisomes when grown in the presence of oleate (Table 3). Peroxisomes that were visible were labeled with the immunogold procedure but appeared to be much smaller than in wild-type cells (Fig. 3e). Peroxisomes in m65, grown in the presence of oleate, were slightly more numerous than in strain OA1; however, they were also smaller than in wild-type cells (Table 3; Fig. 3f). In both OA1 and m65, there was a greater accumulation of lipid droplets than seen in the wild-type strain (compare Fig. 3a with Fig. 3e and f). This is not surprising, as it is expected that these mutant strains would be unable to metabolize the oleic acid present in the growth medium.

Complementation of mutants by *OAF1*. We tested the mutants isolated by our screening strategy to determine whether any of them carried a mutation in the *OAF1* gene, which encodes a transcription factor required for oleate induction of *POX1* (25). For this purpose, a copy of the *OAF1* gene was integrated into the *his3* locus of each of the yeast mutants. Transformants were then analyzed for β-galactosidase activity in cells grown in YPG and YPGO media. In mutant m7, oleate induction of the *POX1-lacZ* construct was restored (Table 4). A cross between an appropriate m7 segregant and strain OA1, in which *OAF1* is disrupted, named m7OA1, had a mutant phenotype for *POX1* induction by oleate (Table 4). This result confirms that *OAF1* is the mutated gene in m7.

In summary, of the mutants isolated thus far, m7, the single member of complementation group 1, is defective in the *OAF1* gene. The remaining mutants, belonging to complementation groups 2 and 3, are defective in different genes.

Cloning of *OAF2* by functional complementation. The mutant strain m65 was chosen as a representative of complementation group 2 and was used to identify the defective gene carried by each member of this group. To clone this gene, m65 was transformed with a genomic library (28). Approximately 50,000 colonies grew, and these were replica plated onto YPGO plates containing X-Gal. Fifty-six transformants that formed dark blue colonies were selected and tested for β-galactosidase activity. Of the transformants selected, three colonies had wild-type levels of activity when grown in YPG medium and a 10-fold induction in activity when grown in the presence of oleate. Restriction analysis of the plasmid DNA isolated from these three clones demonstrated that they were identical. Further analysis was carried out on one of the plasmids, named pm65. The plasmid contained a 16-kb insert which was able to restore *POX1* induction by oleate when reintroduced into m65 cells. To subclone the smallest DNA fragment that was able to complement the mutation in m65, pm65 DNA was partially digested with *Sau*3A and was resolved by agarose gel electrophoresis. A region corresponding to a size of approximately 5 to 7 kb was excised, and the purified DNA was subcloned into a centromeric plasmid. Pooled subclones were transformed back into m65 and tested for *POX1* induction by oleate. The smallest fragment with the ability to restore *POX1* induction was found to be 6.5 kb. Approximately 200 nucleotides at either end of this insert were sequenced and compared to known sequences in the yeast genome database (Stanford Genomic Resources). The search revealed a perfect match with a genomic region of chromosome XV, which contains a 2,988-bp open reading frame between positions 1,020,217 and 1,023,204. The predicted 996-amino-acid sequence encoded by this open reading frame has high homology to Oaf1p, a transcription factor involved in the oleate-induction of *POX1* (25).

Upon sequencing the carboxy-terminal region of *OAF1*, we noticed two discrepancies between our sequence and the *OAF1* sequence contained in the yeast genome database, which consisted of extra G's at positions 3124 and 3130. Correction of these errors allowed us to conclude that the actual stop codon is located after the codon for amino acid 1047 (Fig. 4a); thus, Oaf1p is 15 amino acids shorter than reported by others (3). The predicted protein sequence of the gene that rescues m65 is 39% identical to the corrected Oaf1p sequence (Fig. 4b). Thus, we refer to this gene as *OAF2* and to the encoded protein as Oaf2p. This protein contains a region homologous to the consensus sequence for the fungal C_6 zinc cluster (amino acids 23 to 52) and a leucine zipper motif (amino acids 320 to 362); thus, it has the capacities both to bind to DNA and to dimerize (19).

Characterization of *OAF2*. To confirm that the mutation in m65 is in the gene that we cloned, we prepared a strain (OA2) in which this gene was disrupted, using the one-step gene

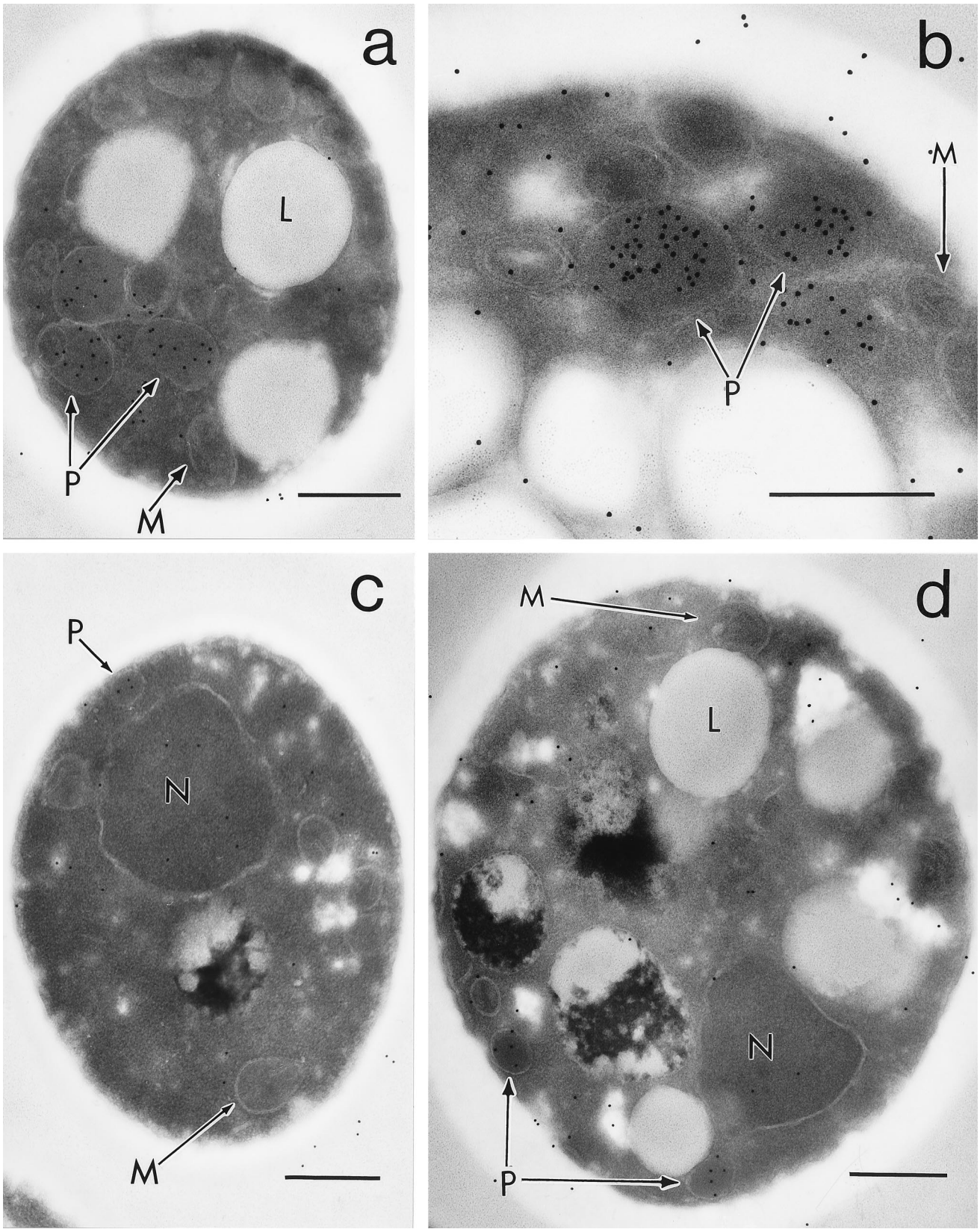

FIG. 3. Electron micrographs of mutant and wild-type cells grown in YPGO and of wild-type cells grown in YPG. All cells were immunocytochemically labeled to detect peroxisomal proteins. (a) Wild-type cells grown in YPGO. Arrows indicate peroxisomes (P) and mitochondrion (M); a lipid droplet is labeled L. (b) Part of a cell different from that shown in panel a, at a higher magnification to demonstrate dense labeling of peroxisomes. (c) Wild-type cell grown in YPG medium. At most, one or two peroxisomes are detectable in each cell. The nucleus is labeled N. (d) m50 grown in YPGO medium. Two peroxisomes are marked with arrows. (e) Strain OA1 (in which *OAF1* is disrupted) grown in YPGO. One small peroxisome is indicated by an arrow. (f) m65 grown in YPGO medium. Bars, 0.5 μm.

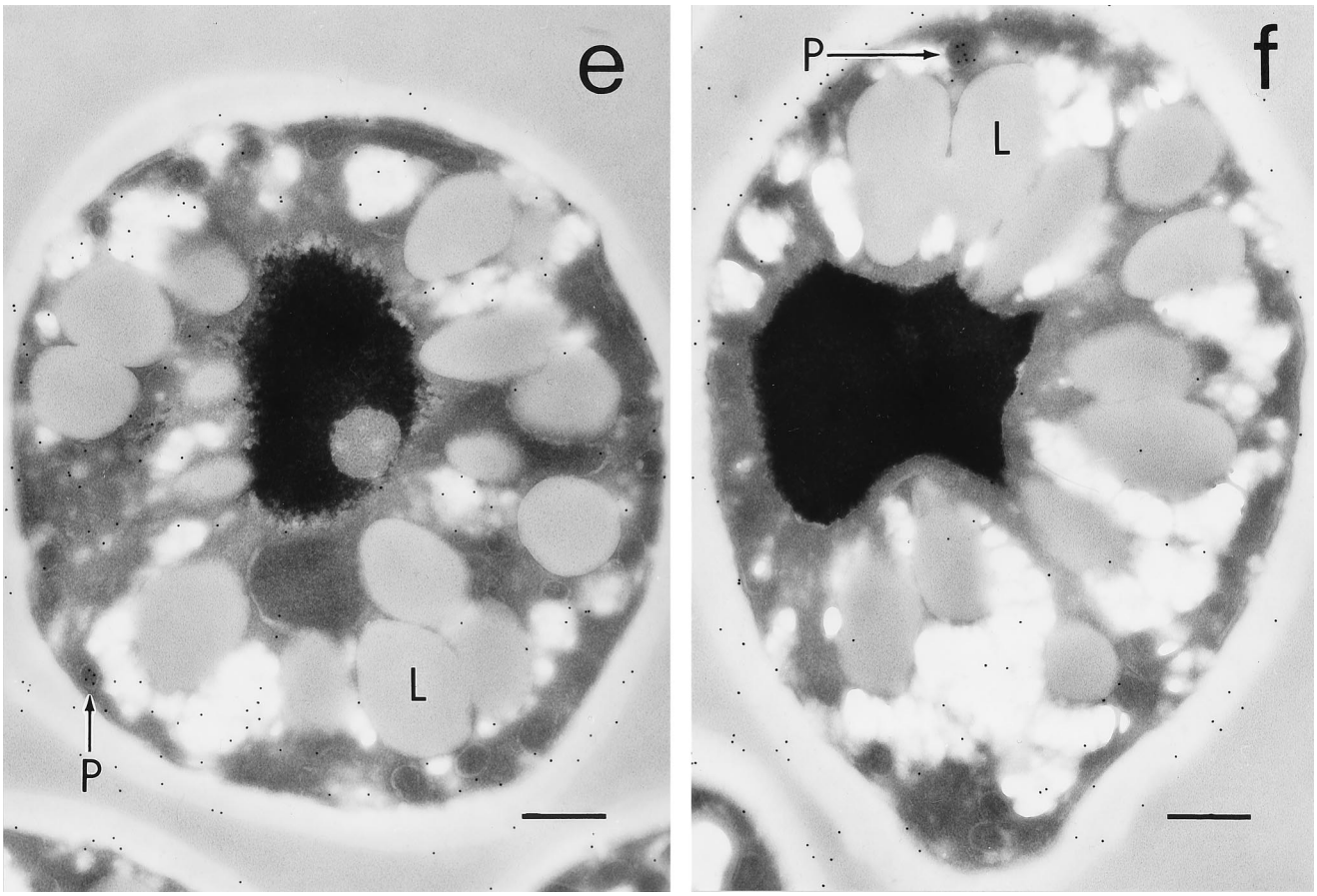

FIG. 3—*Continued.*

disruption procedure (30). A 538-bp *Bam*HI/*Cla*I fragment of the open reading frame of *OAF2* was replaced with the yeast *HIS3* gene as described in Materials and Methods (Fig. 5a). The Oaf2::HIS3 construct was excised on a linear DNA fragment and transformed into *S. cerevisiae* W3031B. Southern blot analysis of total genomic DNA isolated from His^+ transformants indicated a correctly targeted chromosomal integration (Fig. 5b).

Growth on oleate failed to induce *POX1* expression in the disrupted strain. Furthermore, oleate induction was not restored in a diploid strain (m65OA2) obtained by crossing OA2 with m65. Ten tetrads from the resultant diploid strain were tested for acyl-CoA oxidase expression. All of the spores from each tetrad failed to show induction of acyl-CoA oxidase when grown in oleate medium, confirming that *OAF2* is the mutated gene in m65.

To be able to determine whether the presence of Oaf2p is required for *OAF1* expression, we added three copies of the influenza virus HA epitope to the extreme carboxyl terminus of the *OAF1* open reading frame. The triple-tagged Oaf1p, termed OAF1-HA_3, was first tested for its functionality by expressing it in strain OA1 in which the endogenous *OAF1* gene is disrupted (25). Using lysates from OA1 and OA1HA_3 cells, we performed DNA band shift and β-galactosidase assays to confirm that the tagged version of the protein formed a specific UAS1-protein complex and restored oleate inducibility of *POX1* (data not shown). Next we expressed OAF1-HA_3 in strain OA2, in which *OAF2* is disrupted (OA2HA_3). Lysates from transformed cells grown in the presence of glucose, glycerol, or oleate were examined by using SDS-PAGE and Western blotting with antibody 12CA5, which recognizes the HA epitope, to determine whether Oaf1p was present. Oaf1p was clearly detectable in cell lysates from all three growth conditions (Fig. 6). This result indicates that while disruption of *OAF2* causes loss of *POX1* induction, it does not abolish *OAF1* expression. This result further demonstrates that *OAF1* alone is not able to facilitate oleate activation of peroxisomal enzymes.

Oaf2p is induced by oleate. The *OAF2* promoter region contains two UAS1-like sequences, located at positions −324

TABLE 4. β-Galactosidase activities in mutants transformed with a wild-type copy of *OAF1*

Strain	β-Galactosidase activity (U/mg of protein)	
	YPG	YPGO
m7	0.117	1.16
m10	0.087	0.091
m16	0.092	0.063
m50	0.013	0.224
m62	0.097	0.095
m64	0.058	0.067
m65	0.122	0.167
m85	0.115	0.119
m7OA1	0.206	0.151
W303-PU	0.090	1.80

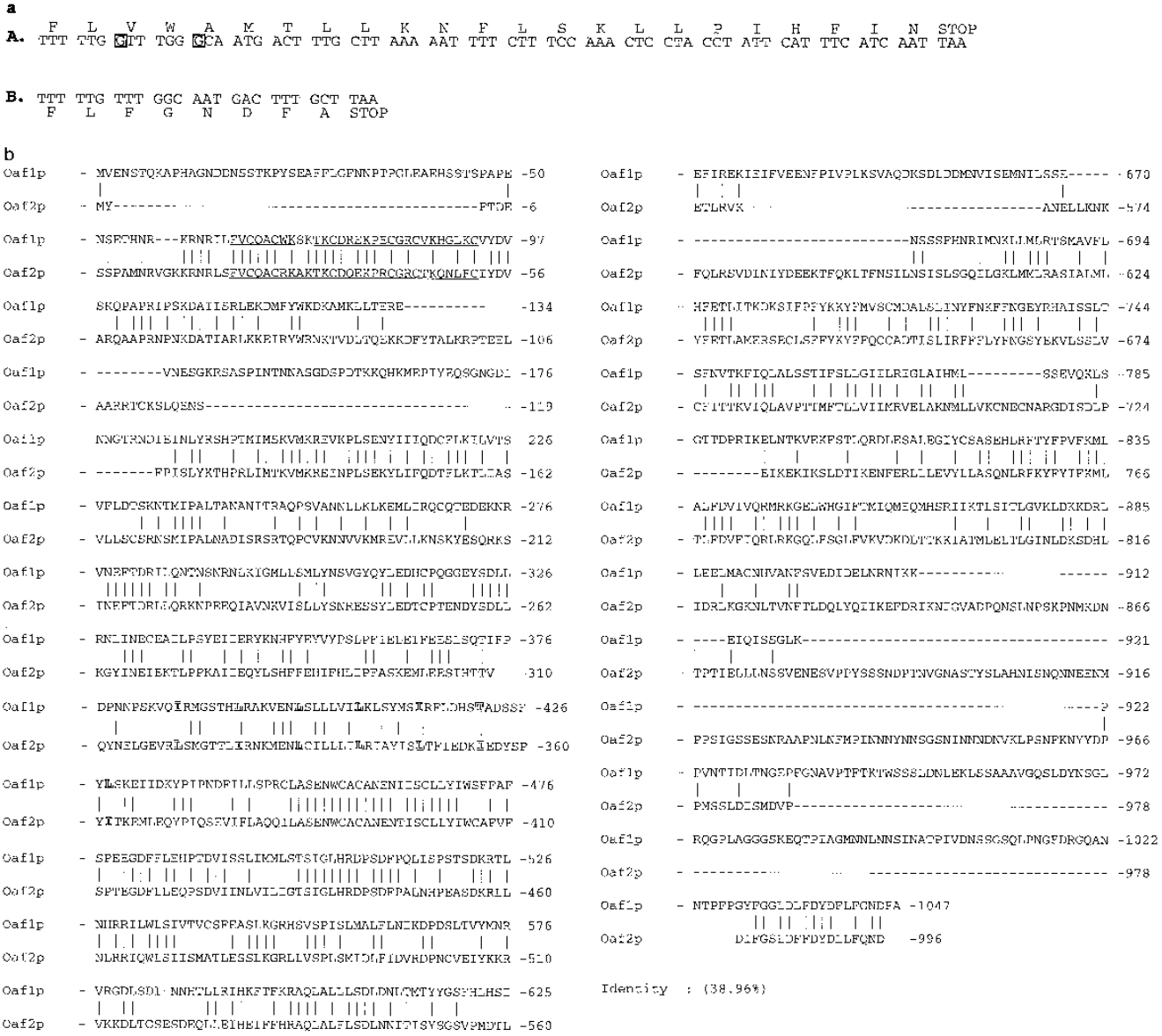

FIG. 4. Predicted amino acid sequence of Oaf2p and homology alignment to Oaf1p. (a) Corrected sequence of the carboxyl terminus of *OAF1*. A, the sequence as previously published. The two extra G's are boxed. B, the corrected sequence and position of the stop codon. (b) Homology alignment of Oaf1p and Oaf2p deduced from the respective nucleotide sequences. The latter sequence was obtained by using a BLAST search of *S. cerevisiae* sequences, which gave the accession number X91991 but no gene name (the gene was subsequently called *PIP2* [31]). The C_6 zinc cluster motif is underlined, and the residues that compose the leucine zipper motif are shown in outline.

to −309 and −156 to −136. To determine whether Oaf2p is induced by oleate, we compared mRNA levels in cells grown in glucose, glycerol, and oleate media. *OAF2* expression was low in cells grown in glucose or glycerol and was induced 7- to 10-fold in oleate-grown cells (Fig. 7a). To confirm that the Oaf2 protein is induced by oleate, we expressed an epitope-tagged version of Oaf2p. A copy of the 10 amino acids EQKL ISEEDL from human c-myc was added to the extreme carboxyl terminus of the *OAF2* open reading frame. Tagged *OAF2* was cloned into a single-copy integrating plasmid (pRS304) that was introduced into strain OA2, in which *OAF2* is disrupted. We confirmed that the tagged version of Oaf2p is functional by performing a DNA band shift assay with lysates from OA2myc cells, expressing Oaf2p-myc, grown in the presence of oleate. A specific band was seen with cells expressing the tagged version of Oaf2p, and this was not present when lysates from OA2 cells were used (data not shown).

Lysates from cells expressing Oaf2p-myc were grown in glucose, glycerol, and oleate media and were subjected to SDS-PAGE followed by Western blotting with antibody 9E10. Under these conditions, the chimeric protein could be detected only in lysates from cells grown in the presence of oleate, indicating that Oaf2p is induced by oleate (Fig. 7b). In a later experiment, the protein was detected by coimmunoprecipitation using lysates from glucose- and glycerol-grown cells, demonstrating that Oaf2p is expressed when cells are grown in these media; however, the expression was lower than in oleate-grown cells (see Fig. 9).

Oaf1p and Oaf2p are both required for the formation of a UAS1-protein complex. Previously we demonstrated the pres-

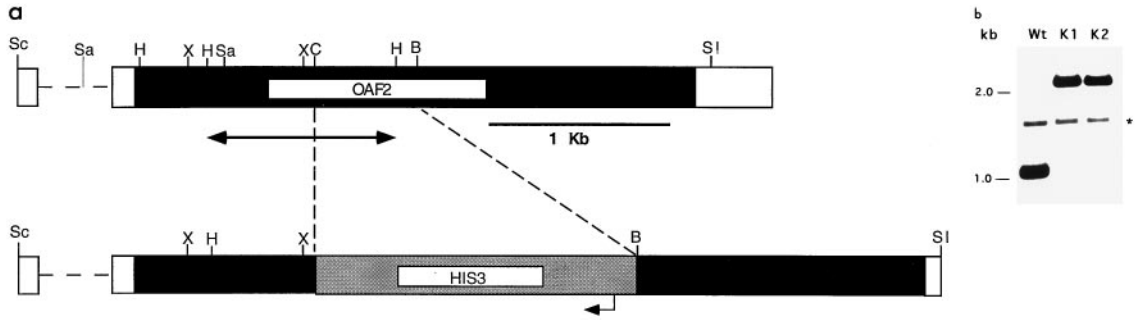

FIG. 5. Disruption of *OAF2*. (a) The *OAF2* DNA sequence (black box) encoding Oaf2p amino acid residues 321 to 501 was replaced by a DNA fragment containing the *S. cerevisiae HIS3* gene (stippled box) and integrated into the *S. cerevisiae* genome (white boxes) by homologous recombination. An arrow marks the start site and direction of transcription of *HIS3*. Sc, *Sac*I; X, *Xmn*I; C, *Cla*I; B, *Bam*HI; H, *Hin*dIII; Sl, *Sal*I; Sa, *Sca*I. The dashed line represents 3.04 kb upstream of the *OAF2* gene. (b) Correct targeting of the *oaf2*::*HIS3* fragment is demonstrated by Southern blot analysis with *Bam*HI/*Sca*I-digested genomic DNA of the wild-type (Wt) and two separate transformants in which *OAF2* was disrupted (K1 and K2). The *Hin*dIII DNA fragment used as a probe is shown by a double-headed arrow in panel a. A *Sca*I fragment common to both wild-type and disrupted strains is marked by an asterisk.

ence of a specific protein-DNA complex when extracts from oleate-grown yeast cells were incubated with DNA containing the UAS1 of *POX1* in a DNA band shift assay (41). This shifted band was not present when extracts from cells in which *OAF1* was disrupted were used in the assay (25). To determine if *OAF2* also binds to UAS1, we used extracts from wild-type cells and from cells carrying the *OAF2* disruption in a band shift assay with UAS1. Extracts from wild-type cells grown in oleate medium gave rise to the expected band containing the UAS1-protein complex (Fig. 8, lane 3). A weak band shift was also seen with extracts from cells grown in glycerol (lane 2). We occasionally see this shift with extracts from glycerol-grown cells, but when present it is much weaker than the shift seen with extracts from oleate-grown cells. No band shift is detected with extracts from glucose-grown cells (lane 1).

In contrast to the results with wild-type cells, there was no specific DNA band shift when extracts from m65 cells were used (Fig. 8, lanes 4 to 6). The DNA bandshift was restored when the assay was carried out with extracts from the m65 strain transformed with plasmid pm65 (lane 9). In addition, there was no band shift when the assay was carried out with extracts from strain OA2 in which *OAF2* is disrupted (lanes 10 to 12). These results, together with our previous studies on *OAF1* (25), suggest that Oaf1p and Oaf2p are both required for the formation of a specific protein-DNA complex in extracts from cells grown in oleate medium.

Oaf1p and Oaf2p coimmunoprecipitate. Due to the similarities between the Oaf1 and Oaf2 proteins, and to the fact that they are both required for the oleate induction of POX1, we postulated that they may interact with each other to facilitate this induction. A strain expressing both OAF1-HA_3 and OAF2-myc ($OA2HA_3$myc) was grown in the presence of glucose, glycerol, or oleate. Cell lysates were prepared and used for immunoprecipitation with monoclonal antibody 12CA5. Following SDS-PAGE and Western blotting with 9E10, tagged Oaf2p was present in immunoprecipitates from all three growth conditions (Fig. 9a, lanes 1 to 3). As expected, no protein was detected in yeast strains that were not transformed with the OAF1-HA_3 construct (Fig. 9a, lane 4). A similar result was obtained when lysates were immunoprecipitated with 9E10 followed by detection with 12CA5 (Fig. 9b). These data suggest that Oaf1p and Oaf2p form a complex which is present in all of the growth conditions tested.

DISCUSSION

We wish to gain an understanding of the mechanisms and factors which regulate induction of peroxisomes and peroxisomal enzymes. Toward this end, we have initiated a genetic approach to isolate mutants in this pathway in the yeast *S. cerevisiae*. Thus far, we have isolated eight mutant strains that fall into three complementation groups. Strains belonging to complementation group 1 carry mutations in the gene encoding the oleate activation factor that we isolated previously (25). We predict that mutations in *OAF1* should be isolated by our screening procedure; therefore, this result underscores the validity of our approach.

Electron microscope examination of the oleate activation mutants grown in the presence of oleate revealed that all have a reduced number of peroxisomes. Furthermore, the peroxisomes in most of these mutants appear to be smaller than those in wild-type cells. These observations, in combination with the peroxisomal enzyme mRNA expression levels reported in this study, suggest that the mechanisms involved in the induction of peroxisomal enzymes and in proliferation of the organelle itself are coupled. Mutant m50 has a phenotype different from those of the other mutants isolated. Peroxisomes in this mutant are almost as numerous as in the wild-type strain; however, the peroxisomal enzyme activities are not fully induced by oleate. We are currently investigating the nature of the mutation in this strain.

In recent years, peroxisome biogenesis mutants in *S. cerevisiae* have been described by several groups (12, 13, 40, 44), leading to the identification of a number of genes essential for

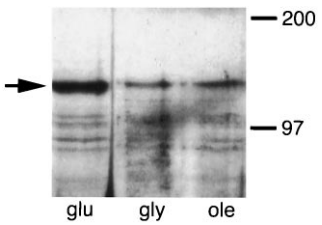

FIG. 6. Oaf2p is not required for the expression of Oaf1p. Extracts from $OA2HA_3$ cells, in which endogenous *OAF2* is disrupted, were cultured in YPD (glu), YPG (gly), or YPGO (ole) and separated by SDS-PAGE. The products were then immunoblotted with monoclonal antibody 12CA5 against HA. Triple-tagged Oaf1p is marked with an arrow.

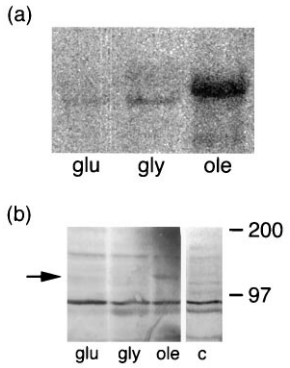

FIG. 7. Oaf2p is induced in cells grown in the presence of oleate. (a) Northern analysis of *OAF2* expression. W303-PU cells were cultured in YPD (glu), YPG (gly), or YPGO (ole) medium. A poly(A)$^+$ fraction was prepared from 500 μg of total RNA, using Oligotex resin as specified by the manufacturer (Qiagen). The mRNA was resolved and transferred as described in Materials and Methods. The 1-kb *Hin*dIII fragment from the *OAF2* coding region (Fig. 5a) was used as a probe. (b) Extracts from OA2myc cells cultured in YPD (glu), YPG (gly), or YPGO (ole) were separated by SDS-PAGE and immunoblotted with monoclonal antibody 9E10 against the human c-myc epitope. Extract from oleate-grown OA2 cells that were not transformed with OAF2-myc was used as a control for nonspecific bands (c). Tagged Oaf2p is marked with an arrow.

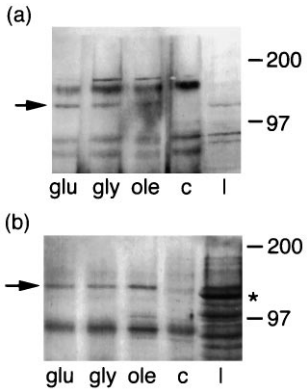

FIG. 9. Oaf1p and Oaf2p form a protein complex. (a) Coimmunoprecipitation of Oaf2-myc and Oaf1p-HA_3 with monoclonal antibody 12CA5. Protein extract (500 μg of protein) prepared from $OA2HA_3$myc cells cultured in YPD (glu), YPG (gly), or YPGO (ole) was incubated with 12CA5 and then precipitated with protein G-agarose. The immunoprecipitated material was separated by SDS-PAGE and immunoblotted with 9E10. Extract from YPGO-grown OA2myc cells that were not transformed with HA-tagged Oaf1p was treated in the same manner as a control (c). One hundred micrograms of cell lysate from YPGO-grown $OA2HA_3$myc cells was subjected to SDS-PAGE and immunoblotted with 9E10 in order to detect the specific band corresponding to Oaf2p-myc (l). Tagged Oaf2p is marked with an arrow. (b) Coimmunoprecipitation of Oaf2p-myc and Oaf1p-HA_3 with monoclonal antibody 9E10. Protein extract was prepared as described above, incubated with 9E10, and then precipitated with protein G-agarose. The immunoprecipitated material was resolved by SDS-PAGE and then transferred to membrane. Extract from YPGO-grown OA2 cells were treated in the same manner as a control (c). One hundred micrograms of total $OA2HA_3$myc cell lysate from YPGO-grown cells was run on the same gel as a positive control for Oaf2p-myc (l). Tagged Oaf1p is marked with an arrow. The membrane was first blotted with 9E10 to confirm that Oaf2p-myc was detectable (*). Oaf2pmyc is clearly seen in total cell lysate and is seen as a faint band in the immunoprecipitated material from each growth condition. The membrane was subsequently blotted, without stripping, with 12CA5 to demonstrate that Oaf1p-HA_3 was coprecipitated (arrow).

this process. These genes encode peroxisome targeting signal receptors (2, 39, 46) as well as a peroxisomal integral membrane protein (16). The search for peroxisome biogenesis mutants has also led to the isolation of some mutants that have low levels of peroxisomal enzyme activities. For example, peroxisome assembly mutant 14 (pas14) was shown to be defective in *SNF1* (40), which encodes a protein kinase involved in the activation of glucose-repressible genes (4). Pas19 was shown to carry a mutation in the *ADR1* gene (12), which was first identified as encoding a protein that regulates transcription of the alcohol dehydrogenase gene *ADH2* (8). Subsequently, *ADR1* was demonstrated to control transcription of several peroxisomal proteins, including catalase and thiolase (35). Thus, it is emerging that previously known regulators of glucose-repressible genes are also involved in the glucose repression and derepression of several peroxisomal proteins. There remains some doubt as to whether these proteins are involved in the regulation of *POX1* (37).

Mutant selection procedures that specifically target the isolation of peroxisome biogenesis mutants have not, to our knowledge, led to the characterization of any genes involved in the oleate induction of peroxisomal proteins. The *RTG* genes, known to affect oleate induction of some peroxisomal proteins, were isolated in a screen for mutants in the retrograde regulation of *CIT2* (24).

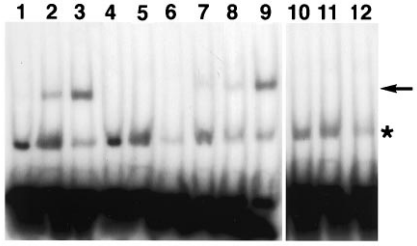

FIG. 8. DNA band shift assay with extracts from wild-type cells (lanes 1 to 3), m65 (lanes 4 to 6), m65 transformed with pm65 (lanes 7 to 9), and OA2 (lanes 10 to 12). Labeled UAS1 was used as a probe with extracts from cells grown in glucose (lanes 1, 4, 7, and 10), glycerol (lanes 2, 5, 8, and 11), or oleate (lanes 3, 6, 9, and 12) medium. An arrow marks the specific shifted band caused by protein binding to UAS1. An asterisk marks a nonspecific band seen in previous experiments (41).

Complementation group 2 contains six of the mutants isolated using our screening strategy. The gene defective in these strains encodes a protein with a calculated molecular mass of 114.7 kDa which is 39% identical to Oaf1p; thus, we named it Oaf2p. During the purification of Oaf1p, we consistently coeluted a doublet of proteins that ran with apparent molecular masses of between 110 and 120 kDa on SDS-polyacrylamide gels (25). Oaf1p was the larger protein of this doublet; it is possible that Oaf2p is the other. Oaf1p and Oaf2p are associated with each other in all growth conditions and are both required for the formation of an oleate-specific UAS1-protein complex. Due to the high homology in the predicted DNA binding regions of these two proteins and their capacity to form a complex, it is likely that they would copurify in the DNA-affinity purification procedure used to isolate Oaf1p (25). We have further shown that Oaf2p is induced by oleate (Fig. 7). Interestingly, Oaf1p expression levels are similar in glycerol- and oleate-grown cells (unpublished data). Whether induction of Oaf2p is sufficient to mediate the activation of β-oxidation enzymes and peroxisome proliferation remains to be determined.

The work reported here together with our previous studies (25, 41) supports the idea that Oaf1p and Oaf2p are transcription factors and that both bind to the UAS1 of *POX1*. We propose a model in which Oaf1p and Oaf2p form a heterodimer and bind to UAS1 as shown in Fig. 10. In this model, we have arbitrarily positioned Oaf1p and Oaf2p binding to the palindromes of the *POX1* UAS1 (they may, of course, bind in

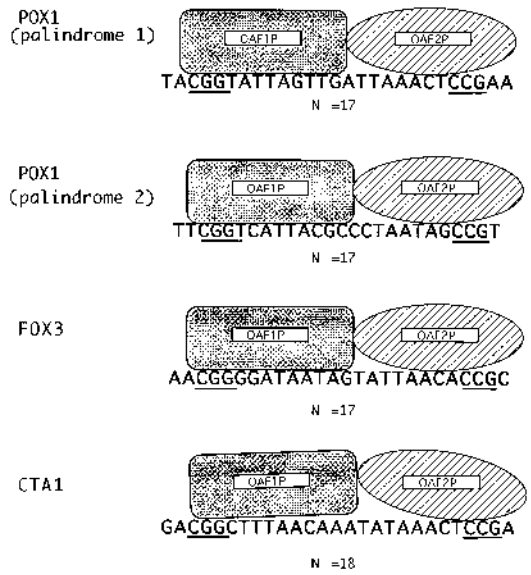

FIG. 10. Model for the binding action of the Oaf1p-Oaf2p heterodimer to the UASs present in genes encoding peroxisomal proteins. In the UASs of *POX1* and *FOX3*, the CGG repeats are 17 nucleotides apart. Both of these genes require Oaf1p and Oaf2p for activation. In the *CTA1* gene, the CGG motifs are separated by 18 nucleotides; this may affect the specific binding of proteins to this DNA.

the opposite manner). This model is consistent with that previously proposed by Einerhand et al., who suggested that protein binding to the UAS of genes encoding peroxisomal proteins would do so in the form of a homodimer or heterodimer (10). The ORE of *FOX3* also contains the $CGGN_{17}CCG$ motif; however, in *CTA1*, the intervening sequence consists of 18 nucleotides (Fig. 10). A report from Reece and Ptashne suggests that the number of nucleotides separating the CGG triplets is an important factor in the specificity of protein binding (27). Thus, it is possible that this difference contributes to the fact that catalase expression is not significantly affected in yeast strains defective in Oaf1p or Oaf2p. The putative UAS1 sequences in the *OAF2* promoter have 10 and 15 separating nucleotides, respectively. We are currently carrying out experiments to determine whether either of these sequences are functional.

While this report was under review, Rottensteiner et al. reported cloning the *OAF2* gene, which they called *PIP2* (31). Their report, which was submitted before our report describing the cloning and characterization of Oaf1p was published (25), suggested that the Pip2 protein (Oaf2p) is the sole transcription factor controlling the induction of components required for proliferation of peroxisomes. They further stated that Pip2p is likely to be a homodimer. Our current findings show that Oaf1p-Oaf1p or Oaf2p-Oaf2p homodimers, if present, are not sufficient to activate *POX1* expression. Therefore, it appears much more likely that, in order to function optimally, these two proteins heterodimerize with each other. This would resemble the mode of regulation for mammalian peroxisomal enzymes. In mammals, two proteins, the peroxisome proliferator-activated receptor α and the retinoic acid X receptor, bind, in the form of a heterodimer, to a response element in the promoters of several genes, including the peroxisomal acyl-CoA oxidase gene, and they cooperatively stimulate gene expression (17). In this system, each receptor alone is able to stimulate gene expression to some extent, whereas in yeast, it appears that both of the proteins are required for gene activation. We are currently performing experiments to further test this model.

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