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# MOLECULAR MECHANISMS REGULATING TRANSCRIPTION OF THE GENE ENCODING PEROXISOMAL ACYL-COA OXIDASE IN *SACCHAROMYCES CEREVISIAE*

By

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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# ABSTRACT


## MOLECULAR MECHANISMS REGULATING TRANSCRIPTION OF THE GENE ENCODING PEROXISOMAL ACYL-COA OXIDASE IN *SACCHAROMYCES CEREVISIAE*

BY

Yi Luo

Adviser: Professor Gillian M. Small

Peroxisomes play an important role in the metabolism of fatty acids. In the yeast *Saccharomyces cerevisiae*, fatty acid β-oxidation occurs in peroxisomes. The first and rate-limiting enzyme of the β-oxidation cycle is fatty acyl-CoA oxidase, which is encoded by a singl copy gene, *POX1*. The transcription of *POX1* is regulated by the carbon source provided for the growth of yeast cells. The gene is repressed in glucose, derepressed in glycerol and induced by oleate. The goal of this study is to analyze the molecular mechanisms regulating the expression of *POX1*.

The regulation of *POX1* expression is controlled by cis-acting elements in the 5' regulatory region of the gene. Previous work in our lab led to the identification of an upstream repression sequence (URS1). By further deletion analysis and DNA band shift assays, another repression sequence, URS2, and an activation sequence, UAS1, were identified. The functionality of these elements was confirmed in a heterologous *CYC1* promoter. By DNA band shift assays, a protein or protein complex, was found to bind to UAS1 in an oleate dependent manner.

As a first step to characterize the oleate induction pathway, a transcription factor (Oaf1p) was purified by standard protein purification methods and was subjected to peptide

sequencing analysis. Using the obtained peptide sequences, Oaf1p was found to be encoded by an open reading frame in yeast chromosome I, and the predicated molecular mass of Oaf1p is approximately 118 kD. By disruption of the *OAF1* gene in yeast, *OAF1* was identified to be involved in the oleate induction of *POX1* and peroxisome proliferation.

The expression of *OAF1* is not induced by oleate. Oaf1p is subjected to regulated phosphorylation in glycerol and oleate growth conditions. The activation domain of Oaf1p is located at the carboxyl terminus and the oleate responsive domain is located in a different region of the protein. Oaf1p appears to form a complex with Oaf2p, a transcription factor which is 39% identical to Oaf1p. A model for the regulation of *POX1* is proposed.

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## TABLE OF CONTENT

# LIST OF TABLES

# LIST OF FIGURES

# CHAPTER I

## HISTORIC REVIEW AND BACKGROUND

### Introduction

Peroxisomes are single membrane bound organelles present in almost all eukaryotic cells [1,2]. They are named based on their function in $H_2O_2$-based cellular respiration [3]. Besides cellular respiration, another major function of peroxisomes is fatty acid β-oxidation (Figure 1-1) [4,5]. In plants and yeasts, fatty acid β-oxidation only occurs in peroxisomes (see review [6]). In higher eukaryotes, fatty acid β-oxidation also occurs in mitochondria. The peroxisomal β-oxidation is thought to have the following functions [6]: (1) supply acetyl-CoA for anabolic functions such as cholesterol synthesis; (2) catabolize substrates that are poorly oxidized by mitochondrial β-oxidation, such as very long chain fatty acids and long chain dicarboxylic fatty acids; (3) catabolize eicosanoids and cholesterol side chain [6]. Four enzymes are involved in the peroxisomal fatty acid β-oxidation cycle: acyl-CoA oxidase (Aox) which is the rate limiting enzyme, bifunctional enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, and thiolase [6].

In mammals, peroxisomes are also involved in cholesterol biosynthesis and bile acid synthesis. One isoform of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, which is the rate-limiting enzyme in cholesterol biosynthesis, has been shown to be located in peroxisomes [7]. Bile acids are generated from cholesterol by hydroxylation of the ring structure and the β-oxidative cleavage of the side chain, these catalytic processes occur in peroxisomes [8,9]. Patients with Zellweger syndrome, a disorder of peroxisome biogenesis, accumulate

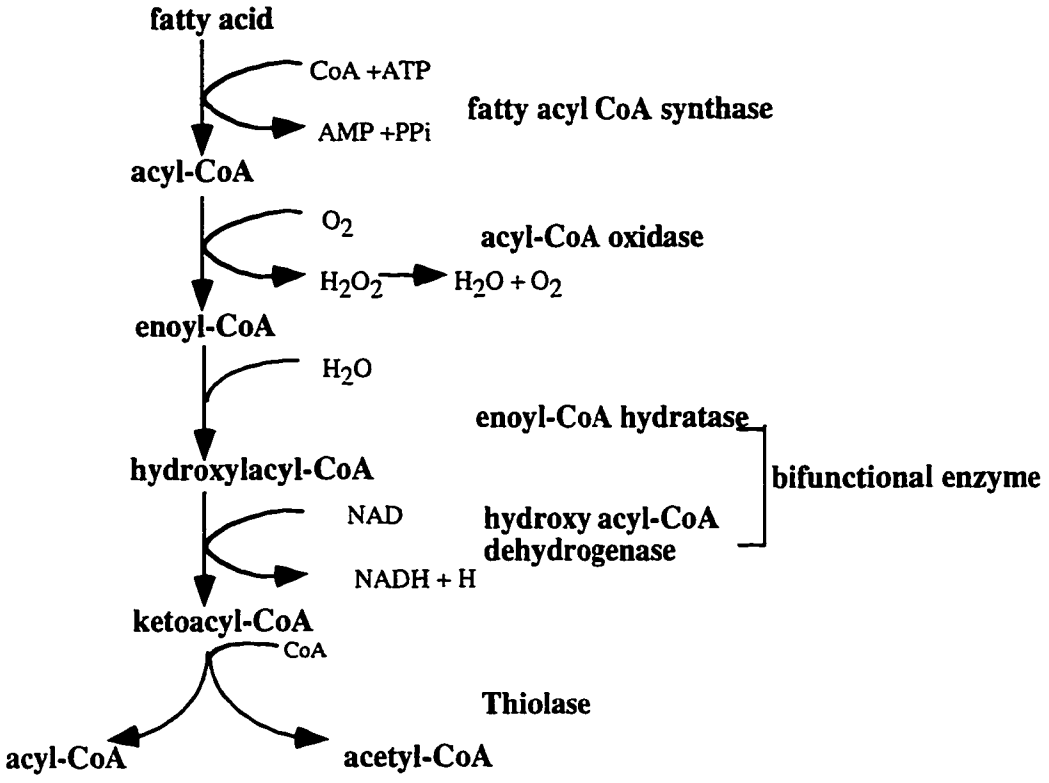



Figure 1-1. Peroxisomal β-oxidation pathway.

intermediates of bile acid metabolism, such as 3α,7α,12α,-trihydroxy-β5-cholestanoic acid (THCA), in their plasma [6]. Peroxisomes also play a critical role in the biosynthesis of plasmalogens, an ether phospholipid [10], which constitute about 5-20 percent of the phospholipids in the cell membrane and are abundant in nerve cells. The synthesis of plasmalogens has been shown to be defective in Zellweger patients [6]. Dihydroxyacetone phosphate (DHAP) acyltransferase and alkyl-DHAP synthetase, which catalyze the first two steps of plasmalogen synthesis, have been found in peroxisomes [10].

## Peroxisome Biogenesis

Peroxisomes do not contain DNA, therefore all peroxisomal proteins are encoded by nuclear genes. Peroxisomes are thought to be formed by the division of preexisting organelles [11,12]. The origin of peroxisomes is not known. All peroxisomal proteins are synthesized in the cytosol on free ribosomes, and then transported into peroxisomes posttranslationally, most of them at their mature size (see review [11]). Peroxisome biogenesis disorders (PBDs), including Zellweger sydrome, which is the most severe type of the PBDs, neonatal adrenoleukodystrophy and infantile Refsum's disease, are caused by a failure in the import of peroxisomal proteins into peroxisomes [6]. Most peroxisomal proteins in cells from patient with Zellweger sydrome remain in the cytosol, where many are rapidly degraded. Peroxisome membrane structures exist in patient cells as empty "ghosts" of the organelle.

Extensive studies have been carried out to understand the mechanisms of peroxisome biogenesis (see review [12-14]). The translocation of proteins into peroxisomes is ATP-dependent [15]. Three kinds of peroxisomal targeting signals have been identified that act to

direct proteins into peroxisomes: (1) Peroxisomal targeting signal type 1(PTS1) is a carboxyl terminal SKL tripeptide, which is present in a majority of peroxisomal matrix proteins [16,17]; (2) PTS2 is located at the amino-terminus of a limited number of peroxisomal proteins, such as rat peroxisomal thiolase [18,19] and the nonspecific lipid transfer protein [20]; (3) An internal signal has been identified in *Candida tropicalis* acyl-CoA oxidase [21] and *Saccharomyces cerevisiae* catalase A [22].

Genetic approaches have been exploited to study the peroxisomal import machinery (see review[23]). A unified nomenclature for the proteins and genes involved in peroxisome biogenesis in different organisms has been proposed [24]. The proteins are termed peroxins, and the genes are named as *PEX*. The signal recognition factors for PTS1 and PTS2 have been identified in *Saccharomyces cerevisiae*, and termed Pex5p (formerly Pas10p) and Pex7p (formerly Pas7p/Peb1p) [25-27]. A human homolog of *PEX5*, which is able to restore peroxisomal protein import in cells from patients with Zellweger sydrome, has been identified [28-30]. Recently, a peroxisomal membrane protein, Pex13p, has been shown to function as a receptor for Pex5p in *S. cerevisiae* and in humans [31-33]. In addition to Pex5p, Pex13p and Pex7p, many factors required for peroxisome biogenesis have been identified in yeast [34-37] and mammals [38-40], however, their functions in this process are not clear. Some peroxisomal proteins have been shown to be imported into the organelle as oligomers [41,42]. The oligomer is probably translocated intact across the peroxisome membrane, this is supported by the observation that PST1-coated gold particals are able to be imported into peroxisomes [43]. The mechanism by which this occurs is not yet understood.

**Peroxisome Proliferation and Tumorgenesis in Higher Eukaryotes**

The number of peroxisomes varies in different cell types. One striking feature of peroxisome biology is that they are inducible. A group of chemicals including hypolipidemic drugs, phthalate ester plasticizers, and trichlorophenoxyacetic acid herbicides have been shown to be able to induce peroxisome proliferation in rat liver (see review [44-46]). These substances are collectively called peroxisome proliferators (PPs). Some natural factors such as high-fat diet [47-49], steroid dehydroepiandrosterone (DHEA) [50] and cold adaptation [51] can also induce peroxisome proliferation, it is likely that this may represent a physiological response to some natural biological stimuli.

In long term studies, many PPs produce liver tumors in rodents [44,52]. Peroxisome proliferators are non-genotoxic carcinogens because they are unable to cause DNA damage directly [53]. The basic mechanisms by which PPs induce tumors in rodents are not known. Several hypothesis have been proposed to explain the hepatocarcinogenesis of PPs (see review [54]): (1) Hydrogen peroxide, produced by increased peroxisomal fatty acid β-oxidation, may result in sustained oxidative stress leading to DNA damage and possibly tumor initiation [55,56]; (2) inhibition of apoptosis, nafenopin has been reported to inhibit apoptosis in rat liver and hepatoma cell line [57,58]; (3) PPs may act as mitogenic factors or tumor promoters [59-61].

Species differences in response to peroxisome proliferators have been reported [46,62,63]. Peroxisome proliferation induced by PPs has been observed in mouse, rat, and hamster but not in guinea pig or monkey [63]. Importantly, no effects have been observed in humans or human cell lines [62,64].

## Regulation of the Genes Encoding Peroxisomal Proteins in Higher Eukaryotes

Peroxisome proliferators have been shown to be able to increase the levels of some proteins in rodents, these include peroxisomal β-oxidation enzymes [5,65]; cytosolic liver fatty acid binding protein (L-FABP) [66] and microsomal cytochrome P450 enzymes [67,68]. Acyl-CoA oxidase (Aox) is used widely as a marker of peroxisome proliferator action, since it catalyzes the rate limiting step in peroxisomal β-oxidation. Aox activities are elevated in rat hepatic cell lines by the treatment with ciprofibrate [69]. The induction of the β-oxidation enzymes by PPs occurs at the transcriptional level [70]. A nuclear receptor, which belongs to the steroid hormone receptor superfamily and which can be activated by peroxisome proliferators, has been cloned by screening a mouse liver cDNA library [71]. This receptor was named mPPAR (mouse peroxisome proliferator-activated receptor)(later referred to as the mPPARα). To date, three isoforms of PPAR, xPPARα,β,γ, have been identified in *Xenopus laevis* [72]; two isoforms, hPPARα and hPPARδ have been identified in human [73,74]; only one isoform, rPPARα has been identified in rats [75]; and six isoforms (mPPARα, mPPARγ, mPPARδ, mPPARγ1, mPPARγ2 and mNUC1) have been identified in mice [71,76-79].

By using chimeric proteins composed of the putative ligand binding domain of PPARs and the DNA binding domain of certain steroid hormone receptors, these PPARs have been shown to be activated by peroxisome proliferators and long chain fatty acids [71,72,74,75]. PPARs are constitutive nuclear proteins and their cytoplasmic-nuclear translocation is independent of exogenous activators [80]. XPPAR and mPPAR have been shown to activate

the promoter of the acyl-CoA oxidase gene in response to peroxisome proliferators [75,81] and physiological concentrations of long chain unsaturated fatty acids [80,82,83]. A cis-element that responds to PPAR, called PPRE (Peroxisome Proliferator Response Element), has been identified in the 5' flanking regions of the genes encoding the acyl CoA oxidase in rats and humans, and the hydratase-dehydrogenase in rats [84-86]. The binding of PPAR to PPRE requires retinoid X receptor (RXR) and is enhanced by 9-cis retinoic acid, which is the ligand of RXR and also regulates the expression of Aox [82,87]. It has been shown that PPAR and RXR form a heterodimer and bind the PPRE to regulate the expression of target genes such as the Aox gene [82]. This indicates that the two regulatory pathways which regulate peroxisomal β-oxidation, starting from fatty acids or retinoids, converge by the dimerization of PPAR and RXR.

PPARα is expressed mainly in liver, which is also the tissue showing the greatest response to peroxisome proliferators. This pattern of expression correlates well with the tissue specific induction of acyl CoA oxidase by peroxisome proliferators [64]. PPARα knockout mice do not respond to peroxisome proliferators. Peroxisome proliferation and the expression of the genes encoding peroxisomal β-oxidation enzymes was abolished by the disruption of PPARα gene. This in vivo result demonstrates that PPARα plays an important role in regulating lipid metabolism and peroxisome proliferation [88]. PPAR-γ, which is highly expressed in adipose tissue, has been shown to function as a key regulator in adipogenesis [89]. A natural ligand of PPARγ has been identified as a prostaglandin derivative [90,91]. Other PPAR isoforms remain orphan receptors. mPPARα/RXR heterodimers have been shown to be functional when expressed in the yeast *Saccharomyces cerevisiae*. The transactivation of the reporter

gene, controlled by PPRE, was independent of exogenously added activators [92], suggesting that yeast might contain endogenous activators of PPARα.

## Peroxisome Proliferation in Yeast

Peroxisome proliferation in yeast has been shown to be regulated by the carbon source used for cell growth. Long chain fatty acids such as oleic acid can induce peroxisome proliferation in *Candida tropicalis*[93] and in *Sacchromyces cerevisiae* [94]. Methanol can induce peroxisome proliferation in the methylotrophic yeast *Hansenula polymorpha* [95]. The induction of peroxisome proliferation is accompanied by the induction of the enzymes required for the catabolism of the inducers [96].

Another aspect of peroxisome biology in yeast is that they are repressed by growing cells in media containing glucose. In *S. cerevisiae*, peroxisomes are almost undetectable by immunofluorescence under glucose repression conditions, as are acyl CoA oxidase and thiolase by Western blot [94,97,98]. The expression of peroxisomal enzymes increases moderately, or is derepressed, when cells are growing in the presence of a non-fermentable carbon source such as glycerol [98,99].

The mechanism of peroxisome proliferation is poorly understood. Recently, Pex11p (former Pmp27p), a fatty acid inducible peroxisomal membrane protein from *S. cerevisiae*, was shown to promote peroxisome proliferation by participating in peroxisome elongation and fission [100,101]. In *H. polymorpha*, an integral membrane protein HpPex10p (Per8p) is required for peroxisome proliferation [102].

## Transcriptional Regulation of the Genes Encoding

**Peroxisomal Proteins in yeast**

The regulation of the genes encoding peroxisomal proteins is correlated with the proliferation of peroxisomes. Thus, a common regulatory mechanism might control and synchronize these processes. Some studies have been carried out to explore the mechanisms of induction and repression of peroxisomal enzymes. In *S. cerevisiae*, the genes encoding acyl CoA oxidase *(POX1/FOX1)*, multifunctional enzyme (*FOX2*), thiolase (*POT1* or *FOX3*) and the peroxisomal $H_2O_2$ decomposing enzyme catalase A (*CTA1*) have been cloned and were shown to be induced at the transcriptional level by oleate [97,103-107]. In *Hansenula polymorpha* the gene encoding peroxisomal methanol oxidase (*MOX*) has been shown to be strongly induced by growing cells in media containing methanol[108,109].

Regulatory elements mediating oleate induction, called oleate responsive elements (ORE), have been identified in the 5' upstream regulatory regions in genes encoding thiolase (*FOX3*) [98,110], acyl-CoA oxidase *(POX1)* (see chapter II) and catalase A (*CTA 1*) [111]. These elements are sufficient and necessary for oleate induction of *FOX3*, *POX1* and *CTA1*. An as yet unknown nuclear protein(s) has been shown, by gel shift assay, to bind to the identified ORE of *FOX3* [110]. Sequences similar to the ORE have been suggested in the regulatory regions of the genes encoding other peroxisomal proteins including the multifunctional enzyme (*FOX2*), Pex11p and Pex1p (Pas1p). These homologous sequences have not been tested to determine if they are functional in regulating gene expression. In *H. polymorpha*, the upstream regulatory sequences mediating the methanol induction of *MOX* have been identified [112].

The search for peroxisome biogenesis mutants has led to the isolation of mutants that have

low levels of peroxisomal enzymes. Peroxisome assembly mutant *pas19* was shown to carry a mutation in the *ADR1* gene [113]. *ADR1* was first characterized as a regulator in the transcription of the *ADH2* gene encoding the glucose-repressible protein, alcohol dehydrogenase II, in *S. cerevisiae* [114]. *ADR1* was also identified as a suppressor of glucose repression of *CTA1*[115]. The expression of *CTA1* in the presence of ethanol, but not oleate, is negatively affected by the *ADR1* null mutation, indicating that *ADR1* may be involved in the derepression of *CTA1*. The effect of *ADR1* on *CTA1* expression is mediated by a regulatory sequence other than ORE. The C-terminal region of *ADR1* plays an important role in the regulation of *CTA1* [116]. *ADR1* also controls the derepression of *FOX2*, *FOX3* and *PEX1 (PAS1)* [115,117], and is required for the formation of peroxisome clusters [117]. A *Pas14* mutant was shown to be defective in the *SNF1*(sucrose non-fermenting) gene [118], which encodes a protein kinase and was shown to be involved in the derepression of the *SUC* genes encoding enzymes for sucrose metabolism [119,120]. It has also been shown that the expression of *FOX3* decreased in *SNF1* and *SNF4* mutants [117]. *SNF4* encodes a protein associated with the Snf1 protein [121]. *SNF1* and *SNF4* but not *ADR1* are required for peroxisome proliferation in yeast [117]. *SNF1* has also been shown to control the *CTA1* expression via both the fatty acid responsive element and the *ADR1* binding element [111]. Mutations in *SNF1* and *ADR1* were previously shown to have little effect on the expression of *POX1* mRNA [122]. However, recent data in our lab suggests that *ADR1* and *SNF1* may also regulate the expression of *POX1* (unpublished data, see Chapter IV).

An upstream activating sequence (UASr) has been identified in the *CIT2* gene encoding peroxisomal citrate synthase. Two proteins, Rtg1p, a basic helix-loop-helix transcription

factor, and Rtg2p, whose function is not known, were shown to be required for the expression of *CIT2*. Rtg1p is able to bind to the UASr in *CIT2* gene [123]. It has been shown that *RTG1* and *RTG2* are required for efficient growth of cells in medium containing oleate. Mutations in *RTG1* and/or *RTG2* also impaired the induction of peroxisome proliferation by oleate. Compared to wild type cells, the oleate induction of *POX1*, *CTA1* and *PEX11* decreased in the *rtg* mutants. The decrease was more severe in the *rtg2* mutant and *rtg1/rtg2* double mutant than in *rtg1* mutant [124]. However, the authors did not compare the expression levels of these genes in the mutant cells under conditions of glucose repression, or glycerol derepression. Thus, it is not known whether the derepression and/or the basal expression of these genes is affected by *RTG1* and *RTG2* mutations. Kos et al showed that the activation of the *FOX3*, *CTA1* and *PEX5*(*PAS10*) promoter by oleate remained unchanged in an *rtg1* mutant [125]. Rtg1p itself does not bind to the *FOX3* ORE. A mutation in *RTG1* does not affect the binding of protein or proteins to the *FOX3* ORE. It is not known whether a mutation in *RTG2* would affect the *FOX3* ORE function. Based on these results, there remains questions about the involvement of *RTG1* and *RTG2* in the induction of *POX1* by oleate.

## Mechanisms Controlling Glucose Repression of the Genes Encoding Peroxisomal Proteins in *S. cerevisiae*

The mechanism for glucose repression of peroxisomal proteins is still not fully understood. However, glucose repression, which is also called catabolic repression, is a general regulatory system in yeast. The expression of a large number of genes that are required for the

metabolism of alternate carbon sources are repressed in cells grown in glucose medium. In *S. cerevisiae*, glucose repression affects the enzymes required for metabolism of the sugars sucrose, maltose and galactose and non-fermentable carbon sources such as glycerol, ethanol and acetate. This glucose control is mostly executed at the level of transcription [120]. Much information has been obtained by genetic studies on glucose repression of *SUC* genes, which encode enzymes for sucrose metabolism, and *GAL* genes encoding enzymes for galactose metabolism [120,126]. Two general types of mutants have been isolated which show defects in the regulation of *SUC2*. Derepression mutants, which do not express *SUC2* in the absence of glucose and are defective in the genes involved in derepression; and repression mutants which are defective in glucose repression. Six genes *SNF1-SNF6* have been isolated and shown to be involved in the derepressional regulation of *SUC* genes. Many genes involved in the glucose repression pathway, such as *HXK2*, *CYC8* (*SSN6*), *TUP1* [127] and *MIG1* have been identified. *HXK2* encodes hexokinase PII and has protein kinase activity and may act as initial sensor for glucose level [128,129]. *MIG1* has been shown to bind to UAS of *SUC2* and inhibit transcription by competing with positive factors for binding [130]. *MIG1* also directs the repression by *CYC8/TUP1* [131].

A negative element called URS1 has been identified in the 5' regulatory region of *POX1* encoding acyl CoA oxidase, and a protein or protein complex was shown to bind to this region. By deletion and mutagenesis analysis, this URS1 was shown to be involved in mediating repression of *POX1*[132]. *MIG1* seems not to be involved in the function of *POX1* URS1[122] or in the glucose repression of *FOX3*[133]. It has been shown that *TUP1*, *CYC8*, *HXK2* are not required for the glucose repression of *POX1*[122]. *CYC8*, *RP-A* (*BUF*), *ABF1*

and *CAR80 (UME6)* have been shown to be required for the glucose repression of *FOX3* [104,133]. *RP-A* and *ABF1* are multi-functional proteins, depending on the context of the binding site, they may act as repressors or activators [134,135]. *CAR80* is required for the URS1 mediated repression of *CAR1* gene, which encodes arginase [136]. The mechanism of glucose repression for *POX1* and *FOX3* appears to be somewhat different, because *CYC8* is required for the repression of *FOX3* but not *POX1* [104,122]. The sequence identical to the URS1 of *POX1* was found in the promoter of *FOX3*, but it appeared not function as a repression sequence in a deletion analysis [133]. The mechanisms that mediate glucose repression of *POX1* remain to be identified.

**Aim of this thesis project**

The goal of my research is to analyze the molecular mechanisms controlling the expression of *POX1* in the yeast *Saccharomyces cerevisiae*. *POX1* encodes acyl-CoA oxidase, which is the first and rate-limiting enzyme of the peroxisomal β-oxidation cycle. Study of its regulation will help us to understand the mechanisms of peroxisome proliferation and gene regulation in yeast and perhaps also in higher eukaryotes. My study is focused on the identification of the fatty acid induction pathway leading to the activation of the *POX1* gene. The following chapters cover the studies I carried out on the identification of the regulatory cis-elements in *POX1*, purification, cloning and characterization of a transcription factor involved in mediating the fatty acid induction of *POX1*.

# CHAPTER II

## MULTIPLE REGULATORY ELEMENTS CONTROLLING THE EXPRESSION OF *POX1* IN *SACCHAROMYCES CEREVISIAE*

### Introduction

In the yeast *Saccharomyces cerevisiae*, peroxisomes are repressed when the cells are grown in medium containing glucose, and are induced by growth on oleate [94]. Peroxisome induction is accompanied by an increase in the activity of peroxisomal enzymes, especially those involved in peroxisomal fatty acid β-oxidation [106]. A single copy gene, *POX1*, which encodes peroxisomal acyl-CoA oxidase in *S. cerevisiae*, has been cloned and sequenced [103]. The expression of the *POX1* gene is regulated at the transcriptional level; *POX1* mRNA is undetectable in cells cultured in glucose medium and is induced by the addition of oleate in the medium.

Regulation of transcription occurs by the binding of regulatory proteins to specific DNA sequences. In order to analyze the molecular mechanisms controlling *POX1* expression, it is necessary to identify the regulatory cis-elements in the promoter region of *POX1*. A region of DNA containing 454 nucleotides upstream from the *POX1* translational initiation codon was found to be sufficient to confer *POX1* regulation [132]. This region was shown to contain a upstream repression sequence (URS1) [132]. However, glucose repression was not completely abolished when the URS1 was mutated, indicating that more cis-elements exist in the remaining promoter, which act to regulate *POX1* expression.

In this chapter, I will describe the studies I carried out to identify other cis-elements

involved in *POX1* regulation. A positive element UAS1 (Upstream Activation Sequence 1) and a negative element (URS2) were identified by deletion analysis, DNA bandshift assay, and were then confirmed by functional analysis.

## Materials and Method

### *Yeast Strains and Culture Conditions:*

*Saccharomyces cerevisiae* strain BMG1-7a (*MAT*a *ade1-100 his4-519 leu-112 ura-52*) was used throughout the studies described in this chapter.

Yeast cells were grown in rich (YPD) or minimal (SD) media as required. YPD medium contains 2% yeast extract, 1% peptone, 2% glucose. SD medium contains 0.67% yeast nitrogen base without amino acids, 2% glucose. Glycerol medium (YPG) contains 1% yeast extract, 2% peptone and 3% glycerol. Induction medium was YPGO, which contains 1% yeast extract, 2% peptone, 3% glycerol, 0.1% oleic acid and 0.2% Tween-40.

### *Plasmid Constructions:*

pP13570, pP1353, pP13532, pP13533, pP13534 and pP13535 were prepared by Dr. Tongwen Wang[132,137].

DNA primers used to make following constructs were synthesized as shown in Table 2-1.

pP13572. Primer 1 and primer 5 were used in PCR reaction with pP13570 DNA as template. The resulting 918 bp DNA fragment containing -316 to 602 of *POX1* was subcloned into pT7blue (Novagen). The resulting plasmid was digested with Pst1/BamH1 and the released DNA fragment was subsequently inserted into the corresponding sites within

Table 2-1

DNA primers used for preparing constructs

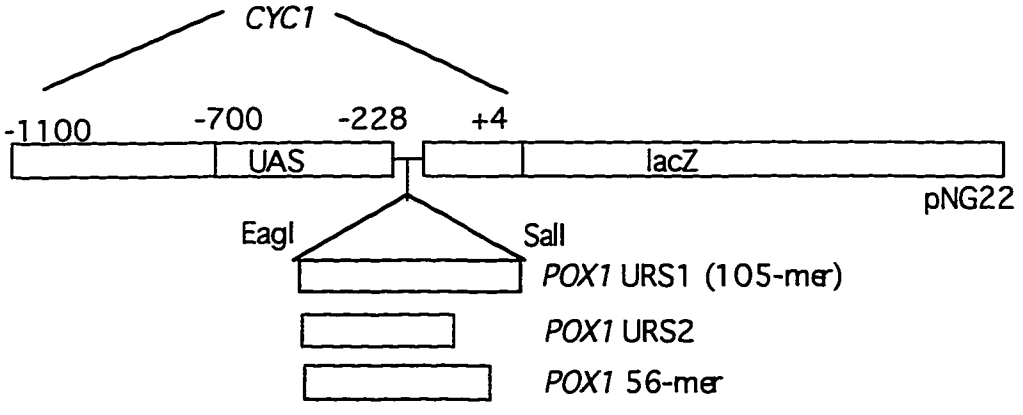
| Primer | Sequence | Region of POX1 |
|---|---|---|
| 1 | 5' CTGCAGCTGGGCAACATTGGA 3' | 582 to 602 |
| 2 | 5' AACGGCTATTAGCCGCTAATGACCG 3' | -275 to -251 |
| 3 | 5' GGTACCGCGTTCAAACCCTGA 3' | -349 to -335 |
| 4 | 5' GGGGTCGACGGTGATTTACCCCAACG 3' | -238 to -254 |
| 5 | 5' GGGGTCGACTTTACGGTATTAGTTGA 3' | -316 to -300 |
| 6 | 5' GGGCTCCGAAGCGAAAGGAAT 3' | -294 to -277 |
| 7 | 5' GGGTCTAGAGGTGATTTACCCCAACGG 3' | -238 to -255 |
| 8 | 5' GGGGCTCGAGAGATCTCGACCAAAAAAA 3' | -454 to -436 |
| 9 | 5' GGGGTCTAGAAAAAAAGGAGGTTTATAA 3' | -350 to -368 |

Figure 2-1. Cloning of the *POX1* cis-elements into the pNG expression vectors.

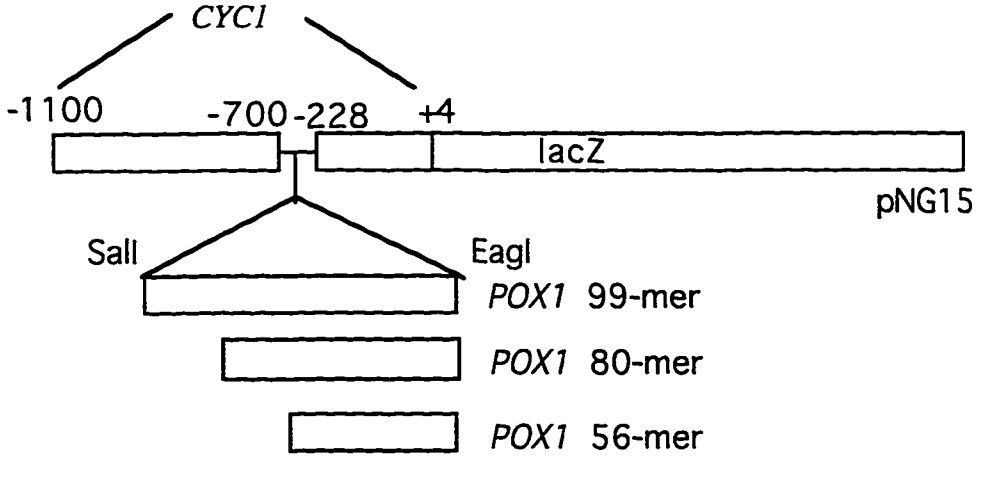
(a). Construction of pNG2256, pNG22URS1 and pNG22URS2. 56 mer (-294 to -238), URS2 (-349 to -314) and URS1 (-454 to -349) were subcloned into the SalI/EagI sites in the pNG22 plasmid.

(b). Construction of pNG1556, pNG1580 and pNG1599. 56 mer (-294 to -238), 80 mer (-316 to-238) and 99 mer (-349 to -251) were subcloned into the SalI/EagI sites in the pNG15 plasmid.

(a). pNG22 constructs




(b). pNG15 constructs



the multiple cloning region of yeast-*E.coli* shuttle vector YIp357 [138]. This resulted in a plasmid containing 316 nucleotides 5' of the ATG and 602 coding bases of POX1 fused in-frame with *lacZ*.

pYL1. A 99 base pair DNA fragment, which contains -349 to -251 region of *POX1*, was obtained by PCR amplification using primer 2 and primer 3 with pP13570 as template. The fragment was treated with T4 DNA polymerase to render the ends blunt and subcloned into the SmaI site of pBluescript II (KS+) (Stratagene) to create pYL1.

pYL2. Primer 6 and primer 7 were used in the PCR with pP13570 to obtain a 56 bp DNA fragment, which contains the *POX1* promoter region from -294 to -238. The DNA fragment was blunt ended by treating with T4 DNA polymerase and subsequently cloned into the SmaI site of pBluescript II (KS+) to form pYL2.

pYL3. An 80 bp DNA fragment containing the *POX1* promoter region from -316 to -238 was obtained by PCR using primers 4 and 5. The ends of the fragment were made blunt and it was then subcloned into the SmaI site of pBluescript II (KS+) to create pYL3.

pYL4. Primers 8 and 9 were used in PCR to generate an 105 bp fragment, which contains the *POX1* promoter region from -454 to -350. The DNA fragment was digested with XhoI/XbaI and subcloned into pBluescript II (KS+) at homologous sites.

pNG plasmids were prepared by cloning fragments of the *POX1* promoter into pNG15 or pNG22 [139] (Figure 2-1). pNG15 contains the *CYC1* promoter, without the *CYC1* upstream activation elements, fused in-frame with *lacZ*. pNG22 is the same construct except that the *CYC1* UAS elements are also present.

pNG1599. A DNA fragment containing the *POX1* promoter region from -349 to -251 and

flanking polylinker DNA was released by digesting pYL1 with XhoI/EagI and subcloned into the SalI/EagI sites of pNG15.

pNG1556. A DNA fragment containing the *POX1* promoter region from -294 to -328 was released by digesting pYL2 with XhoI/EagI and it was subcloned into the SalI/EagI sites of pNG15.

pNG1580. A DNA fragment, which corresponds to the bases from -316 to -238 in the *POX1* promoter, and was released from pYL3 by digesting with XhoI/EagI, and was subsequently ligated into the SalI/EagI sites of pNG15.

pNG2256. The XhoI/EagI fragment excised from pYL2 was subcloned into the SalI/EagI sites of pNG22 to create pNG2256.

pNG22URS1. A DNA fragment, which contains the *POX1* promoter region from -454 to -350, was released from pYL4 by digesting with XhoI/EagI and it was subcloned into the corresponding sites in pNG22 to create pNG22URS1.

pNG22URS2. Two complementary oligonucleotides, YL2-1 (5' GGCCGGCGTTCAA ACCCTGACATTTTAAGCCCTATATTTC 3') and YL2-2 (5'GGCCGAAATATAGGGCT TAAAATGTGTCAGGGTTTGAACGCC 3') consisting of bases -349 to -314 of *POX1* flanked by EagI sites, were synthesized and annealed by heating to 65 °C and slowly cooling to room temperature. The annealed DNA fragment was subcloned into the EagI site of pNG22.

All of the constructs described above were sequenced to verify their identity.

***Transformation:***

Standard protocols were used for the transformation of *E. coli* and yeast [140]. The

*POX1-lacZ* fusion constructs were integrated in the *ura3* locus by first digesting the plasmids at the unique NcoI site in the *URA3* gene. Selection for uracil prototrophs was carried out on minimal medium supplemented with the appropriate amino acids.

***Preparation of Yeast Cell Extracts:***

Yeast cells were grown to stationary phase in YPD medium and were then transferred to YPG, YPGO or YPD medium and allowed to grow for a further 18 hours. Cells were harvested, washed with sterile water and resuspended in extraction buffer (200 mM Tris-HCl, pH 8.0, 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 7 mM 2-mercaptoethanol, 1 mM PMSF, 1 µl/ml protease inhibitor mixture containing 10 µg/µl leupeptin, antipain, chymostatin and pepstatin). The cells were disrupted by vortexing in the presence of glass beads (0.5 mm diameter), and cell debris was removed by centrifugation at 10,000 g for 1 hour. Cell extracts were aliquoted and stored at -80 °C .

***DNA Band Shift Assays:***

The DNA band shift assay were carried out as described by Fried and Crothers [141], with some modifications [142]. Cell extracts (1-5 µl, approximately 30 µg protein) were mixed with 1 µl of $^{32}$P-end labeled DNA fragment (approximately 5000 cpm or 0.02 pmol) in binding buffer (12 mM Hepes pH 7.5, 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris-HCl, pH 8.0, 0.6 mM DTT, 10 % glycerol, 0.26 µg/µg poly(dI-dC), 0.3 µg/µl bovine serum albumin). The binding reactions (approximately 20 µl) were incubated at room temperature for 20 min, and protein-DNA complexes were resolved in 5% polyacrylamide gels at 150 V. For the competition experiments, 30-60 molar excess of unlabeled competitor DNA relative to probe DNA was added to the reaction before the addition of the probe. The DNA fragments used

for band shift assays are shown in Table 2-2 (also see Figure 2-3).

The 184-mer was obtained by PCR as described previously [132], the resulting DNA fragment was cloned into pBluescript II (KS+) and was subsequently excised by digesting the plasmid with BamHI/HindIII. The 99-mer, 80-mer and 105-mer were excised from pYL1, pYL3 and pYL4, respectively, by digesting with BamHI and HindIII. The 70-mer and 29-mer were obtained by digesting the 99-mer with EcoRI. PL and PR of UAS1 (Figure 2-3) were obtained by digesting the 80-mer with EcoRI. The fragments were labeled with [a-$^{32}$P]dATP using Klenow fragment of DNA polymerase I.

uUAS1 fragment, corresponding to the upstream palindrome of *POX1* UAS1 was obtained by annealing two complementary oligonucleotides: YL2-3 (5' GATCTACGGTA TTAGTTGATTAAACTCCGAAG 3'), and YL2-4 (5' GATCCTTCGGAGTTTAATCAACT AATACCGTA 3'). Mutated UAS1 fragment, mUAS1, was obtained by annealing two complementary oligonucleotides YL2-5 (5' GATCTATAAGATGAGTTGATTAAACTAA TGA 3') and YL2-6 (5' GATCTCATTAGTTTAATCAACTCATCTTATA 3'). The mutated bases are underlined. All oligonucleotides were synthesized by the DNA core on an Applied Biosystems DNA synthesizer.

*Assays:*

β-galactosidase activities were measured as described by Wang et al [132] with the exception that the specific activity was calculated according to the Worthington Manual [143]. Protein concentration were determined by the method of Bradford [144], using γ-globulin as a standard.

Table 2-2

DNA fragments used in band shift assays

| Fragment identification | Region of *POX1* |
|---|---|
| 184-mer | -349 to -165 |
| 105-mer | -454 to -349 |
| 99-mer | -349 to -251 |
| 80-mer | -316 to -238 |
| 70-mer | -349 to -279 |
| 56-mer | -294 to -238 |
| 29-mer | -279 to -251 |
| URS2 | -349 to -314 |
| uUAS1 | -313 to -286 |
| mUAS1 | -313 to -286 |

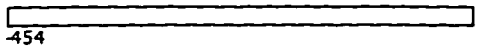
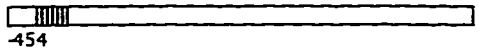
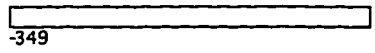
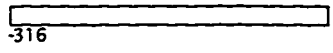
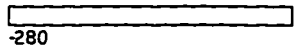
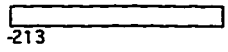
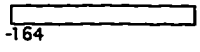
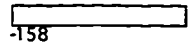
## Results

### *Identification of regulatory elements in the POX1 promoter:*

The transcription of *POX1* is repressed in cells grown in glucose medium, depressed when cells are grown in medium containing a non-fermentable carbon source such as glycerol and induced in cells grown in medium containing long chain fatty acids such as oleate. Previous studies in our laboratory had led to the identification of a negative regulatory element URS1 within -434 to -410 of the *POX1* 5' upstream regulatory region, which is involved in the transcriptional control of this gene [132]. A reporter construct consisting of the *POX1* upstream regulatory region fused in frame with the *lacZ* gene from *E. coli* was used in these studies. Deletion or mutation of URS1 in this reporter construct led to higher β-galactosidase activity in cells, but glucose repression and oleate induction were still evident, indicating that one or more cis-elements were still present and could act to regulate *POX1* expression. In order to identify other cis-elements responsible for oleate induction and glucose repression of *POX1*, a series of *POX1-lacZ* fusion constructs containing various truncated versions of the *POX1* promoter fused in frame with *lacZ* were prepared as described in the Materials and Methods (Figure 2-2). These constructs were introduced into *S. cerevisiae* strain 1-7a by integrating at the *ura3* locus. β-galactosidase activity was measured in extracts from cells grown in medium containing glucose, glycerol or oleate (Figure 2-2). In cells transformed with pP13570, which contains the 454 bp upstream region, the β-galactosidase activity was undetectable in extracts from cells grown in glucose medium. Measurable amounts of activity were present in extracts from cells grown in medium

Figure 2-2. Deletion analysis of the *POX1* promoter region using *POX1-lacZ* reporter constructs.

The constructs were introduced into a *S. cerevisiae* strain BMG 1-7a. The β-galactosidase activity was measured in extracts of cells grown in glucose (YPD), glycerol (YPG) or oleate (YPGO) media. The hatched box represents a mutation in URS1. UD, undetectable. Numbers are the mean of two experiments. The numbers in the two experiments vary slightly.

| Construct | | ß-Galactosidase U/mgprotein YPD | YPG | YPOG |
|---|---|---|---|---|
| p13570 |   | U.D | 0.058 | 0.876 |
| p1353 |   | U.D | 0.626 | 4.563 |
| p13532 |   | 0.001 | 2.70 | 5.058 |
| p13572 |   | 0.001 | 1.335 | 2.85 |
| p13571 |   | 0.005 | 1.83 | 2.80 |
| p13533 |   | 1.00 | 0.663 | 1.92 |
| p13534 |   | 2.117 | 1.615 | 2.678 |
| p13535 |   | U.D | U.D | U.D |

containing glycerol, and approximately 10-fold induction was observed when oleate was supplied as the carbon source. The production of β-galactosidase was greater in glycerol- and oleate-grown cells transformed with pP1353 which contains point mutations in URS1, confirming that URS1 serves as a negative element. These results were obtained by Dr.T.Wang.

When the upstream -349 to -454 region was deleted, as seen in the yeast cells transformed with pP13532, the β-galactosidase activity was higher than that of the transformants bearing pP13570 or pP1353 in glucose and glycerol culture conditions. This result indicates that either the point mutations did not totally abolish the function of URS1, or that another URS may exist in the deleted region. Alternatively, deletion of this 105 bp region may have interfered with the functioning of a putative URS in an adjacent region. In this construct, which has high β-galactosidase activity in the presence of glycerol, there was less than a 2-fold induction by oleate.

With further deletion of the regulatory sequence from -349 to -213 (pP13572, pP13571, pP13533 ), the β-galactosidase activity increased significantly in cells grown in glucose medium, suggesting that there may be another negative element in the deleted region.

Cells transformed with pP13534, which contained the proximal promoter TATAA box (-160 to-164) but no other regulatory elements, did not show repression or induction, and the activities in cells grown in all three media were similar, suggesting the TATAA box alone is sufficient for unregulated expression of *POX1*. Deletion of the TATAA box resulted in the complete abolishment of *POX1* promoter activity (pP13535).

***Specific protein-DNA interactions correlate with the regulation of POX1 expression:***

Since the upstream regulatory cis-elements usually function by the binding of trans-regulatory factors, DNA retardation assays were exploited to analyze the protein-DNA interactions in the *POX1* regulatory region. In order to analyze the protein binding sites, a series of fragments corresponding to the putative *POX1* regulatory regions were used in DNA mobility shift assays (Figure 2-3 and Table 2-2). A 184 bp fragment, spanning from -165 to -349, which includes the region upstream of the TATAA box and the putative negative and positive elements, was labeled and used in DNA band shift assays. When cell extracts from glucose-grown cells were incubated with the labeled 184 mer, two distinct bands were observed (Figure 2-4a, lane 2). The specificity of the two bands was examined by various competition experiments (Figure 2-4a, lanes 3-5, and 7-9). The upper band (C1) was abolished by addition of excessive unlabeled 184 mer, but the lower band was not, indicating the lower band was nonspecific (Figure 2-4a, lane 3). When cell extract from cells cultured in oleate medium was incubated with labeled 184 mer, a weak C1 band was present and also a more retarded band (C2) was seen (Figure 2-4a, lane 6). The unlabeled 184 mer effectively abolished these two bands (Figure 2-4a, lane 7). This result suggests that the 184 mer may contain two distinct protein binding sites, or alternatively the C2 band may be due to the binding of protein(s), unique to the oleate grown cells, at the same region. In order to explore these two possibilities, smaller fragments in the 184 mer were used in competition assays. A 70 bp fragment from -279 to -349 specifically competed out both C1 and C2 in glucose- and oleate- grown cell extracts (Figure 2-4a, lane 4 and 8), whereas an 80 bp fragment from -238 to -316 only abolished the C2 band but did not affect the C1 band (Fig 2-4a, lane 5 and 9), suggesting the presence of two separate protein binding sites within the

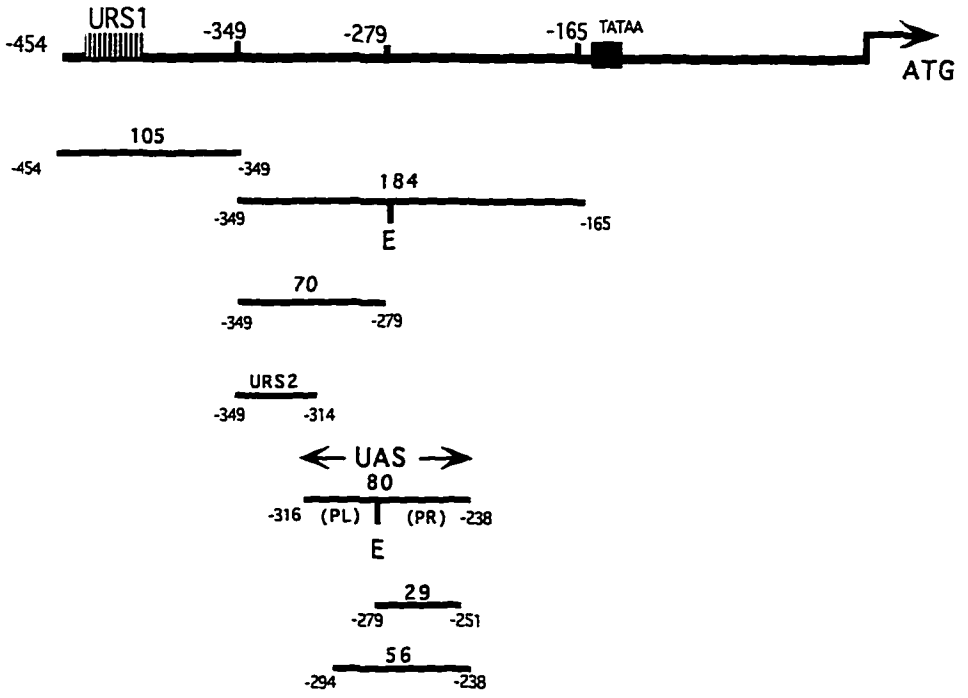


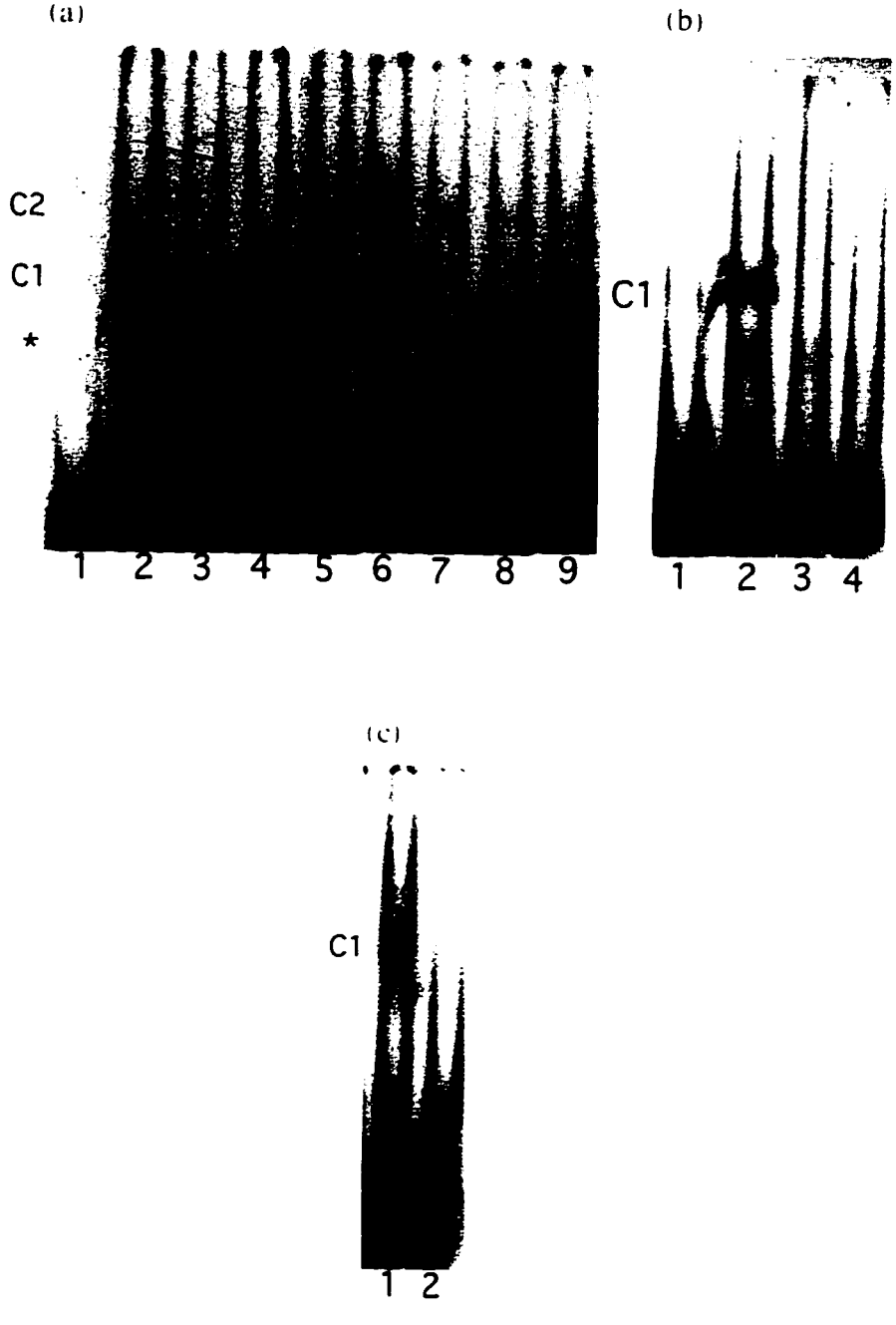

Figure 2-3. DNA fragments from the *POX1* promoter region that were used in DNA band shift assays.

Figure 2-4. DNA band shift analysis of the 5' regulatory sequence of *POX1*.

(a). A 184-bp radiolabeled DNA fragment (-349 to -165) was incubated with the yeast extracts containing approximately 30 μg of proteins from cells grown on glucose (lane 2-5) or oleate (lane 6-9) media. The assay was carried out in the absence (lane 2 and 6) or the presence of 100-150 ng of unlabeled 184-mer (lane 3 and 7), 70-mer (lane 4 and 8) or 80-mer (lane 5 and 9). Lane 1 contained the labeled DNA in the absence of protein. The DNA-protein complexes (C1 and C2) were resolved from free DNA by nondenaturing polyacrylamide gel electrophoresis and were revealed by autoradiography. The asterisk indicates a nonspecific band.

(b). Cell extracts from cells grown in glucose medium were incubated with the labeled 184-mer in the absence (lane 2) or presence of 100 ng of unlabeled 105-mer (lane 3) or double stranded oligonucleotide URS1 (lane 4). Lane 1 was as in (a).

(c) Labeled 70-mer was incubated with cell extracts from glucose-grown cells in the absence (lane 1) or presence of (lane 2) of 100 ng of unlabeled doubled stranded oligonucleotide URS2 (-349 to -314).



184 mer. The finding that the 80 mer could not compete for protein(s) binding at the C1 site led us to conclude that this C1 protein(s) binding site resides in the region upstream of position -316.

The C1 band, produced by labeled 184 mer in gel shift assays, was successfully competed out by an unlabeled 105 bp fragment (-454 to -349) which contains the previously characterized URS1 (Figure 2-4b, lane 3) and by a 25 bp oligonucleotide fragment containing the URS1 protein binding site [132](Figure 2-4b, lane 4). This suggests that the same protein or protein complex that bind to the URS1 may also bind to the 184 mer and gave rise to the C1 band in cell extracts from glucose-grown cells. This raises the possibility that the C1 binding site may serve as an another upstream repression element (URS2). To confirm the presence of the putative URS2 protein binding site, double stranded oligonucleotides corresponding to bases -349 to -316 were used to test their ability to act as competitor in a gel shift assay using labeled 70 mer (-349 to -279). The 70 bp fragment gave rise to a single band with extracts from glucose -grown cells (Figure 2-4c, lane 1) which was successfully competed out by unlabeled oligonucleotides comprising the putative URS2 (Figure 2-4c, lane 2).

***Functionality of the negative response elements URS1 and URS2:***

In order to test whether the URS1 and URS2 serve as negative elements, their functionality was tested in a heterologous *CYC1* promoter, using the pNG22 plasmid, which contains the *CYC1* promoter and UAS cloned in frame with *lacZ* [139]. The *CYC1* UAS in this plasmid mediates induction by heme and non-fermentable carbon sources [145]. The *POX1* URS1, URS2 and fragment 56-mer (See Figure 2-3 for the corresponding region of

these elements in the *POX1* promoter) were cloned downstream of the *CYC1* UAS (Figure 2-1). The plasmids containing the *POX1* regions -454 to -349 (pNG22URS1) and -349 to -316 (pP22URS2), as well as controls pNG2256 containing 56 bp DNA fragment (-279 to -241) or pNG22 alone were introduced into *S. cerevisiae* strain 1-7a. Extracts prepared from cells grown in glucose, glycerol or oleate media were assayed for β-galactosidase activity. An increase in the activity in cells transformed with control plasmid pNG22 or pNG2256 were observed when cells were grown in glycerol or oleate media compared with that of glucose-grown cells (Table 2-3). This confirms that the *CYC1* UAS elements were functioning in this context and insertion of an unrelated DNA fragment did not affect the function of the *CYC1* UAS. Cells transformed with plasmid (pNG22URS1 or pNG22URS2) containing the *POX1* URS1 or URS2 had low levels of activity regardless of growth conditions, confirming that these sequences are functional repression elements and are able to repress the action of the *CYC1* UAS (Table 2-3).

***Characterization of an oleate responsive activating sequence (UAS1):***

The C1 band was seen in gel shift experiments when the 184 mer was incubated with extracts from cells grown in glucose, glycerol and oleate media, whereas the C2 band was only seen with extracts from oleate-grown cells (Figure 2-4a, compare lane 2 and 6). This suggests that the C2 band may due to the binding of an oleate-dependent DNA binding protein(s) to the DNA. To further define the region on which the protein(s) binds, a labeled 70 mer (-349 to -279) was used in a gel shift assay. The C1 band was present in experiments with cell extracts from cells cultured in all three media (Figure 2-5, lane 2-4), whereas the C2 band was only present with cell extracts from oleate grown cells (Figure 2-5, lane 4). Both

Table 2-3

β-galactosidase activities of strains harboring *CYC1-lacZ* constructs in pNG22. The plasmid alone or plasmids containing *POX1* promoter sequences, were introduced into yeast strain BMG 1-7a and the cells were grown in glucose, glycerol or oleate media. Cell extracts were prepared and assayed for β-galactosidase activity. The numbers are mean of two experiments. The numbers in two experiments vary slightly

| pNG22 constructs | β-galactosidase activity (mU/mg protein) | | |
|---|---|---|---|
| | glucose | glycerol | oleate |
| pNG22 | 48.4 | 252.0 | 349.0 |
| pNG2256 (-294 to -138) | 6.8 | 300.0 | 413.0 |
| pNG22URS1(-454 to -349) | 3.0 | 3.9 | 3.2 |
| pNG22URS2 (-349 to -314) | 6.0 | 12.6 | 9.1 |

C1 and C2 were competed out in the presence of excess unlabeled 184 mer and 70 mer (Figure 2-5, lane 5, 6), whereas only C2 was abolished by excess 80 mer (Figure 2-5, lane 8). A third band, which sometimes appears in the experiments was not abolished by any competitor DNA fragment, indicating that this band is non-specific. These results suggest that the C2 binding site is in the region from -316 to -279. Furthermore, when the C2 band was specifically competed out by unlabeled DNA, the C1 band appears stronger (Figure 2-5, compare lane 4 and 7, 8). This suggests a possible reciprocal inhibition of DNA binding activity of the two DNA binding proteins.

The C2 band produced by labeled 70 mer in the gel shift experiment was not only abolished by excess unlabeled 70 mer (Figure 2-5, lane 6), but also by an adjacent 29 bp DNA fragment (-279 to -251) which does not overlap with the 70 mer (Figure 4, lane 7). Analysis of the DNA sequence in the C2 binding site revealed the presence of two incomplete palindromes spanning from -316 to -251. The 70 mer contains the upstream palindrome and the 29 mer contains the downstream one. The consensus sequence of these four repeats is (A/T)A(A/T)NNCCG(A/T)AT (Figure 2-6a). To test the requirement of these four repeats for the binding of C2 protein(s), labeled 80 mer containing all of the repeats was used in a gel shift assay with extracts prepared from glucose-, glycerol- and oleate-grown cells (Figure 6a). A strong band was seen with extracts from oleate-grown cells, but not with those from glucose- or glycerol- grown cells (Figure 2-6a, lane 2-4), this band was specifically competed out by excess unlabeled 80 mer (Figure 2-6a, lane 5), but not with the 105 mer containing URS1 (Figure 2-6a, lane 6). Each of the two palindromes were labeled and tested in gel shift assays. The results showed that the downstream palindrome (PR, -279 to -251) or the

Figure 2-5. A protein-DNA interaction that is specific for cell extracts induced by oleate. Labeled 70-mer was incubated with cells extracts form cells grown on glucose (lane 2), glycerol (lane 3), or oleate (lanes 4-8). Lanes 5-8 were carried out in the presence of 100-150 ng of unlabeled 184-mer (lane 5), 70-mer (lane 6), 29-mer (lane 7), or 80-mer (lane 8). Lane 1 was a control containing labeled 70-mer in the absence of cell extract. The asterisk indicates a nonspecific band.

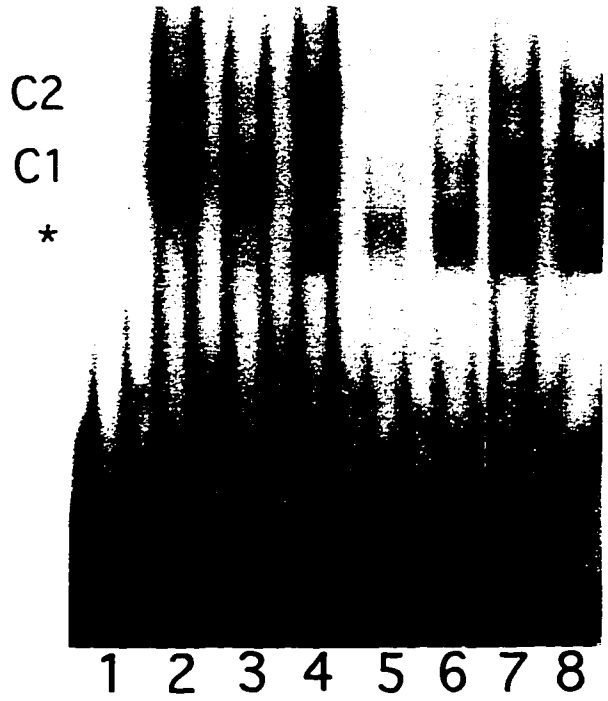
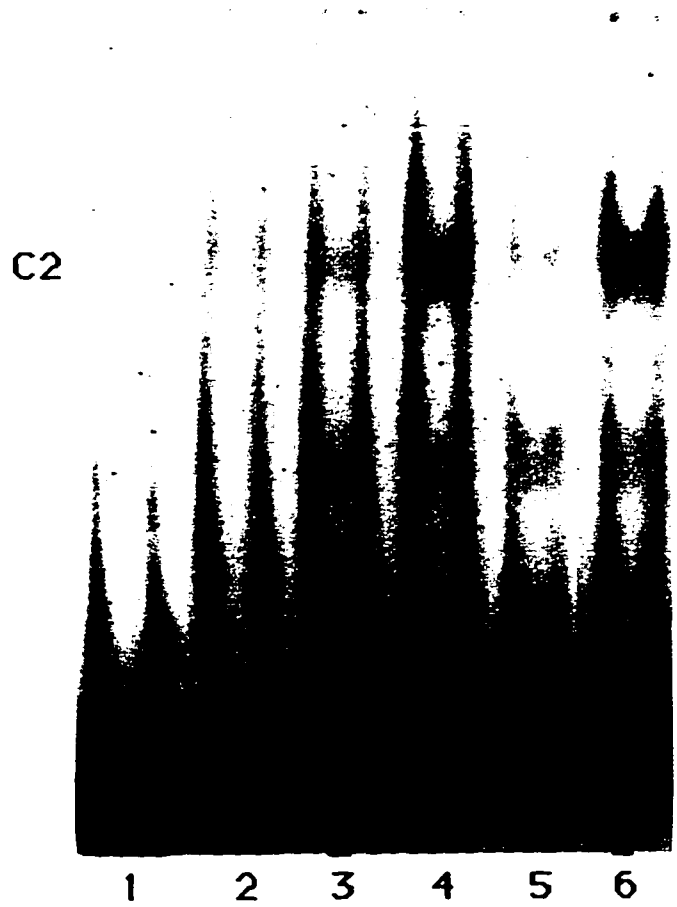


Figure 2-6. Characterization of an oleate response element by DNA band shift assays.

(a). Labeled 80-mer (-316 to -238) was incubated with extracts from cells grown in glucose (lane 2), glycerol (lane 3) or oleate (lanes 4-6) media. Lanes 5 and 6 were carried out in the presence of 100 ng of unlabeled 80-mer (lane 5) or 105-mer (lane 6). The 65-nucleotide sequence within the 80-mer that contains two palindromes is shown below. The conserved CGG triplets in the four repeats is underlined.

(a)




TTTACGGTATTAGTTGATTAAACTCCGAAGCGAAAGGAATTCGGTCATTAGCGGCTAATAGCCGT

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| ATGCCATAAT | AAACTCCGAA | AAGCCAGTAA | AATAGCCGT |

5' 1. TAATACCGTA
2. AAACTCCGAA
3. AATGACCGAA
4. AATAGCCGTT

upstream one ( PL, -279 to -316) were able to give rise to the C2 band (Figure 2- 6b, lanes 2 and 3), respectively. Furthermore, the two palindromes are able to compete with each other for protein binding, as shown in Figure 2-6c and 2-5. The C2 band obtained with cell extracts from oleate induced cells and labeled 29-mer, consisting of the downstream palindrome, was competed out by 70-mer, which contains the upstream palindrome (Figure 2-6c, lane 1 and 3), and the C2 band given by labeled 70-mer was shown to be abolished by excessive 29-mer (Figure 2-5, lane 7). This reciprocal competition suggests that the same protein or protein complex binds to the two palindromes. These results indicate that these two palindromes may act as an oleate responsive element (ORE) or upstream activation sequence (UAS1), and the oleate activated protein(s) may bind to either or both palindromes, perhaps in the form of a dimer. The most conserved sequences in the four repeats of the two palindromes are the CGG triplets, which are also conserved in the oleate response element present in other genes encoding peroxisomal β-oxidation enzymes in *S. cerevisiae* [111]. In order to test whether the CGG triplets are important for the protein(s)-UAS1 interaction, oligonucleotides YL2-5 and YL2-6, corresponding to the upstream palindrome of the putative UAS1, in which each CGG sequence is mutated to TAA were synthesized. These oligonucleotides, when annealed and labeled (mUAS1), do not give rise to a specific band in DNA band shift assay with cell extracts from oleate-grown cells (Figure 2-7, lane 4). Furthermore, the mUAS1 could not compete out the shifted band given by labeled uUAS1, which corresponds to the upstream palindrome of the putative UAS1 and which was obtained by annealing oligonucleotides YL2-3 and YL2-4 (Figure 2-7, lane 2).

Figure 2-6

(b). Extracts from oleate-grown cells were incubated with labeled DNA fragments PR (-279 to -238) (lane 2) or PL (-316 to -280) (lane 3). Lane 1 and 4 contain PR or PL in the absence of extracts.

(c). Cell extracts from cells grown in oleate medium were incubated with labeled 29-mer (-279 to -251) (lanes 1-4). Lanes 2-4 were carried out in the presence of unlabeled 99-mer, 70-mer or 29-mer, respectively. The asterisks indicate nonspecific bands.

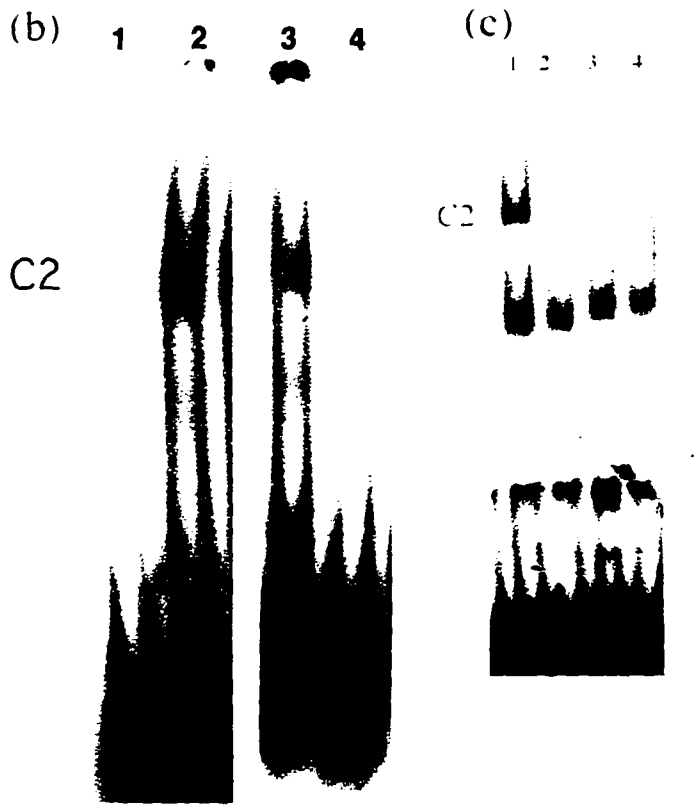
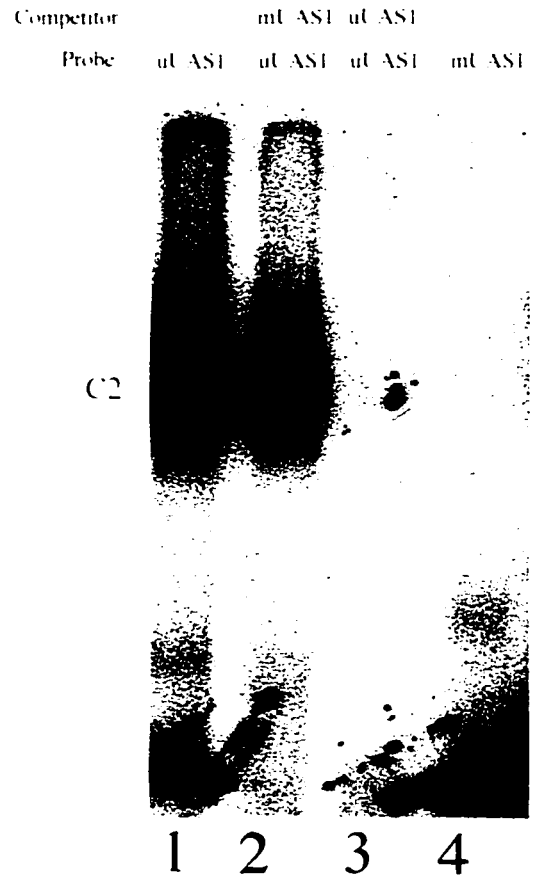


Figure 2-7. The CGG triplets are important for the interaction of the *POX1* UAS1 with its binding protein.

A DNA band shift assay was carried out with cell extracts from cells grown in oleate medium and labeled uUAS1 (lanes 1-3), which corresponds to the upstream palindrome of the *POX1* UAS1, or mUAS1, which contains mutations in the CGG triplets in the upstream palindrome of UAS1 (lane 4). Lanes 2 and 3 contain competitor DNA consisting of mUAS1 or uUAS1, respectively.



***Function of the POX1 UAS1 in a heterologous CYC1-lacZ reporter gene:***

In order to test the positive UAS function of the oleate responsive element defined by mobility shift assays, its ability to promote transcription was tested in a heterologous *CYC1* promoter. The DNA fragments covering the two palindromes, or part of them, were subcloned into an expression vector pNG15 which contains the promoter of the *CYC1* gene, lacking the *CYC1* UAS element, fused in frame with *lacZ* [139]. These constructs were introduced into *S. cerevisiae* strain 1-7a, and β-galactosidase activities were measured in cells grown in glucose, glycerol or oleate medium. The control pNG15 gave a low level of activity in all conditions tested (Table 2-4). When a 56 bp fragment (-294 to -238) containing one palindrome was inserted in front of *CYC1/lacZ* (pNG1556), the β-galactosidase activity was increased approximately 100 fold in oleate-grown cells, compared with that of the control pNG15. When the 80 bp fragment (-316 to -238), which includes the complete putative UAS, was present in the upstream of *CYC1/lacZ* (pNG1580), the β-galactosidase activity in oleate-grown cells increased about 200 fold over background. The activity was reduced to 50 fold above background in cells transformed with pNG1599 which contains the complete UAS, but also URS2. A similar trend was seen in transformants grown in glycerol medium, but with approximately 5-10 fold less induction compared to that in oleate-grown cells. As expected, the transformants grown in glucose medium did not show induced activity. These results demonstrate that the ORE identified by protein-DNA interaction can confer oleate activation for the expression of the reporter gene in a heterologous promoter.

Table 2-4

β-galactosidase activities of strains harboring *CYC1-lacZ* constructs in pNG15. Cells were cultured in glucose, glycerol or oleate media. Cell extracts were prepared and assayed for β-galactosidase activity. The results are mean of two experiments, in which the numbers vary slightly.

| pNG15 constructs | β-galactosidase activity (mU/mg protein) | | |
|---|---|---|---|
| | Glucose | Glycerol | Oleate |
| pNG15 | 0.3 | 0.6 | 0.6 |
| pNG1556 (-294 to -238) | 0.2 | 8.9 | 61.1 |
| pNG1580 (-316 to -238) | 0.2 | 15.6 | 130.0 |
| pNG1599 (-349 to -251) | 0.7 | 0.9 | 33.6 |

## Discussion

By the DNA band shift assays and functional analysis described above, two new regulatory elements were identified in the *POX1* promoter region. These studies, together with previous findings in the *POX1* promoter, are summarized in Figure 2-8. There are at least three cis-acting elements involved in mediating the regulation of *POX1*. All of these regulatory elements were found in a region beginning about 180 bp upstream of the *POX1* transcription initiation site (-55 and -69, upstream from the transcription initiation codon), and are clustered in a 200 bp region. The transcription of *POX1*, as shown by the expression of the *lacZ* reporter gene in pP13570 (Figure 2-2), is repressed when cells are grown in glucose, derepressed in glycerol and induced (about 10 fold over the expression in glycerol) when a long chain fatty acid such as oleate is supplied as the carbon source. The *FOX3* gene, which encodes peroxisomal 3-oxoacyl-CoA thiolase in *S. cerevisiae*, has been shown to exhibit a similar regulation pattern [98], indicating that a general mechanism might act to regulate peroxisomal β-oxidation in *S. cerevisiae*.

There are at least two upstream repression sequences (URS1 and URS2) in the *POX1* promoter. From sequence analysis, the URS1 protein(s) binding site sequence is a complete inverted repeat of the sequence, 5'AGGGT(A/T)A(T/A)3', whereas the URS2 consists of an incomplete direct repeat of the following sequence: 5'AGGG(C)TT(A/T)NA 3', separated by seven nucleotides. One half of the inverted repeat in URS1 has been shown to be sufficient for protein(s) binding, and AGGG are critical nucleotides for protein binding to occur [132]. URS1 and URS2 are similar but in a reverted orientation and the AGGG is conserved, so it

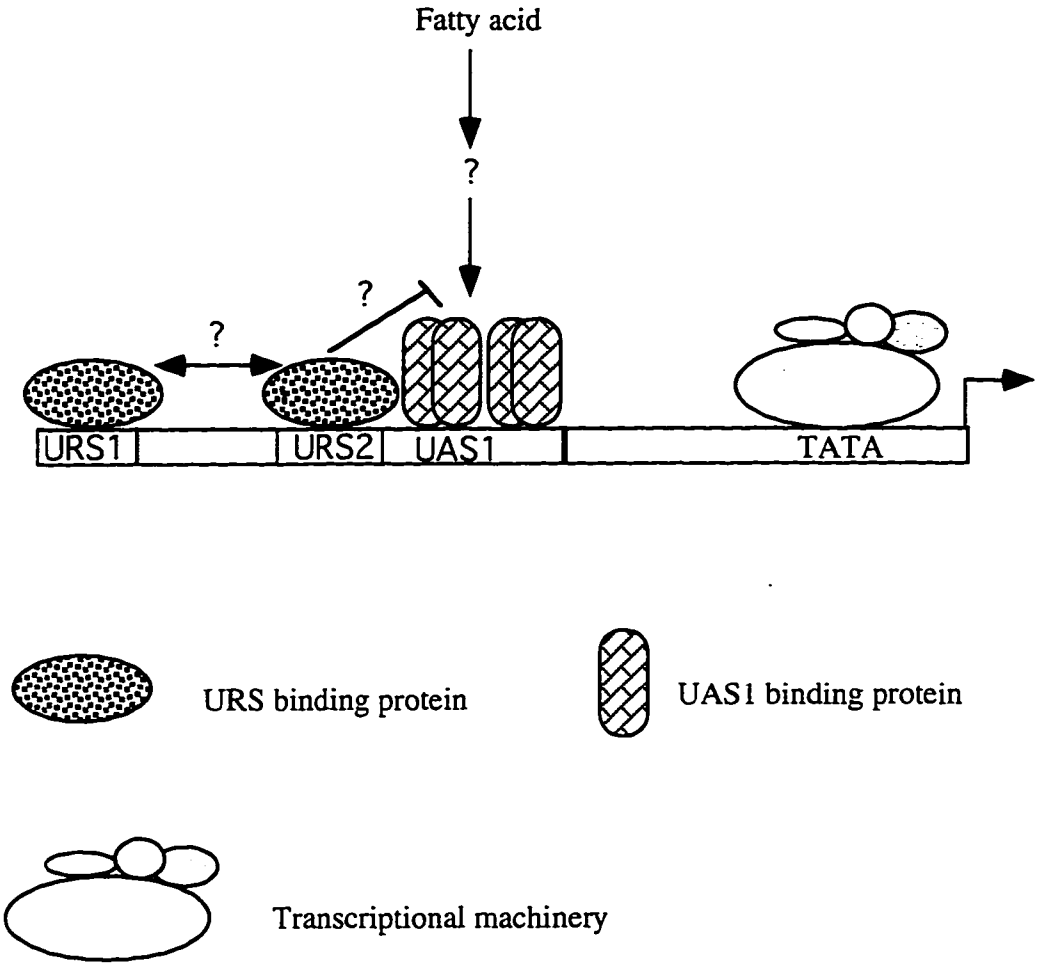



Figure 2-8. A model for the transcriptional regulation of *POX1*. See text for details

is possible that the same protein(s) binds to these two elements. This is supported by the finding that the interactions between trans-acting factors and URS2 were competed away by addition of the URS1 fragment (Figure 2-4b).

Deletion of all of the regulatory region of *POX1* led to high β-galactosidase activity in cells grown in all conditions (Figure 2-2, pP13534). This implies that URS1 and URS2 may function as general repression sequences and might maintain the expression of *POX1* at low levels in all growth conditions. This is supported by the fact that deletion of URS1 and URS2 led to increased expression of the reporter gene when cells were grown on glucose or glycerol medium (Figure 2-2, pP13532 and pP13572). Furthermore, URS2 interferes with the function of the *POX1* UAS1 in a heterologous *CYC1* promoter in all growth conditions tested, as shown in Table 2-4 (pNG1599). The complex regulation of *POX1* might explain why we were unable to identify the UAS1 by simple deletion analysis, however, an internal deletion in UAS1 has been shown to abolish the oleate induction of the *POX1-lacZ* reporter gene (unpublished data by Dr. Karpichev). In this case, the β-galactosidase activity was low when cells were grown in glycerol medium, and there was no increase in the presence of oleate.

URS1 and URS2 fragments are equally capable of repressing the action of the *CYC1* UAS, in the context of the *CYC1* promoter, under all of the growth conditions tested (Table 2-3). However, in the context of the *POX1* promoter, the presence of the URS's, and its binding protein, did not completely block the function of the UAS1 and its binding protein when cells are grown in the presence of oleate. This functional difference is probably due to the different position of the *POX1* URS's in the *POX1* promoter and in the pNG22 constructs. In the

native *POX1* promoter, the two URS's are located upstream of the *POX1* UAS, whereas, they are located in the downstream of the *CYC1* UAS in the pNG22 constructs. The URS1 and URS2 binding protein(s) is able to bind to the URS's whether the cells are repressed or induced, as shown by band shift assays with cell extracts from cells grown in glucose, glycerol and oleate media [132] (Figure 2-5). Thus, in pNG22URS1 and pNG22URS2, the URS's and their binding protein(s) located downstream of the *CYC1* UAS might block the interaction between the *CYC1* UAS binding protein(s) and the transcription initiation complex, and inhibit the function of the *CYC1* UAS in cells cultured in all conditions. An alternative possibility for the different actions of the *POX1* URS's, in the native *POX1* promoter and in pNG22 constructs, is that the URS's and *POX1* UAS binding proteins may compete with each other to bind to the elements in the native *POX1* promoter. Reciprocal inhibition was suggested by the observation that when DNA fragments containing both URS2 and UAS (184-mer and 70 mer) were used in band shift assays with oleate induced cell extracts, the C1 and C2 bands were both apparent, but there was not a third band of slow mobility, which would be present if two different proteins were binding to the same DNA fragment. Thus, in oleate conditions, there appear to be two aspects involved in the *POX1* activation by UAS1 binding protein: (1) UAS1 binding protein may activate the transcription initiation complex; (2) UAS1 binding protein may relieve repression mediated by URS2, and this may be achieved by preventing the binding of the URS2 binding protein.

Expression of the *POX1-lacZ* fusion gene increased 5-10 fold in glycerol- and oleate-grown cells when URS1 was mutated, and a further increase was seen when it was deleted ( Figure 2-2, pP1353 and pP13532). However, when URS2 was deleted ( pP13572 ), the β-

galactosidase activity of glucose-grown cells did not increase (Figure 2-2, compare pP13532 and pP13572). It is possible that URS2 binding protein may function by interacting with upstream cis-acting element such as URS1. Alternatively it is possible that the deletion construct pP13532, in which the region between -454 to -349 was deleted, may have already lost part of the URS2 region required for the optimal function of URS2 in the context of the *POX1* promoter, this would also explain why the activity of pP13532 is higher than pP1353. The observation that deletion of URS2 (-349 to -316) decreased the β-galactosidase activity of oleate-grown cells (pP13572) suggests that in order to function optimally as an activating sequence, UAS1 may require adjacent cis-elements.

The UAS1 sequence identified in *POX1* is similar to a sequence present in several genes encoding peroxisomal proteins, such as *FOX3* (encoding thiolase), *FOX2* (encoding the trifunctional enzyme) and *CTA1* (encoding catalase A). The conserved sequence has been termed the ORE [110]. This was proposed to be the potential binding site for an unknown trans-acting factor responsible for the oleate-specific induction. The presence of the ORE in these genes indicates that the factor(s) binding to this element may be involved in the coordinated expression of oleate-inducible genes. A single palindrome consisting of two repeats was identified in the 5' regulatory region of *FOX3*, and has been shown to mediate the oleate induction of *FOX3* [110]. The finding of two palindromes consisting of four repeats in the *POX1* promoter may reflect the important regulatory role of acyl-CoA oxidase, the rate limiting enzyme in the peroxisomal β-oxidation pathway. This complex regulation of *POX1* resembles the regulation of the *GAL* genes in *S. cerevisiae*. The regulation of these genes, which encode the enzymes responsible for converting galactose to glucose-6-phosphate, has

been studied extensively [126]. The binding site for transcription factor Gal4p is an inverted repeat of CGGA(G/C)GAC, the highly conserved and important bases appear to be the CGG triplets which are also the important bases in the *POX1* UAS1 (Figure 2-7). Some genes such as *GAL1/GAL10* have four such binding sites of which just two are required for gene expression [126].

In the deletion analysis in Figure 2-2, deletion of the whole UAS1 (-316 to -238) and the downstream 25 bp sequence (pP13533) caused a decrease in the expression of the *lacZ* reporter gene in cells grown on oleate medium, but an unexpected increase in the expression in glucose grown cells was observed (compare pP13572 and pP13533). This suggests that a negative element may exist in the region from -213 to -280, however no protein(s) has been identified to bind to this region in band shift assays using cell extracts from cells grown in glucose medium. It is possible that a protein, which may be present in low levels, could bind to the region from -280 to 213 in glucose-grown cells and mediate glucose repression. An alternative possibility is that the defined UAS1 could mediate both glucose repression and oleate induction. In glucose grown cells, a protein of low abundance, which is not detectible in *in vitro* band shift assays, could bind to the UAS1 and mediate the glucose repression of *POX1*. In oleate-grown cells, a different factor would be activated and bind to the same region and activate the transcription of *POX1* by an "anti-repression" mechanism. Thus, this regulatory element might have a dual function. This theory is supported by the finding that the inserted 56-mer in the construct pNG2256, which contains the downstream part of the UAS1, caused repression in glucose-grown cells (Table 2-3, compare activities of pNG22 with pNG2256). It is possible that the *POX1* UAS1 plays a role in both derepression and

oleate induction. This was suggested by the result that UAS1 in a heterologous *CYC1* promoter caused an increase in the expression of the reporter gene in glycerol culture conditions, compared with glucose conditions (Table 2-4, pNG2256 and pNG2280).

*ADR1*, *SNF1* and *SNF4* have been implicated in the regulation of *CTA1* and *FOX3*. It has been suggested that *SNF1* and *ADR1* affect peroxisome proliferation via separate signal pathways [117]. These genes have been reported to have little effect on the oleate induction of *POX1* [122]. The identification of the *POX1* UAS1, and the finding that a trans-acting factor(s) binds to the UAS1 in an oleate-dependent manner, provides the way to isolate and characterize the trans-acting factor(s) which bind to this sequence.

# CHAPTER III

## PURIFICATION AND PROPERTIES OF AN OLEATE-ACTIVATED TRANSCRIPTION FACTOR THAT MEDIATES THE INDUCTION OF *POX1* AND PEROXISOME PROLIFERATION

### Introduction

In the previous chapter, I described the studies that led to the identification of the regulatory elements in the *POX1* promoter. Two upstream repression sequences (URS1 and URS2) and one upstream activating sequence (UAS1) have been identified. A protein or protein complex has been shown to bind to UAS1 in an oleate-dependent fashion, and this might lead to the activation of *POX1*.

Proteins involved in the oleate induction pathway leading to the activation of the genes encoding peroxisomal proteins are totally unknown. The identification of the oleate-dependent UAS1 in *POX1*, that serves as a binding site for specific transcription factors involved in *POX1* regulation, allowed me to explore this pathway. My next goal was to purify and characterize the UAS1 binding protein(s). In this chapter, I describe the purification and molecular identification of a UAS1 binding, oleate-activated transcription factor (Oaf1p). I demonstrate that the amount of protein binding to UAS1 progressively increases during growth on oleate. Furthermore, the disruption of the *OAF1* gene abolishes the oleate induction of *POX1* and oleate-induced peroxisome proliferation.

## Materials and Methods

***Yeast strains and Culture conditions*:**

The *S. cerevisiae* strains 3A (*ura3, trp1, arg4, ctt1*), BMG1-7a (*leu2, ura3, ade1, his4*), and W3031A (*leu2, ura3, trp1, ade2, his3*) were used for the experiments in this chapter. Yeast cells were cultured in YPD, YPG or YPGO medium as described in chapter II. YNO medium contains 0.05% yeast extract, 0.67% yeast nitrogen base without amino acids, 0.1% oleic acid and 0.2% Tween 40.

***Oaf-DNA binding as a function of growth in oleate medium:***

In the time course study cells (BMG1-7a) were grown for 8 hours in YPD medium and were then shifted to YPGO medium. Samples were taken at each of the following time points: 2, 4, 6, 8, 12, 14 and 18 hour. Band shift experiments were carried out with labeled 80 mer containing the whole UAS1 or with a 184-mer containing both URS2 and UAS1 (Figure 2-2). The intensity of the shifted band was quantified using a Phosphorimager (Molecular Dynamics).

***Preparation of a UAS1-oligonucleotide affinity column:***

The oligonucleotides YL2-3 and YL2-4 (see chapter II), which contain the upstream palindrome of the *POX1* UAS1, were annealed. Approximately 200 μg of annealed oligonucleotides were phosphorylated at the 5' ends with polynucleotide kinase (Biolab, 100 U), self-ligated and coupled to cyanogen bromide-activated-Sepharose 4B (Pharmacia Biotech) according the method described [147].

***Purification of the oleate-activated DNA-binding protein:***

Yeast cells (strain 3A) were cultured in YPD to stationary phase and then transferred to YPGO medium and grown for 18 hours. Approximately 60 g of oleate induced cells were harvested, washed with sterile water and resuspended in 60 ml of extraction buffer (200 mM Tris-HCl, pH 8.0, 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 7 mM 2-mercaptoethanol, 1 mM PMSF, 1 µl/ml protease inhibitor mixture containing 10 µg/µl leupeptin, antipain, chymostatin and pepstatin).

All further operations were performed at 4°C. An amount of glass beads (0.5 mm diameter) equivalent to half of the cell suspension volume was added, and the cells were disrupted by vortexing for 20 min. Cell debris was removed by centrifugation at 100,000 g for 1.5 hours. A saturated solution of ammonium sulfate was added to the supernatant to give a final concentration of 25%. Following a 10-30 min incubation, the precipitated proteins were recovered by centrifugation and resuspended in buffer A (20 mM Hepes-NaOH, pH 7.9, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF).

Cell extract was then passed through a cation exchange SP-Sepharose column (Pharmacia Biotech). Proteins were eluted using a linear KCl gradient (0.2-0.7 M prepared in buffer A). Fractions containing the highest DNA binding activity of Oaf (as judged by specific binding activity in a DNA band shift assay) were pooled and concentrated using a centriprep-10 centricon (Amicon). The concentrate was adjusted to 0.1 M KCl and then loaded onto a calf thymus double-stranded DNA cellulose column. Elution of proteins bound to the column was achieved using a KCl gradient, as above, and fractions with highest levels of Oaf1p activity were loaded onto a 2-ml UAS1-oligonucleotide affinity column (about 100 µg DNA/ml Sepharose) pre-equilibrated with buffer B (25 mM Hepes-NaOH, pH 7.9, 0.1% Nonidet P-

40, 0.1 mM PMSF) containing 0.25 M KCl. The column was then washed with 30 ml of equilibrating buffer. Bound proteins were eluted with a linear KCl gradient (0.25-1.0 M in buffer B). The fractions with highest DNA binding activity of Oaf were pooled and concentrated using a microcon-30 spin column (Pharmacia Biotech) in the presence of 0.1% SDS.

***Amino acid sequence analysis of tryptic peptides from Oaf1p:***

The proteins eluted from the affinity column were resolved by SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membrane in standard Towbin buffer. The membrane was stained with 1% Ponceau S in 5% acetic acid. The portion of the nitrocellulose paper containing the upper band of the doublet of interest, at approximately 120 kD, was excised. The protein was sent to the Protein Sequence Core in Mount Sinai School of Medicine for tryptic digestion, and the resulting peptides were purified by HPLC, as described in Luo et al [148]. Amino acid sequences of peptides were determined by Dr. Ron Kohanski by automated Edman degradation using a Beckman model 2090E gas phase sequencer [148].

***PCR amplification of the gene encoding Oaf1p:***

In order to obtain a DNA fragment encoding the 118.2 kD putative transcription factor, oligonucleotidesYL3-1 (5' CAGCTTCTTGTCTCCACAGTAC 3') and YL3-2 (5' TTGAGC ACTTATATTATCGGTC 3'), which were complementary to the 5' and 3' ends of the DNA sequence obtained from GenBank data base, were used in a PCR amplification with genomic DNA isolated from *S. cerevisiae* strain W3031A.

***Disruption of the OAF1 gene:***

To confirm that the cloned gene encodes the purified UAS1-binding protein, the genomic allele of this gene was disrupted using a one-step gene disruption procedure [149]. The DNA product (about 3.3 kb) obtained from the PCR amplification described above was cloned into pBluescrip II (KS+), and a 1.3 kb SphI fragment, containing part of the open reading frame, was excised. The ends of the plasmid were rendered blunt with T4 DNA polymerase, and a 1.8 kb SmaI/HincII fragment containing the *HIS3* gene (gift from Dr. Jeanne Hirsh) was inserted. The 3.8 kb *OAF1*::*HIS3* fragment was then excised and introduced into yeast strain W3031A, which had been transformed with a plasmid (pPOXlz) containing the *lacZ* reporter gene under control of the *POX1* promoter. The resulting transformant containing a disrupted copy of *OAF1* was called OA1.

***Cloning of the genomic copy of OAF1:***

In order to rescue the *OAF1* disrupted strain, a genomic copy of the *OAF1* gene was obtained by Dr. I.V. Karpichev and Mr. Murl Casey in our laboratory, by screening a yeast genomic library [150] with the PCR product described above. A positive clone was identified, and a 4.8 kb HincII fragment containing the *OAF1* open reading frame, 1.4 kb 5' upstream sequence from the ATG, and 70 bp 3' downstream sequence, was subcloned into the EcoRV site of pT7Blue (Novagen) to create pOAF1. A SmaI/XbaI fragment was then subcloned into the corresponding site in pRS306 [151] to produce pRSOAF1.

***UV cross-linking in a gel support:***

The complementary region of oligonucleotides YL3-3 (5'GATCCTTCGGAGTTTAA TCAACTAATACCGTA 3') and YL3-4 (5'GGGGTCGACTTTACGGTATTAGTTGA 3') (the complementary bases of the two oligonucleotides were underlined) containing the

upstream palindrome of *POX1* UAS1 were annealed, and the noncomplementary, overhanging region were filled in with [$^{32}$P]dCTP using Klenow fragment and substituting bromo-2'-deoxyuridine triphosphate (BrdU) for thymidine in order to increase the cross-linking efficiency [152]. 1 µl of the labeled DNA (approximately 10,000 cpm) was incubated with 4 µl of a fraction containing UAS1 binding activity eluted from SP cation exchange column or the UAS1 affinity column in the presence of 1 µg of a competitor DNA, poly(dI-dC). The mixture was separated in a 5% nondenaturing polyacrylamide gel. Following electrophoresis, the gel was subjected to UV radiation (3000 uw/cm2) for 5 min at 4°C and was then exposed to film for 1 hour. The shifted band, due to the formation of a specific protein-DNA complex, was located by lining up the gel with the film, and the gel slice in the region was excised. The gel slice was then incubated with 40 µl of SDS sample buffer (120 mM tris, pH 6.8, 4% SDS, 20% sucrose, 0.002% bromphenol blue and 20 mM DTT) for 15 min at 68 °C, loaded into the well of an 8% SDS-polyacrylamide gel, and electrophoresed for 2 hours at 150 volts. The gel was subsequently dried and subjected to autoradiography.

***UV cross-linking in solution:***

A binding mixture that contained 10 µl binding buffer [132], 5 µl of the fraction with highest UAS1 binding activity eluted from an SP action exchange column, 1 µl of a uniformly labeled UAS1-DNA fragment (prepared as described above), and 2 µg of competitor poly(dI-dC) was prepared. This solution was directly exposed to UV radiation (3000 µw/cm2) for 7 min. The sample was treated with DNase I (2 units) for 20 min at 37 °C. SDS sample buffer was then added to the sample and the sample was boiled for 5 min and then electrophoresed on an 8% SDS-polyacrylamide gel.

***Immunoelectron microscopy:***

Electron microscope immunolocalization was carried out according to [153] by Mr. Vladimir Protopopov in the microscope center. Rabbit anti-serum 10-324 raised against total peroxisomal proteins from *Candida tropicalis* [154] and protein A conjugated to 15 nm gold were used. 10-324 cross reacts with several peroxisomal proteins from *S. cerevisiae* (unpublished data, see [155]).

***Other methods:***

RNA purification and Northern analysis were done by Dr. I.V. Karpichev [148]. DNA band shift assays, protein assays and β-galactosidase assays were performed as described in chapter II. Yeast genomic DNA was prepared as described [140].

## Results

***Oaf binding as a function of growth in oleate medium:***

In order to determine the kinetics of activation of the oleate activated transcription factor (Oaf), when cells were shifted from glucose to oleate medium, a time course study was carried out, in which the levels of the shifted labeled UAS1, resulted from the binding of Oaf, were measured. Cell extracts from cells cultured in oleate medium for various hours were used in band shift assays, either with 184-mer, which contains both URS2 and UAS1 (Figure 3-1a), or an 80-mer containing UAS1 alone (Figure 3-1b), as DNA probes (the sequence of 184-mer and 80-mer were shown in Figure 2-3). The intensity of the shifted band caused by protein binding to UAS1 was low at the 2-hour time point (Figure3-1a, C2 band). However,

Figure 3-1. Oaf binding as a function of growth in oleate medium.

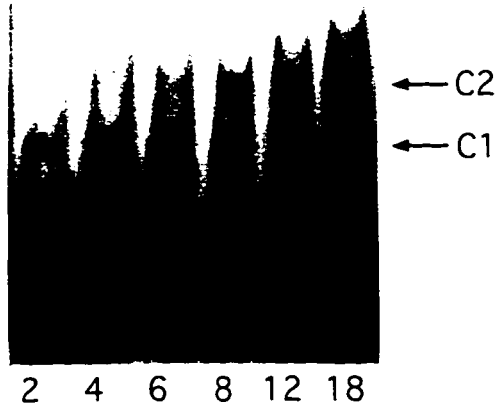
DNA binding assays were carried out with extracts from cells grown in oleate medium for 2-18 hours.

(a). The band shift assay was carried out with approximately 30 μg of cell extract protein and a $^{32}$P-labeled 184-bp fragment that contains URS2 and UAS1.

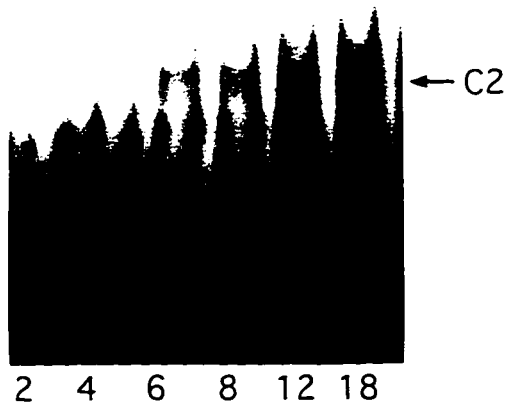
(b). A labeled 80-bp fragment containing UAS1 was used in DNA band shift assay.

C1 refers to the complex formed by protein binding to URS2, and C2 refers to the complex formed by binding of Oaf to UAS1. The intensity of the shifted bands was quantified by PhosphorImager analysis.

(a)




(b)



a band shift with high intensity due to a protein binding to URS2 was observed at the 2-h time point (Figure 3-1a, C1 band). Thus, the protein that binds to this repression element appears to be present in all growth conditions. The intensity of the C2 shifted band, caused by protein binding to UAS1, increased 8-fold over 18 hours, whereas the intensity of the C1 band caused by protein binding to URS2 approximately doubled after 4 hours induction and then remained fairly constant (2.8-fold increase over 18 hours). The time-dependent increase in the intensity of the C2 shifted band occurred irrespective of whether the 184-mer, containing both URS2 and UAS1, or the 80-mer containing only UAS1 (Figure 3-1b), were used in the assay. These results suggest that either the expression of Oaf itself is induced by oleate, or another factor(s) which is required for the activation of Oaf is induced by oleate.

***Temporal induction of POX1 expression:***

In order to determine whether the kinetics of *POX1* induction correlated with the binding of Oaf to UAS1, a BMG 1-7a strain pP13570, which contains the *lacZ* gene under the control of the *POX1* promoter (see Figure 2-2), was used in the time course study shown in Figure 3-1. Thus, the temporal expression of *POX1* and the activity of β-galactosidase driven by the *POX1* promoter could be measured. The *POX1* promoter-dependent expression of β-galactosidase, represented by β-galactosidase activity, in cells induced for various hours, revealed an intriguing pattern (Figure 3-2a). The β-galactosidase activity was very low in glucose-grown cells but was increased following the shift of the cells into oleate medium. The activity increased during the first 2-6 hour induction period and reached a maximum value at 6 hour of induction, after which the activity decreased until the 12 hour time point. However, by 18 hour time point the activity increased slightly. Northern blot analysis of the

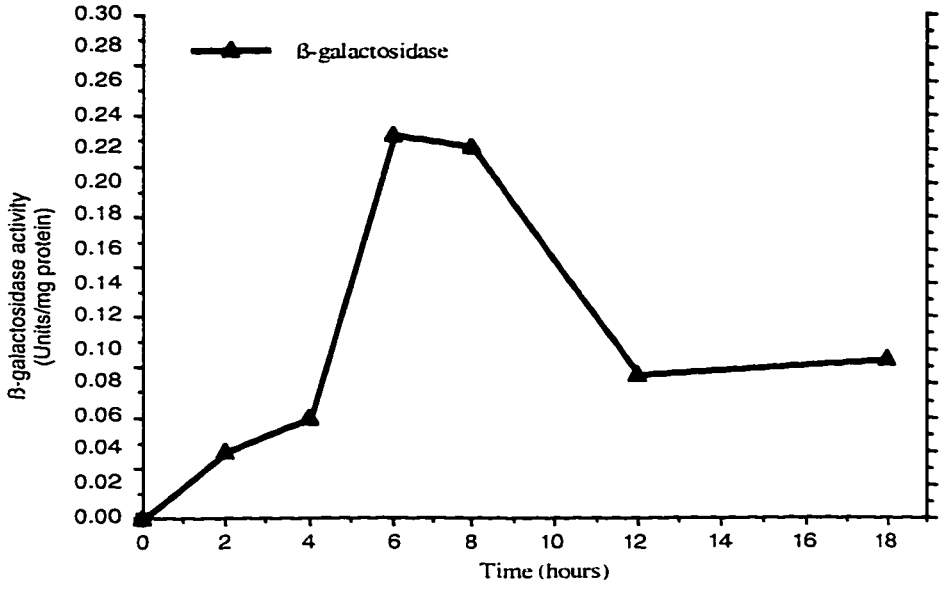
Figure 3-2. Temporal induction of *POX1* in cells shifted from glucose to oleate medium for 0-18 hours.

(a). *POX1* promoter-dependent β-galactosidase activities were measured in extracts prepared from cells grown in glucose medium (YPD) for 8 hours and transferred to oleate medium (YPGO) and grown for 2-18 hours. β-galactosidase activity is the mean of two experiments, in which the numbers vary slightly.

(b). Northern blot analysis of the *POX1* mRNA. Approximately equal amounts of RNA (20 μg) isolated from the same cells used in (a) were loaded.

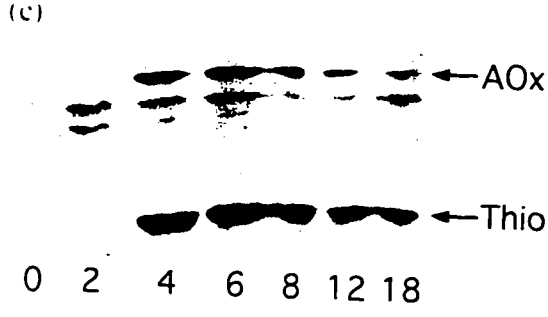
(c). Immunoblot analysis of the temporal expression of acyl-CoA oxidase and thiolase. Approximately 50 μg of cell extract protein from cells used in (a) was separated by 10% SDS-polyacrylamide gel electrophoresis followed by immunoblot with antisera against *S. cerevisiae* acyl-CoA oxidase and thiolase.

(a)




(b)


(c)



*POX1* mRNA level, carried out by Dr. I.V. Karpichev, followed a similar pattern to the β-galactosidase activity. Equal amounts of RNA from each time points were loaded on an agarose gel and transferred to membrane. Probing the membrane with labeled *POX1* DNA resulted in a *POX1* mRNA pattern that rapidly increased during the first 4 hours of induction (Figure 3-2b). Acyl-CoA oxidase (Aox) and thiolase protein levels at each time point, followed the same pattern, as shown by Western blot analysis using anti-seria against *S. cerevisiae* acyl-CoA oxidase or thiolase (Figure 3-2c). Neither of the proteins could be detected until the 4 hour time point, and the maximal levels were detected after 6 hours, beyond this point, the levels of protein decreased. Thus, the point at which the peroxisomal proteins are first expressed correlated with the time that a *POX1* UAS1-protein complex is first detectible. However, while the intensity of the shifted band containing the UAS1-protein steadily increased, the *POX1* message of expressed protein decreased. Whether these fluctuations are caused by protein and/or mRNA degradation or due to transcriptional control is not clear.

***Purification of the UAS1-binding protein, Oaf:***

*POX1* expression is induced by growing yeast cells in oleate medium (YPGO). This induction is correlated with the binding of a protein or protein complex to UAS1 in *POX1* promoter. As a first step toward understanding the mechanisms by which the *POX1* UAS1 mediates the oleate -specific induction of this gene, I developed a procedure to purify the protein that binds to UAS1. The protein purification procedure is shown in Figure 3-3. The ammonium sulfate precipitated sample was first subjected to SP-Sepharose chromatography, which is a cation exchanger (Figure 3-4). Approximately 17% of the total protein, including

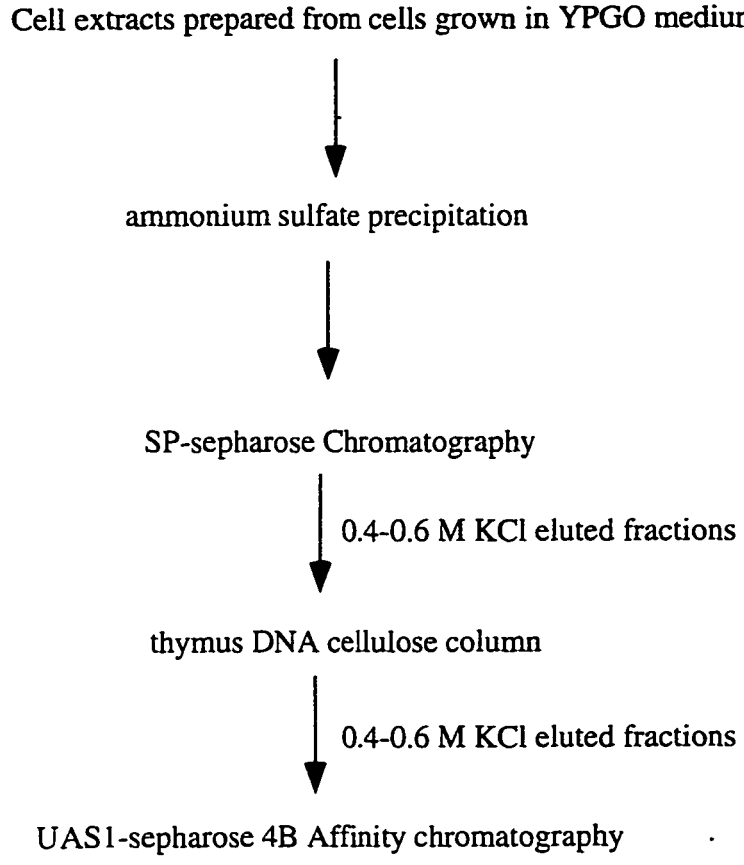


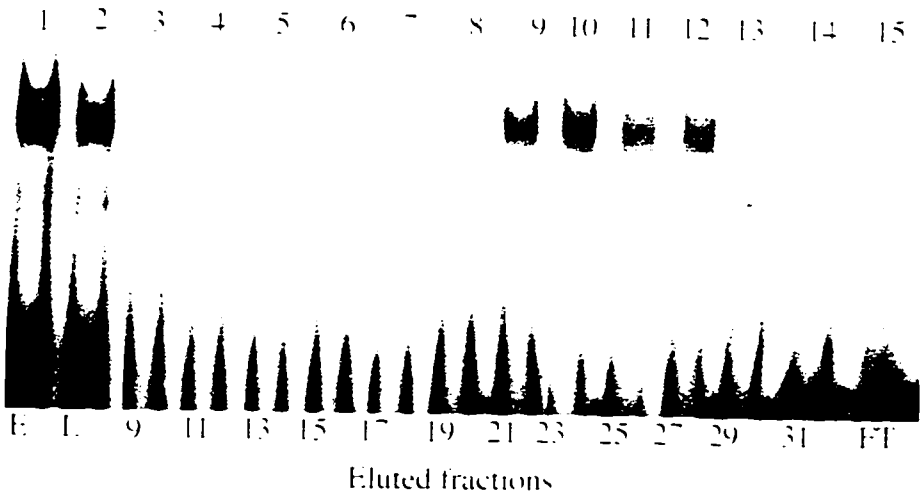

Figure 3-3. Procedure for the purification of the *POX1* UAS1 binding protein, Oaf

Figure 3-4. Elution profile of Oaf obtained from SP-sepharose chromatography.

(a). Elution profile of the SP-sepharose column as detected by a DNA band shift assays carried out with 1 μl out of approximately 120 ml of total cell lysates (lane 1), 1 μl out of 150 ml of ammonium sulfate precipitated sample (lane 2), 1 μl of flow-through (lane 15), and 1 μl of alternate fractions from 9 to 31 (8 ml each) eluted with a linear KCl gradient (0.2-0.7 M) and approximately 10 fmol of labeled uUAS1 (lanes 3-14).

(b). Protein and DNA binding activity profile. The protein concentration of alternate fractions eluted from a SP-sepharose column were determined by measuring the absorbance at 280 nm. Oaf DNA binding activity measured in (a) is represented by the radioactivity of the shifted band, which was quantified using a PhosphorImager ImageQuant software.

(a)




(b)

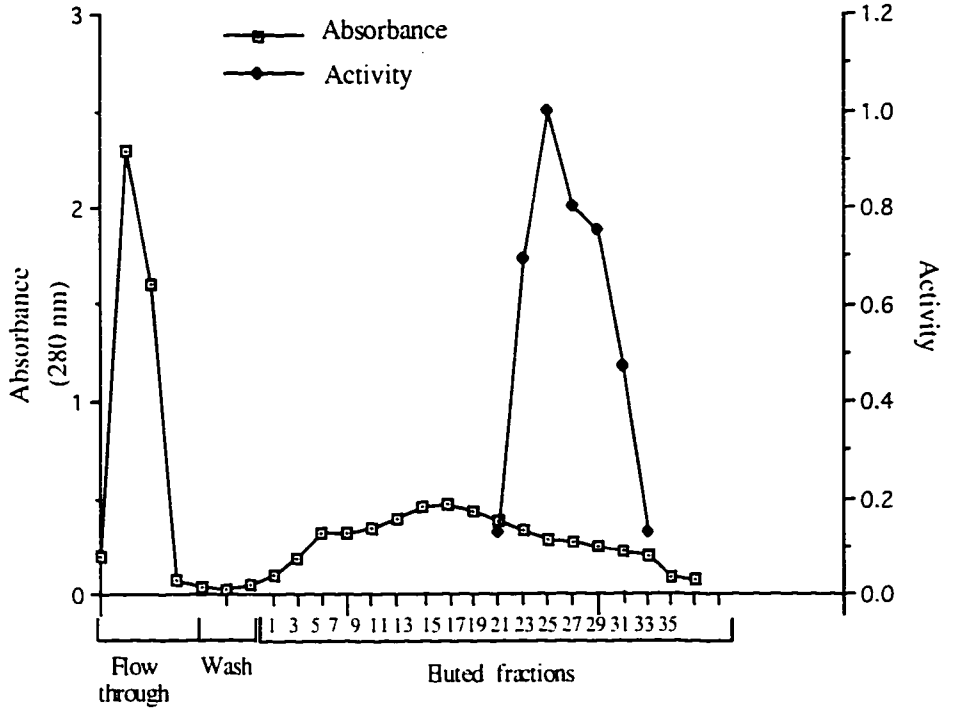
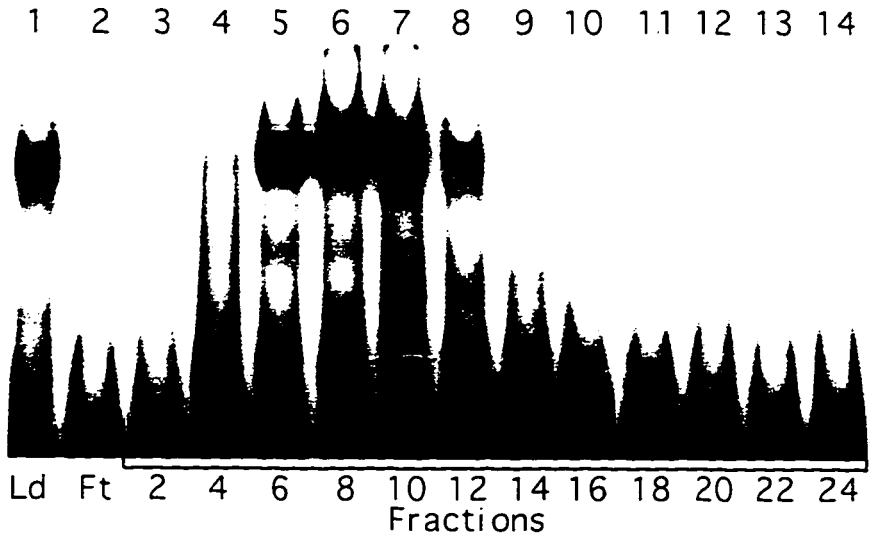


Figure 3-5. Elution profile of Oaf from thymus DNA cellulose chromatography.

(a). Elution profile of the double stranded thymus DNA cellulose column as detected by a DNA band shift assay, which was carried out with 1μl out of 40 ml column loading sample (lane 1), 1 μl of flow-through (lane 2), or 1 μl of alternate fractions (1.3 ml each) eluted as described in Figure 3-4 and approximately 10 fmol of labeled uUAS1 fragment (lane 3-14).

(b). Protein and DNA binding activity profile. The protein concentration was represented by the absorbance at 280 nm. Oaf DNA binding activity was obtained by quantifying the radioactivity in the shifted band in a DNA band shift assay shown in (a).

(a)




(b)

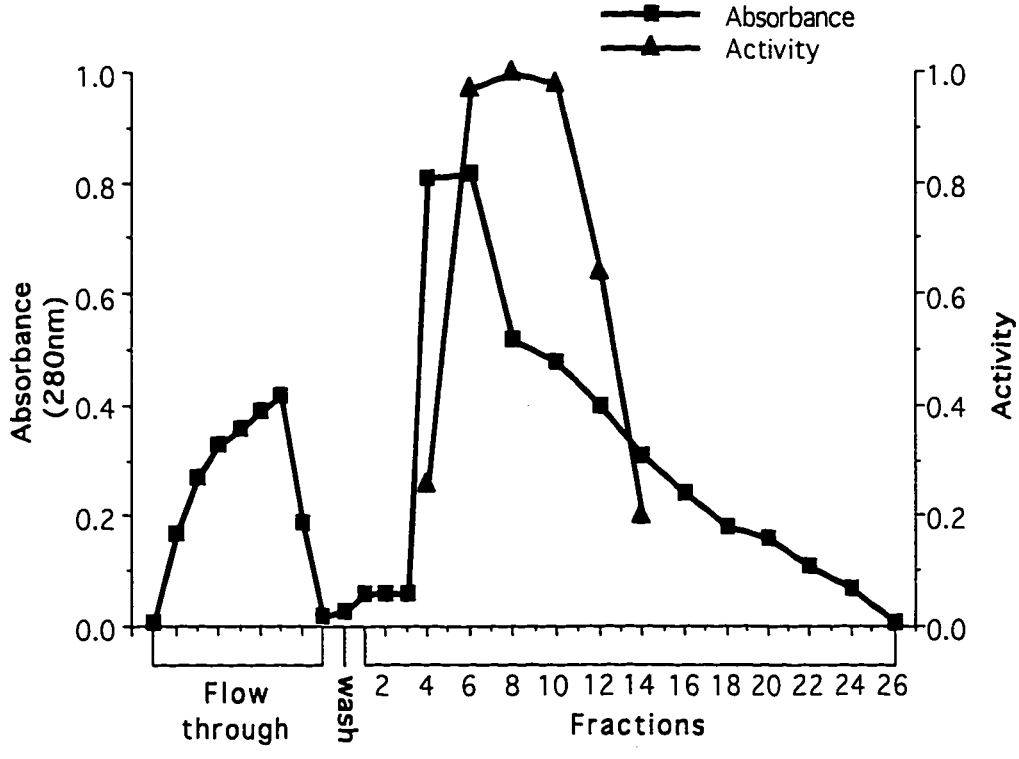
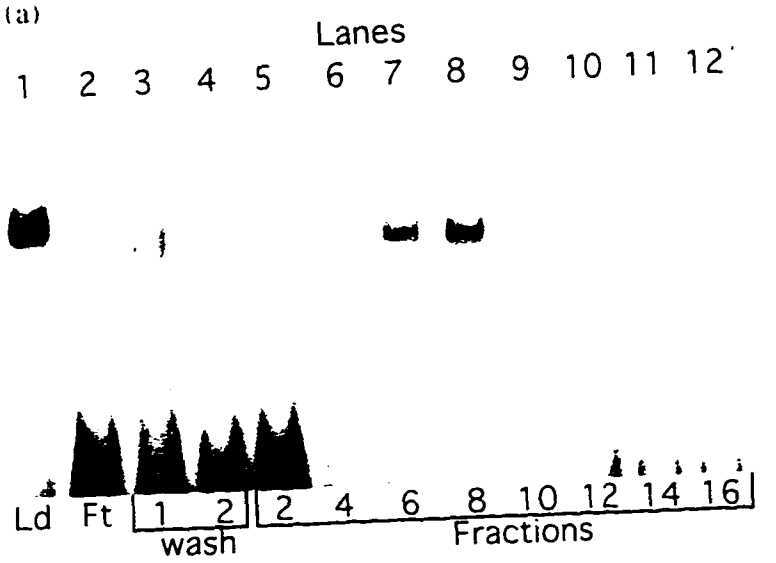
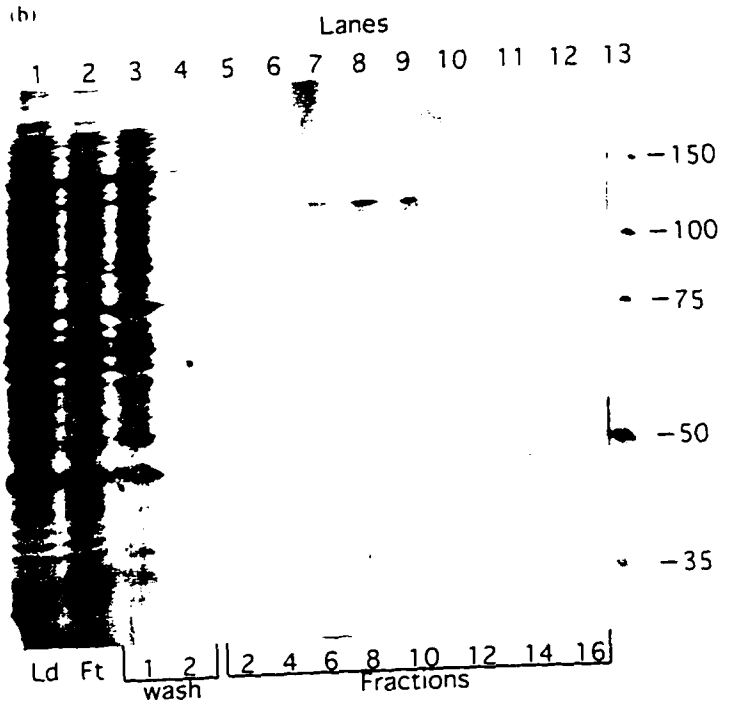


Figure 3-6. Elution profile of Oaf from a UAS1 affinity chromatography.

(a). Oaf DNA binding activity analysis of the fractions eluted from a UAS1 affinity column. DNA band shift assays were carried out with 10 fmol of labeled uUAS1 fragment and 1 μl of each of the following samples: loading sample (concentrated samples eluted from the thymus DNA column) (lane 1), flow-through (lane 2), 0.25 M KCl wash (lane 3 and 4), and alternate fractions eluted from the column by a linear KCl gradient (0.25-1.0 M KCl) (lane 5-12).

(b). Protein analysis of the fractions eluted from the UAS1 affinity column. 20 μl of loading sample (lane 1), flow-through (lane 2), 0.25 M KCl wash (Lane 3 and 4), and alternate eluted fractions were loaded on a SDS-polyacrylamide gel, electrophoresed and visualized by silver staining. Molecular weights were approximated by using "perfect protein markers" (Novagen).



Oaf, bound to this column, while the remainder was recovered in the flowthrough. The bound proteins were then eluted with linear salt gradient. DNA band shift assays, using labeled uUAS1, corresponding to the upstream palindrome of UAS1, and alternate eluted fractions, were performed to measure the Oaf DNA binding activity (Figure 3-4a). The absorbance and the profiles of the DNA binding activity of Oaf in the flowthrough and eluted fractions from this column were shown in Figure 3-4b. Fractions, 19 to 29, which contained the high DNA-binding activity, were pooled and loaded onto a double stranded calf thymus DNA cellulose column. The column was subsequently eluted with a gradient as described previously. Oaf DNA binding activity was followed by DNA band shift assay and the highest activity was recoverd in fractions 6-10 (Figure 3-5a and b).

Fractions 6-10 from the DNA cellulose column were concentrated and loaded onto a UAS1-Sepharose affinity column. The bulk of the total proteins flowed through the column, whereas UAS1-binding activity was retained and eluted at a KCl concentration between 0.4-0.6 M in fractions 4-10 (Figure 3-6a, lanes 6-9). The protein composition of the affinity-purified fraction was determined by SDS-polyacrylamide gel electrophoresis and silver staining (Figure 3-6b). A protein band that appears to consist of a doublet, with an apparent molecular mass of approximately 110-120 kDa, was detected in fractions 6-10 (Figure 3-6b, lane 7-9). These fractions also have high DNA-binding activity. This protein band was not detected in fraction 4 even though there was high DNA binding activity in this fraction (compare Figure 3-6a and b). The reason for this discrepancy is probably due to experimental error. Much less protein sample was used in the DNA band shift assay than was loaded onto the SDS gel (1 μl compared with 20 μl), thus a volume slightly greater than 1 μl inadvertently

Table 3-1

Purification of Oaf

One unit of activity is defined as the amount of Oaf required to bind 1 fmol of labeled UAS1-DNA under conditions of 100-fold excess probe. The activity was measured by performing a band shift assay and quantifying the radioactivity in the band containing the shifted probe using a phosphorImager.

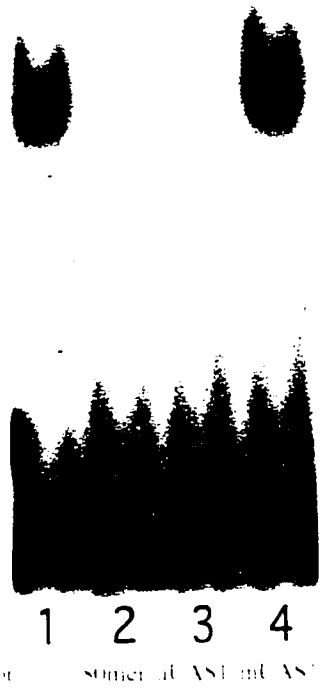
| | Specific activity | Total protein (mg) | Fold purification | Recovery (%) |
|---|---|---|---|---|
| Lysate | 16 | 2700 | 0 | 100 |
| $(NH_4)_2SO_4$ | 24 | 1960 | 1.5 | 108 |
| SP-Sepharose | 610 | 60 | 38 | 84 |
| DNA-cellulose | 750 | 20 | 47 | 34 |
| UAS1 affinity | 296,000 | 0.01[a] | 18,500 | 7 |

[a] Estimated by comparison with the protein markers on a silver-stained gel

Figure 3-7. Identification of the UAS1 binding protein by photoaffinity cross-linking.

(a). DNA band shift assays carried out with fractions eluted from an SP column and $^{32}$P-labeled BrdU-UAS1. The assay was carried out in the absence (lane 1) or the presence (lane 2-4) of competitor DNA. 100-fold excess of unlabelled DNA was used as a competitor for the binding of Oaf to UAS1 as follows: an 80-mer which contains the whole UAS1 (lane 2), uUAS1 which corresponds to the upstream palindrome of UAS1 (lane 3), and a mutated version of upstream palindrome of UAS1, mUAS1 (lane 4).

(a)

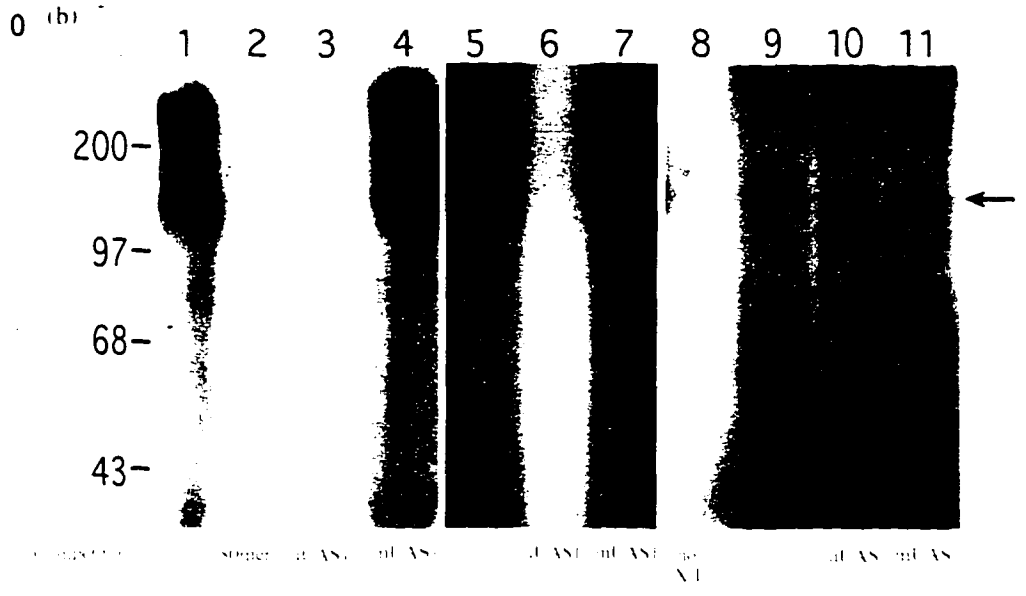



Competitor [illegible] AS1 and AS

3-7

(b). The gel shown in (a) and a second gel, in which a DNA band shift assay was carried out using a fraction eluted from an UAS1 affinity column, were exposed to UV radiation (3000 $\mu w/cm^2$) followed by exposure to film. The protein-DNA complexes were excised and electroeluted into an 8% SDS-polyacrylamide gel followed by electrophoresis and autoradiography. Lanes 1-4 show the result of UV cross-linking the corresponding material from the gel described in (a). Lanes 5-7 show the result of UV cross-linking with protein purified from affinity column in the absence of competitor (lane 5) or in the presence of 100-fold excess uUAS1 (lane 6) or mUAS1 (land 7). The band shift reaction was also carried out and the products was separated on the gel without prior cross-linking (lane 8, no x-l). The binding assay samples from the SP column were also exposed to UV radiation in solution, and the products were separated directly on the SDS-polyacrylamide gel (lanes 9-11). Competitor uUAS1 (lane 10) and mUAS1 (lane 11) were used to determine the specificity. The specifically labeled polypeptide-DNA complex is indicated by an arrow.

3-7



added to the band shift assay may cause an error. Nevertheless, the strongest silver-stained band was recoverd in fraction 8, which was also the fraction with the highest DNA binding activity. The protein concentration of these fractions was too low to measure by standard methods, thus the protein concentration was estimated by comparing the intensity of the bands on the silver-stained gel with that of a known concentration of protein from the molecular weight standards. Approximately 7% of the Oaf activity from the original lysate was recovered following affinity purification. This gave approximately an 18,000-fold purification (Table 3-1).

***Photoaffinity cross-linking of UAS1 binding protein(s) to UAS1:***

As further confirmation that the major 110-120 kDa protein purified by DNA affinity chromatography is the UAS1 binding protein, Oaf, a UV cross-linking method was used. UV crosslinking has been widely used to identify DNA binding protein and estimate their molecular mass [152,156,157]. A uniformly [$^{32}$P]dCTP-labeled UAS1 probe containing bromodeoxyuridine (BrdU) was incubated with fractions eluted from the SP or affinity column in the presence or absence of competitor DNA, and DNA-protein complex was resolved by gel shift (Figure 3-7a). BrdU was substituted for thymidine in order to promote the efficiency of cross-linking, and this substitution did not interfere with protein-DNA complex formation. Competition experiments confirmed the specificity of the shifted band. An 80-mer containing the whole UAS1 or annealed oligonucleotide, uUAS1, which corresponds to the upstream palindrome of UAS1 (see chapter II for the sequence), successfully competed for the binding of labeled UAS1 (Figure 3-7a, lane 2-3), whereas the mutated UAS1 (see chapter II) could not compete the band (lane 4). The DNA band shift gel

was subsequently exposed to ultraviolet light, and protein-UAS1 complexes were excised and electroeluted into an 8% SDS-polyacrylamide gel. Radiolabeled DNA-protein complexes were identified by autoradiography (Figure 3-7b, lane 1-7). A similar result was obtained whether the fractions from the SP column (lane 1-4) or the affinity column (lane 5-7) were used in the cross-linking experiment. Two specific bands were visible, one had a molecular mass of approximately 136 kDa. Adjusting for the presence of associated DNA, the molecular mass of the protein(s) in the DNA-protein complex was approximately 120 kDa. The second band had a mass of greater than 200 kDa. Both of these bands were specific for UAS1 because they were not present in lanes 2, 3 and 6, in which excess unlabeled UAS1 had competed for the binding of labeled UAS1. The high molecular weight band is probably due to a dimerized form of the Oaf-UAS1 complex.

In order to verify the results obtained above, a similar cross-linking experiment was carried out, in which the mixture of labeled DNA and an Oaf containing fraction from the SP column was exposed to UV radiation in solution. This reaction was carried out in the presence or absence of competitor DNA, and following exposure to UV, the mixture was then treated with DNase I to remove any DNA that was not protected by Oaf. Following separation of the products by SDS-PAGE, several cross-linked bands appeared (Figure 3-7b, lane 9-11). However, only the protein-DNA complex at approximately 136 kDa was specifically competed out by UAS1 (lane 10) but not with mutated UAS1 (lane 11). Thus, the DNase I treatment did not affect the mobility of this protein-DNA complex, suggesting that most of this DNA sequence is protected by bound Oaf.

Taken together, these results strongly indicate that the 110-120 kDa protein purified by

Table 3-2

Amino acid sequences of tryptic peptides from Oaf1p

These two sequences are identical to the indicated residues in the hypothetic protein described in Genbank data base (access number P39720)

| Amino acid sequence of fragments | Identical residues in P39720 |
|---|---|
| XWSSSLDNL | 946-953 |
| XXXAAAVGQSLDYNS | 959-970 |

affinity chromatography is the UAS1-binding protein, Oaf. It is still possible that Oaf consists of two different proteins or protein subunits that have similar molecular masses rather than of a single protein, which could give rise to a dimerized form of greater than 200 kDa.

***Microsequence analysis of Oaf1p:***

The protein, corresponding to the upper band of the doublet, at approximately 120 kDa, from the affinity purified fraction was subjected to tryptic digestion, and the resulting peptides were sequenced by Dr. Ron Kohanski as described [148]. Two peptide sequences were obtained, as shown in Table 3-2. Each peptide sequence was used to search the GenBank data base, and both showed 100% identity to the amino acid sequence of a hypothetical protein from yeast with a molecular weight of 118.2 kDa. The amino acid sequence of this protein was deduced from an open reading frame in the yeast genome, which was obtained by the yeast genome sequence project.

The 118.2 kDa hypothetical protein from yeast was described in the GenBank sequence data base as a "putative 118.2 kDa transcriptional regulatory protein " (GenBank accession number P39720). However, the protein had not been purified or functionally characterized. It is highly likely that this 118.2 kDa protein is the *POX1* UAS1 binding protein, thus the gene encoding this 118.2 protein was termed *OAF1*, which is localized in yeast chromosome I [158], and the gene product was named Oaf1p. The amino acid sequence of Oaf1p is shown in Figure 3-8a. Upon sequencing the carboxy-terminal region of a cloned genomic copy of *OAF1*, two discrepancies were noticed between the obtained sequence and the *OAF1* sequence in the yeast genome data base. These consisted of extra Gs at positions 3124 and 3130. After correction, the actual stop codon is located after the codon for amino acid 1047

Figure 3-8. Predicted protein sequence analysis of Oaf1p.

(a). Predicted amino acid sequence of Oaf1p. The amino acid sequence is deduced from the yeast genome sequence obtained by yeast genome project. The Cys6 zinc cluster DNA binding domain (66-95) is underlined, the conserved cysteine residues are in bold. A putative dimerization motif (112 to 132) consisting of 3 heptad repeats, in which the first and forth positions are occupied by hydrophobic amino acids (in bold), in underlined. A putative leucine sipper motif is located from residues 386 to 428, and the residues that compose this motif are shown in outline. The C-terminal 6 residues were corrected according to the sequence shown in (b).

(a)

MVENSTQKAPHAGNDDNSSTKPYSEAFFLGFNNPTPGLEAEHSSTSPAPE -50

NSETHNRKRNRILFVCQACWKSKTKCDREKPECGRCVKHGLKCVYDVSKQ -100

PAPRIPSKDAIISRLEKDMFYWKDKAMKLLTEREVNESGKRSASPINTNN -150

ASGDSPDTKKQHKMEPIYEQSGNGDINNGTRNDIEINLYRSHPTMIMSKV -200

MKREVKPLSENYIIIQDCFLKILVTSVFLDTSKNTMIPALTANANITRAQ -250

PSVANNLLKLKEMLIRQCQTEDEKNRVNEFTDRILQNTNSNRNLKIGMLL -300

SMLYNSVGYQYLEDHCPQGGEYSDLLRNLINECEAILPSYEIIERYKNHF -350

YEYVYPSLPFIELEIFEESLSQTIFPDPNNPSKVQIRMGSTHLRAKVENL -400

SLLLVILKLSYMSIRFLDHSTADSSFYLSKEIIDKYPIPNDFILLSPRC -450

LASENWCACANENIISCLLYIWSFFAFSPEEGDFFLEHPTDVISSLIMML -500

STSIGLHRDPSDFPQLISPSTSDKRTLNHRRILWLSIVTVCSFEASLKGR -550

HSVSPISLMALFLNIKDPDSLTVYMNRVRGDLSDINNHTLLRIHKFTFKR -600

AQLALLLSDLDNLTMTYYGSFHLHSIEFIREKIEIFVEENFPIVPLKSVA -650

QDKSDLDDMNVISEMNILSSENSSSFHNRIMNKLLMLRTSMAVFLHFETL -700

ITKDKSIFPFYKKYFMVSCMDALSLINYFNKFFNGEYRHAISSLTSFNVT -750

KFIQLALSSTIFSLLGIILRIGLAIHMLSSEVQKLSGTTDPRIKELNTKV -800

EKFSTLQRDLESALEGIYCSASEHLRFTYFPVFKMLALFDVIVQRMRKGE -850

LWHGIFTMIQMEQMHSRIIKTLSITLGVKLDKKDRLLEELMACNHVANFS -900

VEDIDELNRNIKKIQISSGLKPPVNTIDLTNGEPFGNAVPTFTKTWSSSL -950

DNLEKLSSAAAVGQSLDYNSGLRQGPLAGGGSKEQTPIAGMNNLNNSINA -1000

TPIVDNSSGSQLPNGFDRGQANNTPFPGYFGGLDLFDYDFLFGNDFA -1047

FIgure 3-8.

(b). Corrected sequence of the carboxyl terminus of Oaf1p. A, the sequence as previously published. The two extra G's are boxed. B, the corrected sequence obtained by sequencing a cloned genomic copy of *OAF1*.

(c). Comparison of the homology of the zinc cluster DNA binding domain of Oaf1p, Cyp1p and Gal4p. The numbers on the left refer to the positions of the first Cys residue shown in the respective proteins. Identical residues are boxed, and a dash indicates positions where a gap has been introduced for the alignment.

(b)

```
   F  L  V  W  A  M  T  L  L  K  N  F  L  S  K  L  L  P  I  H  F  I  N  STOP
A. TTT TTG GTT TGG GCA ATG ACT TTG CTT AAA AAT TTT CTT TCC AAA CTC CTA CCT ATT CAT TTC ATC AAT TAA

B. TTT TTG TTT GGC AAT GAC TTT GCT TAA
   F  L  F  G  N  D  F  A  STOP
```

(c)

```
Oaf1  66 CqaCwksKtKCdrekPeCgrCvKhglk--CvY--d
Cyp1  64 CtiCrkrKvKCdklrPhCqqCtKtgvahlChYmeq
Gal4  11 CdiCrlkKlKCskekPkCakClKhnwe--CrYspk
```

(Figure 3-8b), thus Oaf1p is 15 amino acids shorter than previously reported [158].

***Oaf1p mediates oleate-specific induction of POX1 and FOX3:***

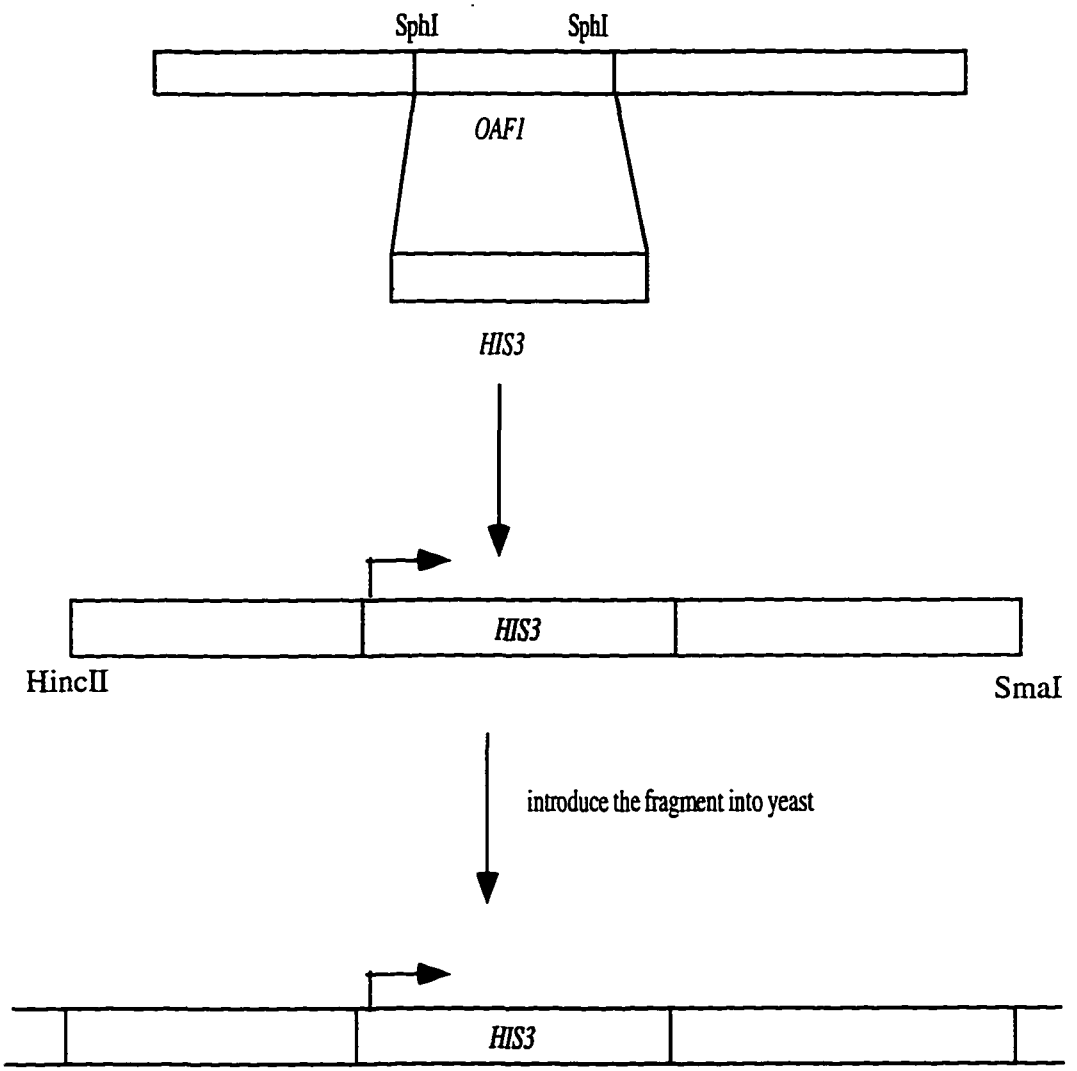
In order to characterize the function of Oaf1p, the genomic allele of the *OAF1* gene was disrupted. By PCR amplification using two primers YL3-1 and YL3-2, which were complementary to the 5' and 3' ends of the *OAF1* sequence in the Genbank data base, a DNA fragment with predicted size (3.2 kb) was obtained. Restriction enzyme and sequencing analysis of the 100 nucleotides at both the 5' and 3' ends confirmed that the fragment encodes *OAF1*.

A 1.3 kb SphI fragment in the open reading frame of *OAF1* was replaced with the yeast *HIS3* gene as shown in Figure 3-9. The genomic *OAF1* gene was disrupted by introducing the *OAF1*::*HIS3* fragment into yeast. The disruption of *OAF1* was confirmed by using genomic DNA from the parental and disrupted strain as a template in a PCR assay with primers YL3-1 and YL3-2. The DNA product from the transformants was approximate 0.5 kb larger than that obtained from the parent strain, confirming that the *OAF1*::*HIS3* fragment was correctly targeted into the yeast genome.

The effect of disrupting *OAF1* was tested in four ways. First, I tested whether the disrupted strain could grow on plates containing oleate as the sole carbon source (YNO medium). Yeast strains that lack functional β-oxidation enzymes are not able to grow on YNO plates [155,159]. The parental strain grew, although slowly, on this medium, whereas the disrupted strain was unable to grow (Figure 3-10a), suggesting that the β-oxidation pathway was not induced in the presence of oleate in this strain. The extracts from both parental and disrupted strains grown in YPG and YPGO medium were used in a DNA band

Figure 3-9. Disruption of *OAF1*.

The *OAF1* sequence (white box) encoding amino acid residues 457 to 891 was replaced by a DNA fragment containing the *S. cerevisiae HIS3* (shaded box) and integrated into the yeast genome by homologous recombination. An arrow marks the start site and direction of transcription of *HIS3*.



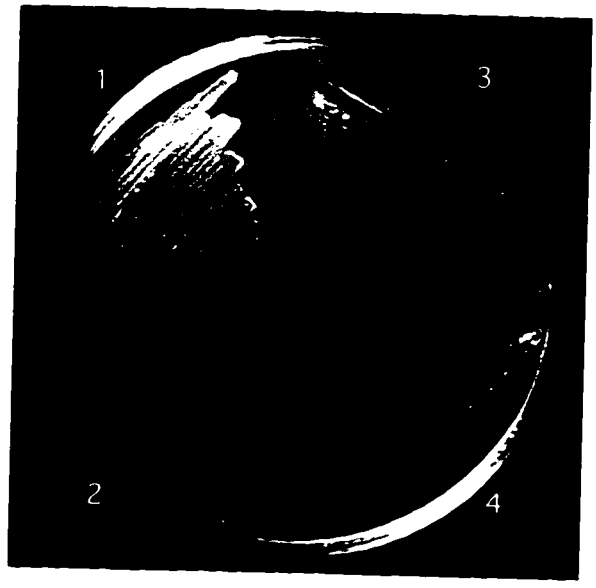

*OAF1* locus in the yeast genome

Figure 3-10. Disruption of the *OAF1* gene prevents the growth of cells on YNO plate and the formation of the *POX1* UAS1-protein complex.

(a). Growth of the parental (1 and 2) and *OAF1* disrupted strain (3 and 4) on a YNO plate which contains oleate as the sole carbon source.

(b). DNA band shift assay with cell extracts from parental (lanes 1 and 2) and *OAF1* disrupted strains (lanes 3 and 4) grown in YPG (G) or YPGO (O) medium.

(a)




(b) 1 2 3 4

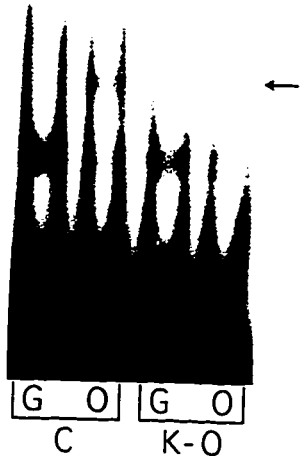


Figure 3-11. *OAF1* is involved in oleate activation of the *POX1* promoter.

(a). Northern blot analysis of *POX1* and *PGK1* mRNA expression in parental (P), *OAF1*-disrupted (K-O), and *OAF1*-rescued (R) strain grown in YPG (G) or YPGO (O) medium.

(b). β-galactosidase activities measured in extracts prepared from the same cells used in (a). The number is the mean of two experiments.

(a)


(b)

ß-galactosidase activity
u/mg

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| 0.45 | 4.6 | 0.32 | 0.11 | 0.32 | 3.3 |

shift assay with labeled UAS1. No specific shifted band was seen with extracts from the disrupted strain grown in the presence of oleate ( Figure 3-10b, lane 4), whereas the expected shift was present with extracts from the parental strain (Figure 3-10b, lane 2). The lower band seen in this gel is non-specific, as shown in chapter II.

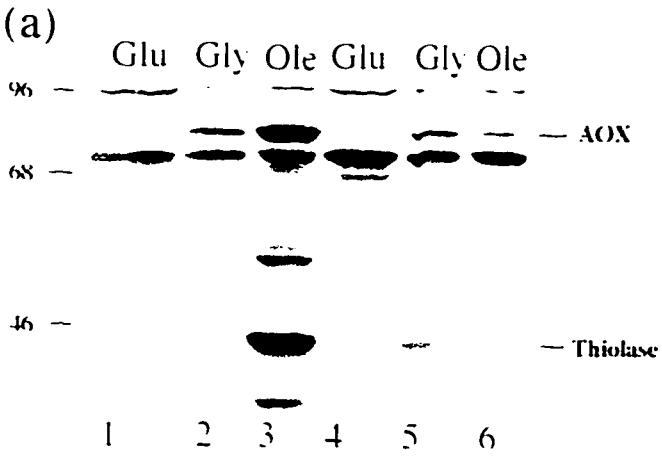
In order to further confirm that the gene encoding the transcription factor responsible for the oleate induction of *POX1* was disrupted, a plasmid pRSOAF1, containing the *OAF1* gene under control of its own promoter, was introduced into the disrupted strain, which has transformed with a *POX1-lacZ* reporter gene. The expression of the reporter gene, as represented by β-galactosidase activity, was analyzed using the parental, disrupted and rescued strains grown in glycerol or oleate medium (Figure 3-11b). The result showed that the *POX1* promoter was not activated by oleate in the *OAF1* disrupted strain but was activated by oleate in both parental and rescued strains. Northern blot analysis of the *POX1* mRNA, performed by Dr. I. V. Karpichev, confirmed that the *POX1* induction by oleate was abolished by the disruption of *OAF1* (Figure 3-11a, lane 4), and the induction was restored by introducing *OAF1* into the knock-out strain (Figure 3-11a, lane 6). Taken together, these results confirm that the *OAF1* gene I cloned encodes the UAS1-binding protein, or one of the UAS1 binding proteins, and disruption of this gene prevents the protein(s) from binding to UAS1 and thus activating *POX1*.

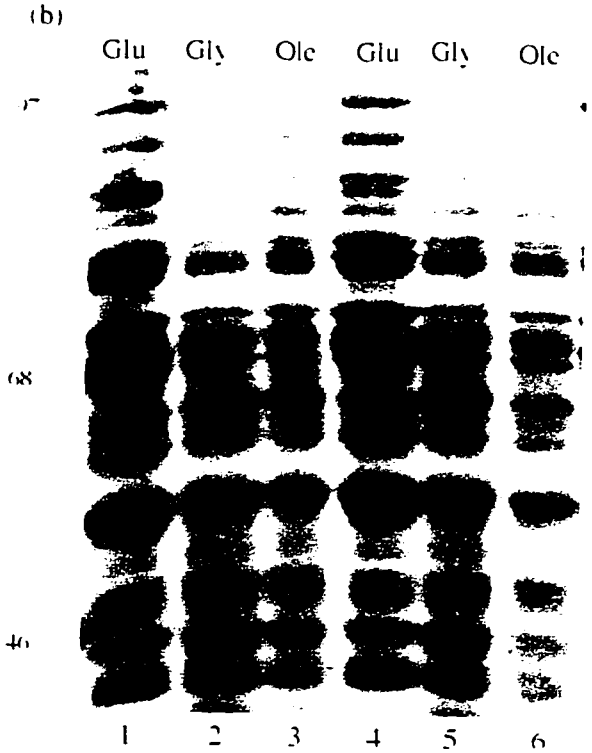
Immunoblot analysis was employed to analyze the expression of thiolase in the *OAF1* disrupted strain. The result showed that the oleate induced expression of thiolase was abolished by the disruption of *OAF1* (Figure 3-12a, lane 6), suggesting that Oaf1p also regulates the induction of *FOX3*. The lower levels of acyl-CoA oxidase and thiolase in lane

Figure 3-12. *OAF1* coordinately regulates the expression of acyl-CoA oxidase and thiolase.

(a). Immunoblot analysis of the expression of acyl-CoA oxidase (AoX) and thiolase in parental (lane 1-3) and *OAF1* disrupted (lane 4-6) strains grown in glucose, glycerol or oleate medium. Approximately 50 μg of cell lysate proteins were separated by SDS-PAGE and immunoblotted with antisera against *S. cerevisiae* acyl-CoA oxidase. The same membrane was subsequently blotted, without stripping, with α-thiolase.

(b) Coomassie blue staining of a duplicate SDS gel as described in (a) to show the proteins loaded on the gel.



6 (oleate) than in lane 5 (glycerol) is probably due to the less loading of proteins in lane 6 (Figure 3-12b). Recently, a mutant strain m7, which carries a mutation in *OAF1*, was obtained by a mutagenesis in our laboratory. In this mutant strain, *POX1* and *FOX3* mRNA induction was abolished [170]. These results suggest that Oaf1p is involved in the regulation of *POX1*, *FOX3*.

***Regulation of peroxisome proliferation by Oaf1p:***

The regulation of peroxisomal β-oxidation enzymes is correlated with the proliferation of peroxisomes, thus peroxisome proliferation was analyzed by immunoelectron microscopy by Vladmir Protopopov, using the *OAF1* disrupted strain and a control strain. Samples were prepared by preculturing cells in YPD medium and then transferring to YPG or YPGO and culturing for a further 18 hour. Peroxisomes were identified under the electron microscope by immunogold staining using a rabbit antiserum 10-234, which recognizes several peroxisomal proteins from *S. cerevisiae* (unpublished, see [155]). A random sample of 100 cells were examined for the presence of peroxisomes on three separate occasions for wild type cells grown in oleate and glycerol medium, and in the *OAF1* disrupted stain grown in oleate medium (Table 3-3).

Wild type cells grown in the presence of oleate exhibited the characteristic clusters of peroxisomes (Figure 3-13a). The peroxisomes were labeled, often densely, with gold particals. In contrast, there were fewer peroxisomes in cells grown in glycerol medium, and the labeling was weak (Figure 3-13b, Table 3-3). In the *OAF1* disrupted strain, very few small peroxisomes were identified in the presence of oleate (figure 3-13c and Table 3-3). In the *OAF1* disrupted strain there was a greater accumulation of lipid droplets than seen in the

Table 3-3

Number of cells containing visible peroxisomes in wild type strain W3031A and *OAF1* disrupted strain OA1.

| Strains | 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 |

Figure 3-13. Electron micrographs of parental strain grown in glycerol or oleate medium, and an *OAF1*-disrupted strain (OA1) grown in oleate. All cells were immunocytochemically labeled to detect peroxisomal proteins.

(a). Parental cells grown in oleate. Arrows indicate peroxisomes (P) and mitochondrion (M); a lipid droplet is labeled L.

(b). Parental cells grown in glycerol medium. Only one peroxisomes is detected in this cell.

(c). OA1 grown in oleate. One small peroxisome is indicated by an arrow.

wild type cells (compare Figure 3-13a and 13c). This may be expected because the *OAF1* disrupted cells could not metabolize the oleate present in the growth medium.

## Discussion

In chapter II, I described the identification of a *POX1* UAS1 and showed that a protein or a protein complex binds to the UAS1 element in the *POX1* promoter in an oleate-activated fashion. In this chapter, I continued the studies on the UAS1 binding protein. A time course study showed that the amount of protein(s) binding to UAS1 increased following a shift of cells from glucose to oleate medium, suggesting that the UAS1 binding protein, Oaf1p, is activated and/or induced continuously in the presence of oleate, or alternatively, another protein, required for the function of Oaf1p, is induced by oleate. The protein that binds to URS2 appears to be present in cell extracts regardless of the growth conditions tested. It seems that the two proteins are bound to different DNA molecules because there is no evidence of a super-shifted band in the DNA band shift assay, which would be expected if both proteins were bound to the same DNA molecule; this is consistent with the result described previously (Figure 2-5). It took about 2-4 hours to be able to detect the Oaf1p binding activity in DNA band shift assays when cells were shifted from glucose to oleate medium. The reason for this long lag period could be that there was still glucose present in the cells when they are shifted into oleate medium, and glucose repression has been shown to be dominant to oleate induction, since no oleate induction was observed when cells were grown in the presence of both glucose and oleate [104]. Thus, Oaf1p will not be activated

until the glucose is depleted. Alternatively, the activated Oaf1p level was too low to be detected within 2 hours induction. Based on these results, a simple model could be proposed: *POX1* is repressed when cells are grown in glucose medium and the repression is mediated by the binding of protein(s) to the URS elements. When cells are shifted to oleate medium, Oaf1p may be induced and/or activated and then binds to UAS1, this would over-ride the repressing activity of the URS protein(s) and cause activation of the gene.

As a first step to characterize the oleate induction pathway, I purified the UAS1 binding protein, using a chromatographic strategy, based on methods described previously with some modifications [147,157]. Approximately an 18,000-fold purification was obtained by UAS1 affinity chromatography. The purified protein appears as a doublet on a silver stained gel and has an apparent molecular weight of approximately 110-120 kDa. Photoaffinity cross-linking data confirmed that the UAS1 binding protein is about 120 kDa. Two peptide sequences, obtained by sequencing the protein corresponding to the upper band of the doublet, were used to search the protein data base, and a 100% identity was found in an open reading frame on the left arm of chromosome I [158]. The hypothetic protein deduced from this open reading frame has a molecular mass of 118.2 kDa, which is similar to that of UAS1 binding protein. Thus the putative gene was termed *OAF1*.

The sequence of Oaf1p from amino acids 66 to 95 has a putative "zinc-finger" DNA binding domain, which shows a high level of structural homology to the DNA-binding domain found in many regulatory proteins, such as *CYP1*[160] and *GAL4* [161] (Figure 3-8c). Comparing the amino acid sequence of Oaf1p with that of the product of *HAP1* (*CYP1*) and *GAL4*, the highest homology is present in the DNA-binding zinc finger-like domain. Thus,

Oaf1p belongs to a family of fungal transcription factors that have complex mechanisms for regulating several genes. Gal4p, Ppr1p and Put3p are representative members of this family. These transcription factors, like Oaf1p, also recognize DNA binding sites containing two palindromic CGG triplets separated by various characteristic spacing nucleotides [162]. Gal4p recognizes CGG triplets separated by 11 nucleotides, Put3p and Ppr1p recognize CGG triplets separated by 10 and 6 bp, respectively [163,164]. In the case of the Oaf1p binding site, the CGG triplets are separated by 17 nucleotides. It has been suggested by Reece and Ptashne that the binding specificity is largely determined by the linker element connecting the DNA binding zinc cluster and the dimerization domain [162]. An amphipathic $\alpha$-helix motif containing 3 heptad repeats, in which the first and the fouth residues are hydrophobic and which is characteristic of coiled coil dimerization domain, is present in Oaf1p (residue 122 to 132). Oaf1p differs from other members of this family by the presence of a putative leucine zipper motif in the region from residue 386 to 428, which comprises two isoleucines and four leucines spaced seven residues apart. This motif, identified in many transcription factors, has been shown to mediate dimerization [165]. To date, only one protein (Sip4p) has been shown to have both C6 zinc cluster and leucine zipper motif in yeast [166]. The palindromic targeting sequence and the presence of putative dimerization domains suggest that Oaf1p probably binds to *POX1* UAS1 as a homodimer, or Oaf1p heterodimerizes with other protein and binds to UAS1 upon activation by oleate. Whether these two putative dimerization domains are functional remains to be tested.

Several members of this transcription factor family have been shown to be activated by the metabolic precursors of the pathways they regulate. Gal4p regulates galactose catabolism

upon galactose stimulation [126], dihydroorotate activates Ppr1p which controls pyrimidine anabolism [164], Put3, which regulates the proline utilization pathway, is activated by proline [163,167]. In this respect, Oaf1p could be considered the regulator of fatty acid metabolism activated by oleate. One striking difference of Oaf1p from other members is that Oaf1p binds to UAS1 *in vitro* only when it is activated by its inducer oleate, however, Gal4p and Put3p are able to bind to targeting sequence in the absence of their corresponding inducers [126,168,169].

Oaf1p not only mediates the induction of *POX1*, but regulates the expression of *FOX3* as well, suggesting that Oaf1p may coordinately regulate the expression of the genes encoding the β-oxidation enzymes. Oaf1p also mediates peroxisome proliferation induced by oleate, this process was abolished by the disruption of *OAF1*. The mechanism for the regulation of peroxisome proliferation by *OAF1* remains to be identified. It is possible that Oaf1p induces the expression of some peroxisomal proteins that are required for the proliferation of peroxisomes, such as Pex11p (Pmp27p) [100], and thus trigger this process.

# CHAPTER IV

## FUNCTIONAL ANALYSIS OF THE ROLE OF Oaf1p IN OLEATE ACTIVATED PATHWAY IN *SACCHAROMYCES CEREVISIAE*

### Introduction

A protein or protein complex has been shown to bind to the UAS1 of *POX1* when cells are induced by oleate. A UAS1-binding, oleate activated, transcription factor (Oaf1p) has been purified and the *OAF1* gene encoding this protein was subsequently cloned. Oaf1p belongs to a family of transcription factors, which includes Gal4p and Hap1p, based on the homology of the N-terminal zinc cluster DNA binding motif. *OAF1* is required for the oleate induction of *POX1* and *FOX3*. The induction, but not the derepression, of *POX1* is abolished by the disruption of *OAF1*. Oaf1p is also required for peroxisome proliferation induced by oleate. Thus, Oaf1p plays a critical role in the regulation of peroxisomal β-oxidation and peroxisome proliferation.

Recently, a gene termed *OAF2*, was cloned by Dr. I.V.Karpichev in our lab, by functional complementation of a yeast mutant defective in oleate induction of *POX1* [170]. *OAF2* encodes a transcription factor which has 39% identity to Oaf1p. *OAF2* has been shown not only to be required for the fatty acid induction of *POX1*, *FOX3*, but also for the proliferation of peroxisomes. A mutation in *OAF2* prevented protein(s) binding to the *POX1* UAS1, suggesting that Oaf2p is also essential for the formation of the UAS1-protein complex.

In order to better understand the role of Oaf1p in the oleate-induction pathway, I analyzed the expression of the *OAF1* gene and the phosphorylation states of Oaf1p in cells cultured in different media. The similarities of Oaf1p and Oaf2p in primary structure and function

Table 4-1 *S. cerevisiae* strains used in this study.

| Strain | Genotype |
|---|---|
| OA1 | MATa, *ade1,leu2, ura3, oaf1::HIS3/his3, trp1[pRS304-TRP1-POX1-lacZ]* |
| OA2myc | MATα, *ade1, leu2, ura3 [YIp357-URA3- POX1-lacZ], oaf2::HIS3/his3, trp1[pRS304-TRP1-OAF2mcy]* |
| OA1HA3 | MATa, *ade1,leu2[pRS305-LEU-OAF1HA3] ura3, oaf1::HIS3/his3, trp1 [pRS304-TRP1-POX1-lacZ]* |
| OA2HA3myc | MATα, *ade1, leu2[pRS305-LEU-OAF1HA3], ura3[YIp357 -URA3- POX1-lacZ], oaf2::HIS3/his3, trp1[pRS304-TRP1-OAF2mcy]* |
| SFY562ΔOAF1 | MATa, *ade1, leu,ura3/GAL1-lacZ, oaf1::HIS3/his3, trp1, lys2, gal4, gal80* (SFY526 is from Clontech) |
| SFY526ΔOAF2 | MATa, *ade1, leu2, ura3/GAL1-lacZ, oaf2::HIS3/his3, trp1, lys2, gal4, gal80* |
| MCY2692plz | MATa, *leu2, ura3[YIp357-URA3-POX1lacZ], snf1-K84R* (MCY2692 was obtained from Dr. Marian Carlson) |
| MCY2692plzΔOAF1 | MATa, *leu2,ura3[YIp357-URA3-POX1lacZ], oaf1::HIS3/his3, snf1- K84R* |

suggest that they may cooperatively regulate gene expression, thus I analyzed the functional relationship of Oaf1p and Oaf2p. In addition, the functional domains of Oaf1p were characterized by domain swapping experiments.

## Materials and Methods

***Yeast strains and culture conditions:***

The yeast strains used in this study are described in Table 4-1. Yeasts were cultured as described previously.

***Plasmids:***

pPC97-OAF1 (2-1047): Primers YL4-1 (5'GGGTCGACCGTCGAAAATAGTACGCA 3') and YL3-2 (See chapter III) were used in a PCR to synthesize a DNA fragment encoding the *OAF1* open reading frame from the second codon to the stop codon. The fragment was subcloned into pT7blue. The resulting construct, ptOAF1, was digested with Sal1/Sma1 and the released fragment was cloned into the corresponding sites of pPC97 [171] to create pPC97-OAF1.

pPC97-1047 (180-1047): A DNA fragment encoding truncated *OAF1*, starting from residue 180 to the stop codon, was obtained by PCR amplification using primers YL4-2 (5' GGGTCGACCAGAAATGATATT GA 3') and YL3-2. The fragment was subcloned into pT7blue and was released by digesting with Sal1 and Sma1. The released fragment was then cloned into the corresponding sites of the pPC97 vector.

pPC97-920 (180-920): A DNA fragment encoding a portion of *OAF1*, starting from residue 180 to 920, was obtained by PCR amplification using primers YL4-2 and YL4-3 (5'

TTACTTCAATCCTGAAGAAA 3'). The fragment was subcloned into the pT7blue vector and was released by digesting with Sall and Smal. The released fragment was then cloned into the corresponding sites of pPC97 .

pPC97-670 (180-670): PCR amplification using primers YL4-2 and YL4-4 (5'TTAT TCTGAAGATAATATAT 3') was used to obtain a DNA fragment encoding a portion of *OAF1* from residue 180 to 670. The fragment was subcloned into the pT7blue vector and was then released and subsequently cloned into pPC97 as described above.

pPC97-128 (920-1047): A DNA fragment encoding the C-terminal 128 amino acids of *OAF1* was obtained by PCR amplification, using primers YL4-5 (5' GGGTCGACCAAGC CGCCTATAAACAC 3') and YL3-2. The fragment was subcloned into pT7 blue, released by digesting with Sall and Smal, and subsequently cloned into the corresponding sites of pPC97.

***Epitope tagging:***

pRSOAF1HA3: The nine amino acid epitope of influenza virus hemagglutinin (HA) (YPYDVPDYA), which can be recognized by the monoclonal antibody 12CA5 [172], was used for tagging the *OAF1* gene product. Three tandem copies of the HA epitope were added to the carboxyl terminus of *OAF1*. Primers YL4-6 (5'-CATTCGGAAATGCTGT TCC-3') and YL4-7 (5' TGGGACGTCGTATGGGTAAGCAAAGTCATTGCCAAACAAA A 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 HA epitope. The underlined bases are complementary to the plus strand encoding HA. The PCR fragment was blunt ended, digested with EagI, and subcloned into the EagI/SmaI sites

in pOAF1 (chapter III). The resulting construct was then digested with AatII and HincII and the released DNA fragment was subcloned into the corresponding sites in pT7peb1HA3 [26]. The resulting pOAF1HA3 construct contains the *OAF1* gene followed by DNA sequence encoding three copies of the HA epitope. The final sequence of the triple-tagged Oaf1p is FLFGNDFAYPYDVPDYAGYPYDVPDYAGSYPYDVPDSPNESI, with the epitope sequence underlined. A DNA fragment encoding OAF1-HA3 was released from this plasmid by digestion with HindIII/SacI, and was subsequently subcloned into the corresponding sites in pRS305 [151] to create pRSOAF1HA3.

pRSOAF2myc: The ten amino acid epitope from human c-myc (EQKLISEEDL), which can be recognized by the monoclonal antibody 9E10 [172], was used for tagging the *OAF2* gene product. The myc epitope was added to the extreme carboxyl terminus of the *OAF2* gene product by PCR amplification with following primers:

YL4-8(5'CTACAAGTCTTCTTCAGAAATAAGCTTTTGTTCGTCGTTCTGGA A AAGTA-3'), underlined bases are complementary to the plus strand encoding the myc epitope with the stop codon in bold; primer YL4-9 (5' TTAAAAACTACTATGAC 3'), which consists of nucleotides 2876 to 2896 of *OAF2*. The resultant 110 bp fragment contained the 3' end of the *OAF2* open reading frame (minus stop codon) followed by the myc sequence. This fragment was blunt ended, digested with Nco1 and subcloned into the NcoI/ blunt-ended SalI site in pOAF27B [170] to create pOAF2-myc. A DNA fragment encoding the complete OAF2-myc protein was released by digestion with SacI/PstI, and was subcloned into the corresponding sites in pRS304 to create pRSOAF2-myc.

***Phosphatase treatment and Immunoblot analysis*:**

Cell extracts were prepared as described previously (chapter II and III). For phosphatase treatment, cell extracts containing 10 μg total protein were incubated at 37°C for 1 hour in the presence of 20 U of calf intestinal alkaline phosphatase (CIP, New England Biolab). Phosphatase inhibitor cocktail (5mM sodium phosphate [pH 7.5], 10mM sodium pyrophosphate and 5mM EDTA) was added as indicated. Mock sample was treated identically but in the absence of CIP or phosphatase inhibitors. The treated samples were separated by SDS-PAGE and subjected to immunoblot analysis with monoclonal antibody 12CA5, against the HA epitope.

The result obtained from the immunoblot analysis was subjected to densitometry analysis to analyse the migration of Oaf1pHA3 in the SDS-gel. The densitometry analysis was carried out using Molecular Dynamic ImageQuant software. A common reference point was used to scan the protein bands.

***Immunoprecipitation*:**

Yeast cell extracts were prepared as described previously, and 100 μl of cell extracts (approximately 500 μg total proteins) were diluted with 500 μl buffer H ( 25 mM Tris-HCl, pH7.4, 15 mM $MgCl_2$, 1 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF and 0.1 μg/ μl sodium metabisulfite, 0.1 μg/μl of chymostatin, pepstatin, leupeptin and antipain). 2 μg of the monoclonal antibody 9E10, which recognizes the myc epitope [172], were added to the extracts. The mixture was incubated for 2 hours at 4°C with gentle rotation. 50 μl Protein G agarose (Boehringer mannheim) were then added followed by 2 hour incubation at 4°C. The agarose beads were collected by centrifugation, and washed three times with buffer H. The bound proteins were eluted by boiling the sample in SDS sample buffer. The

beads were pelleted by centrifugation, the supernatant were loaded on a SDS-polyacrylamide gel, and was then subjected to electrophoresis and immunoblot analysis.

***Other Methods*:**

RNA purification and Northern blot analysis was carried out by Dr. I.V.Karpichev as described [170]. β-galactosidase activity and DNA bandshift assays were performed as described previously (chapter II). In the band shift assay containing antibody, following a 10 min incubation of the cell extracts with the labeled DNA, 12CA5 or 9E10 antibody were added as indicated, followed by a further 15 min incubation. 10 μg of HA peptide, 2 pmol of uUAS1 and mUAS1 fragments were used in the competition assays. Western blot analysis was carried out using standard procedures [140].

## Results:

***Oaf1p is not induced by oleate:***

Oaf1p is activated and binds to the *POX1* UAS1 in the presence of oleate. To determine whether *OAF1* expression is regulated by glucose and/or oleate, Oaf1pHA3, which contains three copies of the hemagglutinin (HA) epitope tag at the carboxyl terminus of Oaf1p, was expressed from the *OAF1* promoter, which consists of a 1.4 kb sequence upstream from the initiation codon, in an integrative plasmid. The Oaf1p-HA3 protein was functional when expressed in OA1, a strain in which *OAF1* is disrupted and which contains a reporter gene.

Figure 4-1. Oaf1pHA3 restores the activation of the *POX1* promoter by oleate in an *OAF1*-disrupted strain, OA1.

Cell extracts from wild type strain (W3031A), OA1 or OA1HA3, which expresses Oaf1pHA3, grown in glycerol or oleate media were analyzed for β-galactosidase activity. These strains were transformed with plasmids containing the reporter gene *POX1-lacZ*. Results are the mean of five experiments.

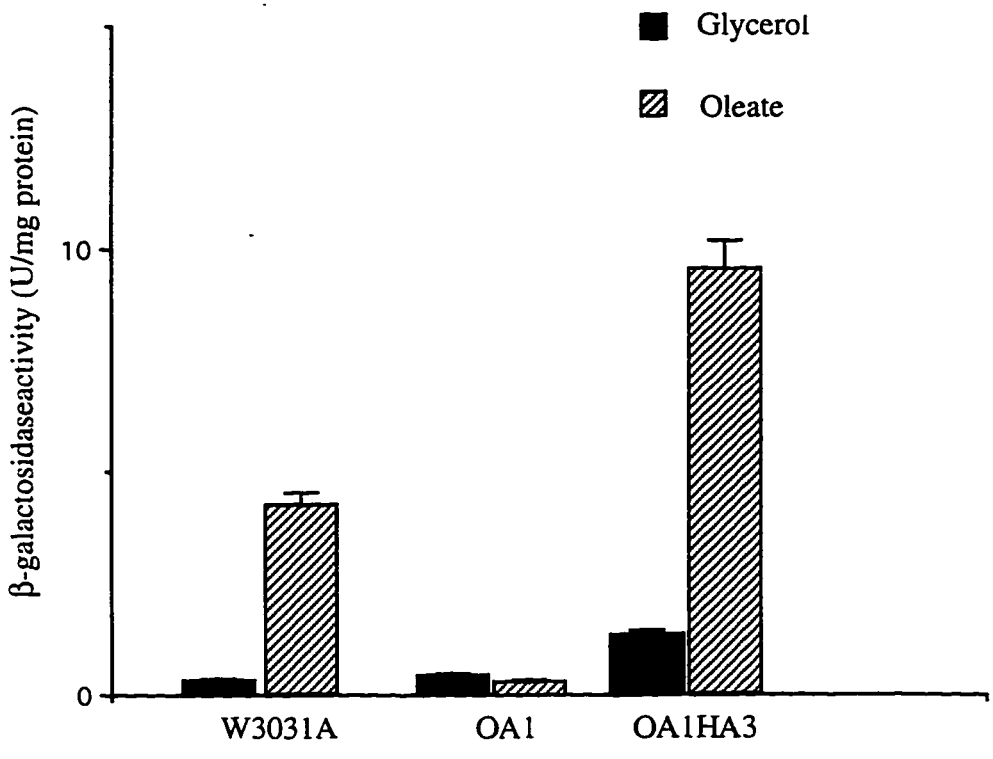
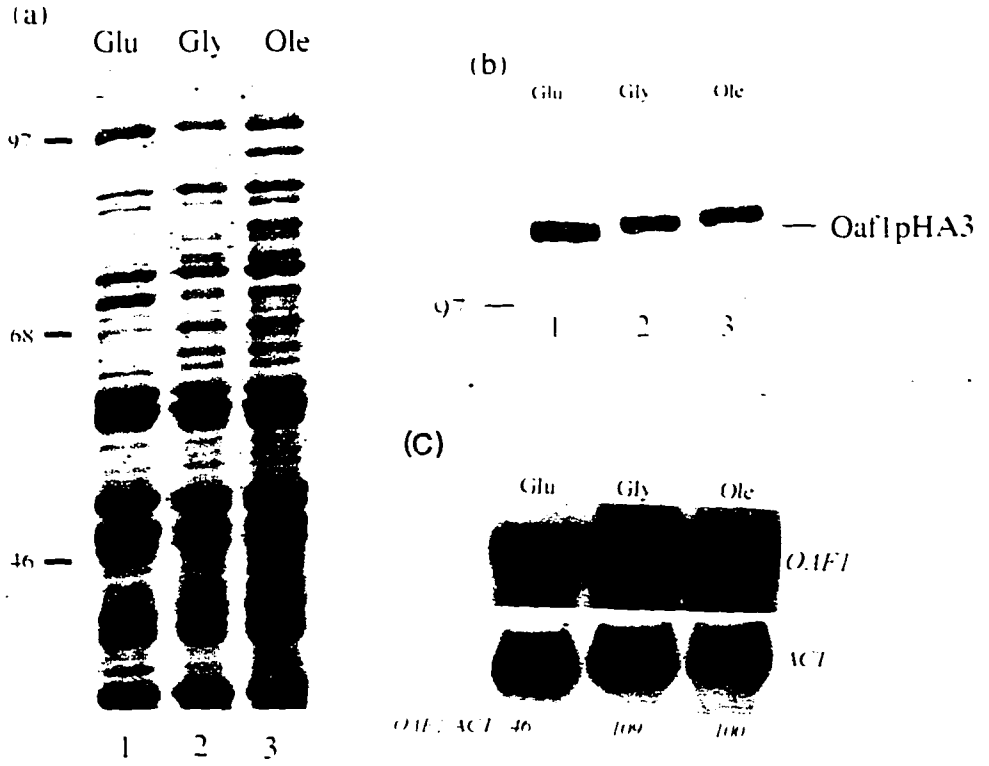


Figure 4-2. Expression of *OAF1* is not induced by oleate.

(a). Coommassie blue staining of an SDS gel to show the proteins loaded on the gel. Cell extracts containing approximately 20 μg of protein (measured by the Bradford's method) from cells grown in glucose medium (lane 1), glycerol medium (lane 3) or oleate medium (lane 3) were separated on 7% SDS polyacrylamide gel.,

(b). Immunoblot analysis for the expression of Oaf1pHA3. A duplicate gel of that described in (a) was subjected to immunoblot analysis with monoclonal antibody 12CA5, recognizing the HA epitope.

(c). Northern blot analysis of the expression of *OAF1* mRNA and actin mRNA in wild type cells grown in glucose (lane 1), glycerol (lane 2) or oleate (lane 3) media. The mRNA levels were quantified using a PhosphorImager ImageQuant software and normalized to the actin mRNA levels. The relative levels of *OAF1* mRNA are expressed as percentage of the level in cells grown in oleate medium, which is set at 100%.



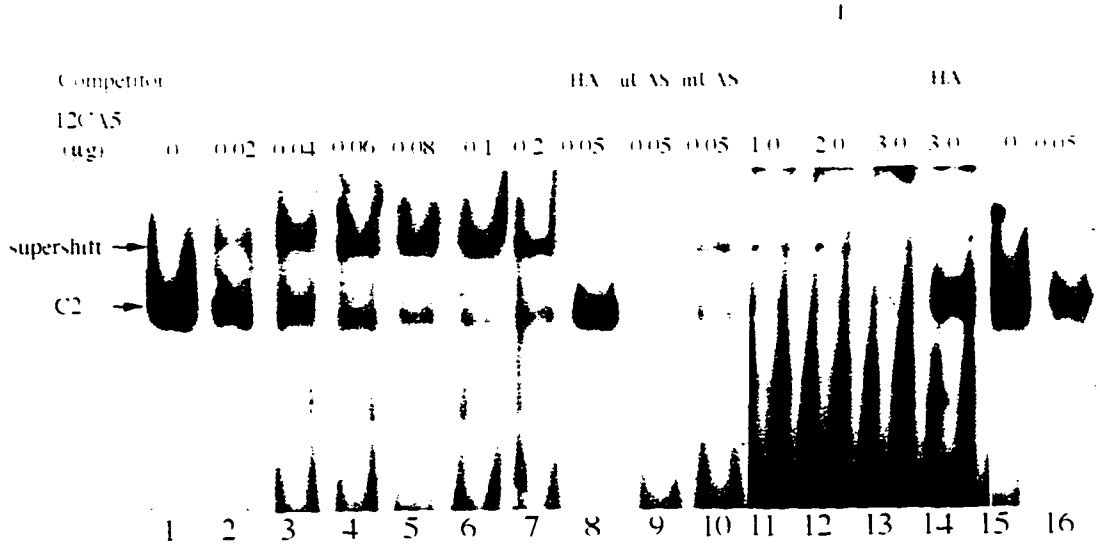
Oaf1pHA3 restored oleate induction of *POX1*, measured by β-galactosidase activity (Figure 4-1). The β-galactosidase activity of cells expressing Oaf1pHA3 were higher than that of wild type cells. The reason for this is not clear, however it may be due to the fact that the triple HA tag introduces negatively charged amino acids (six aspartic acids) and makes the c-terminus of the protein more acidic. Many members of the Gal4p transcription factor family have C-terminal activation domains, which are acidic [126], thus increased acidity in this region may lead to increased activity.

In order to analyze the expression of Oaf1p, total protein extracts from glucose-, glycerol- and oleate-grown cells, expressing Oaf1pHA3, were separated by SDS-PAGE and were subjected to immunoblot analysis using 12CA5 antibody, which recognizes the HA epitope. The protein concentration measured by Braford assay in extracts from oleate grown-cells was generally found to be overestimated by approximately 40% compared to that from glycerol-grown cells when compared to Coomassie blue-stained gels. This is probably due to interference by oleate or tween-40 with the Braford protein assay. Samples were adjusted accordingly so that they were approximately equal loaded, as shown in Figure 4-2a. The results show that Oaf1pHA3 is constitutively expressed in cells cultured in glucose, glycerol or oleate medium (Figure 4-2b).

Northern blot analysis of the *OAF1* mRNA level, carried out by Dr I.V.Karpichev showed that *OAF1* mRNA was expressed in all conditions tested (Figure 4-2c). The *OAF1* mRNA levels were normalized to that of actin. The mRNA level of *OAF1* in glucose-grown cells was approximately 50% of that in oleate grown cells, and *OAF1* mRNA levels in glycerol and oleate cultured cells were similar, suggesting that *OAF1* transcription is not significantly

Figure 4-3. Oaf1pHA3 is a component in the UAS1-protein complex.

A DNA band shift assay was carried out using cell extracts from OA1HA3 cells grown in oleate media and labeled uUAS1 (lanes 1-15). Lanes 2-14 and 16 were carried out in the presence of various amount of 12CA5 antibody as indicated. 10 μg of HA peptide was included in the reaction in lanes 8 and 14. 2 pmol of competitor DNA uUAS1 and mUAS1 were added in reactions in lanes 9 and 10, respectively. A control carried out in the presence of 0.14 μg of 9E10 antibody, which recognizes the myc epitope, is shown in lane 15. Lane 16 is performed with cell extracts from oleate-grown wild type cells that do not express Oaf1pHA3, the reaction also contains 0.05 μg 12CA5.



repressed by glucose, and is not induced by oleate.

***Oaf1p is a component of the POX1 UAS1-protein complex:***

Oaf1pHA3 is able to restore the formation of a *POX1* UAS1-protein complex in a band shift assay using cell extracts from cells grown in oleate medium (Figure 4-3, lane 1). Addition of a small amount of the monoclonal antibody 12CA5 (0.02-0.1 μg), which recognizes the HA epitope, in the binding reaction resulted in an additional super-shifted band (Figure 4-3, lanes 2-7). Such a band was not observed when cell extracts from oleate-grown wild type cells, which do not express Oaf1pHA3, were used in the assay (lane 16). This new band was abolished by addition of an HA peptide, indicating that it is a super-shifted band caused by the binding of antibody to the protein-DNA complex (Figure 4-3, lane 8). Monoclonal antibody 9E10, recognizing myc epitope tag, does not give rise to such band (Figure 4-3, lane 15). Competition using a uUAS1 or a mutated UAS1 fragment showed that the super-shifted band is specific (lanes 9 and 10). The intensity of the shifted or supershifted band appears not as strong as the shifted band in the absence of antibody or in the presence of HA peptide (lane 1 and 8). Furthermore, when higher amount of 12CA5 is added in the binding reaction (0.2-3 μg), in addition to the formation of a faint super-shifted band, 12CA5 antibody also reduced or abolished the band corresponding to the UAS1-protein complex (Figure 4-3, Lanes 11-13), this was prevented in the presence of HA peptide (lane 14). One possibility for the inhibition of the binding of Oaf1HA3 to UAS1 by high concentrations of antibody is that occupation of all three HA epitopes by antibody may interfere with the binding of Oaf1p to UAS1. These data confirm the involvement of Oaf1p in the complex of *POX1* UAS1-protein(s).

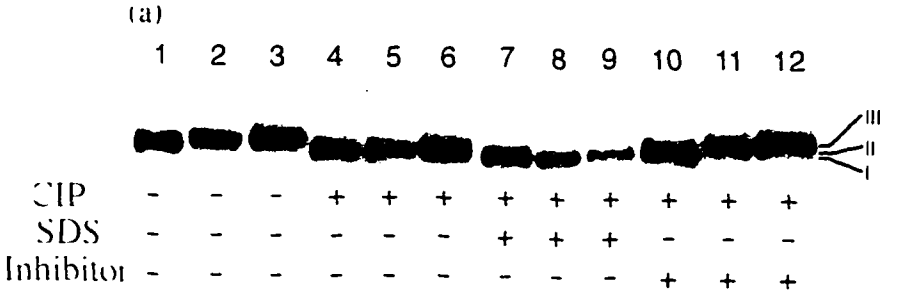
***Regulated phosphorylation of Oaf1p:***

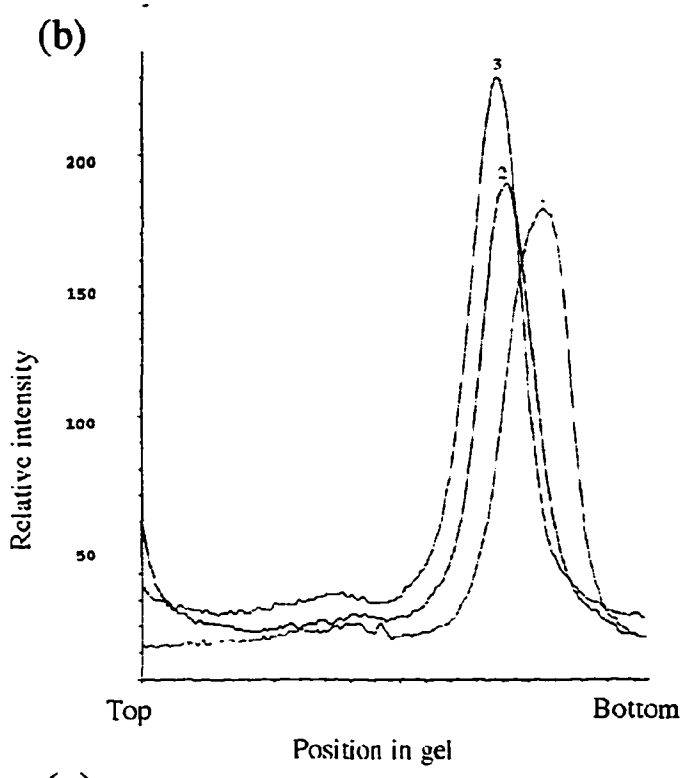
Expression of the *OAF1* gene itself is not induced by oleate, therefore it is possible that Oaf1p may be activated by posttranslational modifications. Immunoblot analysis of Oaf1pHA3 showed that Oaf1pHA3 in extracts from cells cultured in glycerol and oleate medium (Fig 4-4a, lane 2 and 3) appeared to migrate slightly slower than that from glucose-grown cells (lane 1). In order to quantitatively analyze the migration of the protein, densitometry analysis was carried out to identifiy the position of the protein bands. The result confirmed that Oaf1pHA3 from glycerol or oleate cultured cells migrated slower than that from glucose grown cells (Figure 4-4b). The decreased mobility of Oaf1pHA3 suggests that the modification states of this protein in glucose-grown cells are different to those in glycerol or oleate grown cells. Phosphorylation has been shown to be required for the activation of many transcription factors, such as Gal4p [173] and heat shock factor (HSF) [174], therefore we postulate that Oaf1p may also be subjected to phosphorylation. In order to test whether Oaf1p is phosphorylated, cell extracts were treated with calf intestinal phosphatase (CIP) (Figure 4-4a). Addition of CIP to the extracts from glucose- and glycerol-grown cells gave rise to a single band Oaf1p (Figure 4-4a, lane 4-5). Densitometry analysis of the peak position of the CIP treated Oaf1p confirmed that the peaks of the CIP treated Oaf1p from glucose and glycerol grown cells were at the same position (Figure 4-4c, peaks 4 and 5), and there was little difference between the peaks for glycerol and oleate-grown cells (peaks 5 and 6). Similar results were obtained when the phosphatase treatment was carried out in the presence of 0.2% SDS (Figure 4-4a, lane 7-9). Addition of phosphatase inhibitors prevented the change in the mobility pattern of Oaf1p-HA3 caused by CIP (Figure 4-4a, lane 10-12).

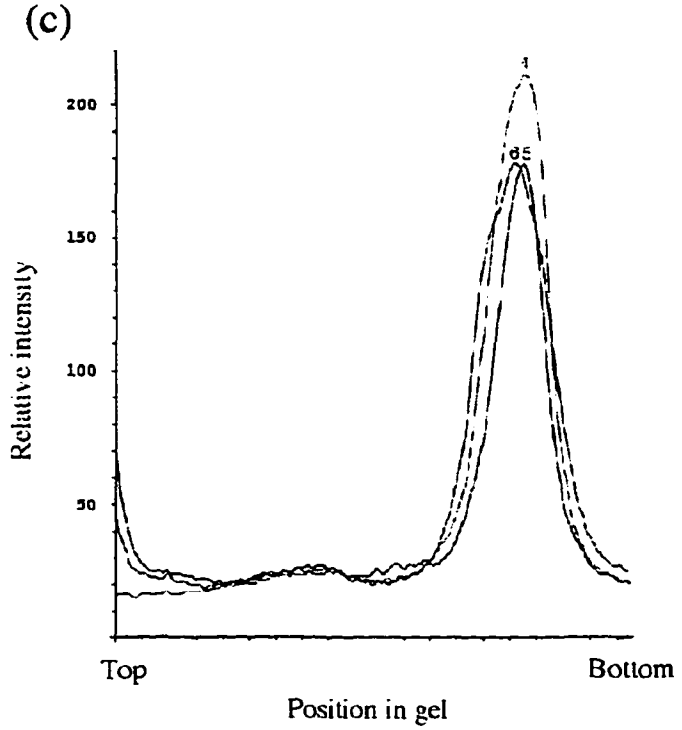
Figure 4-4. Oaf1pHA3 is phosphorylated in cells grown in glycerol or oleate medium.

(a). Phosphatase treatment of Oaf1pHA3. Reactions were carried out with cell extracts, which contain approximately 20 μg protein, from glucose- (lanes 1, 4, 7 and 10), glycerol- (lanes 2, 5, 8 and 11) or oleate- (lanes 3, 6, 9 and 12) grown cells. The addition of CIP (alkaline calf intestinal phosphatase), phosphatase inhibitor cocktail and 0.2% SDS is indicated below each lane. Reaction mixture were incubated at 37 $^{0}$C for 1 hour and was then separated on 7% SDS polyacrylamide gel followed by immunoblot with 12CA5 antibody recognizing the HA epitope.

(b) and (c). Densitometry analysis of the result obtained from the immunoblot analysis in (a) to identify the positions of the protein bands corresponding to Oaf1pHA3 from cells grown in different conditions. Peaks 1, 2 and 3 in (b) correspond to lanes 1, 2 and 3 in (a), respectively. Peaks 4, 5 and 6 in (c) correspond to lanes 4, 5 and 6 in (a).





These results suggest that phosphorylation accounts for the reduced mobility of Oaf1pHA3 in cells grown in glycerol and oleate medium.

***SNF1 is not essential for the phosphorylation of Oaf1p:***

*SNF1* kinase has been implicated in the derepression of *CTA1* and *FOX3* [117] and has been shown to mediate the phosphorylation of Sip4p, a C6 zinc cluster transcriptional activator with unknown function [166]. Therefore I analyzed whether *SNF1* could phosphorylate Oaf1p and regulate the expression of *POX1*, using a yeast strain MCY2692 [166], which carries a mutation in the *SNF1* gene. The expression of *POX1* was examined by transforming MCY2692 with a plasmid containing a *POX1-lacZ* reporter gene. Although we did not have the isogenic wild type strain, the expression of the reporter gene in MCY2692 was lower than in our *SNF1* wild type strain (W3031A) (compare wild type in Figure 4-1 and MCY2692 in Figure 4-5). However, in each case, β-galactosidase activity was induced approximately 10-fold in response to oleate. The induction was abolished by the disruption of *OAF1*. This raised the possibility that *SNF1* might be involved in the derepression of *POX1*, but is not essential for the oleate induction this gene. Electrophoretic migration of Oaf1p, which indicates the phosphorylation state of the protein, was examined by expressing Oaf1pHA3 in MCY2692. The migration pattern of Oaf1pHA3 in the *snf1* mutant was similar to that in wild type cells (Figure 4-6), suggesting that *SNF1* is not essential for the phosphorylation of Oaf1p. However, the possibility that Snf1p plays some role in the phosphorylation of Oaf1p, but does not cause a detectable change in its migration in SDS-PAGE, can not be ruled out.

***Oaf1p and Oaf2p form complex:***

Figure 4-5. Expression of the *POX1-lacZ* reporter gene in a strain carrying a mutation in *SNF1*.

The β-galactosidase activity was measured using cell extracts from strain MCY2692plz, which carries a mutation in the *SNF1* gene and is transformed with a plasmid containing the *POX1-lacZ* reporter gene, and MCY2692plzΔ*OAF1*, in which the *OAF1* gene is disrupted. Cells were grown in media containing glucose, glycerol or oleate. Numbers are the mean of four experiments.

Figure 4-6. Expression of Oaf1pHA3 in a *snf1* mutant.

Cell extracts from strains MCY2692HA3 which expresses Oaf1pHA3 (lanes 1-3), and OA1HA3 (lanes 4-6) which is wild type for *SNF1* grown in glucose (lanes 1 and 4), glycerol (lanes 2 and 5) or oleate (lanes 3 and 6) media were separated on a 7% SDS polyacrylamide gel followed by immunoblot with 12CA5 against the HA epitope. Lanes 1-3 contains approximately 50 μg proteins and exposed for 5 seconds. Approximately 6 μg protein was loaded in lanes 4-6, and the membrane is exposed for 30 seconds

Figure 4-5

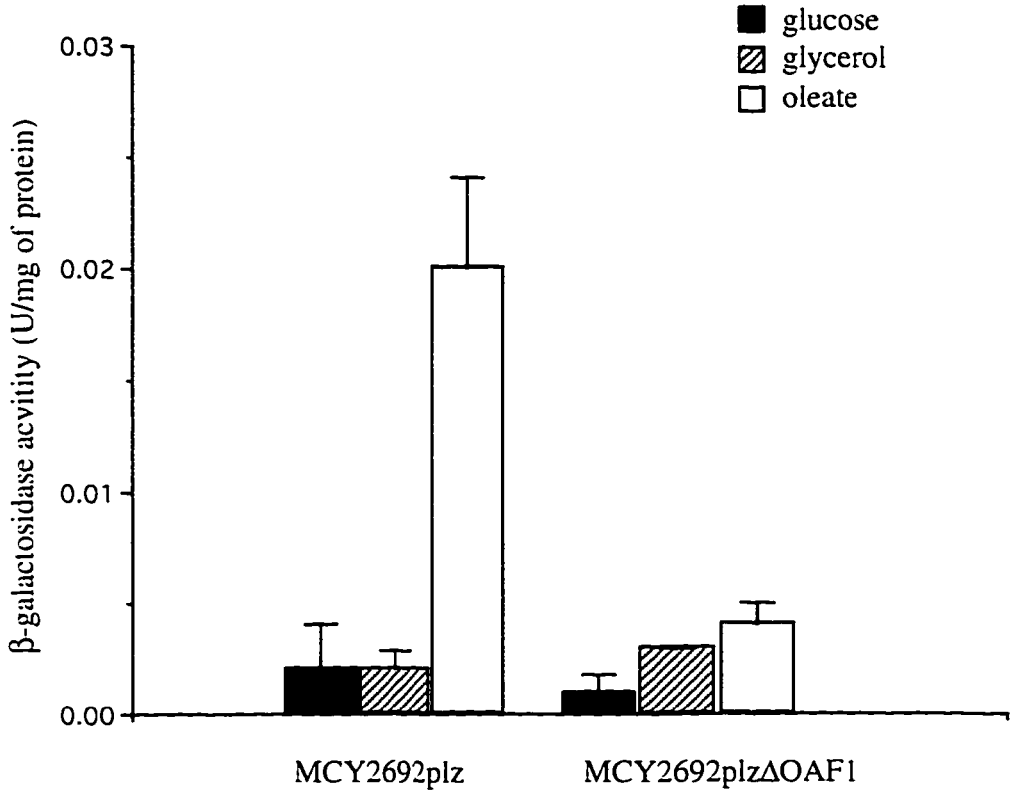



Figure 4-6

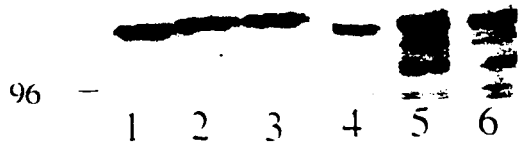


Figure 4-7. Oaf1p and Oaf2p form a complex.

Coimmunoprecipitation of Oaf1pHA3 and Oaf2pmyc with monoclonal antibody 9E10, recognizing the myc epitope. Protein extracts (approximately 500 μg of protein) prepared from OA2mycHA3 cells cultured in glucose, glycerol or oleate media were incubated with 2 μg of 9E10 followed by precipitation with 50 μl protein-G agarose. The immunoprecipitated material was separated by SDS-PAGE and immunoblotted with monoclonal antibody 12CA5 recognizing the HA epitope (lanes 1-3). Extracts from OA2myc (Lanes 4 and 5) or OA2HA3 (lanes 6 and 7) cells, which express either Oaf2pmyc or Oaf1pHA3, grown in glucose (lanes 4 and 6) or oleate (lanes 5 and 7) media were treated in the same manner as controls (lanes 4-7). 50 μg of of total protein from OA2mcyHA3 cells grown in oleate medium was subjected to SDS-PAGE followed by immunoblot with 12CA5 (lane 8).

OAF2, a gene recently cloned by Dr. Karpichev in our laboratory, is also required for the oleate induction of *POX1*. The expression of OAF2 is low in glucose or glycerol grown cells, and is induced in the presence of oleate. The functional and structural similarities between Oaf1p and Oaf2p, and the requirement of both of them for the oleate induction of *POX1*, suggest that they may interact with each other to facilitate this induction. In order to test this possibility, a yeast strain, OA2HA3myc, which expresses both Oaf1pHA3 and Oaf2pmyc was used to determine whether the two epitope tagged proteins interact. Cell lysates were prepared from cells grown in the presence of glucose, glycerol or oleate, and were used for immunoprecipitation with the 9E10 antibody which recognizes the myc epitope [172]. The immunoprecipitated material was then analyzed by SDS-gel electrophoresis and western blotting using 12CA5, which recognizes HA epitope, to detect the coimmunoprecipitated Oaf1pHA (Figure 4-7). Tagged Oaf1p was coimmunoprecipitated with Oaf2pmyc from all three growth conditions (Figure 4-7, lanes 1-3), but was not precipitated from strain OA2myc, which expresses Oaf2pmyc but not Oaf1pHA3 (Figure 4-7, lanes 4 and 5), or strain OA2HA3, which expressed Oaf1pHA3 but not Oaf2pmyc (Figure 4-7, lanes 6 and 7). These data suggest that Oaf1p and Oaf2p may form a complex in all growth conditions tested.

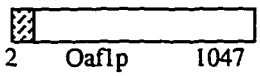
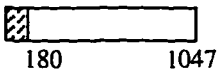
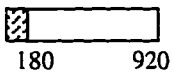
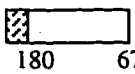
***The activation domain of oaf1p is located at the carboxyterminus:***

In order to identify the activation domain of Oaf1p, a series of constructs containing truncated versions of Oaf1p fused, in frame, with the DNA binding domain of the Gal4 protein (GDB) were prepared. Plasmids containing the hybrid gene were introduced into yeast strain SFY526 ΔOAF1, which carries a *GAL1-lacZ* reporter gene and in which *OAF1* is disrupted. This strain also carries mutations in *GAL80* and *GAL4* to eliminate

Figure 4-8. Transcriptional activation of the *GAL1-lacZ* reporter gene in cells expressing fusion proteins consisting of the Gal4p DNA binding domain and various truncated versions of Oaf1p.

The constructs expressing the fusion proteins were transformed into a yeast strain SFY526Δ*OAF1*, which carries a *GAL1-lacZ* reporter gene and mutations in *GAL4*, *GAL80*, and in which *OAF1* is disrupted. β-galactosidase activity was measured using extracts from the transformants grown in glucose, glycerol or oleate media. Results are the mean of four experiments.

β- galactosidase activity (U/mg protein)

| | Glucose | Glycerol | Oleate |
|---|---|---|---|
|  GDB | 0.002 ± 0.000 | 0.032 ± 0.006 | 0.016 ± 0.001 |
|  2 Oaf1p 1047 | 0.124 ± 0.014 | 0.289 ± 0.031 | 3.675 ± 0.361 |
|  180 1047 | 0.888 ± 0.088 | 2.416 ± 0.588 | 9.320 ± 0.728 |
|  180 920 | 0.004 ± 0.000 | 0.002 ± 0.000 | 0.002 ± 0.000 |
|  180 670 | 0.002 ± 0.001 | 0.016 ± 0.001 | 0.017 ± 0.001 |
|  920 1047 | 1.929 ± 0.010 | 6.337 ± 0.045 | 5.373 ± 0.026 |

interference with the reporter gene expression. The function of the hybrid proteins were examined by their ability to activate the expression of the reporter gene (Figure 4-8). The presence of the fusion proteins GDB-Oaf1$^{2\text{-}1047}$ or GDB-Oaf1$^{180\text{-}1047}$ caused an increase in the expression of the reporter gene in all conditions tested, compared to the level found in control cells. However, expression of the reporter gene in cells expressing fusion proteins GDB-Oaf1$^{180\text{-}920}$ or GDB-Oaf1$^{180\text{-}670}$, in which the C-terminal regions of Oaf1p were deleted, was the same as for the control. These results suggest that the C-terminal 128 amino acids may be required for the transcriptional activation capacity of Oaf1p. The possibility that the deletion of the C-terminal region of Oaf1p may interfere with the binding of the fusion protein to the *GAL1* promoter can not be ruled out at this point.

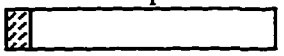
Expression of fusion protein GDB-Oaf1$^{920\text{-}1047}$, which consists of the C-terminal 128 amino acids of Oaf1p fused with the Gal4p DNA binding domain, results in an increase in the expression of the reporter gene in all growth conditions tested, compared with control. This fusion protein is fully activated when cells are grown in glycerol medium, suggesting that the Oaf1p region from residues 920 to the stop codon is able to activate transcription in an oleate-independent fashion, when it is fused with a functional DNA binding domian. Thus, the C-terminal region of Oaf1p functions as an activation domain.

The fusion protein GDB-Oaf1$^{2\text{-}1047}$ responds to oleate, resulting in a 10-fold induction of the reporter gene (Figure 4-8, compare lane glycerol and oleate). The fusion protein GDB-Oaf1$^{180\text{-}1047}$ also responded to oleate, in this case, the expression of the reporter gene in all growth conditions tested was higher than in the former case, however, the oleate induction was only approximately 4 fold. In contrast, the hybrid protein GDB-Oaf1$^{920\text{-}1047}$, which

Figure 4-9. Activation of Oaf1p does not require the presence of Oaf2p.

Constructs expressing fusion proteins consisting of the Gal4p DNA binding domain and various truncated versions of Oaf1p were transformed into a yeast strain SFY526ΔOAF2, in which *OAF2* is disrupted. β-galactosidase activity was measured using extracts from cells cultured in glucose, glycerol or oleate media. Results are the mean of four experiments.

β-galactosidase activity (U/mg protein)

| | Glucose | Glycerol | Oleate |
|---|---|---|---|
|  GDB | 0.003 ± 0.000 | 0.015 ± 0.002 | 0.017 ± 0.002 |
| 2 Oaf1p 1047  | 0.142 ± 0.013 | 0.479 ± 0.010 | 3.176 ± 0.205 |
| 180 1047  | 1.288 ± 0.150 | 1.853 ± 0.182 | 8.467 ± 0.860 |
| 920 1047  | 3.050 ± 0.212 | 12.982 ± 1.665 | 9.369 ± 0.692 |

contains only the C-terminal 128 residues, exhibited high activity in all growth conditions tested and did not respond to oleate. Taken together, these results suggest that the N-terminal 180 amino acids of Oaf1p, which includes the putative zinc cluster DNA binding domain and amphipathic dimerization domain, might contribute to the oleate response of Oaf1p, and that the central domain of Oaf1p (181 to 920) plays a critical role in this oleate-dependent response.

***Oaf2p is not required for the activation of Oaf1p:***

Oaf1p and Oaf2p appear to form a complex in cells cultured in all conditions, however, the protein binding to the UAS1 and induction of *POX1* only occurs in the presence of oleate. In order to determine whether the oleate-dependent activation of Oaf1p itself requires the presence of Oaf2p, the fusion proteins consisting of the Gal4 DNA binding domain and various truncated versions of Oaf1p were expressed in strain SFY526 ΔOAF2, which contains the *GAL1-lacZ* reporter gene and in which the genomic copy of *OAF2* is disrupted. The activities of these fusion proteins in the absence of *OAF2* were similar to those in the presence of *OAF2* (compare Figure 4-8 and 4-9). These results revealed that the fusion proteins GDB-Oaf1p$^{2\text{-}1047}$ and GDB-Oaf1p$^{180\text{-}1047}$ were able to activate transcription in an oleate dependent manner in the absence of Oaf2p.

## Discussion

In this chapter, I described the functional characterization of Oaf1p and its interaction with Oaf2p. Oaf1p was shown to be expressed whether cells are grown in glucose, glycerol or

oleate medium, however Oaf2p is expressed at very low levels in glucose-grown cells, derepressed in glycerol grown cells and induced in oleate-grown cells [170,175]. Oaf1p and Oaf2p are both required for the oleate induction of *POX1*, disruption of either *OAF1* or *OAF2* abolishes the UAS1-protein complex formation and the induction of *POX1*. This suggests that an Oaf1p or Oaf2p homodimer, if present, is not able to bind to the *POX1* UAS1 and mediate *POX1* induction *in vivo*. However, Rottensteiner et al recently showed that a fusion protein GST-Oaf2p(1-179), which consists of the Cys6 zinc cluster of Oaf2p fused in-frame with glutathione S-transferase (GST), was able to bind to the *FOX3* ORE. Their capability to detect the *FOX3* ORE-GST-Oaf2p(1-179) complex could be due to the fact that they used partially purified recombinant protein in the DNA band shift assay, thus the presence of a high concentration of recombinant protein allowed the detection of the low affinity binding activity of the recombinant protein. Coimmunoprecipitation of Oaf1p and Oaf2p suggests that Oaf1p and Oaf2p appear to form complexes (Figure 4-7), this raises the possibility that Oaf1p and Oaf2p may function by forming a heterodimer. Oaf2p expression is induced by oleate, however, similar amounts of Oaf1pHA3 coimmunoprecipitated with Oaf2pmyc in lysates from cells cultured in all growth conditions. This could be due to the following possibilities: (1) The antibody was not added in excess; (2) The presence of oleate or its derivatives in the cell extracts may interfere with the formation of the antibody-antigen complex.

Oaf1p is not significantly regulated at the transcriptional level by oleate, but it appears to be modified by differential phosphorylation in different growth conditions. The phosphorylation state of Oaf1p in glycerol and oleate cultured cells appears to be different to

that in glucose cultured cells, as shown by the phosphatase treatment experiments.

The transcriptional activation domain of Oaf1p, which resides in the C-terminus, is negatively charged, and there are 13 acidic residues and 5 basic residues in the C-terminal 128 residues. The location of the Oaf1p activation domain is similar to that of other members of the Cys6 zinc cluster transcription factor family, such as Gal4p, Hap1p and Put3p, which also have C-terminal negatively charged activation domains [160,168,176]. The activation domain of Oaf1p is fully activated in cells cultured in glycerol medium, as shown with the fusion protein GDB-Oaf1$^{920\text{-}1047}$. The Oaf1p activation domain exhibited lower activity in glucose-grown cells, it is possibile that some phosphorylation of Oaf1p in glycerol cultured conditions might occur in this domain and result in its activation.

The Oaf1p activation domain, when fused with the Gal4p DNA binding domain, showed lower activity in the presence of glucose, suggesting that the Oaf1p activation domain may have low activity in glucose cultured conditions. This low intrinsic activity of the Oaf1p activation domain might contribute to the activity of the fusion proteins GDB-Oaf1$^{2\text{-}1047}$ or GDB-Oaf1p$^{180\text{-}1047}$ to activate the expression of the *GAL1-lacZ* reporter gene in cells grown in glucose medium. These two fusion proteins showed higher activity in cells grown in media containing glycerol, this might be caused by the activation of the Oaf1p activation domain in this culture medium. GDB-Oaf1p$^{180\text{-}1047}$ showed higher activity, in all conditions tested, than GDB-Oaf1p$^{2\text{-}1047}$. The reason for this is not clear, one possibility is that the presence of the N-terminal 180 amino acids of Oaf1p in the fusion protein may alter the conformation of the protein and this may affect its activity. Alternatively, this region may mediate the repression of Oaf1p in the absence of oleate.

Upon oleate activation, the hybrid protein GDB-Oaf1$^{2\text{-}1047}$ leads to a 10-fold induction in the expression of the reporter gene, whereas, GDB-Oaf1$^{180\text{-}1047}$ only results in a 4-fold induction although in this case the β-galactosidase activity in all growth conditions was higher than that in the former case (Figure 4-8). These data suggest that the N-terminal putative DNA binding domain of Oaf1p might also contribute to the oleate response, possibily by maintaining Oaf1p in a conformation that has low activity in the absence of oleate. The central domain (181 to 920) may keep the protein in a conformation such that it is not fully activated in the absence of oleate. In the absence of this domain, the activation domain is fully activated in glycerol-grown conditions, suggesting that the central domain is involved in mediating the oleate response. A possibile model for the regulation of Oaf1p is that the N-terminal portion (1-920) of Oaf1p blocks the function of the Oaf1p activation domain in glycerol-cultured cells; this region responds to oleate and might lead to the activation of the DNA binding domain, this may cause a conformational change and result in the exposure of the activation domain to the transcriptional machinery, thus leading to the induction of *POX1*.

Both Oaf1p and Oaf2p are required for the formation of a *POX1* UAS1-protein complex and *POX1* activation. However, the fusion protein GDB-Oaf1$^{2\text{-}1047}$ is activated by oleate in the absence of Oaf2p, suggesting that Oaf1p is able to receive the signal transmitted from oleate, and activated Oaf1p is then able to mediate transcriptional activation once positioned on promoter. Thus, Oaf2p might only be required to mediate the *POX1* UAS1 binding of oleate activated Oaf1p. It is not yet known whether Oaf2p alone is activated by oleate or if Oaf2p activity is only controlled at the transcriptional level.

Stanway et al suggested that a *SNF1* mutation had little effect on the expression of *POX1*

[122], however, the present study showed that the overall expression of the *lacZ* reporter gene, controlled by the *POX1* promoter, is reduced in a *snf1* mutant, although there is still approximately a 10-fold induction in the presence of oleate. This result indicates that *SNF1* may be required for *POX1* derepression, but it is not essential for oleate induction. Alternatively, *SNF1* might act on the basal transcription of *POX1*. The presence of oleate induction in a *snf1* mutant is consistent with the finding that *SNF1* is not essential for the phosphorylation of Oaf1p in cells cultured in glycerol and oleate medium. It is not clear how *SNF1* regulates *POX1* depression.

# CHAPTER V

## SUMMARY AND FUTURE STUDIES

### Summary of the results

In the yeast *Saccharomyces cerevisiae*, peroxisome proliferation is regulated by the carbon source provided for cell growth. This process is induced by fatty acids, such as oleate, and is repressed in the presence of glucose. The induction of peroxisome proliferation is accompanied by the induction of the β-oxidation enzymes. Repression or induction of peroxisomal β-oxidation is regulated at the transcriptional level. In order to understand the mechanisms controlling peroxisome proliferation and the expression of peroxisomal proteins, I have carried out a study on the transcriptional regulation of *POX1*, which encodes peroxisomal acyl-CoA oxidase in *S. cerevisiae*. In this chapter, I will summarize the results presented in the previous chapters.

In chapter II, I described studies on the identification of two regulatory cis-elements responsible for *POX1* regulation. A negative cis-element, URS1, had been identified within -434 to -410 [132]. Further deletion analysis of the *POX1* promoter and DNA band shift analysis, using a number of DNA fragments from region -213 to -349, led to the identification of an additional negative cis-element, URS2, which is located between -349 and -316, and a positive oleate responsive cis-element, UAS1, which resides in region from -316 to -238. URS1 and URS2 are able to compete with each other for protein(s) binding, suggesting that same protein or protein complex binds to both of the URS's. The URS1 protein binding site consists of a complete inverted repeat of a sequence, 5' AGGGT(A/T)A(T/A) 3', whereas

URS2 contains an incomplete direct repeat of a sequence, 5 AGGG(C)TT(A/T)NA 3'. The nucleotides AGGG, which have been shown to be critical for protein binding [132], are conserved in both URS1 and URS2. The functionality of URS2 was confirmed by demonstrating that they were able to repress the function of the *CYC1* UAS in a heterologous *CYC1* promoter. The data we obtained suggest that URS1 and URS2 might function as general repression sequences

A protein or protein complex was shown, by DNA band shift assay, to bind to the *POX1* UAS1 in an oleate-dependent manner. The URS2 binding protein(s) and UAS1 binding protein(s) could not bind simultaneously to a DNA fragment containing both of these elements, because if the two proteins were both bound to the same DNA fragment, they would give rise to a "supershifted" band, but this was not observed in the band shift assays with the 184-mer or the 70-mer (Figure 2-4a and 2-5). Thus, the URS2 binding protein and the UAS1 binding protein appear to compete with each other to occupy the promoter region. *POX1* UAS1 consists of two palindrome sequences. Each of the palindromes is able to bind protein(s), and also they can compete with each other for protein binding, suggesting that the same protein or protein complex bind to each of the palindromes. The palindromic structure of the UAS1 indicates that the UAS1 binding protein may bind to DNA in the form of a dimer. The consensus sequence of the four repeats in both palindromes, (A/T)A(A/T)NNCCG(A/T)AT, is homologous to the oleate-responsive elements (ORE) of *FOX3* (encoding thiolase) and *CTA1* (encoding catalase A) [110,111]. Similar sequences are also present in several other genes encoding peroxisomal proteins, such as *FOX2* (encoding bifunctional enzyme), *PEX11* (*PMP27*), *PEX5* (*PAS10*), *PEX6* (*PAS8*)and *FAA2* (encoding

acyl-CoA synthetase) [111,175]. These genes contain one single palindrome consisting of two repeats of the consensus sequence. The regulation of *POX1* by two palindromic sequences might reflect the important role that acyl-CoA oxidase plays in the peroxisomal β-oxidation pathway. The most conserved sequences in the ORE are the CGG triplets, which are separated by 17 nucleotides in *FOX3* and *POX1*, and by 18 nucleotides in *CTA1*. Mutation of the CGG triplets in the *POX1* UAS1 abolished the interaction of the protein(s) with the DNA, confirming that the triplets are critical for protein binding. The function of the *POX1* UAS1 as a positive oleate responsive cis-element was confirmed by demonstrating that the *POX1* UAS1 was able to activate transcription in an oleate dependent fashion in the context of the *CYC1* promoter.

The regulation of a gene by multiple regulatory cis-elements is a general feature for yeast genes, whose expression is regulated. *SUC2* encoding invertase and *CAR1* encoding arginase, are controlled by an upstream repression sequence and an upstream activation sequence [120,139]. *CYC1* encoding iso-1-cytochrome c is controlled by an upstream activation site consisting of two independent UASs. *CYC1* UAS1 mediates heme-dependent activation by the binding of Hap1p, and *CYC1* UAS2 mediates the binding of Hap2p, Hap3p and Hap4p complex binding and derepression in the presence of a non-fermentable carbon source [177,178]. *ADH2*, which encodes glucose repressibile alcohol dehydrogenase II, is controlled by two UASs, the UAS1 and UAS2 activate gene expression synergistically [179]. The *GAL1* gene, which encodes galactokinase, is controlled by $UAS_G$ which mediates galactose induction by the binding of Gal4p, and $URS_G$, which is reponsive to glucose repression [126,180]. The glucose repression of *GAL1* genes is controlled by two mechanisms, one is

mediated through $URS_G$, the other inhibits the activity of Gal4p [180].

In chapter III, I reported studies concerning the properties and purification of a UAS1 binding protein. I demonstrated that the binding of the UAS1 binding protein to UAS1 is correlated with the induction of *POX1*, thus the UAS1 binding protein(s) is a critical factor in the oleate induction pathway leading to the transcriptional activation of peroxisomal proteins. Several factors, such as *ADR1*, *SNF1*, *RTG1* and *RTG2*, have been reported to be involved in the regulation of the genes encoding peroxisomal proteins, however, none of them has been shown to specifically mediate oleate induction of the genes [117,125]. As a first step towards characterizing the oleate induction pathway, I purified the UAS1 binding protein. A major protein with molecular mass of approximately 110-120 kDa was purified by UAS1 affinity chromatography. The purified protein was subjected to peptide sequencing analysis, and two peptide sequences were obtained. Using the obtained sequences, a protein with molecular mass of 118.2 kDa was identified by searching the protein data base. We named the gene encoding this protein Oleate-Activated transcription Factor 1, *OAF1*, and the gene product is Oaf1p. A zinc cluster DNA binding motif, which is homologous to the DNA binding domain of Hap1p and Gal4p, is located at the amino-terminus of Oaf1p. Thus, the Oaf1p protein belongs to the family of transcription factors containing zinc cluster DNA binding domain, which includes Gal4p, Hap1p and Put3p, which bind to targeting sequences consisting of CGG triplets separated by various numbers of nucleotides. Gal4p, Put3p and Ppr1p recognize palindromic CGG triplets separated by 11 bp, 10 bp and 6 bp, repectively [163, 164,181]. Certain mutations in the internal 11 bp separating the CGG triplets in Gal4p binding site moderately reduce the affinity for protein binding [182]. However, the identity

of the internal 6 bp in Ppr1p binding sites do not influence the binding of Ppr1p [182]. Hap1p binds to an asymmetrical, direct repeat containing two direct CGG triplets separated by 6 bp [183]. Recently, a novel DNA motif, an everted repeat, in which the CGG triplets are oriented in opposite directions, was identified for the zinc cluster proteins, Leu3p and Pdr3p. Leu3p, which regulates genes encoding enzymes involved in branched-chain amino acid synthesis [184], and Pdr3p, which is involved in the regulation of multidrug resistance genes [185], recognize the everted CGG triplets separated by 4 bp and 0 bp, respectively [186]. By using chimeric proteins made of Gal4p, Put3p and Ppr1p, Reece and Ptashne showed that the linker region, which connects the zinc cluster and the amphipathic dimerization domain, specifies the binding of the proteins to their corresponding targeting sequences [162].

Gal4p and Put3p are able to bind to their respective regulatory sequence irrespective of the presence of corresponding inducers [168,169]. In contrast, strong binding of Oaf1p to the *POX1* UAS1 is only observed *in vitro* when it is activated by oleate. However, a faint shifted band was occasionally seen in experiments using labeled *POX1* UAS1 and lysates from glycerol-grown cells (Figure 2-6). Whether Oaf1p binds to *POX1* UAS1 *in vivo* in the absence of oleate is not known. The oleate-dependent activation of the DNA binding activity of Oaf1p resembles that of Hap1p, which binds to its targeting sequence in a heme-dependent manner [178].

In order to analyze the function of *OAF1*, the genomic allele of *OAF1* was disrupted. The disruption of *OAF1* abolished oleate induction of *POX1* and *FOX3*, and in addition, abolished oleate-induced peroxisome proliferation, suggesting that Oaf1p is involved in coordinately regulating the expression of peroxisomal proteins and proliferation of the

organelle itself.

In chapter IV, I described the functional analysis of Oaf1p. The expression of *OAF1* is not significantly regulated by glucose or oleate. Phosphatase experiments described in this chapter suggest that Oaf1p appears to be phosphorylated in cells grown in glycerol or oleate media, but not in glucose-grown cells. This result may indicates that the phosphorylation of Oaf1p in glycerol-grown cells might be a part of the process which may make Oaf1p ready to be activated by oleate. It is not yet clear whether the conformation and/or the phosphorylation states of the oleate activated Oaf1p is different to that from glycerol-grown cells.

In order to understand how Oaf1p functions to activate *POX1* expression, I dissected the functional domains of Oaf1p. An activation domain of Oaf1p (920-1047) was identified at its carboxyl-terminus and is negatively charged. The presence of negatively charged C-terminal activation domains are common to the members of the transcription factor family, in which Oaf1p belongs, such as Gal4p and Put3p [160,161]. The activation domain of Oaf1p, once positioned on a promoter element, is able to activate transcription, in an oleate independent manner. The inability of the Oaf1p activation domain to respond to oleate resembles Put3p, in which the activation domain does not respond to its inducer [176]. However, the Gal4p C-terminal activation domain, when fused to a functional DNA binding domain, is able to activate transcription in a galactose-dependent fashion [187], this response is mediated by Gal80p, a negative regulator which binds to the C-terminus of the activation domain, and which is inactivated in reponse to galactose and therefore allows the activation domain to function [187]. The N-terminal (1-180) and central region (181-920) of Oaf1p

play an important role in the activation of Oaf1p by oleate. The oleate responsive domain remains to be further defined.

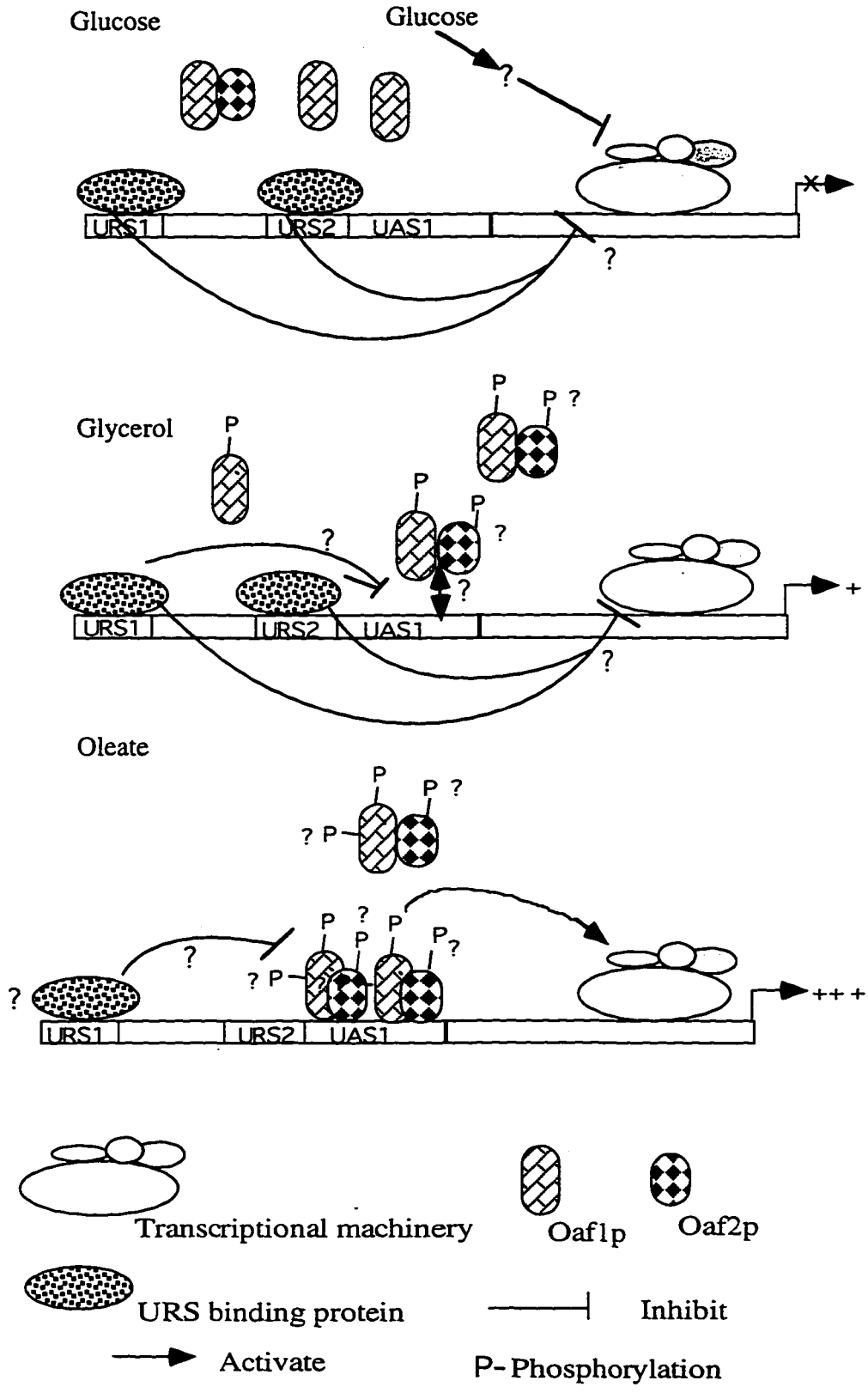
*OAF2* was recently cloned in our and another laboratory by functional complementation of a yeast mutant defective in the induction of *POX1* by oleate [170,175]. Oaf2p, which is 39% identical to Oaf1p, also has a C6 zinc cluster at its amino-terminus and a putative leucine zipper motif (residue 320 to 362). Oaf2p is also required for the oleate induction of *POX1*, *FOX3* and *CTA1* [170,175] and the formation of the UAS1-protein complex. Oaf2p is not required for the expression of Oaf1p, therefore Oaf2p does not function indirectly on the formation of UAS1-protein complex and induction of *POX1* by affecting the expression of Oaf1p [170]. Coimmunoprecipitation experiments showed that Oaf1p and Oaf2p appear to form a complex in cells cultured in all conditions tested, raising the possibility that Oaf1p and Oaf2p may bind to UAS1 in the form of a heterodimer. Oaf1p, when fused with the GAL4 DNA binding domain, is able to respond to oleate and mediate oleate induction of reporter gene *GAL1-lacZ* in the absence of Oaf2p, suggesting that Oaf2p may only be required for the formation of the *POX1* UAS1-protein complex, and not for the activation of Oaf1p. It is not known whether Oaf2p is also phosphorylated and activated by oleate in the absence of Oaf1p or whether Oaf2p activity is only controlled at the transcriptional level. The possibile involvement of the Oaf1p-Oaf2p heterodimer in the *POX1* expression appears to resemble the mode of regulation for mammalian peroxisomal enzymes. In mammals, two proteins, PPARα and RXR heterodimerize and bind to PPRE in the promoter of several genes, including the peroxisomal acyl-CoA oxidase gene, and cooperatively stimulate gene expression in response to peroxisome proliferators [82].

Based on the results I described above, I propose a model for the regulation of *POX1* in *S. cerevisiae*, which is illustrated in Figure 5-1. The mechanisms that mediate glucose repression of *POX1* are unknown. In the presence of glucose, Oaf1p does not appear to be phosphorylated and Oaf2p is expressed at a low level. This may maintain Oaf1p and Oaf2p in inactive forms. The cis-element(s) involved in mediating glucose repression of *POX1* remain to be further defined.

When cells are shifted to glycerol medium, the basal transcriptional machinery is able to turn on the transcription of *POX1* at low rate and lead to moderate expression of the gene. The URS1 and URS2 binding protein(s) may contribute in maintaining the transcription at a low rate. At the same time, the activity of Oaf1p may be derepressed by phosphorylation. The activation domain of Oaf1p appears to be fully active when fused with the Gal4p DNA binding domain in glycerol cultured cells, suggesting that derepression of Oaf1p may involve activation of the activation domain. The mechanisms by which *SNF1* regulate *POX1* derepression are not known. *SNF1* has been shown to alleviate transcriptional repression of the *SUC2* gene by modifying the factors involved in glucose repression pathway [119]. The kinase activity of *SNF1* increases during derepressing condition [188]. However, *SNF1* does not appear to be essential for the expression of *OAF1* or for the phosphorylation of Oaf1p. *SNF1* may regulate the derepression of *OAF2*, which may in turn affect the expression of *POX1*. Whether an Oaf1p-Oaf2p complex binds to UAS1 *in vivo* is not yet known.

When oleate is included in the medium, the affinity for the Oaf1p-Oaf2p complex to bind to the *POX1* UAS1 appears to increase. This binding results in the activation of the transcription machinery, thus leading to the induction of *POX1*. There are several possibilities

Figure 5-1 A model for the transcriptional regulation of *POX1* in *S. cerevisiae*. See text for detail.



for the mechanism by which activation of the Oaf1p-Oaf2p complex is mediated: (1) Oleate, possibly through intermediate signal molecules, may induce a conformational change of the phosphorylated Oaf1p. This conformational change may result in the activation of the DNA binding domain and exposure of the activation domain to the transcriptional machinery. (2) Oleate may induce additional phosphorylation on Oaf1p and/or Oaf2p, which in turn may activate the DNA binding and transcriptional activation activity of Oaf1p-Oaf2p. (3) Oleate activation of the Oaf1p-Oaf2p complex may involve the inactivation of a negative regulator, which could bind to Oaf1p and inhibit the function of the DNA binding domain and/or the activation domain. (4) An activator, activated by the oleate induction pathway, may bind to Oaf1p and activate its function. There are many questions which remain to be answered. Future studies on the identification of the phosphorylated residues, the kinase and the putative negative regulator or activator would help to determine the nature of the mechanism by which Oaf1p and Oaf2p are activated.

## Future Studies

Results ontlined in this thesis suggest that Oaf1p and Oaf2p are involved in the induction of *POX1* by oleate. The mechanisms by which Oaf1p is activated are not known. Future studies described below might improve our knowledge about the oleate induction pathway.

***Identification of the oleate reponsive domain:***

The results obtained to date suggest that the central region of Oaf1p (180 to 863) is

critical for oleate response. In order to further indentify the oleate reponsive domain, fusion proteins, consisting of the Gal4p DNA binding domain and various N-terminal truncated versions of Oaf1p, would be expressed in the yeast strain SFY526, which has *GAL1-lacZ* reporter gene. These experiments might lead to the identification of the region required to mediate the oleate induction.

***Identification of the phosphorylated residues on Oaf1p:***

Oaf1p is phosphorylated in cells grown in glycerol and oleate medium. In order to determine whether the phosphorylation state of Oaf1p in glycerol cultured cells is the same as that in oleate cultured cells and whether phosphorylation is a prerequisite for the activation of Oaf1p, the identification of the phosphorylated residues could be helpful. Phosphoamino acid analysis [189] of Oaf1p from glycerol and oleate cultured cells could be carried out to show whether serine, threonine or tyrosine is phosphorylated. This result might also tell us whether oleate induction leads to additional activating phosphorylation on derepressed Oaf1p at different kind of residues. Phosphopeptide mapping could be used to compare the phosphorylated peptides from derepressed and activated Oaf1p, this might tell us whether different peptides are phosphorylated upon oleate activation.

Phosphopeptide mapping would also show which region of Oaf1p is phosphorylated in cells cultured in glycerol and oleate. The putative phosphorylated residues (serine, threonine or tyrosine) in the region could be mutated by site directed mutagenesis. Thus, the phosphorylated residues would be identified by the mutations that lead to alteration in the migration of the protein in SDS gel, or the disappearance of phophoamino acid in phosphoamino acid analysis. Identification of the residues subjected to phosphorylation in

glycerol or oleate cultured conditions would show whether Oaf1p is differentially phosphorylated in these growth conditions. The transcriptional activity and the DNA binding activity of the mutated proteins could be examined by the activation of *POX1* or a *POX1-lacZ* reporter gene and by band shift assays. These experiments would demonstrate whether phosphorylation on certain residues is required for the activation and DNA binding of Oaf1p.

***Identification of the proteins interacting with Oaf1p-Oaf2p:***

Identification of the proteins associated with Oaf1p or Oaf2p would help us to understand the regulation of Oaf1p and to characterize the oleate induction pathway. Several approaches could be used to identify such interacting factors. (1) Biochemical approach: Oaf1p-GST or Oaf2p-GST fusion proteins could be expressed in yeast cultured in glucose, glycerol or oleate medium. A glutathione affinity column would be used to purify the fusion proteins from cell lysates. Any associated proteins might then be purified along with Oaf1p-GST or Oaf2p-GST. However, this approach would not work well if the interaction of the protein with Oaf1p or Oaf2p is temporary and weak. (2) Functional suppression: Yeast strains containing mutations in *OAF1* or *OAF2* could be subjected to EMS mutagenesis. This could lead to the isolation of suppressors that suppress the mutation of Oaf1p and Oaf2p. The suppressors could be a negative regulator, or an activator. (3) Two hybrid system: Yeast cells expressing a fusion protein containing a truncated form of Oaf1p or Oaf2p (without the activation domain) fused in frame with the Gal4p DNA binding domain would be transformed with yeast cDNA libraries, which contain fusions between the GAL4 activation domain and yeast cDNA fragments. In order to isolate proteins interacting with Oaf1p or Oaf2p in all

cultured conditions, the transformants would be grown on plates containing glucose, glycerol or oleate. Negative regulators, a protein kinase or activators could be identified by this approach.

In summary, the expression of the genes encoding the β-oxidation enzymes, such as *POX1* and *FOX3*, is controlled by multiple regulatory elements. The induction of the genes by oleate is mediated by a complicated process involving the transcription factors Oaf1p and Oaf2p. Oaf1p and Oaf2p couple the regulation of the expression of these genes to the regulation of oleate induced peroxisome proliferation. The identification of Oaf1p and Oaf2p provides a way for the complete identification of the factors involved in the oleate induction pathway in *S. cerevisiae*.

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