January 1997

A gene required for expression of methanol-utilization pathway enzymes in the yeast Pichia Pastoria

Laurie Germana Godfrey

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A GENE REQUIRED FOR EXPRESSION OF METHANOL-UTILIZATION PATHWAY ENZYMES IN THE YEAST PICHIA PASTORIS

Laurie Germana Godfrey
B.S., Trinity College, 1976

A thesis submitted to the faculty of the Oregon Graduate Institute of Science and Technology in partial fulfillment of the requirements for the degree Master of Science in Molecular Biology

January, 1997
The thesis "A gene required for expression of methanol-utilization pathway enzymes in the yeast Pichia pastoris" by Laurie Germana Godfrey has been examined and approved by the following Examination Committee:

James M. Cregg, Advisor
Professor

Michael H. Gold
Institute Professor

Matthew S. Sachs
Associate Professor
ACKNOWLEDGMENTS

The assistance, support and patience of numerous people are behind the completion of this thesis. My most heartfelt gratitude must go to my advisor, Dr. James M. Cregg, for providing an environment in which I could work on this project at my own pace. His guidance, support, teaching and criticisms were consistently given with a generous spirit and were always appreciated.

A thank you to Dr. Matthew Sachs for the advice on Sib selection and other lab guidance. I am also grateful to Dr. Michael Gold for his help with this document.

I was fortunate to work in a laboratory full of helpful, interesting and congenial people, all of whom assisted me in countless ways. At the head of the list must come Kimberly Russell and Hans Waterham who have done favors for me that allowed me to complete my work despite my absence from the lab. For technical help, guidance and camaraderie I would like to thank Barbara Smith, Monique Johnson, Yne de Vries, Xuqiu Tan, Henry Liu, Kathy Hartrum, Michael Freitag and Weiqiao Xie.

The support staff has been first rate and essential to this project, particularly with tedious tasks regarding formatting and publication. I would like to acknowledge Nancy Christie and Terrie Hadfield for their many hours of work on my behalf.

I must thank Elsa, George and the nearly completed Owen for 3 years of letting Mommy do her thing. My sister and mother were always supportive and helpful, particularly with the kids and lightening my stress load. And finally I must thank my dear husband, Bruce. Without his scientific/computer-oriented brain, challenging nature expeditions, and soothing temperament I could never have made it through.
DEDICATION

This thesis is dedicated to my mother, Nancy Tanner. Her high standards of kindness, morality and hard work have set an example worth following. Her enthusiasm for all my attributes and interests have sustained me through the highs and lows of life and given me the courage to persevere. A parental reward for you.....thanks Mom.
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ABSTRACT

A Gene Required for Expression of Methanol-Utilization Pathway Enzymes in the Yeast *Pichia pastoris*

Laurie Germana Godfrey, M.S.
Supervising Professor: James M. Cregg

This thesis describes the characterization of a *Pichia pastoris* mutant, JC131, and the cloning and partial characterization of a candidate for the affected gene, *MXRI*. The JC131 strain was isolated during a search for peroxisome-deficient mutants of *P. pastoris*. JC131 cells are defective in methanol- and oleate-utilization (Mut^- and Out^-). Biochemical experiments demonstrate that two enzymes required for methanol metabolism, alcohol oxidase and dihydroxyacetone synthase, are absent in this mutant, thus providing a potential explanation for the Mut^- phenotype. The reason for the Out^- phenotype was not determined. Activity and protein levels for selected peroxisomal enzymes were determined after subcellular fractionation of JC131 cell homogenates into supernatant and crude organelle pellet fractions. Results demonstrated that thiolase, acyl-CoA-oxidase, catalase from oleate-grown cells, and catalase from methanol-grown cells were enriched in pellet fractions, indicating that JC131 cells have normal import-competent peroxisomes. This, combined with the absence or low levels of Mut pathway enzymes, suggests JC131 may be defective in a gene whose product is important for the expression of methanol-utilization-pathway enzymes.

To gain insight into the nature of the defect in JC131 cells, the affected gene was cloned and its product partially characterized. The gene, *MXRI*, transcribes a message of ~3,500 bases and encodes a large polypeptide of 1156 amino acids.
Northern blots demonstrate that the message is constitutively expressed at low levels in glucose and methanol-grown cells. The DNA sequence of MXR1 was determined, and the predicted amino acid sequence was compared to others in the data banks. Mxr1p shows strongest similarity to *Saccharomyces cerevisiae* Adr1p, a transcription factor that is required for growth of this yeast on ethanol and oleic acid, and is known to play a role in the induction of peroxisomal proteins. Both proteins contain a zinc-finger domain that is nearly identical; however, there is little similarity over the remaining portions of the two proteins. Based on the similarity of the proteins and mutants, we hypothesize that Mxr1p may be a transcription factor required for induction of methanol-utilization-pathway enzymes in *P. pastoris*.
CHAPTER 1
INTRODUCTION

1.1 Morphological and Biochemical Overview of the Peroxisome

The peroxisome is a small (typical size = 0.5 μm) electron-dense organelle that is bound by a single thin membrane (7-8 nm). First characterized in 1954 by Rhodin in mouse kidney cells, it was classified as a type of microbody and has been found in all eukaryotic cells (Rhodin, 1954). Biochemical evaluations performed primarily by de Duve in the 1960s established peroxisomes as a distinct subset of microbodies (de Duve et al., 1960; de Duve and Baudhuin, 1966). Glyoxysomes in plants and fungi (Breidenbach and Beevers, 1967) and glycosomes in certain parasites (Opperdoes and Borst, 1977) are modified forms of peroxisomes. The organelle almost always contains at least one flavin-containing oxidase that produces hydrogen peroxide (H$_2$O$_2$) and catalase to degrade the H$_2$O$_2$ (de Duve et al., 1960; de Duve and Baudhuin, 1966). A specific type of β-oxidation distinct from the β-oxidation of mitochondria is common to most peroxisomes (Cooper and Beevers, 1969; Blum, 1973; Lazarow and de Duve, 1976; Opperdoes, 1987; Veenhuis and Harder, 1987). Numerous other biochemical reactions also occur in this organelle (see Table 1.1). The specific enzymatic pathways it contains are dependent upon tissue, organism, and substrates being metabolized (Tolbert, 1981; van den Bosch et al., 1992).

The primary function of the peroxisome is thought to be the protection of other cell contents from damage that may be caused by the presence of H$_2$O$_2$ and highly reactive oxygen free radicals such as superoxide (O$_2$•$^-$) and the hydroxyl radical (•OH$^-$) (Chance and Oshino, 1971). Free radical damage has been associated with degenerative diseases, such as atherosclerosis, cancer, and neurological disorders. Because of this, peroxisomes are also being studied in reference to ageing as well as
Table 1.1 IMPORTANT BIOCHEMICAL PATHWAYS FOUND IN PEROXISOMES*

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<td>(Methanol utilization as growth</td>
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* Pathways not found in all organisms.
Taken from Voet, 1990; van den Bosch, 1992; Moser, 1993; Wanders, 1995.
the prevention and treatment of cancer and other diseases (Masters and Crane, 1995). Another potential function in certain instances is the concentration of enzymes and intermediates for optimal reaction rates. Peroxisomes also allow for selected and efficient responses to environmental changes. For example, yeasts will change the enzyme content of their peroxisomes to metabolize certain carbon and nitrogen sources (van den Bosch et al., 1992). The organelle's enzymes play a key role in anaplerotic pathways which allow cells to generate Krebs Cycle intermediates from non-carbohydrate substrates.

1.1.1 Common peroxisomal metabolic pathways

Since more than fifty enzymes are known to be located in peroxisomes, an exhaustive recitation of all known peroxisomal reactions is beyond the scope of this thesis. Only those pathways of direct relevance to the present work will be discussed. To illustrate the broad utility of the peroxisome, selected pathways are summarized in Table 1.1. Also, this subject has been extensively reviewed (Veenhuis and Harder, 1987; van den Bosch et al., 1992).

1.1.1.1 β-oxidation of fatty acids. The β-oxidation pathway begins with "activation"—the addition of Coenzyme A (CoA) to the carboxyl end of fatty acid chains, forming an acyl-CoA (Shindo and Hashimoto, 1978). This is catalyzed by an acyl-CoA synthase (found on the outside of the peroxisomal membrane) and requires CoA and ATP (Mannaerts et al., 1982). After transport into the matrix of the peroxisome, the activated fatty acids are oxidized by acyl-CoA oxidase. The enzyme generates 2-enoyl-CoA and H$_2$O$_2$ (Osumi and Hashimoto, 1978). The bifunctional enzyme converts the 2-enoyl-CoA to 3-hydroxyacyl-CoA and then to 3-oxoacyl-CoA (Furuta et al., 1980). In the final step of the pathway, the fatty acid is reduced in size by the cleavage of two carbons in a thiolysis reaction with CoA and the enzyme 3-oxoacyl-CoA thiolase (Miyazawa et al., 1981). The final products are acetyl-CoA and an acyl-CoA species that is two carbons shorter than the original substrate. Catalase removes the H$_2$O$_2$ generated by acyl-CoA oxidase (and other H$_2$O$_2$-generating reactions) in either of two ways. It can combine two molecules of H$_2$O$_2$
and produce H$_2$O and O$_2$, or it can utilize certain peroxidation substrates such as ethanol, methanol, or formate in a process known as peroxisomal respiration in which the -OH group of the substrate is oxidized to =O and two molecules of H$_2$O are generated (Chance and Oshino, 1971; Chance et al., 1979).

In plants, fungi, and yeast, the $\beta$-oxidation reactions are exclusively peroxisomal (van den Bosch et al., 1992), a fact which is exploited for isolating peroxisomal mutants in yeast (see Section 1.4.1). Fatty seeds and yeast growing on oleate will retain the acyl-CoA in their glyoxysomes or peroxisomes, respectively, for use in the glyoxylate cycle and gluconeogenesis (Section 1.1.1.2).

In mammals the complete $\beta$-oxidation of fatty acids requires that certain metabolites transfer between peroxisomes and mitochondria. Peroxisomal $\beta$-oxidation is distinguished from the mitochondrial process largely by the chain length of their acyl-CoA substrates. The basic chemical reactions are similar, but the more complex and larger substrates such as those with branches or more than 24 carbons are sent to the peroxisomes first, where they are degraded to a length of approximately 6–8 carbons (Kawamura et al., 1981; Pace-Asciak and Granstrom, 1983; Yamada et al., 1984; Singh et al., 1990). These are converted to an acyl-carnitine form and transported out of the organelle by the enzyme carnitine transferase (Farrell and Bieber, 1983). They are then conveyed to the mitochondria for completion of the oxidative process. Peroxisomal $\beta$-oxidation generates two less ATP per two-carbon cycle than the mitochondrial version, due to the generation of H$_2$O$_2$ rather than FADH$_2$.

1.1.1.2 Glyoxylate cycle. Glyoxysomes in germinating fatty seeds (such as castor bean), and the peroxisomes of yeasts growing on alkanes or fatty acids contain enzymes of the $\beta$-oxidation pathway as well as enzymes essential for the glyoxylate cycle, including isocitrate lyase and malate synthase (Fukui and Tanada, 1979; Tolbert, 1981). The latter metabolic pathway, collaborating with mitochondrial and cytosolic enzymes, generates biomass by a net conversion of two acetyl-CoAs to oxaloacetate (Tolbert, 1981). The oxaloacetate can then enter gluconeogenesis. This
pathway is also critical for yeast and fungal utilization of various two-carbon compounds (e.g., acetate and ethanol) (Maxwell et al., 1977; Zwart, 1983).

1.1.1.3 Cholesterol metabolism. Of particular interest to the medical field is the regulation of cholesterol levels in the body. The principal point of regulation in the biosynthesis of cholesterol is 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), which is located in the peroxisomal matrix as well as the endoplasmic reticulum (ER) membrane (Brown and Goldstein, 1980; Keller et al., 1985; Keller, et al., 1986). Other peroxisomal enzymes for this pathway include thiolase, mevalonate kinase (Thompson and Krisans, 1990; Stamellos et al., 1992), and steroid-8-isomerase (Appelkvist et al., 1990). Recently it has been demonstrated that all enzymes necessary to synthesize cholesterol from mevalonate are peroxisomal (Biardi and Krisans, 1996). Bile acid conjugates are the only means available for an organism to excrete excess cholesterol. The crucial β-oxidation steps in bile acid synthesis are exclusively peroxisomal (Pedersen and Gustafsson, 1980; Krisans et al., 1985). The organelle also contains enzymes for the hydroxylation of cholesterol (Kase and Bjorkhem, 1989). Cholesterol levels are severely compromised in patients with peroxisomal disorders (Hodge et al., 1991), indicating that peroxisomes are essential for proper cholesterol balance.

1.1.1.4 Methanol metabolism in yeasts. Yeast utilization of methanol is of key interest to this thesis as peroxisomes contain several enzymes essential to the pathway (Veenhuis et al., 1976; Veenhuis et al., 1983; Douma et al., 1985). The capacity to exploit methanol for all carbon and energy needs of the organism is unique to only a few yeast species. These include Hansenula polymorpha, Candida boidinii, and Pichia pastoris (Anthony, 1982; Veenhuis et al., 1983). The one-carbon nature of the substrate requires a larger output of energy for generating biomass than other sources such as glucose, and only two NADH$_2$ molecules are generated per molecule of methanol oxidized. Overall there is a small net gain to the cell that allows growth. The full methanol utilization pathway is diagrammed in Figure 1.1.
Figure 1.1. The methanol pathway in yeasts. (1) Alcohol oxidase; (2) catalase; (3) formaldehyde dehydrogenase; (4) formate dehydrogenase; (5) dihydroxyacetone synthase; (6) dihydroxyacetone kinase; (7) fructose 1,6-bisphosphate aldolase; (8) fructose 1,6-bisphosphatase.
After diffusion into the peroxisome, methanol is oxidized to formaldehyde by the enzyme alcohol oxidase (AOX). This reaction generates H$_2$O$_2$ which is degraded by catalase (CAT). Some of the formaldehyde is utilized directly to make energy. In the cytosol it combines with glutathione (GSH) and is then oxidized to formate, generating an NADH$_2$. The GSH is released to cycle again and the formate is further oxidized to CO$_2$, generating a second NADH$_2$. The rest of the formaldehyde remains in the peroxisome to generate biomass via the xylulose 5'-phosphate cycle (Xu5P cycle). Dihydroxyacetone synthase (DHAS) is the peroxisomal enzyme that converts formaldehyde and Xu5P into GAP and DHA (Douma et al., 1985). These two three-carbon molecules exit to the cytosol where combination and rearrangement reactions yield a single molecule of glyceraldehyde-3-phosphate (GAP) for every three turns of the cycle (Anthony, 1982; Veenhuis et al., 1983). GAP is utilized as a starting material by a variety of biosynthetic pathways.

1.2 Human Diseases of the Peroxisome

When any aspect of peroxisome function is compromised in humans, it externalizes as a grave and often lethal disease (Moser et al., 1991; van den Bosch et al., 1992). Failures can be due to a defect involving a specific enzyme, as seen in primary hyperoxaluria type I (Danpure, 1993), or to a defect in the import of needed substrates and/or cofactors for an enzyme's activity, as is postulated for X-linked adrenoleukodystrophy (X-linked ALD) (H. W. Moser et al., 1995). Recently, two genes have been cloned in *Saccharomyces cerevisiae* that are considered candidates for an import defect that could be a cause of X-linked ALD. *PAT1* and *PAT2* appear to function as components of the import machinery for very-long-chain fatty acids (Hettema et al., 1996). Primary hyperoxaluria type I is another peroxisomal disease due to an import defect. In these patients, peroxisomal alanine:glyoxylate amino transferase (AGT) is mislocalized to the mitochondria (Purdue et al., 1990). Patients with this disease have elevated levels of oxalate in their tissues and urine. The oxalate forms a salt with calcium that deposits in the kidneys and causes them to fail (Danpure et al., 1989). The peroxisomes are normal in morphology and enzyme
content, with the exception of AGT (Iancu and Danpure, 1987). From studies of disease-causing mutations, it is theorized that an amino acid change near the N-terminus results in an amphipathic α-helix which targets the enzyme for mitochondria. Another mutation at a more internal location is at a crucial site that may also play a role as part of a peroxisomal targeting signal (Danpure, 1993). Recent experiments show that AGT is normally imported via the PTS1 import pathway (see Section 1.3.2) (Motley et al., 1995). The significance of the import process is demonstrated by the lethal effect of Zellweger syndrome (ZS), a family of diseases that appear to be the result of defects in peroxisomal protein import in which patients' cells are without functional peroxisomal compartments (Bowen et al., 1964; Goldfischer et al., 1973; Santos et al., 1988b; Braverman et al., 1995; Lazarow and Moser, 1995; Wanders et al., 1995) (Table 1.2).

Patients with Zellweger syndrome are of particular interest to researchers studying peroxisome biogenesis, because their cells lack normal peroxisomes (Goldfischer et al., 1973). Peroxisomal enzymes are synthesized normally by the cells of ZS patients, but are located in the cytoplasm where they are active in some instances (e.g., catalase) (Lazarow and Moser, 1989) or inactive and rapidly degraded in others (e.g., acyl-CoA oxidase) (Schram et al., 1986). As a consequence, virtually all peroxisomal metabolic pathways are defective in cells of ZS patients, resulting in multiple birth defects and death shortly after birth. Although ZS patients die, their cells can be cultured in vitro (Goldfischer, 1988). Membrane vesicles that include peroxisomal integral membrane proteins are found in ZS cells (Santos et al., 1988b; Small et al., 1988a; Suzuki et al., 1989; Gartner et al., 1991). The presence of these vesicles or ghosts suggests that ZS patient cells are defective in the import of peroxisomal proteins or some other aspect of peroxisome biogenesis (Santos et al., 1988b). Genetic studies of ZS cells indicate that mutations in any one of at least ten different genes will result in ZS (A. B. Moser et al., 1995; Poulos et al., 1995).
### Table 1.2 PEROXISOMAL DISEASES

#### Type A:
- Peroxisome deficiency and generalized loss of peroxisomal functions
  - Zellweger syndrome (ZS)*
  - Neonatal adrenoleukodystrophy (NALD)*
  - Infantile refsum disease (IRD)*

*ZS cell lines fall into ten complementation groups. NALD and IRD are thought to be, slower progressing forms of ZS.

#### Type B:
- Loss of multiple peroxisomal functions, normal peroxisome morphology
  
  Rhizomelic chondrodysplasia punctata (RCDP)

#### Type C:
- Single enzyme deficiencies, normal peroxisome morphology. Deficiency may be due to lack of functional enzyme or improper targeting of enzyme or substrate(s).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Enzyme</th>
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<tbody>
<tr>
<td>X-linked adrenoleukodystrophy</td>
<td>Very long chain fatty acyl-CoA synthetase</td>
</tr>
<tr>
<td>Acyl-CoA oxidase deficiency (pseudo NALD)</td>
<td>Acyl-CoA oxidase</td>
</tr>
<tr>
<td>Bifunctional protein deficiency</td>
<td>Bifunctional enzyme</td>
</tr>
<tr>
<td>Peroxisomal thiolase deficiency</td>
<td>Thiolase</td>
</tr>
<tr>
<td>DHAP acyl-transferase deficiency (pseudo RCDP)</td>
<td>DHAP-AT</td>
</tr>
<tr>
<td>Alkyl-DHAP-synthase deficiency (pseudo RCDP)</td>
<td>Alkyl-DHAP-synthase</td>
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<tr>
<td>Glutaric aciduria type III</td>
<td>Glutaryl-CoA oxidase</td>
</tr>
<tr>
<td>Hyperoxaluria type I transferase</td>
<td>Alanine:glyoxylate amino transferase (AGT)</td>
</tr>
<tr>
<td>Acatalasemia</td>
<td>Catalase</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis (ALS)</td>
<td>Zn/Cu superoxide dismutase</td>
</tr>
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</table>

*a Taken from: Shimozawa, 1993; Moser, 1995; Wanders, 1995.*
1.3 Peroxisome Biogenesis

Basic questions about peroxisome biogenesis have been hard to address, due to difficulties in isolating and experimenting with the fragile organelles. In particular, an *in vitro* protein import system such as that available for studies of ER and mitochondria (Gasser and Schatz, 1983) has not been established for peroxisomes despite much effort. However, even with such impediments, an overall picture has emerged. The peroxisome biogenesis process begins with the transcriptional induction of messages for selected peroxisomal enzymes and structural components. These proteins must then be targeted to the organelle for import and assembly. The proliferation of induced peroxisomes in both size and number has also been studied. These topics are covered in more detail in the following sections.

1.3.1 Transcriptional regulation of peroxisome proliferation

1.3.1.1 β-Oxidation pathway and peroxisome induction in *S. cerevisiae.*

Peroxisome induction by oleic acid in *S. cerevisiae* has been shown to be controlled at the transcriptional level (Dmochowska et al., 1990; Einerhand et al., 1991; Simon et al., 1991). Transcription of peroxisomal enzymes and biogenesis gene products is repressed in the presence of glucose, derepressed in the absence of glucose, and fully induced only when glucose supplies have been exhausted and a β-oxidation-inducing carbon source such as oleate is present (Einerhand et al., 1991). Research has elucidated some of the particulars of this elaborate oleate-response machinery.

Examination of DNA sequences 5' to the transcribed regions of genes encoding β-oxidation enzymes in *S. cerevisiae* has revealed complex promoter regions with sites for binding numerous transcription factors (Einerhand et al., 1992). Among these is a site that binds to alcohol dehydrogenase 2 regulatory factor I (Adr1p). Adr1p was first characterized as a transcription factor that plays a pivotal role in the regulation of the transcription of alcohol dehydrogenase 2 (Adh2p) (Denis et al., 1981). Adh2p is an enzyme that is needed to produce carbohydrates from ethanol, and wild-type cells increase translation of this enzyme under conditions of glucose starvation (derepression) (Lutsdorf and Megnet, 1968). Cells lacking Adr1p
(adr1 mutants) display variable phenotypes, depending upon the carbon source being utilized and the specific region of deleted or altered amino acids. In cells with deletions C-terminal to the zinc-finger region of Adr1p, CTA1, FOX2 (trifunctional enzyme), and FOX3 are all expressed at far lower levels when grown on ethanol compared to wild type. However, when induced on oleate, these mutants produce levels of these enzymes that are nearly equal to those of wild-type cells. Despite adequate enzyme levels, these mutant cells do not grow on oleate. Small single peroxisomes are present in these mutant cells in total numbers comparable to the larger clustered peroxisomes produced in wild-type cells growing on oleate. This implies that C-terminal deletions of Adr1p have an effect on size and distribution of the organelles, but not on the proliferation process. Mutant cells lacking the entire sequence of ADR1 (adr1 null mutants) are unable to grow on oleate and do not produce CTA1, FOX2, or FOX3 mRNAs (Simon et al., 1991, 1995). Experiments have demonstrated that peroxisomes could not be detected in snf1 and snf4 mutant cells (Simon et al., 1992). SNF1 (CAT1 or CCR1) and SNF4 (CAT3) are known to play a role in the regulation of glucose derepression (Gancedo and Gancedo, 1986; Entian, 1986; Carlson, 1987) and are apparently also critical for peroxisome biogenesis and proliferation.

The primary sequence of Adr1p contains two type I zinc-finger domains (C2H2 type) that have been shown to be essential to its function (Hartshorne et al., 1986; Blumberg et al., 1987). Each of these domains recognizes a particular nucleotide triplet in the specific upstream activation sequence (UAS1) to which Adr1p binds in a zinc-dependent manner (Eisen et al., 1988; Thukral et al., 1989). Experiments show that the zinc fingers also serve to activate transcription independently of their ability to bind DNA (Cook et al., 1994b). UAS1 is an inverted repeat of 22 base pairs (5'-TCTCCAACTTA TAAGTTGGAGA-3') (Shuster et al., 1986), each half of the sequence binds a monomer of Adr1p, and two monomers must bind for transcriptional activation (Yu et al., 1989; Thukral et al., 1991a). Sequences similar to UAS1 are also found upstream of many genes encoding peroxisomal enzymes such as thiolase (FOX3) and catalase A (CTA1) and biogenesis proteins such
as peroxisome assembly factor-1 (PAF-1) (Simon et al., 1991). Detailed analysis of UAS1 has allowed a more general consensus binding site to be composed (Cheng et al., 1994).

Adr1p’s effects on gene expression are complex and variable and appear to involve numerous activating and inhibiting domains within the protein that interact with DNA and other as yet unidentified factors (Cook et al., 1994a). When glucose is present, Adr1p may be phosphorylated at residue Ser-230 (C-terminal to the zinc-finger domains) by a cAMP-dependent protein kinase (cAPK) that is activated by high glucose levels (Cherry et al., 1989). However, more recent evidence suggests that a repressor may bind in the region of this potential phosphorylation site and thus prevent Adr1p from activating transcription (Denis et al., 1992; Cook et al., 1994a). In any case, significantly reduced expression of ADH2 and peroxisomal genes is the result, particularly on ethanol, even though the protein remains bound to UAS1 in the inhibited state (Taylor and Young, 1990). Mutations that delete residues 227-239 or alter the Ser-230, or other crucial residues proximal to Ser-230, result in adrlc mutants. These mutants may not be phosphorylated properly or may be sterically altered in such a way that an Adr1p repressor cannot bind. Such mutants produce Adh2p in a constitutive manner, regardless of the carbon source being utilized (Denis and Gallo, 1986; Simon et al., 1991; Denis et al., 1992; Cook et al., 1994a).

Upstream of UAS1 in the ADH2 promoter region is UAS2 (Yu et al., 1989) which contains a sequence that shows specific but low affinity binding for Adr1p (consensus sequence 5’-AGGAGA-3’). Evidence suggests that another glucose-regulated transcription factor binds to UAS2, possibly displacing Adr1p, and that Adr1p (bound at UAS1) may interact with this other factor to fully activate transcription of ADH2 (Donoviel et al., 1995).

An oleate response element (ORE), first characterized in the promoter FOX3, is a DNA sequence responsible for full induction of many β-oxidation enzymes and peroxisome biogenesis genes (Einerhand et al., 1991; Filipits et al., 1993; Wang et al., 1994). This site is an imperfect inverted repeat (consensus sequence 5’-CGGNNNTNA-3’) that will only activate transcription when bound to ORE-binding protein (ORE-bp) (Einerhand et al., 1993). This protein has recently been cloned,
sequenced, and named the peroxisome induction pathway 2 gene (*PIP2*) (Rottensteiner et al., 1996). Binding of a protein specifically to ORE DNA is evident on DNA band-shift assays, and this band shift does not occur using extracts from a *pip2* null mutant (Einerhand et al., 1993; Rottensteiner et al., 1996). These mutants grow poorly on oleate, and cannot induce proliferation of peroxisomes or matrix enzymes needed for oleate utilization. However, *pip2* cells contain a small number of unclustered peroxisomes typical for derepressed cells. Northern blot analysis of several peroxisomal proteins indicates that *pip2* null mutant cells do not produce transcripts of these proteins beyond their wild-type derepressed levels when grown on oleate (Rottensteiner et al., 1996). This indicates that Pip2p is a transcription factor specifically required to give maximal induction of peroxisomal protein transcripts when *S. cerevisiae* is grown on oleate.

Upstream from the ORE is a region of DNA that has been shown by deletion analysis to be involved in the repression of the expression of peroxisomal structural and β-oxidation enzyme genes (Einerhand et al., 1991). Recent research demonstrates that autonomously replicating sequence (ARS) binding factor-1 (ABF-1p) and hetero-oligomer replication protein (RP-Ap) can each bind independently or together to consensus sequences upstream of the ORE in the *FOX3* gene (Einerhand et al., 1995). The proteins remain bound to the DNA in both repressed and induced states. As with Adr1p, the phosphorylation state of ABF-1p and RP-Ap may modulate their effect on transcription, as these proteins are known to be phosphorylated in other cases (Einerhand et al., 1995). Similar sequences are evident in the promoter regions of *CTA1, FOXI, FOX2,* and *PASI* (Einerhand et al., 1991). The varied complexities of peroxisomal gene promoter regions reflect the differing transcriptional responses of these genes to a change in carbon source. For example, derepression due to growth on glycerol medium leads to maximal expression of *S. cerevisiae PAS3* that encodes a peroxisomal membrane protein, while CAT is present at only 20% of its maximum level in these cells (Kos et al., 1995).
1.3.1.2 Peroxisome induction in mammals. As seen with *S. cerevisiae*, the levels of peroxisomal enzymes in mammals are controlled at the transcriptional level (Furuta et al., 1982; Reddy et al., 1986). Much of the transcriptional control pathway has been elucidated as a result of studies with peroxisome proliferator drugs such as clofibrate and Wy-14-643. These drugs induce liver enlargement and elevate the levels of many peroxisomal enzymes and the number of peroxisomes per cell (proliferation) (Hess et al., 1965; Reddy et al., 1986). They are of significant medical interest because they lower cholesterol levels in the blood (Havel and Kane, 1973). Unfortunately, they also induce cancer in rats by a nongenotoxic mechanism (see below) (Reddy and Qureshi, 1979; Reddy and Lalwani, 1983).

Peroxisome proliferator-responsive elements (PPREs) are DNA sequences that are often thousands of base pairs upstream from genes encoding peroxisomal proteins. These sequences function in either orientation when fused to a reporter gene, indicating that they function as transcriptional enhancers (Osumi et al., 1991; Dreyer et al., 1992; Tugwood et al., 1992; Zhang et al., 1992). The PPRE regions of various peroxisomal enzyme-encoding genes were found to include the consensus sequence 5'-AGGTCA-3', which suggests that proteins from the intracellular hormone receptor family (type II) may bind to them (Evans, 1988; Umesono et al., 1991). They are often composed of imperfect inverted repeats, implying that the binding proteins are dimers (or larger) (Glass, 1994).

Genes encoding PPRE-binding proteins have been cloned from several organisms and are called peroxisome proliferator-activated receptors (PPARs) (Issemann and Green, 1990; Dreyer et al., 1992; Schmidt, et al., 1992; Kliewer et al., 1994). As expected, their deduced amino acid sequences show similarity to other members of the intracellular hormone receptor family. PPRE-binding proteins contain two type-II zinc fingers (C2C2 type) which allow for very specific DNA binding (in contrast to Adr1p of *S. cerevisiae* with two type-I zinc fingers), as well as a ligand-binding domain and a dimerization domain (Evans, 1988). Numerous experiments have established that at least one ligand-activated PPAR dimerizes with the activated retinoid-X receptor (RXR-α), another member of the intracellular receptor superfamily (Mangelsdorf et al., 1990; Kliewer et al., 1992; Miyata et al., 1994;
Mangelsdorf and Evans, 1995). The activating ligand for RXR-α and its isoforms (Mangelsdorf et al., 1992) is 9-cis-retinoic acid, a metabolite of fat-soluble vitamin A that is responsible for triggering specific responses that vary by developmental stage and cell type (Dreyer et al., 1992; Heyman et al., 1992; Marcus et al., 1993). Tissue-specific isoforms of PPAR are known, and these can each form heterodimers with other members of the RXR family, allowing for a wide range of potential responses (Dreyer et al., 1992; Marcus et al., 1993; Kliewer et al., 1994; Tontonoz et al., 1994a).

The best characterized isoform, PPAR-γ, is found almost exclusively in fat tissue and has a key role in adipose cell differentiation (Tontonoz et al., 1994b). Fibroblasts with ectopically expressed PPAR-γ in the presence of lipids and lipid-like PPAR activators will differentiate into adipocytes (Tontonoz et al., 1994c). In the presence of its recently identified ligand, 15-deoxy-Δ12,14-prostaglandin J2 (Forman et al., 1995; Kliewer et al., 1995), PPAR-γ dimerizes with RXR-α (forming a complex known as ARF6) (Tontonoz et al., 1994a) and, along with other factors, induces transcription of genes involved in the transformation of pre-adipocytes to adipocytes. The ligands for the other isoforms of PPAR have not been established. However, since β-oxidation is not required for the activation of PPAR, the ligands are speculated to be unsaturated fatty acids, their Co-A esters, or some other derivative of activated fatty acids (Bocos et al., 1995).

There are currently two theories to account for the nongenotoxic mechanism leading to cancer in rats that have been dosed with peroxisome proliferator drugs. The first hypothesizes that the drugs may activate a signal transduction pathway leading to PPAR and RXR or may serve as ligands for certain PPAR isoforms. This leads to a 10–15 fold induction of several peroxisomal oxidases but only a 1.4-fold induction of CAT (Reddy et al., 1986). The excess H₂O₂ generated by the oxidases is believed to escape from peroxisomes and generate other activated oxygen species. These may lead to oxidative and free radical damage to DNA and other cellular components, possibly resulting in the development of cancer (Reddy and Lalwani, 1983; Kasai et al., 1989). An alternative model suggests that peroxisome proliferator drugs may activate genes that induce liver growth by some mechanism. The
increased levels of DNA synthesis that are known to be induced by these drugs may thus lead to the promotion of tumors (Marsman et al., 1988).

PPAR-\(\alpha\)/RXR-\(\alpha\) dimers are also implicated in viral activation of both hepatitis B and human immunodeficiency virus-1 (HIV-1). Hepatitis B virus enhancer element-1 contains sequences that can bind and be transactivated by PPAR-\(\alpha\)/RXR-\(\alpha\) (Huan et al., 1995). HIV has a long terminal repeat that contains a region with numerous binding sites for nuclear receptors (NRRE). Within the NRRE is a cluster of binding sites for RXR-\(\alpha\) and, in the presence of 9-cis-retinoic acid or clofibrate PPAR-\(\alpha\)/RXR-\(\alpha\) dimers, will induce transcription of HIV-1 genes that are under control of the long terminal repeat region (Ladias, 1994). Clearly, unlocking all the functional particulars of these proteins will be beneficial.

1.3.2 Protein import

Once a cell has induced the expression of genes encoding peroxisomal enzymes and biogenesis proteins, these proteins must be properly targeted and imported in order to assemble the complete organelle. All peroxisomal proteins are encoded by nuclear genes, translated on free cytoplasmic ribosomes, and then post-translationally translocated (Goldman and Blobel, 1978; Subramani, 1993). ATP and cytosolic factors including chaperones are necessary for translocation; however, the proton gradient that exists across the peroxisomal membrane does not seem to be required for protein import (Imanaka et al., 1987; Wendland and Subramani, 1993; Walton et al., 1994).

The molecular machineries responsible for import of peroxisomal matrix and membrane proteins appear to be distinct. ZS cell lines and certain yeast peroxisome-deficient mutants are unable to import most or all matrix proteins but continue to correctly insert membrane proteins, as evidenced by the presence of peroxisomal ghost vesicular structures that contain peroxisomal membrane proteins (Santos et al., 1988a,b; Small et al., 1988a; Suzuki et al., 1989; Spong and Subramani, 1993; van der Leij et al., 1993; Liu et al., 1995; Kalish et al., 1996; Waterham et al., 1996). In addition, peroxisomal matrix protein import requires ATP while membrane protein insertion does not (Diestelkotter and Just, 1993). Progress has been made in
understanding certain aspects of the targeting and import of peroxisomal enzymes into the matrix. However, far less is known about peroxisomal membrane protein targeting and insertion. Recent experiments have defined a 20-amino acid internal membrane-spanning region of *C. boidinii* PMP47 that is essential for sorting and insertion of this protein into the organelle’s membrane (McCammon et al., 1994). Experiments show that the N-terminal 40 amino acids of *P. pastoris* Pas2p are sufficient to target a fusion reporter protein to the peroxisomal membrane (Wiemer et al., 1996). The peroxisomal membrane targeting signals (mPTS) of each of these proteins contain a high number of acidic residues which may be important to their function.

Two peroxisomal targeting signals (PTSs) for matrix proteins have been defined, PTS1 and PTS2. PTS1 is a conserved sequence of amino acids located at the carboxy-terminus of many peroxisomal matrix proteins including the yeast methanol pathway enzymes AOX and DHAS (Gould et al., 1988, 1989). The signal is a tripeptide of sequence SKL (Gould et al., 1987) and other conservative variants (e.g., AKL, SRL, SHL) (Gould et al., 1989; Subramani, 1992). Recently, the four COOH-terminal amino acids (KANL) of human CAT have also been shown to function as a PTS1 in both human and yeast cells (Purdue and Lazarow, 1996). PTS2 is located near the amino terminus of some peroxisomal matrix proteins including thiolase, malate dehydrogenase, and amine oxidase (Bruinenberg et al., 1989; Gietl, 1990; Swinkels et al., 1991; Faber et al., 1995). Its consensus sequence is (R/K)(L/V/I)(X)₅(H/Q)(L/A) (Swinkels et al., 1991; Gietl et al., 1994; Glover et al., 1994b). In higher eukaryotes, an amino-terminal peptide containing PTS2 is cleaved by a peroxisome-specific protease upon reaching the peroxisomal matrix (Bodnar and Rachubinski, 1990; Hijikata et al., 1990), but in yeast the peptide is not cleaved (Swinkels et al., 1991). A matrix protein known to contain both PTS1 and PTS2 is encoded by *H. polymorpha* PERI. The function of this gene is unknown, but it may play a role in the import competence of newly formed peroxisomes (Waterham et al., 1994). A third type of matrix PTS with an internal location has been hypothesized for *Candida tropicalis* acyl-CoA oxidase. To date, this signal is poorly characterized but is not similar to PTS1 or PTS2 (Small et al., 1988b; Kamiryo et al., 1989).
Other matrix-targeting signals are presumed to exist because there are peroxisomal enzymes with no apparent PTS1- or PTS2-like signal, such as the multifunctional β-oxidation protein of *Neurospora crassa* (Fossa et al., 1995). An interesting case is presented by *S. cerevisiae* carnitine acetyl transferase, which has a mitochondrial signal at its N-terminus, a PTS1 at its C-terminus, and an internal peroxisomal targeting signal that is recognized by the PTS1 receptor (see below). This single gene produces the variably targeted proteins by initiating transcription at different points (Elgersma et al., 1995).

Receptors for PTS1 and PTS2 have recently been identified and characterized in yeast and human cells. Mutants that are selectively deficient in their ability to import proteins containing either a PTS1 or PTS2 signal have been central to these discoveries. *P. pastoris* pas8 mutants have peroxisomal remnants that retain the ability to import thiolase, an enzyme with a PTS2. However, PTS1 enzymes are found in the cytosolic fraction of this mutant (McCollum et al., 1993). Pas8p (and other homologues) has been shown to bind PTS1 with high affinity and has been designated as the PTS1 receptor (McCollum et al., 1993; Terlecky et al., 1995). The section of its sequence that is necessary for this function shows homology to the tetratricopeptide repeat family (TPR), a 34-amino acid repeat that is rigid in the number of amino acids but shows variation in sequence (Sikorski et al., 1990; Brocard et al., 1994; Dodt et al., 1995; Terlecky et al., 1995). The phenotype of pas8 mutants is identical to the phenotype of ZS cell line complementation group 2 (Walton et al., 1992; Motley et al., 1994). This fact led to the cloning of *PXRI* (aka *PTS1R*), the human homologue of *PAS8*, which is another member of the TPR family (Wiemer et al., 1995). In addition to sequence and functional homologies between *PXRI* and *PAS8*, a Pas8–Pxr1 fusion protein has been shown to partially complement a *P. pastoris* pas8 mutant (Wiemer et al., 1995). Homologues to *P. pastoris* *PAS8* have also been cloned in *S. cerevisiae* (*PAS10*) (van der Leij et al., 1993), *H. polymorpha* (*PER3*) (Klei et al., 1995), and *Yarrowia lipolytica* (*PAY32*) (Szilard et al., 1995). Research from three different laboratories has recently identified another critical component of the PTS1 import machinery. The gene, cloned in human, *P.*
pastoris and *S. cerevisiae*, is named *PEX13* (peroxin 13), using the new nomenclature for peroxisome biogenesis genes (Distel et al., 1996). Pex13p has been shown to be an integral membrane protein that recognizes and interacts exclusively with the PTS1 receptor. The protein contains an Src-homology 3 (SH3) domain that is critical to its function and is hypothesized to be the actual recognition and binding site to the PTS1 receptor. Cells lacking Pex13 are unable to import PTS1 or PTS2 proteins. The reasons for this mutant phenotype are unclear, since the assays used could not demonstrate that Pex13p interacts with the PTS2 receptor. However, the results do imply that Pex13p is part of a common import machinery for the two PTS pathways (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996). Membrane proteins were inserted normally, confirming earlier findings that the molecular insertion system for these proteins is distinct from the matrix protein system. These findings also show that the peroxisomal matrix import system may be similar to the systems for ER and mitochondria in that a cytoplasmic factor recognizes the protein. These form a complex which is then targeted to the appropriate organelle and binds to a docking protein for subsequent import.

The *S. cerevisiae* pas7 (pebl) mutant is selectively defective in thiolase import, a PTS2 enzyme. However, it has normal peroxisomal structures that contain PTS1 enzymes and is thought to have a defect in the PTS2 receptor (Marzioch et al., 1994; Zhang and Lazarow, 1995). This phenotype is identical to cells from patients with rhizomelic chondrodysplasia (RCDP) group I, another serious peroxisome disorder (Motley et al., 1994). Recent experiments have shown that Pas7p (Peb1p) interacts with PTS2 and is the receptor for proteins bearing the PTS2 signal (Rehling et al., 1996; Zhang and Lazarow, 1996). Although a human homologue of *S. cerevisiae* *PAS7* has not yet been identified, it could be a gene responsible for RCDP. Pas7p is a member of the WD40 repeat family (Voorn and Ploegh, 1992). The WD-40 repeat proteins, with their conserved core of approximately 31 amino acid residues, participate in diverse intracellular functions which may include the assembly of protein complexes (Neer et al., 1994). Interestingly, some WD40 proteins have been shown to interact with functionally related TPR proteins, and there is recent evidence using the GAL4 two-hybrid system that PTS1 (TPR family) and PTS2 (WD-
40 family) may indeed interact (Rehling et al., 1996). Experiments intended to identify intracellular locations of these receptors have given different results in different organisms, so a clear picture of how and where they work has not emerged (Rachubinski and Subramani, 1995).

Although there are mutants incapable of importing matrix proteins with one signal or the other, there are far more that result in peroxisomal ghosts that are incapable of importing any enzymes. This indicates that the import machinery may consist of separate receptor systems that converge on a common translocation complex (McCollum et al., 1993; Subramani, 1993). *P. pastoris* per3 mutants (*H. polymorpha* homologue *PER1*) (Waterham et al., 1994) are partially defective in PTS1 import but are competent for PTS2 import. Since the deletion mutant does not import proteins with either type of signal, this indicates that Per3p may be part of this common machinery (Liu et al., 1995). Other genes whose products are involved in the import and assembly process have also been isolated from yeasts and mammals. Several of these contain cysteine-rich motifs that may be involved in protein-protein interactions.

Peroxisome assembly factor-1 (Paf1p) is a 35-kD integral membrane protein that has been cloned in human, rat, and CHO cells (Tsukamoto et al., 1991; Shimozawa et al., 1992; Thieringer and Raetz, 1993). A mutation in human *PAF-1* that introduces a stop codon within the first third of its transcript has been shown to cause ZS in complementation group F, but its function remains unclear (Shimozawa et al., 1992). Recently yeast homologues have been cloned and characterized in *P. pastoris* (*PER6*) (Waterham et al., 1996) and *Yarrowia lypolitica* (*PAY5*) (Eitzen et al., 1996). Features include two putative membrane-spanning domains and a RING-finger domain (C3HC4 type) in its C-terminal region that may be essential to its function (Lovering et al., 1993; Tsukamoto et al., 1994; Liu et al., 1996). Mutants are compromised in the import of both PTS1 and PTS2 proteins, indicating that PAF1p (Per6p) may be part of a common import machinery (Braverman et al., 1995; Waterham et al., 1996). In the fungus *Podospora anserina*, mutants defective in a homologue of *PAF-1* lack normal peroxisomes and lose the ability to utilize oleate as a carbon source. Interestingly, these mutants were first isolated because of their inability to fuse nuclei for sexual reproduction (Berteaux-Lecellier et al., 1995). Another gene coding for a
peroxisomal integral membrane protein with a RING-finger motif has recently been cloned in *P. pastoris*. Cells lacking a functional *P. pastoris* PAS10 gene have a phenotype similar to *P. pastoris* PER6 cells. Pas10p is also thought to be part of the translocation machinery (Kalish et al., 1996).

A gene with mutations known to cause ZS in complementation group C has been cloned recently from human cells by using the *P. pastoris* PAS5 sequence to screen a data base. This gene has been named *PXAAA1*. The mutant cells' phenotype indicates an import defect similar to PAF-1 mutants. Pxaa1p appears to be cytosolic and may be involved with the function or stability of the PTS1 receptor, as Pxr1p is unstable in this mutant (Yahraus et al., 1996). As a member of the AAA protein family (ATPases associated with diverse cellular activities), it shows similarity to several other gene products. Yeast homologues include *P. pastoris* PAS5 (Spong and Subramani, 1993), *S. cerevisiae* PAS8 (Voorn-Brouwer et al., 1993), and *Y. lipolytica* PAY4 (Nuttley et al., 1994). It is speculated that Pxaa1p may play a role in actively transporting proteins from the cytoplasm to the peroxisome (Yahraus et al., 1996).

The mechanism by which matrix proteins are translocated into the organelle has also been the subject of much study [see McNew and Goodman (1996) for a recent review]. Thiolase, a dimer, is efficiently imported even if only one subunit contains a PTS2. However, thiolase dimers that are made up of monomers without a PTS2 are not translocated (Glover et al., 1994a). Thus, it appears that thiolase must fold and dimerize outside the organelle prior to import. Proteins that are pre-folded and stabilized by disulfide bonds such as immunoglobulin G, and even gold particles up to 9 nm, will get into peroxisomes provided that they contain PTS1 or have been decorated with PTS1, respectively (Walton et al., 1995). Since there is no evidence for large pores in the peroxisomal membrane, it is hypothesized that certain proteins may get into the organelle by an endocytotic or pinocytotic mechanism (McNew and Goodman, 1994). In any case, the import process for peroxisomes is clearly different from that for chloroplasts and mitochondria, which involves the import of unfolded monomeric proteins.
1.3.3 Proliferation

In response to various cues such as a change in carbon source or certain drugs such as clofibrate, peroxisomes in higher and lower eukaryotes will increase in number (Hess et al., 1965; Veenhuis et al., 1979). Lipids for peroxisomal membranes are thought to originate in the ER where they are often seen in close association. However the mechanism of this acquisition is unknown (Beevers, 1977; Veenhuis and Harder, 1987; Sulter et al., 1993). Current models theorize that new peroxisomes bud off of other organelles, although there is some evidence for de novo synthesis (Waterham et al., 1993; Lazarow and Moser, 1995). The mechanism by which peroxisomes proliferate is poorly understood but appears to involve two processes that operate independently of each other—increase in size and increase in number. The order in which these steps occur can vary, depending on the organism. *H. polymorpha* cells that have been shifted to methanol medium will get very large peroxisomes before the organelles divide (Veenhuis et al., 1979), whereas in *C. boidinii* there is an increase in peroxisome numbers first (Veenhuis and Goodman, 1990). Yeast genes with a possible role in peroxisome proliferation include *H. polymorpha PER8* and *S. cerevisiae PMP27*. Overexpression of either of these genes leads to a significant increase in the number of peroxisomes per cell, whereas deletion of the gene results in either a lack of peroxisomes (*PER8*) or one or two oversized peroxisomes (*Pmp27*) (Erdmann and Blobel, 1995; Marshall et al., 1995; Tan et al., 1995).

1.4 Yeast as a Model System for Peroxisome Studies

1.4.1 Advantages of yeasts

In addition to their general advantages of small size, rapid growth rate and inexpensive growth media, yeast offer a way to combine genetic and biochemical approaches to the study of peroxisome biogenesis. From a genetic standpoint, the haploid/diploid life cycles can be manipulated to great benefit. The haploid phase is useful for mutant isolation, as phenotypes are revealed immediately. The diploid phase is used for genetic analysis. The ease of transformations, small genome and
lack of introns makes it easier to clone and analyze genes. Gene replacement techniques allow the researcher to construct strains with specific mutations and deletions (Rothstein, 1991). The phenotypes of such mutants can provide clues about the function of the targeted gene. Also, the physiology of certain yeasts enables them to grow on oleate and/or methanol (Anthony, 1982; Zwart et al., 1983), substrates which induce the cells to produce large and numerous peroxisomes. The induced organelles are easy to observe by light or electron microscopy (Veenhuis et al., 1983; Veenhuis and Harder, 1987), and they can be purified for biochemical experiments (Douma et al., 1985).

Screening for peroxisomal mutants is based on a straightforward technique that takes advantage of the organisms' inability to grow on certain carbon sources without a functional peroxisome compartment (Cregg et al., 1990; Liu et al., 1992). AOX and thiolase are two peroxisomal enzymes that are required for growth on methanol and oleate, respectively. Without peroxisomes, these enzymes and others are mislocalized to the cytosol where they may be degraded or are otherwise unavailable for metabolism. For *P. pastoris*, the yeast used in this study, the peroxisome-requiring carbon sources are oleate and methanol. After mutagenesis treatment, *P. pastoris* peroxisome biogenesis (*per*) mutants can grow on glucose and ethanol, where peroxisomal function is not required for survival, but not on methanol or oleate. Electron microscope examination of these *per* mutant cells reveals that they are without apparent peroxisomal structures or are grossly deficient in peroxisomal morphology or numbers. Peroxisome deficient (*per* or *pas*) mutants have been isolated in five yeast species. In all these mutants, normal peroxisomes are absent. Most appear to harbor anomalous membrane vesicles that are likely to be peroxisomal remnants. In all yeast *per* or *pas* mutants, levels of most peroxisomal enzymes are comparable to wild type, but the enzymes are mislocalized to the cytosol (Gould et al., 1992; Kunau and Hartig, 1992; Liu et al., 1992; Veenhuis et al., 1992; Nuttley et al., 1993).

To date, numerous different genes essential for yeast peroxisome biogenesis have been cloned, but the specific function for most are unknown. Two genes for which there is good evidence for specific function are *PAS8* of *P. pastoris* (McCollum
et al., 1993) and *PAS7* of *S. cerevisiae* (Marzioch et al., 1994). These genes have been established as receptors for peroxisomal targeting signals 1 and 2, respectively (Terlecky et al., 1995) (Section 1.3.2).

1.4.2 Peroxisome biogenesis in yeasts and humans

Yeast peroxisomal functions and mutant phenotypes parallel the human system, making them a useful model to study human peroxisome disorders. Similar import signals are used (Section 1.3.2), and the ghost structures evident in many yeast mutant phenotypes are a hallmark of ZS (Santos et al., 1988b). There are several examples of peroxisomal biogenesis yeast gene products that show strong similarity to their human counterparts, lending further credibility to the hypothesis that yeast peroxisome biogenesis machinery is similar to that in humans. It is well established that yeast is an easy, effective, and inexpensive organism to use for the rapid cloning and characterization of genes that are essential components of peroxisome biogenesis (Lazarow, 1995). These organisms will continue to be utilized as a tool to identify and understand the components and mechanics of the peroxisomal compartment and human diseases associated with the organelle.

1.5 Thesis Summary

This thesis describes the characterization of a *P. pastoris* mutant JC131 and the cloning and partial characterization of the affected gene, *MXRI* (methanol enzyme expression regulator 1). The mutant was isolated during a search for peroxisome deficient (*per*) mutants of *P. pastoris*. Like *per* mutants, JC131 is specifically defective in its ability to grow on the peroxisome-requiring substrates methanol and oleic acid. However, unlike *per* mutants, JC131 has morphologically normal-appearing peroxisomes. Biochemical studies of methanol-induced JC131 cells revealed that the major peroxisomal methanol pathway enzymes AOX and DHAS are absent and that CAT is present at low levels. However, in oleate-induced JC131 cells, the β-oxidation pathway enzymes acyl-CoA oxidase, thiolase and CAT are present at normal levels. Furthermore, the β-oxidation pathway enzymes from oleate-
induced cells and the CAT from methanol-induced cells are found in crude organelle pellet fractions, indicating that these enzymes are imported normally into peroxisomes. These results indicate that JC131 has normal peroxisomes but may be defective in a gene whose product is important for the expression of peroxisomal methanol-utilization pathway enzymes. The reason for the mutant’s inability to grow on oleate remains unclear.

To gain insight into the nature of the defect in JC131 cells, the affected gene was cloned and partially characterized. The gene which we have named MXRI codes for an open reading frame of 3468 bases. It is constitutively expressed at low levels and encodes a large polypeptide of 1156 amino acids. We have determined the DNA sequence of MXRI and compared the predicted amino acid sequence to sequences in the data banks. These comparisons suggest that the MXRI product has high similarity to several known transcription factors including S. cerevisiae Adr1p (Hartshorne et al., 1986). Adr1p is required for full induction of peroxisomal β-oxidation pathway enzymes and structural components in response to oleate. Based on these similarities, we propose that Mxr1p may be a transcription factor required for induction of methanol-utilization-pathway enzymes in P. pastoris.
CHAPTER 2
MATERIALS AND METHODS

2.1 Media, Strains, and Transformation Techniques

*Pichia pastoris* and *Escherichia coli* strains used in this study are listed in Table 2.1. *P. pastoris* cells were cultured in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or minimal YNB medium (0.67% yeast nitrogen base without amino acids) supplemented with either 0.4% glucose, 0.5% methanol, or 0.1% oleic acid and 0.5% Tween 40 (to solubilize the oleate). Oleate-induced cultures that were used for differential centrifugation experiments were also supplemented with 0.05% yeast extract. Histidine (50 µg/ml) was added to cultures requiring support of auxotrophic growth. All growth of *P. pastoris* strains was at 30°C. Transformations of *P. pastoris* were done by either the spheroplast method (Cregg et al., 1985), the whole-cell polyethylene glycol 1000 method (Klebe et al., 1983), or the electrotransformation method (Becker and Guarente, 1991).

*E. coli* cells were cultured in LB medium (0.5% yeast extract, 1% glucose, 1% NaCl) at 37°C. Ampicillin (0.1 mg/ml) was added to LB medium (LB amp) as required for plasmid selection. Transformation and other standard recombinant DNA techniques used in this study for *E. coli* follow techniques described previously (Sambrook et al., 1989).

2.2 Cell Fractionation, Enzyme Assays, and Immunoblotting

Crude extracts and subcellular fractionations were prepared according to procedures described previously (Liu et al., 1992). Enzyme assays for peroxisomal CAT (Fujiki et al., 1984), AOX (van der Klei et al., 1990), acyl-CoA oxidase
Table 2.1 *P. pastoris* and *E. coli* Strains Used in This Study

<table>
<thead>
<tr>
<th><em>P. pastoris</em> Strains</th>
<th>Genotype</th>
<th>Source of reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JC131</td>
<td>mxr1</td>
<td>Liu et al., 1992</td>
</tr>
<tr>
<td>JC132</td>
<td>mxr1 his4</td>
<td>Liu et al., 1992</td>
</tr>
<tr>
<td>JC100</td>
<td>wild type</td>
<td>NRRL Y-11430</td>
</tr>
<tr>
<td>GS115</td>
<td>his4</td>
<td>Cregg et al., 1985</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>E. coli</em> Strains</th>
<th>Genotype</th>
<th>Source of Reference</th>
</tr>
</thead>
</table>
| MC1061            | hsdR mcrB araD139
Δ(araABC-leu)7679
ΔlacX74 galU galK rpsL
thi | Meissner et al., 1987 |
| DH5-α             | supE44 hsdR17 thi-1
recA1 endA1 gyrA relA1 | Sambrook et al., 1989 |
(Dommes et al., 1981), and mitochondrial cytochrome c oxidase (Douma et al., 1985) were performed at 30°C using standard protocols.

Protein concentrations were determined using the Bio-Rad protein assay kit (Hercules, CA) according to manufacturer's instructions and using bovine serum albumin as a standard. Cytosolic and pellet fractions containing 25 μg of protein from differential centrifugations were loaded onto stacked sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) for electrophoresis (Laemmli, 1970). Proteins were then transferred onto nitrocellulose using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) according to the supplied instructions. Immunoblots were done using polyclonal antibodies to AOX, CAT, DHAS, and thiolase, and visualized with the Tropix Western-Light Kit (Bedford, MA).

2.3 Isolation of Plasmid pYL1

Plasmid pYL1 was isolated from a P. pastoris genomic DNA library using the sib selection procedure (Lederberg and Lederberg, 1952; Cavalli-Sforza and Lederberg, 1956). The library is constructed from an E. coli-P. pastoris shuttle vector (pYM8) (Figure 2.1), with fragments of P. pastoris genomic DNA inserted at a unique BamHI site in the vector (Liu et al., 1995). The library was transformed into E. coli MC1061, and cells were spread onto ten 150 × 15 mm LB amp plates. Each of these master plates contained 1000–2000 transformed colonies which were then replica plated onto LB amp. The replica plates were incubated overnight at 37°C, and the colonies from each plate were collected by washing the plate with 15 ml of LB amp. Cells were inoculated into 200 ml liquid cultures of LB amp and incubated at 37°C overnight. Plasmid DNA was purified from each of these cultures using a Qiagen kit (Qiagen, Inc., Chatsworth, CA). This DNA (~1–2 μg) was then used to individually transform ten sets of P. pastoris JC131 cells using the spheroplast method (Cregg et al., 1985). Transformed yeast cells were first grown on YNB glucose plates to select for His⁺ prototrophy and then replica plated to YNB methanol to further select those cells that were also Mut⁺. Two of the ten sets of JC131
Figure 2.1. pYM8 Library plasmid. PARS = autonomous replication sequence (P. pastoris); Amp = ampicillin resistance gene (E. coli); ORI = origin of replication (E. coli); HIS4 = HIS4 gene (S. cerevisiae).
transformants yielded Mut$^+$ colonies, indicating that the two sub-libraries used to transform these sets were likely to contain at least one plasmid carrying a gene to correct the JC131 mutation. *E. coli* colonies from one master plate were picked and streaked to fresh LB amp plates (100 streaks/plate). After growing streaks overnight, each second-generation master plate was replica plated to LB amp and grown overnight. The replica-plated colonies from each plate were collected by washing with 5 ml of LB amp, and plasmid DNA was prepared from each sample. These plasmid preparations were used to individually transform eight sets of *P. pastoris* JC131 cells by the spheroplast method, and transformants were selected as before (Cregg et al., 1985). Colonies from one *E. coli* master plate whose plasmid DNA yielded Mut$^+$ yeast transformants were picked and streaked onto LB amp plates in groups of 10. The process of replica plating, plasmid preparation and transformation was repeated a third time. Finally, plasmid DNA was extracted from each of six individual *E. coli* strains and transformed into JC131 (Klebe et al., 1983). One plasmid, pYL1, transformed JC131 to Mut$^+$ at high efficiency. A map of this plasmid is shown in Figure 2.2.

### 2.4 Subcloning of pYL1

In order to determine what portion of the *P. pastoris* DNA in pYL1 contained *MXR1*, sub-fragments from the vector were prepared and tested for the ability to confer the Mut$^+$ phenotype. Some regions were examined by deletion of other regions of pYL1 by cutting the vector with selected restriction enzymes that cut the insert region in two places and then ligating the plasmid closed again. Three of these linearized and religated plasmids were made: pYL1$\Delta$Spe (pYL1 with a 7-kb SpeI fragment missing), pYL1$\Delta$Eag (pYL1 with a 0.9-kb EagI fragment missing), and pYL1$\Delta$Stu (pYL1 with a 2-kb Stul fragment missing). Another subclone vector, pYL3, was made by ligating a 2.0-kb EcoRV fragment from pYL1 into the EcoRV site of pYM8. All subclones are diagrammed in Figure 3.2.
Figure 2.2. pYL1 Plasmid containing MXRI. PARS = autonomous replication sequence (P. pastoris); Amp = ampicillin resistance gene (E. coli); ORI = origin of replication (E. coli); HIS4 = HIS4 gene (S. cerevisiae); MXRI = open reading frame for MXRI gene (within sequenced region of pYL1 insert). Sequenced region of pYL1 insert indicated by shaded area.
2.5 Northern Blotting

Plasmid DNA digested with restriction enzymes and used for restriction mapping, hybridization probes, and cloning of sub-fragments were separated on 0.8% agarose gels (Sambrook et al., 1989). DNA fragments were purified from agarose gels by using Schleicher and Schuell NA45 DEAE membrane (Schleicher and Schuell, Keene, NH) or by use of a Qiagen kit (Qiagen, Inc., Chatsworth, CA). Restriction enzymes were purchased from New England Biolabs, Inc. (Beverly, MA). The probe for *P. pastoris AOX* was a 2.0-kb *BamHI/StuI* fragment from pPG4. The probe for *P. pastoris GAP* was a 300-base pair *SalI* fragment from pGAP9.

Yeast RNAs were prepared by a standard procedure (Ausubel et al., 1994). Samples containing 7.5 μg of total RNA were loaded into the wells of 1.5% agarose/denaturing formaldehyde gels and electrophoresed overnight at 15-20 mA for clearest separation. Transfer of RNAs to nitrocellulose membranes, crosslinking, prehybridization, hybridization, high stringency washing and imaging were all performed following standard procedures (Sambrook et al., 1989). DNA fragments were labeled utilizing a random priming kit from Amersham (Arlington Heights, IL), with α-32P-dCTP as the radioactive label. Labeled DNAs were separated from unincorporated nucleotides by centrifugation through Sephadex G-25 or G-50 columns at 3,000 × g for 5 min. Between 10^6 and 10^7 counts of denatured DNA fragments were added as hybridization probes to RNA blots (Sambrook et al., 1989).

2.6 DNA Sequencing and Analysis

A 2-kb *EcoRV* fragment from pYL1 was inserted in both orientations into the *EcoRV* site of pBSIISK-(pBS) to generate plasmids pBL1 and pBL2. A third plasmid, pBL3, was made by inserting a 1.5-kb *SacII/HpaI* fragment from pYL1 into pBS cut with *SacII* and *EcoRV*.

DNAs were sequenced at the DNA core facilities at either Oregon State University in Corvallis or the Oregon Regional Primate Research Center in Beaverton. The protein sequence of Mxr1p was compared to other protein sequences
for similarities using the BLAST Network Service of the National Center for Biotechnology Information (Bethesda, MD). The alignment of *MXRI* and *S. cerevisiae ADR1* sequences and the search for potential biologically significant sequences in both proteins was performed using PC/Gene software (release 6.8, Intelligenetics, Mountain View, CA). Oligonucleotides for DNA sequencing were purchased from Integrated DNA Technologies (Coralville, CA).
CHAPTER 3
RESULTS

3.1 Isolation and Characterization of *Pichia pastoris* Strain JC131

The *P. pastoris* strain JC131 was one of several potential peroxisome biogenesis mutants isolated in this lab (Liu et al., 1992). Since methanol and oleate are carbon sources that require functional peroxisomes, it was theorized that mutants that were defective in both methanol utilization (Mut\(^{-}\)) and oleate utilization (Out\(^{-}\)) would have a strong chance of being defective in peroxisome biogenesis (Pex). Electron microscopy confirmed that all but one of the Mut\(^{-}\) and Out\(^{-}\) mutants were also peroxisome deficient and mislocalized peroxisomal enzymes to the cytoplasm. The one exception was JC131, which was Per\(^{+}\). Genetic recessive analysis showed that the mutant had a single mutation.

As a first step in the biochemical characterization of JC131, we determined the levels of activities for selected peroxisomal enzymes in induced JC131 cells (Table 3.1). Cell-free crude extracts were assayed for CAT and AOX activity in methanol-induced cells. CAT was present in JC131 cells, although at a level that was 16.7\% of the wild-type level. AOX activity was not detectable. Cell-free crude extracts from oleate-induced cells were assayed for CAT and acyl-CoA oxidase activities and found to contain levels comparable to wild-type cells.

The location of peroxisomal enzymes in induced cells of JC131 was determined by differential centrifugation (Figure 3.1). Methanol- or oleate-induced cultures were protoplasted, lysed in an osmotically supportive buffer, and centrifuged at 20,000 \( \times g \). The resulting crude organellar (pellet) and cytosolic (supernatant) fractions were then assayed for activity and/or protein levels of key peroxisomal enzymes. CAT activity and protein were detected in the organellar pellet fraction of
Table 3.1 ACTIVITIES OF SELECTED Peroxisomal Enzymes
IN CELL-FREE EXTRACTS

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>JC131</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methanol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>98</td>
<td>16</td>
</tr>
<tr>
<td>AOX</td>
<td>1.5</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Oleate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>179</td>
<td>83</td>
</tr>
<tr>
<td>Acyl-CoA Ox</td>
<td>0.020</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Activity units:

CAT, $\Delta \varepsilon_{240}$/min/mg protein
AOX, acyl-CoA oxidase, $\mu$moles product/min/mg protein
Table 3.1. Specific activity and relative protein concentration of enzymes in post-20,000 x g supernatant (S) and organellar pellet (P) after subcellular fractionation.

- **Methanol**
  - **Catalase**
    - P: 98
    - S: 102
    - P/S ratio: 0.96
  - **AOX**
    - P: 1.22
    - S: 1.16
    - P/S ratio: 1.05
  - **CytCOx**
    - P: 1.4
    - S: 0.07
    - P/S ratio: 19.6

- **JC131**
  - **Catalase**
    - P: 3.4
    - S: 6.8
    - P/S ratio: 0.50
  - **AOX**
    - P: 0.00
    - S: 0.00
    - P/S ratio: 0.00
  - **CytCOx**
    - P: 0.94
    - S: 0.01
    - P/S ratio: 94

- **Oleate**
  - **CAT**
    - P: 45
    - S: 32
    - P/S ratio: 1.4
  - **Acyl-CoA Ox**
    - P: 0.94
    - S: 0.04
    - P/S ratio: 23
  - **CytCOx**
    - P: 1.0
    - S: 0.01
    - P/S ratio: 102

- **JC131**
  - **CAT**
    - P: 40
    - S: 31
    - P/S ratio: 1.3
  - **Acyl-CoA Ox**
    - P: 0.32
    - S: 0.01
    - P/S ratio: 32
  - **CytCOx**
    - P: 0.13
    - S: 0.01
    - P/S ratio: 13

**Figure 3.1.** Quantity and location of selected peroxisomal enzymes in subcellular fractions. Specific activity and relative protein concentration of enzymes in post-20,000 x g supernatant (S) and organellar pellet (P) after subcellular fractionation. In immunoblots, each lane contains 25 µg protein. Activity units: CAT, ΔA240/min/mg protein; AOX, cytochrome c oxidase, acyl-CoA oxidase, µmoles product/min/mg protein.
both wild-type and JC131 cells, with a significant amount of CAT also present in the supernatant fraction due to typical leakage of this enzyme from peroxisomes. As with the crude extract results, the total level of CAT activity in methanol-grown mutant cells was much lower than in wild-type cells. No AOX activity or protein and no DHAS protein were detected in either fraction from JC131 cells grown in methanol. Oleate-induced JC131 cells also showed sedimentable CAT, acyl-CoA oxidase, and thiolase with mutant enzyme levels comparable to wild-type levels. Activity for the mitochondrial enzyme, cytochrome c oxidase, was assayed as a control for organelle intactness and was found to be primarily present in the pellet of both wild-type and JC131 cells.

The results demonstrate that the two methanol pathway-specific enzymes, AOX and DHAS, are entirely absent in this mutant, while CAT expression is greatly reduced. The observation that several enzymes are affected simultaneously suggests that the mutation involves some aspect of transcriptional regulation of methanol pathway enzymes, rather than biogenesis. The enzyme profile of JC131 cells does not provide an explanation for why the cells cannot grow on oleate. Attempts to assay enzyme activity for the remaining oleate pathway enzyme (bifunctional enzyme) were unsuccessful.

### 3.2 Isolation of Plasmid pYL1 and Localization of the MXRI Gene

A plasmid complementing the JC131 mutant was isolated from a *P. pastoris* genomic DNA library by functional complementation of the JC131 mutant using a cloning strategy known as Sib selection (Cavalli-Sforza and Lederberg, 1956). It was necessary to use the Sib selection method, because repeated attempts to recover the plasmid by transformation of DNA from complemented JC131 cells were not successful. Using Sib selection, a single colony carrying a complementing plasmid can be isolated from a large population of *E. coli* cells transformed with a genomic library. A series of transformations of mutant cells is done using samples of library plasmid DNA prepared from progressively smaller numbers of colonies.
The complementing plasmid was named pYLI (Figure 2.2). This plasmid contains approximately 8 kb of pYM8 sequence and 11 kb of *P. pastoris* genomic DNA insert for a total size of approximately 19 kb. Transformation of JC131 with pYLI restored wild-type methanol and oleate growth rates to JC131 mutant cells.

In order to estimate the approximate location of the *MXRI* sequence in pYLI, we determined what portion of the 11-kb insert was essential for complementation of the JC131 mutant. For this, sections of the insert were deleted and the resulting plasmids were used for transformation of JC131. The structure and transformation results with these subclones are summarized in Figure 3.2. Two deletion subclones that retained ability to transform JC131 cells to Mut⁺ were pYLIΔStu, in which a 2-kb section of the insert was deleted, and pYLIΔSpe, in which a 7-kb section of the insert was deleted. In a third subclone, pYLIΔEag, a small fragment (0.9 kb) was deleted. This plasmid was unable to restore a Mut⁺ phenotype to JC131, indicating that this fragment contained sequences needed to correct the mutation in JC131. A fourth subclone, pYL3, was constructed using a 2-kb EcoRV fragment from pYL1 inserted into the EcoRV site of pYM8. pYL3 was able to restore a Mut⁺ phenotype to JC131 cells at a low frequency. When His⁺ pYL3 transformants were replica plated to methanol, only a few cells within each colony would grow on methanol. This suggests that only those transformed cells in which the vector integrated were able to correct for the mutation and that cells that retained pYL3 as an autonomous plasmid were not able to complement. This suggests that the EcoRV fragment did not contain the entire *MXRI* coding sequence but that it did contain the mutated region of the gene.

Northern blots were used to determine the message sizes and approximate locations and regulation patterns of transcribed genes within pYL1. RNA extracted from wild-type cells grown on YNB glucose or on YNB methanol was hybridized with labeled DNA fragments from the pYLI insert (Figure 3.3). A 0.9-kb EagI fragment (P2) hybridized to an approximately 4.0-kb message that was expressed at a slightly higher level in methanol. As a control, we hybridized with a labeled DNA fragment encoding for glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme. The *GAP* gene is expressed at a slightly higher level on glucose than on
Figure 3.2. Location of MXRI gene in pYL1. Deletion and subfragment clones were made from pYL1 and tested for complementation of JC131 to Mut⁺. (A) Linear map of pYL1 insert (11,000 bp) showing restriction sites used to generate subclones. Sequenced region indicated by open rectangle; MXRI ORF indicated by dark arrow. (B) Boxes represent fragments tested for complementation. 1, pYL1ΔEag; 2, pYL1ΔStu; 3, pYLΔSpe; 4, pYL3. Dark boxes indicate no complementation. Light boxes indicate positive complementation.
Figure 3.3. Location and size of mRNAs on pYLI. (A) Sequenced region within pYLI is represented by open box. The MXR1 ORF is represented by dark arrow. Probe 1 (P1), 0.6-kb EcoRv/PstI fragment; probe 2 (P2), 0.9-kb EagI fragment; probe 3 (P3), 3.3-kb HpaI fragment. (B) Northern blots of P. pastoris wild-type RNA probed with P1, P2, P3, AOX, and GAP.
methanol (Waterham and Cregg, unpublished results). Other probes from pYL1 revealed that at least three additional messages may originate, at least partially, within this plasmid. A *PstI/EcoRV* fragment (~600 bp) (P1) hybridizes to a single band of approximately 1 kb with stronger expression in glucose than in methanol. A 3.3-kb *HpaI* fragment (P3) hybridized to three messages. The top band at ~4 kb corresponds to the band seen with the *Eagl* fragment probe. A second band at 3.5 kb shows strong expression on glucose and very low expression on methanol. The third band at 1.4 kb shows stronger expression on methanol.

These results, combined with the results from subcloning, allow certain conclusions to be drawn. Since the *Eagl* fragment hybridizes to a single 4.0-kb message and pYL1ΔEag, which lacks this fragment, does not transform JC131 to Mut+, the 4.0-kb message is a strong candidate for the *MXR1* transcript. pYL1ΔSpe, which lacks a large fragment of pYL1, transforms JC131 to Mut+. The *HpaI* fragment is part of the DNA deleted in pYL1ΔSpe, indicating that the two other messages to which the *HpaI* fragment hybridizes (3.5 and 1.4 kb) are not likely to be from genes that correct the JC131 mutation. The *PstI* fragment does not hybridize to the 4.0-kb message. Therefore, *MXR1* is likely to be an ~4.0-kb gene located between the *PstI* site at approximately 1.9 kb and the *HpaI* site at approximately 7.6 kb of pYL1.

### 3.3 Characterization of the *MXR1* Gene and its Product

The DNA and predicted amino acid sequences of *MXR1* and Mxr1p, respectively, are given in Figure 3.4. *MXR1* has an open reading frame of 3468 bp. Database searches revealed that an ~80-amino acid portion of Mxr1p has strong similarity to *S. cerevisiae* Adr1p. Adr1p is a transcriptional regulator that controls the expression of *ADH2* and other genes, including β-oxidation genes and *PEX* genes (Simon et al., 1991) (Figure 3.5). This region of similarity is predicted to encode two C2H2-type zinc fingers (amino acids 34–92) which in Adr1p is known to be essential for the zinc-dependent binding to the promoter region of the *ADH2* gene.
ATCACCTTTCTCCTGATCGTAATCGTATCGTAAACCCGGTGTCATCAAGTGGCGTACTGCAA~TTTTGCGTGGGG
TTTAGCGTATTTGCTCCTCTCAACGGTTGTGTAGTTGACGTATAGTTGGTTGTCGTGGCCCCTTTTTTTCTCTAAGCAC
ACCACATGATTTCTATCCCTCTTTTTCCGCATTTTGCCTCCCGTTCGGCCATAACCTCTGCTGCTCCTAATCCCAAA
CCTCTTTTCCACGCGGCGGACCGCCCTCGCAATAAAGAATGGCAACGTTTTCACATCTAGAATTTTTTCCATTGTGATACGAAGACTAA
AAAAACCATCAAAAAAAAAAGATCCTGGACGAAAGACTTTTTTATACATTACCTTCTTTTCTTATACCCAGCA
CCACCTCCTATTAAATTTCTGCTTGGTCTATTGCTGACTTTATCCACGCCAC
549/1 579/11
ATG AGC AAT CTA CCC CCA ACT TTT GGT TCC ACT AGA CAA TCT CCA GAA GAC CAA TCA CCT
Met ser asn leu pro pro thr phe gly ser thr arg gln ser pro glu asp gln ser pro
609/21 639/31
CCC GTG CCC AAG GAG CTG TCA TTC AAT GGG ACC ACA CCC TCA GGA AAG CTA GGC TTA TTT
pro pro lys glu leu ser phe asn gly thr thr pro ser gly lys leu arg phe
669/41 699/51
GTC TGT CAG ACA TGT ACT CTA GTA TTT GCT GAG GAA GAG AAA CGA AAC AGG
val cys cys thr cys thr ala phe ala ala glu glu arg
729/71
TCT CAC ACC AAG GAG AAA CCT TTC AGC TGC GGC ATT TGT TCT GTG AAA TCC AGT CCA
ser his lys glu val asp pro pro thr ser ser asp ala
789/81 819/91
GAT CTG TTA TTG AGA CAT GCC CAA AAA CTG CAT GGC TGT GCT CAA AGC TGA ACT GCC
asp leu leu leu arg glu cys cys ser arg asp asp pro
849/101
AGA CTA AGG CGC AAG ACT GTG ACC GCC TCT CAA GAA CAA CAA CAA ACT CCA CAA
arg leu pro glu pro gln cys cys cys pro pro pro pro
909/121 939/131
ACT CGG GTG ACA AAT AAC AAT CTG TCA TTC CAA AGA CCA CAA CAA CAA ACT CCA
asn ser met thr lys phe pro pro pro pro pro
999/141 1029/161
CAG AAA CTG GCC GCC GCC GGC GAG TCA AAT GAG CAA AAG AAA CTG CAA CTG CAA CAA CAA CAA
aspt cys pro pro pro cys glu ser asp cys
1029/161 1059/171
CTA CAG CAA CAA CAG TGG CAA TAC CAA CAA CTC AGT CAA GAT CAT CAA AAT GAA CTA
Figure 3.4. DNA sequence and amino acid translation of MXR1. Zinc-finger region is in bold face and underlined (base pairs 672–821). EagI restriction sites are underlined (base pairs 66 and 984). EcoRV restriction site is underlined (base pair 1115). SpeI restriction site is underlined (base pair 1811). The potential phosphorylation site is underlined (base pairs 1176–1208).
Figure 3.5. Amino acid sequence comparison between *P. pastoris* Mxr1p and *S. cerevisiae* Adr1p. The two zinc-finger domains of each protein are in bold face. The potential cAMP protein kinase phosphorylation sites of Adr1p and Mxr1p have been underlined. Other regions of similarity include the following: Mxr1p 249-336 to an Adr1p transactivation domain; Mxr1p 341-417 to an Adr1p region critical for glycerol metabolism; Mxr1p 544-913 to an Adr1p region critical for oleate metabolism. The character "|" shows that two aligned residues are identical. The character "." shows that two aligned residues are similar. Amino acids defined as similar are: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,N; S,N; D,N; E,Q; Q,R; E,K; N,H. Sequence comparison was done using PCGENE release 6.8 (Intelligenetics, Mountain View, CA), combined with results from Genebank Database searches.
The sequence C-terminal to the zinc fingers shows far less similarity. However, the C-terminal 520 amino acids of Mxrlp show more similarity to Advlp than to any other protein in the database. There are three C-terminal regions of Adrlp with similarity to Mxrlp. The first is part of a transcription activation domain. The second plays a critical role in glycerol metabolism. The third includes a portion of sequence known to be essential and specific for the induction of oleate metabolism genes by Adrlp. Deletion of this region prevents \textit{S. cerevisiae} growth on oleate, but leaves ethanol growth unaffected (Cook et al., 1994; Simon et al., 1995).

Other potential biologically significant sites within the sequence of Mxrlp and Adrlp were analyzed. A potential cAMP-dependent protein kinase phosphorylation site in Adrlp (LTRRASFSAQS) is found between amino acids 225–235. A similar kinase site is also found in Mxrlp (GLRRASFSAVS) at about the same location (210–220) (Figure 3.5). Also of possible interest are two potential protein kinase C sites located in both Adrlp and Mxrlp. The first is located on the N-terminal side of the first zinc finger of both Mxrlp and Adrlp in a region known to play a role in Adrlp DNA-binding specificity (Thukral et al., 1989; Camier et al., 1992; Cook et al., 1994b). The second lies within the second zinc finger of both proteins. The amino acid sequence of Adrlp also contains a bipartite nuclear targeting sequence (Robbins et al., 1991) at amino acids 208–224 and 534–550. The Mxrlp sequence revealed no known nuclear targeting sequence.

An open reading frame (ORF) analysis of the entire sequenced region can be seen in Figure 3.6. Frame 3 revealed the large ORF that we named \textit{MXRI}. Reading frame 2 may contain approximately 360 bp of the 3' end of an ORF. This ORF is not considered as a candidate for the mutated gene in JC131 cells, because the pYL1\DeltaSpe subclone, which lacks this section of the pYL1 insert (Figure 3.2), was still able to transform JC131 cells to Mut\(^{+}\). This potential ORF does not overlap with the ORF of \textit{MXRI}. Since Northern blot results indicate the presence of at least three other messages within the insert of pYL1, these results are not unexpected.
Figure 3.6. Open-reading-frame analysis of sequenced region of pYL1. Reading frame is indicated in margins. *MXRI* ORF is in reading frame 3. Short mark = start codon. Tall mark = stop codon. Figure generated using DNA Strider software version 1.1.
CHAPTER 4
DISCUSSION

4.1 Biochemical Characterization of the JC131 Mutant of *Pichia pastoris*

The JC131 mutant of *P. pastoris* has a phenotype of Mut-, Out-, and Per+ that cannot be explained by the loss of any single enzyme. In order to further understand the nature of this mutation, a more detailed biochemical profile of the mutant was developed. The levels and location of peroxisomal methanol- and β-oxidation-pathway enzymes provide a distinct picture of the effect that the JC131 mutation has on the organism. Our results show that the mutant cells induced on oleate contain nearly wild-type levels of CAT, thiolase, and acyl-CoA oxidase, and that all three of these enzymes are present in post-differential centrifugation pellet fractions in normal proportions; therefore, they are likely to be localized to peroxisomes. It is not clear why JC131 cells cannot grow on oleic acid. One possibility is that the bifunctional enzyme, which we could not assay, is very low. Other possibilities are that Mxrlp is essential for synthesis of (1) one or more glyoxylate pathway enzymes which are needed for growth on oleate, or (2) a substrate transport protein such as for FAD, acyl-CoA, or acetyl-CoA. Further studies are needed to understand this Out− phenotype.

JC131 mutant cells induced on methanol are entirely different from wild type with respect to their enzyme profile. CAT activity and protein are present and sedimentable, but at a significantly reduced level compared to wild-type methanol-induced cells. The two other methanol pathway enzymes, AOX and DHAS, could not be detected by activity assay (AOX) or by immunoblotting (AOX and DHAS). These results explain JC131's inability to grow on methanol. Peroxisomes of JC131 mutant cells appear to be import competent, because the oleate pathway enzymes
tested were pelletable, and CAT is pelletable on both peroxisome-requiring carbon sources. One explanation for the multiple missing enzymes of the methanol pathway is that the mutation is affecting transcription of methanol pathway enzymes. The enzyme profile of JC131 mutant cells is similar to the enzyme profile of \textit{S. cerevisiae} \textit{adr1} mutants (Simon et al., 1992). On ethanol, \textit{adr1} mutant cells produce a significantly reduced level of CAT compared to wild type. On oleate, \textit{adr1} mutant cells have CAT and thiolase levels comparable to wild type.

4.2 Cloning of \textit{MXR1}, Characterization, and Sequence Analysis

The gene most likely affected in JC131, \textit{MXR1} (methanol enzyme expression regulator 1), codes for a protein of 1155 amino acids. A short region of 80 amino acids near the N-terminus has high similarity to the product of the \textit{S. cerevisiae} \textit{ADR1} gene. Thus, in addition to the similarity of their mutant phenotypes, regions of the predicted amino acid sequences for both proteins are also similar. As described above, Adr1p plays an important role in the induction of \textit{ADH2} and other peroxisomal genes, such as \textit{CTA1}, \textit{FOX2}, and \textit{FOX3} (Simon et al., 1991). Mxr1p and Adr1p each contain two C2H2-type zinc fingers in the region of high similarity. All residues within the zinc fingers that have been shown to be critical for recognizing and binding to the specific DNA sequence, UAS1, to which Adr1p binds, are identical in Mxr1p (Thukral et al., 1991b; Taylor et al., 1995). The first zinc finger of Mxr1p is nearly identical to the first zinc finger of Adr1p, and the second finger of each protein is highly similar. Other residues proximal to the zinc-finger regions that play a role in zinc-finger conformation and stability are also highly conserved between the two proteins (Figure 4.1). Other similarities between Mxr1p and Adr1p include three potential phosphorylation sites. The potential cAMP-dependent protein kinase phosphorylation site of Adr1p has been shown to be essential for the optimal functioning of Adr1p (see Section 1.3.1.1). Phosphorylation is known to play a role in deactivating Adr1p, but it is not clear if Adr1p is actually phosphorylated at this particular site or if a repressor may bind there (Cherry et al., 1989; Taylor and
**Figure 4.1.** Comparison of zinc-finger region of Mxr1p and Adr1p. Alignment shows that amino acid residues known to be critical to the function of Adr1p are similar in Mxr1p.

Bold face = zinc finger-DNA contacts (per Taylor et al., 1995).
Underlined = amino acid residue that may play a role in contacting DNA, stability, and/or conformation of zinc-finger domain.
+ = exact match of critical residue between Mxr1p and Adr1p.
: = near match of critical residue between Mxr1p and Adr1p.
- = no match of critical residue between Mxr1p and Adr1p.
A = residue not critical for DNA binding, but needed for transactivation.

* Note that 3' half site is represented 3' to 5'.

Taken from: Thukral et al., 1991; Camier et al., 1992; Cheng et al., 1994; Taylor et al., 1995.
Young, 1990; Denis et al., 1992; Cook et al., 1994a). Regardless of the actual mechanism, mutations at this site produce Adh2p and CAT constitutively (Denis and Gallo, 1986; Simon et al., 1991). Mxrlp contains a nearly identical potential cAMP-dependent protein kinase phosphorylation sequence in approximately the same location relative to its zinc fingers. Two other potential protein kinase C phosphorylation sites are also common to the two proteins. These are located on the N-terminal side of and within the zinc-finger regions of both proteins. The functional significance of these potential phosphorylation sites in Adrlp are not known. However, the region N-terminal to the zinc-finger region of Adrlp is known to play a role in DNA binding; therefore, the similarity of the two proteins in this region may be significant (Thukral et al., 1989; Camier, et al., 1992; Cook et al., 1994b). Sequence analysis for potential functional domains revealed that Mxrlp does not contain any known nuclear targeting sequence. However, Adrlp contains a bipartite nuclear targeting sequence. Very little similarity or identity is seen between Mxrlp and Adrlp on the C-terminal side of the zinc-finger domains.

Northern blots show that the 4.0-kb message of \textit{MXRI} has nearly equivalent expression levels on methanol and glucose. This differs from other known peroxisomal structural and enzymatic genes, which show low or no expression on glucose and markedly higher expression on an inducing carbon source (Liu et al., 1995; Waterham et al., 1996). However, the expression pattern of \textit{MXRI} is consistent with a transcription factor like \textit{ADR1}, which is present and bound to DNA regardless of the carbon source being utilized (Taylor and Young, 1990). The amount of \textit{ADR1} message remains the same in cells grown on either glucose or ethanol. In contrast, the level of Adh2p in ethanol-grown cells increases as much as 100-fold over the level on glucose (Denis and Gallo, 1986).

4.3 Evidence that \textit{MXRI} Is the Affected Gene in JC131 Mutants

The results of subcloning experiments provide evidence that \textit{MXRI} is the gene affected in the JC131 mutant. The deletion of a small fragment of DNA between two \textit{EagI} sites eliminates the ability of plasmid pYL1 to complement JC131 mutant cells.
This suggests that the JC131 mutation lies between or very near these two sites. The sequence of the Eagl fragment is almost entirely contained within the large ORF we believe is MXRI. Northern blots show that, of the four potential ORFs on pYL1, the Eagl fragment probe only hybridizes to one—a 4.0-kb message which corresponds closely in size to the putative MXRI ORF.

The message of MXRI clearly extends well into the region of the fragment deleted in the pYL1ΔSpe subclone (Section 3.2 and Figure 3.2), indicating that either a recombination event could have occurred with this plasmid to generate a wild-type gene or that the C-terminal region of the message is not critical to the function of Mxr1p for growth on methanol (growth on oleate was not tested). Either explanation could account for why this subclone was able to restore the Mut+ phenotype to the JC131 mutant. It has been demonstrated that an adrl mutant that is composed of only the N-terminal 505-amino acid residues (out of 1323 residues), containing the two zinc fingers and some flanking sequence, will grow at wild-type rates on ethanol (Hartshorne et al., 1986). The pYL1ΔSpe subclone contains sequences encoding the N-terminal 421-amino acid residues of the putative MXRI ORF plus the promoter. These include both zinc fingers and 330 residues downstream. The fact that pYL1ΔSpe can successfully transform JC131 to Mut+ is consistent with the ORF we selected being MXRI and eliminates the ORFs downstream from MXRI as candidates for the affected gene in this mutant.

Although these experimental results strongly indicate that the ORF we have chosen as MXRI is the gene that carries the JC131 mutation, they are not conclusive. There is a possibility that an ORF 5' to the putative MXRI ORF (Figures 3.3 and 3.6) may be the affected gene. There is also the formal possibility that the ORF we have identified encodes a suppressor gene and not the same gene mutated in JC131. The putative MXRI ORF can be confirmed as the affected gene by experiments with a deletion strain. This is discussed in more detail in Section 4.5.
4.4 Potential Function of Mxr1p

The phenotype and biochemical profile of JC131 mutant cells suggests a regulatory malfunction since expression levels of several peroxisomal proteins are shown to be affected on methanol. Null mutants of *S. cerevisiae* ADRI display phenotypes that are strikingly similar to the *P. pastoris* JC131 mutant. Both are Out− and Per+ with numerous peroxisomal functions compromised. Experiments have shown that Adr1p affects the expression of peroxisomal enzymes and structural genes in *S. cerevisiae*, such as CAI, FOX2, and FOX3 (Simon et al., 1991). The phenotypic effect of a mutation in a regulatory protein can be complex, since the expression of numerous genes may be affected (Simon et al., 1992). This complexity is evidenced by the levels of CAT in JC131 cells. On methanol, CAT levels are drastically reduced compared to wild type in this mutant, whereas on oleate the CAT levels are comparable to wild type. This is also characteristic of adrJ null mutants (Simon et al., 1991). Although it was not possible to determine why JC131 cells cannot grow on oleate, it is reasonable to speculate that if MXRI is a regulatory protein it could be having an effect on some aspect of oleate metabolism for which it has not yet been tested. In Section 4.1 there is a more detailed discussion of this question.

The sequence of the predicted MXRI product is evidence that it is a transcription factor. Similar zinc fingers are found in other known transcription factors such as SP1 (human), Zif268 (mouse), and Mig1p (yeast) (Kadonaga et al., 1987; Christy et al., 1988; Nehlin and Ronne, 1990), and their presence in Mxr1p implies a DNA-binding function related to transcriptional regulation (Evans and Hollenberg, 1988). A search of the BLAST database for amino acid sequences homologous to the zinc-finger region of Mxr1p revealed many DNA-binding proteins and transcription factors. No other types of protein appeared on the list. All amino acid residues known to be critical for DNA-binding specificity within the zinc-finger domains are identical between Adr1p and Mxr1p (Thukral et al., 1991b; Taylor et al., 1995). This is significant because a single amino acid substitution within an Adr1p zinc finger or in the region immediately upstream from the zinc fingers can interfere
with the protein's ability to bind DNA, alter its binding specificity, and/or form a proper zinc-finger domain (Thukral et al., 1991b; Cook et al., 1994b).

The promoter of the _P. pastoris AOX1_ gene contains a sequence that conforms to the consensus sequence for UAS1 to which Adr1p binds (Koutz et al., 1989) (Figure 4.2). The _P. pastoris AOX2_ promoter region does not contain a complete binding site, although two 3' half sites are present. A sequence resembling a 5' half site is in close proximity to one of these. _P. pastoris AOX1_ is expressed at a much higher level in methanol-grown cells than _AOX2_. A lack of good binding sites for Mxr1p could explain this difference. The promoter regions for _P. pastoris DAS1_ and _DAS2_ (dihydroxyacetone synthase genes) were also examined for Adr1p consensus binding sites. Half sites (5' and 3') for the consensus sequence were found between -490 and -600 of the _DAS1_ promoter (Figure 4.2). However, these half sites are separated by a larger number of nucleotides than the consensus sequence permits. A close variation of the 5' consensus half site exists at an acceptable distance from one of the 3' half sites and, therefore, may be utilized for Mxr1p binding. Another set of half sites separated by a large number of nucleotides is found in the DNA sequence between the two adjacent _DAS_ promoters. The _DAS2_ promoter does not contain a complete binding site. However, there is a 5' half site situated close to an approximate 3' half site. The two _DAS_ genes are thought to be expressed at similar levels (J. M. Cregg, personal communication).

Adr1p has been shown to bind to the _H. polymorpha MOX_ gene promoter and to function as a transcription regulator when the _MOX_ promoter is 5' of a heterologous reporter gene in _S. cerevisiae_. The _MOX_ promoter expresses protein at only 50% of the level of wild type in an adr1 mutant. These results indicate that _H. polymorpha_ may also contain a transcription factor for the _MOX_ promoter that is similar to Adr1p (Pereira and Hollenberg, 1996). The ability of _S. cerevisiae_ to regulate the _MOX_ promoter through Adr1p is surprising, since _S. cerevisiae_ cannot utilize methanol as a carbon source. The region of the _MOX_ promoter that binds Adr1p does not conform to the consensus sequence for Adr1p UAS1. It contains only a partial 5' half site. In spite of this, experiments show that two monomeric proteins
**Figure 4.2.** *ADRI* UAS elements in selected yeast promoters. Promoter regions of selected yeast genes that code for enzymes of methanol metabolism were scanned for matches to the consensus sequence for *ADRI* UAS1. Bases or spacer regions that do not match consensus sequence are in lower-case italics or parentheses, respectively. N = any nucleotide.

Taken from: Shuster et al., 1986; Koutz et al., 1989; Cheng et al., 1994; Pereira et al., 1996; Cregg, unpublished.
bind to this region. The authors speculate that Adrlp may heterodimerize with another transcription factor or that the consensus sequence is not entirely correct (Pereira and Hollenberg, 1996). A lack of sequence similarity outside the zinc-finger region is common among transcription factors of this type (Nehlin and Ronne, 1990). Although the putative DNA-binding domains of different transcription factors can be highly similar, such factors can function differently. For example, some repress transcription while others activate it. Some require other proteins to regulate activity; others may contain their own regulatory domain. Some remain bound to DNA at all times while others bind only in the presence (or absence) of a ligand (Struhl, 1995; Svetlov and Cooper, 1995). The phenotype similarity of S. cerevisiae adrl and P. pastoris JC131 mutants and the sequence similarity within the zinc-finger regions strongly suggest that Mxrlp is a transcription factor that functions in a similar manner to Adrlp, specific for methanol pathway enzymes.

4.5 Further Experiments

The first important experiment is to formally confirm that MXRI is the defective gene in the JC131 mutant of P. pastoris, and not a suppressor gene or one of the other genes within pYL1. To do this, a null mutant of MXRI must be constructed and crossed against JC131. If MXRI is the defective gene in the JC131 mutant, certain experimental results can be predicted. First, the mxmlA strain will no longer produce the MXRI 4.0-kb message. Second, the strain should grow on glucose medium, but not on methanol or oleate. Its phenotype should be similar to that of the JC131 mutant. However, the mxmlA phenotype could be more severe than JC131. A cross between JC131 and the mxmlA strain should not produce any cells that are Mut+ or Out+, nor should any spores produced from such a cross be able to grow on the restrictive carbon sources. Results of such a cross would confirm that the mutated gene of the JC131 strain and mxmlA are mutant alleles of the same gene and that MXRI is not a suppressor of the JC131 mutation.
The enzyme profile of JC131 cells supports the hypothesis that peroxisome morphology is normal. However, due to the lack of AOX and DHAS in this strain, it is possible to predict that the organelles are smaller than normal in methanol-induced cells. Peroxisomes on oleate would be expected to be nearly normal in appearance. A more detailed EM study on the JC131 mutant and mxr1Δ should be done to observe any differences between their peroxisomes on oleate versus methanol and compare them to wild type.

Recently, the promoter region for methanol oxidase (MOX) in H. polymorpha has been found to interact with Adr1p in S. cerevisiae cells (Pereira and Hollenberg, 1996). This implies that the induction mechanisms for utilization of alternate carbon sources have been conserved across species. To investigate whether Adr1p can at least partially replace Mxr1p, and vice versa, mxr1Δ could be transformed with a plasmid expressing the ADRI gene, and an adr1 null mutant could be transformed with a plasmid carrying the MXRI gene. If the two genes are functionally conserved, they may be able to complement a null mutant of the other species.

DNA band shift assays and footprinting will establish promoter sequences to which Mxr1p binds and in which genes. It would also be useful to determine the mutation in MXRI that exists in JC131. It could shed light on a functional domain and how it interacts with DNA or other proteins. Evidence from this thesis suggests that the defect is within the first 496 bases of the putative MXRI ORF in a region that encodes the zinc fingers of Mxr1p. This crucial domain is likely to harbor a mutation that could disable its function.

It would also be interesting to investigate the potential cAMP-dependent protein kinase phosphorylation site within the MXRI sequence. Site-directed mutagenesis could be used to alter residue 215, which is normally Ser. If this is a key phosphorylation site or a repressor binding site critical to the function of Mxr1p (as in Adr1p), an mxr1Δ mutant might result with a phenotype that displays unregulated AOX and DHA expression. Such mutants would be expected to contain equally high levels of these enzyme messages on methanol and glucose.
Another obvious line of inquiry would be why the JC131 mutant does not grow on oleate. The presence of other oleate pathway enzymes, such as bifunctional enzyme and acyl-CoA-synthase, should be examined for JC131 cells via enzyme activity assays or western blots. If an enzyme is not missing, it is possible that some β-oxidation-pathway enzyme, substrate, or cofactor is not being induced or imported properly in this mutant.

The experiments outlined above would establish MXRI as the gene responsible for the defect in the JC131 mutant and give an overall picture of how Mxr1p functions in cells of *P. pastoris*. They would also undoubtedly raise more questions to be answered by further research.


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BIOGRAPHICAL SKETCH

The author was born on November 11, 1954, in Portland, Oregon. She attended Trinity College in Hartford, Connecticut, graduating cum laude, high honors in Biology, with a B.S. in Biology in 1976. As a senior, she completed an independent study project with her advisor, Dr. James Brewer, on the response of coelenterate polyps to amino acids and cell extracts. After working on an air pollution project at the Harvard School of Public Health for a year, she attended the University of Minnesota in Minneapolis/St. Paul, Minnesota. There she worked as a teaching assistant and completed course work towards a Ph.D. in Plant Biochemistry. Due to various life circumstances, including marriage, children, and other familial responsibilities, she curtailed her pursuit of a degree for 15 years. In 1994 she returned to academic life, working in the lab of Dr. James Cregg at the Oregon Graduate Institute of Science and Technology. There she worked on the cloning and characterization of a gene required for methanol utilization in the yeast Pichia pastoris and completed her M.S. in Molecular Biology.